HUMAN KALLIKREIN-RELATED PEPTIDASE 6 (KLK6) AND 13 (KLK13) ARE INVOLVED IN OVARIAN CARCINOMA PATHOGENESIS

NICOLE WHITE


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

Human Kallikrein-Related Peptidase 6 (KLK6) and 13 (KLK13) are Involved in Ovarian Carcinoma Pathogenesis

By

©Nicole White

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Division of BioMedical Sciences
Memorial University of Newfoundland
June 2010

St. John's *Newfoundland and Labrador*

Abstract

It is estimated in 2009 that 2500 Canadian women were diagnosed and 1750 women lost their lives to epithelial ovarian cancer. This malignancy has a high mortality rate because the majority of women are diagnosed in late stage disease where the 5 year survival rate is only 20%. Late diagnosis is a result of the lack of an effective screening marker. Currently, CA125 is the only marker that is used for ovarian cancer patients and it is used primarily to monitor disease recurrence after treatment. Unfortunately, CA125 lacks the sensitivity and specificity to be used for early detection of ovarian cancer. Recently, a new group of genes, the human kallikrein-related peptidase (KLK) family, has been implicated in ovarian cancer and are being investigated as potential new biomarkers for the malignancy. In particular, KLK13 has been shown to have increased expression in ovarian cancer. KLK13 has increased expression in the ovarian cancer cell lines CAOV-3, OVCAR-3, and SKOV-3 when compared to the IOSE cell line and is involved in cell motility. Increased KLK13 expression increases migration in the epithelial cell lines IOSE and Mv1Lu. Also, when KLK13 expression was decreased in the ovarian cancer cell line SKOV-3, which has high endogenous KLK13 expression levels, there was a decrease in cellular migration. Increased KLK13 expression in IOSE cells increased cellular invasion through the basement membrane. These data together suggest KLK13 plays a role in ovarian carcinogenesis and may be a potential therapeutic target. In order to see if KLK expression had any prognostic significance in ovarian cancer patients, paraffin embedded ovarian cancer samples were analyzed for *KLK*6 and *KLK13* mRNA expression. High expression levels of both *KLK6* and *KLK13* were associated with

invasive ovarian cancer. Also, high *KLK6* expression was associated with late stage ovarian cancer and serous histological type. Both *KLK6* and *KLK13* were also shown to be markers of poor prognosis as patients with high kallikrein expression were more likely to have a recurrence than patients with low KLK expression. When *KLK6, KLK13* and *Muc16* were assessed for the ability to detect ovarian cancer, the genes detected 56%, 50%, and 56%, respectively, early stage (Stage I and II) ovarian cancer patients. When all three markers were used in combination, the sensitivity of the test improved to 84%. There was no significant change in the specificity or positive predictive value, but the negative predictive value increased from 33% using the individual markers to 58% when all three markers were combined. These data together suggest KLK6 and KLK13 are involved in ovarian cancer tumorigenesis. Both KLK6 and KLK13 are potential new markers and possible therapeutic targets for ovarian carcinoma.

Acknowledgements

First, I would like to thank my supervisor, Dr. Jules Doré, who has taken the many hours and much effort to train and guide my through my graduate studies. Dr. Doré volunteered to take me on as a graduate student under very difficult circumstances. I began in his lab part-way through my program which created many challenges for him and his lab. But, through his patience, guidance and advice throughout my studies, I have been able to successfully complete my program.

I would also like to thank the members of my supervisory committee, Dr. Gary Paterno and Dr. Amrah Pirzada, have taken the time out of their busy schedules to provide encouragement and advice throughout the completion of my project. I would also like to thank them for taking the time and effort to read and provide comments on my thesis.

I would also like to thank the late Dr. John Desmond Robb. Dr. Robb first saw potential in me and gave me the opportunity to begin graduate studies under his supervision. I am lucky that I had the pleasure of meeting and being supervised by him and I learned a great deal from him in the short time I knew him.

I would also like to thank the members of Dr. Robb's lab, Dr. George Yousef and Cathy Searle, who taught me many techniques, provided assistance, and helped me technically when required in both the early days and later years of my program.

I would also like to thank the members, past and present, of the Doré lab; Danielle Ings, Danielle French, Andrea Darby-King, and Alex Cohen, for helpful input and suggestions, as well as comic relief, throughout the entirety of my project.

I would like to thank the members of the MacPhee Lab, our "sister lab," throughout my program who were always available when needed. I would also like to thank Bryan White for spending the time to teach me certain techniques during my studies.

I would not be here today without the support from my family. To my parents, Eleanor and Bill White, and my sister, Roxanne White, thank you for all the support, both financially and emotionally, through my entire academic career. You have been here since the beginning of my program and your influence in my life has helped make my studies a success.

I would also like to thank all my friends, from the 5th floor research crew, to the 5th floor nurses, to my buddies from Torbay, for being there for physical or refreshment breaks and emotional support throughout my studies.

Finally, I thank CIHR for funding the Doré lab that provided me with the needed resources for the completion of my work.

Table of Contents

List of Figures

List of Figures

List of Tables

List of Abbreviations

αACT	α_1-antichymotrypsin
α_2AP	α_2-antiplasmin
α_2M	α_2-macrogloublin
APC	adenomatous polyposis coli
ATIII	antithrombin III
ATCC	American Type Culture Collection
ARE	androgen response element
ATM	ataxia telangiectasia mutated
BCA	bicinchoninic acid
BL	borderline
BRCA1	breast cancer susceptibility gene 1
BRCA2	breast cancer susceptibility gene 2
CA125	cancer antigen 125
CA19-9	carbohydrate antigen 19-9
CDSN	corneodesmosin
CFU	colony forming unit
CI	confidence interval
CMV	cytomegalovirus
C_T	threshold cycle
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DSC1	desmocolin 1
DSG1	desmoglein 1
E	estrogen
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
EMT	epithelial to mesenchymal transition
FCS	fetal calf serum
FFPE	formalin fixed, paraffin embedded
FIGO	International Federation of Gynaecology and Obstetrics
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCT	granulosa cell tumor
HNF-1β	hepatocyte nuclear factor-1β
HNPCC	hereditary non-polyposis colorectal cancer
HOSE	human ovarian surface epithelium
HR	hazard ratio
HRT	hormone replacement therapy
KLK	kallikrein-related peptidase

List of Abbreviations

KLK6	kallikrein-related peptidase 6
KLK13	kallikrein-related peptidase 13
LH	luteinizing hormone
LOH	loss of heterozygosity
M-CSF	macrophage-colony stimulating factor
MOI	multiplicity of infection
MOPS	3-(N-morpholino) propanesulfonic acid
miRNA	microRNA
MISRII	Müllerian inhibitory substance type II receptor
mRNA	messenger RNA
MUC16	mucin 16
NEAA	non-essential amino acids
NOE	normal ovarian epithelium
NPV	negative predictive value
OAS	oligoadenylate synthase
OS	overall survival
OSE	ovarian surface epithelium
P_4	progesterone
PI3K	phosphatidylinositol 3-kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
PNGaseF	Peptide: N-glycosidase F
PPV	positive predictive value
PSA	prostate specific antigen
pro-uPA	pro-urokinase plasminogen activator
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidine diflouride
qRT-PCR	quantitative reverse transcription polymerase chain reaction
Rb1	retinoblastoma 1
RFU	relative fluorescence units
RFS	recurrence free survival
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SgI	seminogelin I
SgII	seminogelin II
SNP	single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SSPE	sodium chloride, sodium phosphate, EDTA
TBST	tris buffer, sodium chloride, tween

List of Abbreviations

TCF	T-cell factor
TGF-β	transforming growth factor β
TGF-βR2	transforming growth factor β receptor 2
uPA	urokinase plasminogen activator

List of Appendices

Chapter 1 The Current Understanding of Ovarian Cancer and Kallikrein-Related Peptidases

Introduction

1.1 Ovarian surface epithelium

The ovarian surface epithelium (OSE), also referred to as the ovarian mesothelium or the normal ovarian epithelium (NOE), is a single layer of flat-to-cuboidal epithelial cells covering the ovary (Bast, Jr. *et al.*, 1998b;Nicosia *et al.*, 1997). The OSE is separated from the ovarian stroma by a basement membrane that overlies a collagenous connective tissue layer, the tunica albuginea (Wong and Auersperg, 2002). The OSE originally arises from the coelomic epithelium (Auersperg *et al.*, 2001). Between ten and twenty weeks of gestation, the human fetal OSE changes from a simple epithelium with a fragmentary basement membrane to a multistratified, papillary epithelium on a well defined basement membrane. It reverts to a monolayer by term, overlaying the now, elongated, lobular ovary (Choi and Auersperg, 2003). The OSE is of great interest in gynaecological oncology because it is thought that approximately 90% of epithelial ovarian cancers arise from this layer of cells (Katabuchi and Okamura, 2003).

The OSE has few distinguishing features. The monolayer of cells is characterized by apical microvilli (Okamura, 1997), a basal lamina, and expression of types 7, 8, 18, and 19 keratin, typical of simple epithelium (Choi and Auersperg, 2003). The OSE can be distinguished from the extraovarian mesothelium by its characteristic protein expression pattern. While the OSE expresses mucin, 17β-hydroxysteroid dehydrogenase and cilia (van Niekerk *et al.*, 1991), it lacks cancer antigen 125 (CA125) expression (Kabawat *et al.*, 1983). Interestingly, CA125 is expressed by the endometrial and endocervical

epithelial, as well as by the pleura, pericardium and peritoneum of adult women (Jacobs and Bast, Jr., 1989). The OSE is the only coelomic epithelial derivative lacking CA125 expression.

Cell to cell adhesion in the OSE is maintained by simple desmosomes, incomplete tight junctions, N-cadherin, and integrins (Auersperg *et al.*, 2001). N-cadherin is a cell surface glycoprotein that functions as a calcium-dependant cell-cell adhesion protein and is normally expressed in mesodermally derived tissues. It is the most abundantly expressed cadherin in OSE. In contrast to the rat OSE, which expresses both N-cadherin and E-cadherin, the human OSE expresses E-cadherin only in regions where the cells assume a columnar shape characteristic of metaplastic epithelium (Maines-Bandiera and Auersperg, 1997). E-cadherin is a marker of differentiation for normal Müllerian epithelium and has been shown to correspond with epithelial differentiation (Marrs and Nelson, 1996). P-cadherin expression is also absent in the OSE, but is present in the epithelium of Müllerian duct derivatives and in ovarian adenocarcinoma cell lines (Wong *et al.*, 1999), indicating P-cadherin expression changes in association with tissue-specific morphogenetic events and pathological processes (Auersperg *et al.*, 2001).

With age, the human ovary assumes increasingly irregular contours and forms OSE-lined surface invaginations called clefts and epithelial inclusion cysts in the ovarian cortex (Wong and Auersperg, 2002). Inclusion cysts are thought to arise from either OSE fragments that are trapped in ruptured follicles at the time of ovulation, or through inflammatory adhesions of OSE at sites of surface invaginations (Scully, 1995). Within

clefs and inclusions, OSE cells have an increased tendency to undergo metaplastic changes, *i.e.,* to take on phenotypic characteristics of Müllerian epithelium, including columnar cell shape and expression of several markers typically found in ovarian cancers, including CA125 and E-cadherin (Choi and Auersperg, 2003).

Epithelial inclusion cysts are thought to be more at risk for neoplastic progression for two main reasons. The first is that the OSE in inclusion cysts is not separated from underlying stroma by the tunica albuginea and therefore has more access to stroma-derived and blood-borne growth factors and cytokines that may promote neoplastic progression (Choi and Auersperg, 2003). This hypothesis is supported by the fact that metaplastic and dysplastic changes tend to be more apparent on the side near the stroma rather than the side adjacent to the tunica albuginea (Scully, 1995). Secondly, the lumen of the inclusion cyst is a secluded site in which the accumulation of tumor-promoting environmental factors may promote neoplastic progression through OSE-derived cytokines and hormones since these agents could accumulate to bioactive levels in this confined site where normally they diffuse into the pelvic cavity when secreted by OSE on the ovarian surface (Choi and Auersperg, 2003).

1.2 Ovarian cancer

1.2.1 Etiology

The initiating events of epithelial ovarian cancer are poorly understood. Currently, there are numerous hypotheses surrounding the origin of ovarian carcinoma. The two theories

that have the most supporting evidence are the incessant ovulation hypothesis and the gonadtropin hypothesis. Other less well supported theories include the inflammation hypothesis and the hormonal theory.

1.2.1.1 The incessant ovulation hypothesis

The "incessant ovulation" hypothesis was first put forward by Fathalla in 1971 proposing ovarian cancer may be caused by the trauma of ovulation (Fathalla, 1971). The ovarian surface is ruptured by the ovulating follicle, damaging the OSE which then requires immediate repair. Over time, this process of repeated damage and OSE proliferation to repair the wound places strain on the OSE, increasing the chances of error during replication where genetic instability would predispose the cell layer to tumorigenesis (Ho, 2003;Riman *et al.*, 1998;Savage *et al.*, 1998).

Supporting this hypothesis are studies demonstrating that the use of oral contraceptives (Narod *et al.*, 1998), parity [number of live births; (Risch *et al.*, 1994)], and prolonged breast feeding (Whittemore *et al.*, 1992), all significantly reduce the risk of ovarian cancer. During oral contraceptive use, ovulation is suppressed by stable estrogen (E) and progesterone (P_4) levels which in turn inhibit the gonadtropins and their ability to stimulate ovulation. Interestingly, it has been shown that the use of oral contraceptives may even reduce the risk of ovarian cancer in women with mutations in the breast cancer susceptibility gene 1 (*BRCA1)* and breast cancer susceptibility gene 2 (*BRCA2)* which are known to cause familial ovarian cancer (Narod *et al.*, 1998). Also, during pregnancy and breast feeding, ovulation is suppressed. The use of oral contraceptives, parity, and breast

feeding all reduce the overall number of ovulations in a woman's lifetime, supporting the incessant ovulation hypothesis. However since these factors provide a reduction in risk for two to three decades after their cessation, they must prevent the triggering biological events that would clinically manifest as cancer years later (Risch, 1998).

There is also evidence that conflicts with the incessant ovulation theory. For example, if ovarian cancer risk is based on a woman's total lifetime ovulations, then women who are infertile should have a very low risk of ovarian cancer. On the contrary, women who have polycystic ovarian syndrome are at an increased risk of developing epithelial ovarian cancer despite the fact they are anovulatory (Schildkraut *et al.*, 1996). Also, women who have never been pregnant because of infertility, have a 40% higher rate of developing epithelial ovarian cancer than women who have never attempted (Rodriguez *et al.*, 1998). Although the incessant ovulation hypothesis is supported by a large number of studies, it does not account for all the risk factors associated with developing epithelial ovarian cancer.

1.2.1.2 The gonadotropin theory

Stadel et al., put forth the "gonadotropin theory" to account for the development of ovarian carcinoma (Stadel, 1975). This hypothesis predicts high levels of pituitary gonadotropins, related to the surge occurring during ovulation, increases estrogenic stimulation of the OSE, which in turn increases the likelihood of malignancy. Gonadotropins can act either directly on the OSE, enhancing transformation, or indirectly by stimulating E production (Mohle *et al.*, 1985).

Supporting this hypothesis is the fact that gonadotropin levels are increased during perimenopause and remain elevated postmenopause, consistent with the median age range for epithelial ovarian cancer of 50-59 years old with 70% of these patients being postmenopausal (Heintz *et al.*, 2003). Also, it has been documented that women with polycystic ovarian syndrome, who hyper-secrete LH, are at a higher risk of developing ovarian cancer (Schildkraut *et al.*, 1996). In addition, pregnancy and oral contraceptive use, which have been shown to reduce the risk of ovarian cancer, suppress circulating levels of both LH and FSH (Risch, 1998).

There also exists evidence contradictory to the gonadotropin theory. For example, lactating women have increased levels of circulating FSH, yet breast-feeding appears to be protective against developing epithelial ovarian cancer (Harris *et al.*, 1992). Also, some ovarian cancer patients have been shown to have lower levels of gonadotropins when compared to their non-cancer counterparts (Ness and Cottreau, 1999;Risch, 1998).

1.2.1.3 Other theories

The development of all ovarian cancers is not accounted for by the previously described theories. The inflammation hypothesis and the hormonal theory have also been suggested as the cause of ovarian cancer. The inflammation hypothesis was suggested based on the increasing incidence of epithelial ovarian cancer among women with pelvic inflammatory disease (Ness and Cottreau, 1999). Also, patients with endometriosis, an inflammatory condition, have been linked to endometrioid and clear cell epithelial ovarian cancers (Sainz *et al.*, 1996;Stern *et al.*, 2001). Also supporting this hypothesis is

the fact that hysterectomy and tubal ligation, which decrease the risk of inflammation, reduce the risk of ovarian cancer (Ness and Cottreau, 1999).

Finally, the hormonal theory was proposed as a cause for epithelial ovarian cancer as growing evidence suggests hormones such as androgen, E, and P_4, have a role in ovarian carcinogenesis. Androgens are produced in the ovary at a higher rate than estrogens and are the principle sex steroids present in the ovary by the developing follicles (McNatty *et al.*, 1979). Although androgens can stimulate proliferation in inclusion cysts and OSE of guinea pigs, they have shown no proliferative effect on human OSE in culture (Bai *et al.*, 2000;Karlan *et al.*, 1995;Silva *et al.*, 1997). However, patients with polycystic ovarian syndrome, who have higher circulating levels of androgens, have an increased risk of epithelial ovarian cancer while patients taking oral contraceptives, which suppress ovarian testosterone production, are protected against ovarian cancer (Schildkraut *et al.*, 1996).

Estrogens have been suspected as etiologic factors of ovarian cancer for some time (Ho, 2003). Ovarian tissue and follicular fluid have much higher E levels than blood and it is likely the OSE and inclusion cysts are exposed to this high E (Lindgren *et al.*, 2002). Also, estrogen has been shown to be genotoxic in breast cancer cells (Yager, 2000). Increased cell proliferation in breast cancer cells caused by E, through E receptor-mediated signalling, is accompanied by an increased probability for mutations to occur during DNA synthesis. Since E has been shown to have a mitogenic effect on both

normal and malignant OSE cells *in vitro* (Syed *et al.*, 2001), this may be reflective of what is occurring with the OSE *in vivo*.

Progesterone, on the other hand, has a protective effect against ovarian cancer. Oral contraceptives, which reduce ovarian cancer risk, contain high levels of P_4. It has been hypothesized that the long-term exposure to P_4 may be the cause for the reduction in ovarian cancer risk (Ho, 2003). Interestingly, the low dose progestin-only pill, which does not suppress ovulation, still decreases the risk of developing ovarian cancer to a level similar to the combination pill (Rosenberg *et al.*, 1994). Also, during pregnancy, protective against ovarian cancer, P_4 levels are high while androgen levels are low. According to the hormonal theory, the protective effect from pregnancy is due to the high levels of circulating P_4 rather than the lack of ovulation (Risch, 1998).

There are numerous theories suggesting the etiology of ovarian cancer. Although each theory has much supporting evidence, there are also facts that disagree, and support other theories. It is therefore more likely that a combination of these theories harbours the true cause of ovarian cancer.

1.2.2 Risk factors

Ovarian carcinoma, known as "the disease that whispers," or, "the silent killer," is the most lethal of all the gynaecological malignancies. In 2009, it was estimated that 2500 new cases of ovarian cancer would occur in Canada while approximately 1750 women would succumb to the disease (Canadian Cancer Society's Steering Committee, 2009).

Ovarian cancer is therefore highly lethal and represents a great clinical challenge in gynaecological oncology because most patients remain asymptomatic until the disease is well advanced. About 75% of patients are diagnosed in late stage disease with a five year survival rate of less than 20%. Reasons for late diagnosis include the anatomical location of the malignancy and the absence of signs and symptoms associated with the early stages of ovarian carcinoma. The disease is treatable and in most cases curable, if diagnosed in the early stages, but still causes the death of more North American women than all other gynaecological malignancies combined.

There are few known risk factors for ovarian cancer. The search for underlying causes of ovarian cancer such as diet and/or lifestyle choices, in order to decrease the incidence of ovarian cancer, is currently a large part of prevention research. The use of oral contraceptives (Narod *et al.*, 1998), parity (Risch *et al.*, 1994), and prolonged breast feeding (Whittemore *et al.*, 1992), significantly reduces the risk of ovarian cancer. These results are consistent with the incessant ovulation hypothesis in that these factors reduce the number of total lifetime ovulations. There seems to be no significant correlations between dietary patterns (Chang *et al.*, 2007;Chang *et al.*, 2008) or alcohol intake (Tworoger *et al.*, 2008) with the occurrence of ovarian cancer.

On the other hand, early menarche (Booth *et al.*, 1989), late menopause (Franceschi *et al.*, 1991), and interestingly, increasing height (Schouten *et al.*, 2003), have been shown to be associated with a increased risk of ovarian cancer. Cigarette smoking has been shown to increase the risk of only mucinous epithelial ovarian cancer (Tworoger *et al.*,

2008). Age, as with many other cancers, appears to be a risk factor for ovarian cancer. The incidence of ovarian cancer climbs dramatically in women during the peri-menopausal period, from less than four in 100,000 women of 30-35 years of age to almost 40 in 100,000 women aged 60-64 years (Public Health Agency of Canada, 2010). Also, Yang et al. (Yang *et al.*, 2008) , found among women with early stage disease (Stage I and II), there was some indication that being overweight in young adulthood, as well as in later years, increased the risk of developing ovarian cancer. The use of hormone replacement therapy (HRT) also increases the risk of ovarian cancer (Riman *et al.*, 2002). Lacey et al. (Lacey, Jr. *et al.*, 2002), observed a significant positive association between estrogen replacement therapy and ovarian cancer among women who used E only therapy for ten years or more. There was no significant association with ovarian cancer and women who used estrogen-progestin for a short period of time. These data support the hormonal theory for the origin of epithelial ovarian cancer. Although all these factors do contribute to ovarian carcinogenesis, the most significant risk factor for ovarian cancer has consistently proven to be family history or genetics (Pharoah and Ponder, 2002).

1.2.3 Familial ovarian cancer syndromes

About 5% to 10% of ovarian cancers are familial while the remaining are sporadic (Boyd and Rubin, 1997). In the general population, the lifetime risk for developing ovarian cancer is 1.6%. Women with one first-degree relative with ovarian cancer have an approximately 5% risk, while women with two first-degree relatives with ovarian cancer

have a 7% risk (Pharoah and Ponder, 2002;Werness and Eltabbakh, 2001). In the United States, 10% to 20% of breast and ovarian cancer patients have a first or second-degree relative with one of these diseases (Madigan *et al.*, 1995). There are three classifications of inherited ovarian cancer: hereditary breast and ovarian cancer syndrome, site-specific ovarian cancer syndrome, and hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch II Syndrome.

1.2.3.1 The breast/ovarian cancer syndrome

The hereditary breast/ovarian cancer syndrome predisposes women to both breast and ovarian cancer. Features of this syndrome include pre-menopausal breast cancer (<50 years), ovarian cancer at any age, bilateral breast cancer, breast and ovarian cancer in the same woman, and male breast cancer (Marshall and Solomon, 2007). This syndrome is linked to germline mutations in both *BRCA1* and *BRCA2* tumor suppressor genes. The frequencies of mutations throughout both genes are relatively uncommon in the general population. In specific ethnic populations, such as the Ashkenazi (Eastern Europe) Jewish population, the frequency of mutation is higher (Roa *et al.*, 1996). The three founder mutations identified in Ashkenazi family ancestry are: 185delAG and 5382insC in *BRCA1*; and 6174delT in *BRCA2*. These mutations are carried by approximately 2.5% of the Ashkenazi Jewish population (Roa *et al.*, 1996). Founder mutations are common in women with ovarian cancer, even without a family history of breast/ovarian cancer.

BRCA1 was originally identified by positional cloning (Miki *et al.*, 1994). *BRCA1* has been identified as a tumor suppressor gene and is composed of 22 exons distributed over

100kb of genomic DNA. When it was noted that approximately 45% familial breast cancer showed evidence of linkage to *BRCA1,* there was a search for another cancer susceptibility gene. The breast cancer susceptibility gene 2 (*BRCA2*), also a tumor suppressor gene, was discovered shortly afterwards (Wooster *et al.*, 1995). This gene is also quite large, as it has 26 coding exons distributed over approximately 70kb of genomic DNA.

Both genes display characteristics of a tumor suppressor such as, inheritance within affected families, autosomal dominant pattern of inheritance and loss of heterozygosity (LOH) at the relevant gene locus in familial tumors (Scully, 2000). Both proteins play a significant role in DNA repair, transcriptional response to DNA damage, and regulation of cell-cycle checkpoints. When DNA suffers a double strand break, BRCA1 is phosphorylated by ataxia telangiectasia mutated (ATM) protein (Cortez *et al.*, 1999). BRCA1 and BRCA2 then co-localize with Rad51 to form complexes that repair double strand breaks and initiate homologous recombination (Scully *et al.*, 1997b).

BRCA1 is thought to play a role in the transcriptional response to DNA damage while the function of BRCA2 as a transcriptional regulator is still unknown. BRCA1 complexes with RNA polymerase II holoenzyme through RNA helicase (Scully *et al.*, 1997a). BRCA1 binds to a large number of transcriptional factors and may mediate signals to RNA polymerase II. BRCA1 also interacts with p53 and may function to stimulate p53 pathways (Buck, 2008).

BRCA1 has also been shown to play a role in regulating cell-cycle checkpoints in response to DNA damage. Cells defective in different DNA damage-responsive proteins have shown that both BRCA1 and ataxia telangiectasia mutated (ATM) are required for effective response to the G2/M checkpoint arrest (Yoshida and Miki, 2004). Expression of BRCA1 variants defective for ATM-mediated phosphorylation were associated with a defect in the G2/M arrest, suggesting that BRCA1 phosphorylation by ATM is required for the G2/M checkpoint response to DNA damage. Although some studies have suggested a role of BRAC2 in cell cycle regulation or checkpoint functions, it remains unclear whether the protein participates.

1.2.3.2 The site-specific ovarian syndrome

The second hereditary syndrome is the site-specific ovarian cancer syndrome that predisposes women to ovarian cancer. A family that has three or more cases of invasive epithelial ovarian cancer at any age and no case of breast cancer diagnosed before age 50 qualifies as a site-specific ovarian cancer family (Lynch and Lynch, 1992). Most of these cancers are caused by a germline mutation in the tumor suppressor gene *BRCA1* and less frequently in the *BRCA2* gene (Antoniou *et al.*, 2000). Hereditary site-specific ovarian cancer syndrome is considered as the same spectrum of disease as the hereditary breast/ovarian cancer syndrome, in which early-onset breast cancer has not yet appeared (Steichen-Gersdorf *et al.*, 1994).

1.2.3.3 The hereditary non-polyposis colorectal cancer syndrome

The third hereditary predisposition to ovarian cancer is hereditary non-polyposis colorectal cancer (HNPCC) syndrome or Lynch II syndrome. HNPCC is characterized by autosomal dominant inheritance, early age at onset of colorectal carcinoma, right-sided predominance, and increased incidence of synchronous and metachronous colorectal cancers (Fitzgibbons *et al.*, 1987). It is associated with germline mutations in several DNA mismatch repair genes: *MSH2*, *MLH1*, *PMS1*, *PMS2* and *MSH6* (Lynch and de la Chapelle, 1999), although 90% of all HNPCC cases are caused by mutations in the *MSH2* and *MLH1* genes (Lynch and de la Chapelle, 2003).

Germline mutations in HNPCC-related genes confer risks for colorectal, endometrial, and ovarian cancer of up to 90%, 60%, and 13%, respectively (Lynch *et al.*, 1998). To date, the single most common *MSH2* mutation is a point mutation (A→T nt943+3) in the 3' splice site of exon 5, resulting in deletion of this exon and a truncated protein. Male carriers of this mutation have a significantly higher risk of colorectal cancer than females (63% *versus* 30% and 84% *versus* 44%, at age 50 and 60 years, respectively) , while female carriers have a higher risk of endometrial (50% at age 60 years) and pre-menopausal ovarian cancer [20% at 50 years; (Froggatt *et al.*, 1999)].

Recently, a single nucleotide polymorphism (SNP) in the promoter region (G→A nt93) of *MLH1* has been associated with a modest, but highly significant risk of ovarian cancer (Harley *et al.*, 2008). This SNP has also been associated with a 1.5-fold increased risk of

developing endometrial cancer (Beiner *et al.*, 2006) and an increased risk of colorectal cancer (Raptis *et al.*, 2007).

The level of ovarian cancer risk associated with HPNCC has not been widely appreciated. The clinical criteria, Amsterdam and modified Amsterdam criteria, for the diagnosis of HPNCC does not include ovarian cancer, but does include stomach cancer that carries essentially the same level of risk as ovarian cancer (Aarnio *et al.*, 1999). Women who have relatives with colon, endometrial, ovarian, breast, or stomach cancer may be at a higher risk for developing ovarian malignancy and should therefore be tested for the presence of a germline mutation in the *BRCA1, BRCA2*, or one of the mismatch repair genes.

1.2.4 Histological classification of ovarian tumors

Human ovarian tumors are divided into three major categories based on the histogenesis of the normal ovary. The histological classification categorizes ovarian neoplasms with regard to their derivation from the coelomic surface epithelium, germ cells, and mesenchymal cells, into epithelial tumors, germ cell tumors, and sex-cord stromal tumors, respectively (Kaku *et al.*, 2003). The aggressiveness of ovarian tumors is assessed by tumor stage and grade. Staging for primary carcinoma of the ovary was defined by The International Federation of Gynecology and Oncology (International Federation of Gynecology and Oncology, 1986). Stage I primary ovarian carcinoma is diagnosed if tumor growth is limited to the ovaries. Stage II ovarian cancer is defined as growth that is involved with one or both ovaries with pelvic extension. Stage III ovarian

cancer is diagnosed as tumor involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. In the case of Stage III ovarian cancer, the tumor is limited to the true pelvis but with histologically proven malignant extension to the small bowel or omentum. Finally, stage IV ovarian cancer is defined as a tumor growth that involves one or both ovaries with distant metastasis. If pleural effusion is present, there must be positive cytology to classify a patient as stage IV ovarian cancer. Patients with parenchymal liver metastasis are diagnosed with stage IV (International Federation of Gynecology and Oncology, 1986).

The aggressiveness of ovarian tumors is also assessed by tumor grade. The grading system for ovarian cancer is based on assigned points for three components; architecture (glandular, papillary, or solid), degree of nuclear atypia, and mitotic index (Silverberg, 1999). The points for each component are added resulting in a total score which determines the tumor grade. Ovarian tumors are called Grade 1, or well differentiated if they represent cells that are more normal looking. These tumors are usually less aggressive. Grade 3, or a poorly differentiated tumor, looks very abnormal, almost unrecognizable, and are the most aggressive. Grade 2, moderately differentiated tumors, are in between the two. Ovarian tumors can also be classified as "borderline" or "low malignant potential." These tumors are early stage and usually do not grow back, or grow back very slowly (Silverberg, 1999).

1.2.4.1 Epithelial ovarian cancer

Epithelial ovarian tumors make up approximately 90% of all ovarian malignancies (Fleming *et al.*, 2006). Neoplasms of the ovarian surface epithelium are classified as serous, mucinous, endometrioid, clear cell, and transitional cell types. These tumors are then subdivided into benign, borderline and malignant categories (Christie and Oehler, 2006). Only the first four types of epithelial ovarian cancer will be discussed as the true prevalence of transitional cell ovarian carcinoma is impossible to ascertain (Soslow, 2008). The malignancy is not diagnosed reproducibly and the genetic changes in transitional cell ovarian carcinomas remain largely unknown.

1.2.4.1.1 Serous ovarian tumors

Approximately 80% to 85% of all ovarian carcinomas diagnosed in Western, industrialized countries are Stage III or Stage IV serous carcinoma (Seidman *et al.*, 2004). It is uncommon to see a Stage I serous carcinoma (Soslow, 2008). Serous ovarian tumors typically differentiate into cells resembling cells of the fallopian tube (Scully, 1987). More recently, studies suggest that a proportion of high-grade serous tumors may originate from the fallopian tube (Vang *et al.*, 2009). Low and high-grade serous tumors appear to arise by mutations through different signalling pathways. Low-grade serous tumors are likely to progress from an adenoma to a borderline tumor to a malignant carcinoma progression via alteration of the RAS-RAF signalling pathway due to mutations in *KRAS* and *BRAF* (Bell, 2005). *KRAS* mutations have been found at a higher rate in serous borderline tumors (27-36%) than in high grade serous tumors [0-12%;

(Sieben *et al.*, 2004;Teneriello *et al.*, 1993)], indicating there may be separate genetic pathways leading to the development of the malignancy. *KRAS* is a member of the RAS family of genes. It encodes a GTPase protein which is involved in the RAS/RAF/MAP kinase growth pathway (Mitin *et al.*, 2005). Mutations in *KRAS* result in constitutive activation of the protein, leading to increased growth signals (Tsukuda *et al.*, 2000). High-grade serous carcinomas, on the other hand, appear to arise from the OSE or inclusion cysts. Approximately 60% of high-grade tumors have mutations or overexpression of p53, an uncommon feature among borderline tumors (Chan *et al.*, 2000). p53 is a tumor suppressor gene involved in cell cycle regulation and DNA repair. High grade serous ovarian tumors are also known to exhibit loss of BRCA1. Press et al. studied 38 high grade ovarian tumors and found that high grade serous carcinomas can be divided into three groups, BRCA1 loss (genetic), BRCA1 loss (epigenetic) and no BRCA1 loss (Press *et al.*, 2008). Tumors with BRCA1 mutations were characterized by decreased phosphatase and tension homolog (*PTEN)* mRNA levels, while those with epigenetic loss of BRCA1 had copy number gain of phosphotidylinositol-3 kinase (PI3K). PTEN is a lipid phosphatase that negatively regulates the PI3K signalling pathway (Stambolic *et al.*, 1998).

1.2.4.1.2 Mucinous ovarian tumors

Mucinous ovarian carcinomas are very uncommon, being less than 3% of all ovarian carcinomas (Seidman *et al.*, 2004). Approximately 50-66% of mucinous tumors diagnosed in industrialized, Western countries are Stage I. Mucinous ovarian tumor cells

resemble cells of the endocervix (Scully, 1987). Approximately 80% of these carcinomas have areas of benign and/or borderline mucinous tumor suggesting they arise via an adenoma to borderline tumor to malignant carcinoma progression (Riopel *et al.*, 1999). These tumors have the highest rate of *KRAS* mutations (Gemignani *et al.*, 2003). Cuatrecasas et al., studied *KRAS* in benign, borderline, and malignant mucinous tumors and found they were 56%, 73%, and 85%, respectively, positive for *KRAS* mutations (Cuatrecasas *et al.*, 1997). The increasing frequency of these mutations supports the progressive pathway.

1.2.4.1.3 Endometrioid ovarian tumors

Endometrioid ovarian tumors resemble cells of the endometrium and endometriosis is thought to be a precursor for this tumor type (Scully, 1987). This tumor type accounts for approximately 10% of all ovarian carcinomas diagnosed at Stage I or II (Leitao, Jr. *et al.*, 2004). Interestingly, patients with endometrioid cancers have a better overall survival than patients with serous cancers (Einhorn *et al.*, 1985). This has been attributed to the fact that patients with endometrioid cancer usually have early stage disease (Tornos *et al.*, 1994). Recently, one study compared a sequential set of patients who were treated for endometrioid or serous adenocarcinoma of the ovary and found that even with Stage III or poorly differentiated tumors, despite similar platinum-based chemotherapy, endometrioid histology was still associated with a better survival when compared to serous cancer (Storey *et al.*, 2008). Mutations in *PTEN* are found in approximately 15% of endometrioid cancers, most being low grade (Obata *et al.*, 1998). Interestingly, *PTEN*

mutations are unique to ovarian endometrioid cancers when compared to other ovarian cancers, suggesting an exclusive pathway for the development of this malignancy. Another protein that appears to be commonly mutated (up to 30%) in endometrioid type cancers, is the *CTNNB1* gene, encoding β-catenin (Moreno-Bueno *et al.*, 2001). β-catenin is involved in embryonic development and cell proliferation. It binds to a T-cell factor (TCF), stimulating cell proliferation. β-catenin is normally degraded by the adenomatosis polyposis coli (APC) protein, but mutant forms are resistant to degradation and accumulate in the nucleus, thereby leading to uncontrolled cell proliferation.

Endometrioid cancers are frequently associated with endometriosis (Stern *et al.*, 2001) and occasionally show a spectrum of changes from typical endometriosis, to endometriosis with atypical hyperplasia, to endometrioid ovarian carcinoma, similar to that seen in carcinomas of the endometrium (Sainz *et al.*, 1996). This stepwise progression model is also supported by the fact that endometrioid cancers frequently have areas of borderline endometrioid tumor (Bell and Kurman, 2000) and borderline tumors often show mutations in the *CTNNB1* gene (Palacios and Gamallo, 1998). High grade endometrioid ovarian carcinomas, on the other hand, have similar changes to the high grade serous carcinomas.

1.2.4.1.4 Clear cell ovarian tumors

Clear cell carcinoma is the third most common ovarian carcinoma in North America, and accounts for approximately 5% of all ovarian tumors. Similar to the detection of endometrioid carcinomas, most women are diagnosed with clear cell ovarian carcinoma

in Stages I and II (Seidman *et al.*, 2004). Clear cell ovarian tumors are composed of cells resembling endometrial epithelium during pregnancy (Scully, 1987). Like endometrioid ovarian cancer, clear cell tumors have been associated with endometriosis (Stern *et al.*, 2001) and have microsatellite instability in approximately 15% of carcinomas (Gras *et al.*, 2001;Moreno-Bueno *et al.*, 2001). Unlike any other ovarian cancer type, clear cell tumors display distinct genetic changes. Gene expression profiling has shown the transcription factor, hepatocyte nuclear factor-1 β (HNF-1β), is over expressed in clear cell carcinomas and may be involved in the pathogenesis of this tumor type (Tsuchiya *et al.*, 2003). Immunohistochemistry has shown HNF-1β is overexpressed in benign, borderline and malignant clear cell tumors, but rarely in other ovarian tumors (Kato *et al.*, 2006). Also, clear cell carcinomas contain mutations in transforming growth factor-β receptor 2 (TGFβR2), which is rarely seen in other ovarian tumors (Francis-Thickpenny *et al.*, 2001).

1.2.4.2 Sex cord-stromal ovarian cancer

The second type of ovarian cancer, sex cord-stromal ovarian cancer is a diverse group of tumors that accounts for approximately 7% of all primary ovarian malignancies (Koonings *et al.*, 1989). These tumors are derived from the sex cord and stromal components of the developing gonad (Scully, 1987). The most common tumor in this category is the thecoma-fibroma, followed by the ovarian granulosa cell tumor (GCT). GCT tumors account for approximately 2-5% of all ovarian tumors (Schumer and Cannistra, 2003). There are both juvenile (Scully, 1988) and adult forms of the

malignancy, characterized by age of onset and morphological features (Savage *et al.*, 1998).

A much rarer sex-cord stromal tumor is the Sertoli–L9eydig cell tumor comprising only 0.5% of all ovarian tumors. It is pathologically characterized by the biphasic proliferation of sertoli and leydig cells in varying degrees of differentiation (Young and Scully, 1988). Approximately 75% of these tumors are encountered during the second to third decade of life (Young and Scully, 1985).

1.2.4.3 Germ cell tumors

The final category of ovarian tumors are classified as germ cell tumors and are most commonly found as benign mature cystic teratomas or dermoid cysts (Scully, 1987). These growths rarely undergo malignant transformation, but when it does occur, squamous cell carcinoma is primarily the result (Bal *et al.*, 2007). Their components derive from all three germ layers: ectoderm, endoderm, and mesoderm, and interestingly have been reported to contain teeth and hair (Devoize *et al.*, 2008).

1.2.5 Ovarian cancer models

1.2.5.1 Cell lines

In order to analyze the onset and progression of ovarian cancer, suitable models for both *in vitro* and *in vivo* studies are required. The capacity to study early events in ovarian carcinogenesis was limited for many years due to the lack of normal ovarian surface

epithelial (OSE) cells in culture. The ability to isolate and culture pure OSE cells from human (Auersperg *et al.*, 1984) and mouse (Kido and Shibuya, 1998), has given investigators the opportunity to systematically modify the genetics of normal cells to define the requirements for tumorigenic transformation (Garson *et al.*, 2005). Although currently, normal OSE cells in culture are the best *in vitro* model we have, one must be aware that genetic changes observed in culture may not properly represent changes in the ovarian surface epithelium *in vivo*. Human ovarian surface epithelium (HOSE) cells are structurally and physiologically different from murine OSE (Auersperg *et al.*, 2001), making the HOSE advantageous when one wants to avoid species differences. Also, the HOSE are more resistant to malignant transformation than murine cells (Hahn and Weinberg, 2002). Inactivation of p53 is sufficient to transform mouse embryo fibroblasts, while human counterparts require inactivation of p53, the retinoblastoma gene (*Rb*), and the activation of telomerase in order to bypass senescence (Garson *et al.*, 2005).

Ovarian cancer cell lines have also proven to be quite useful when studying ovarian carcinogenesis. The largest resource for cell lines is the American Type Culture Collection (ATCC) repository. Cancer cell lines require little resources and man power for daily maintenance and are relatively simple models when testing hypotheses. They are a controlled system where the effect of alterations in hormones, growth factors, or further genetic manipulations of these malignant cells can be accurately measured. Ovarian cancer cell lines originate from tumor or ascites from patients and histologically, they are similar to the patients' original tumor. Cell lines that have been developed after

primary chemotherapy treatment are useful for investigating the significance of novel treatments and the mechanisms of drug resistance in ovarian cancer. The OVCAR-3, for example, was established from the malignant ascites of a patient with ovarian adenocarcinoma after chemotherapy with cyclophosphamide, adrimycin and cisplatin (Hamilton *et al.*, 1983). Because cell lines exist in such a controlled environment, results and findings may be specific to that particular cell line. It is important that before these findings are generalized, similar experiments are tested in more than one cell line.

The cells models used for this work were the immortalized ovarian surface epithelial (IOSE)-398 cells and the ovarian cancer cell lines, CAOV-3, OVCAR-3, and SKOV-3. The IOSE cells were a kind gift from Dr. Nelly Auerspreg and the Canadian Ovarian Tissue Bank. These cells are derived from ovarian surface epithelial cells (Auersperg *et al.*, 1984). Although they are immortalized with Simian virus 40, they are not truly immortal and will senesce at approximately passage 20. The ovarian cancer cell lines CAOV-3, OVCAR-3, and SKOV-3 were purchased from ATCC. Both the CAOV-3 and OVCAR-3 cell lines were derived from ovarian adenocarcinomas and are adherent cells. The OVCAR-3 cell line was established in 1982 from malignant ascites if a patient with progressive adenocarcinoma of the ovary (Hamilton *et al.*, 1983). When these cells are injected into nude mice tumors develop within 21 days with 100% frequency. The cell model is an appropriate cell model to study drug resistance and other biological processes in ovarian cancer. The SKOV-3 cell line was derived from the ascites of a metastatic ovarian adenocarcinoma. When injected into mice, these cells form moderately to well differentiated adenocarcinomas that are consistent with primary ovarian cancers. The

SKOV-3 cell line is resistant to tumor necrosis factor and to several cytotoxic drugs including diphtheria toxin, cisplatinum, and adrimycin (Blagosklonny *et al.*, 1997). The cell line is also know to be migratory and invasive (Whitley *et al.*, 2007). Similar information for the CAOV-3 cell line is not currently available.

1.2.5.2 Xenografts

Xenografts have proven useful for ovarian cancer research, as they are derived from naturally occurring malignancies, are quite representative of specific tumor types, and are reproducible with a genetically defined tumor (Shaw *et al.*, 2004). Xenografts are usually injected into immune-deficient mice under the bursa membrane surrounding the ovary. The bursa provides a microenvironment that has been identified to influence cancer cell behaviour (Shaw *et al.*, 2004). The bursa provides a good environment to study early ovarian cancer, but the inability of cells to disseminate outside the bursa limits its use as a model of late stage metastatic ovarian cancer.

1.2.5.3 Animal models

Animal models are a large part of cancer research and have historically complemented the discovery of disease etiology and progression by making it possible to examine events that are difficult to study in humans (Stammer *et al.*, 2008). Ovarian cancer research has largely been impeded by the limitations of a suitable model of spontaneous ovarian cancer.

One animal model used to study ovarian cancer is the rhesus monkey, *Macaque mulatta*. The rhesus monkey has a reproductive physiology similar to humans, including cycle length, hormone profiles, and ovarian structure (Stouffer *et al.*, 1993). Interestingly, among mammals, only primates develop epithelial ovarian cancer (Moore *et al.*, 2003). Wright et al., using the rhesus monkey model, examined the OSE at different stages of the ovarian cycle and found that the primate OSE, similar to the human OSE, undergoes a proliferative repair process and may therefore contribute to the etiology of epithelial cancer (Wright *et al.*, 2008). Although similar to humans, this model has not been widely used as many research institutions do not have the proper facilities to house and care for these animals.

Two other well known models for ovarian cancer are the laying hen and the mouse. The laying hen is the only other spontaneous model of ovarian cancer. The occurrence of epithelial ovarian cancer is rare in most animals. The mouse is the most widely used model for ovarian cancer as genetic manipulations are attainable and housing and care are available at most institutions.

1.2.5.3.1 Laying hen

Gallus domesticus, the laying hen, has significant similarities to human ovarian cancer making it a functional model to study the malignancy. The incidence of ovarian cancer is high in hens (up to 40% by age 6) and similar to ovarian malignancy in humans, in that it is age dependant (Fredrickson, 1987). Also, hormone cycles, hormone regulation, and ovulation in hens are similar to women. Hens ovulate approximately 250 eggs a year,

which is approximately 20 years of ovulating in humans, given the relative lifespans (Lewis and Long, 1992). In addition, the common histological subtypes seen in humans, such as serous, endometrioid, mucinous, and clear cell ovarian cancers, are represented in hens (Barua *et al.*, 2007). On a molecular level, the hen is similar to humans as they have similar markers expressed in both organisms, including CA125 (Jackson *et al.*, 2007), p27, proliferating cell nuclear antigen and erbB-2 (Rodriguez-Burford *et al.*, 2001). Recently, hen models have verified that the expression of selenium-binding protein 1 mRNA is decreased in ovarian cancer tissues when compared to normal ovarian surface epithelium (Stammer *et al.*, 2008). The same expression pattern is found in humans (Huang *et al.*, 2006). Furthermore, Urick et al. have used the laying hen model to identify COX-1 as a potential target for ovarian cancer treatment and prevention (Urick and Johnson, 2006).

1.2.5.3.2 Mouse

Mice have been successfully used as models in many different diseases. Because mice do not normally spontaneously form epithelial ovarian tumors, they can be used to study genetic differences when a tumor is induced. There have been two main systems used to induce carcinogenesis in mouse OSE. First, Flesken-Nikitin et al. used an intra-bursal adenovirus delivery and Cre-loxP mediated gene inactivation to render OSE cells deficient in *p53* and retinoblastoma (*Rb1*), two key tumor suppressor genes (Flesken-Nikitin *et al.*, 2003). They showed that concurrent inactivation of both genes is sufficient for reproducible induction of epithelial ovarian carcinoma in mice. The tumors were

similar to tumor development in humans as they formed ascites and metastasized to the lung and liver. These results identified a genetically defined immunocompetent mouse model of sporadic epithelial ovarian cancer (Flesken-Nikitin *et al.*, 2003).

Dinulescu et al. used a similar method to study both endometriosis and endometrioid cancer (Dinulescu *et al.*, 2005). They delivered a recombinant adenovirus expressing Cre recombinase into the bursal cavity that encloses the ovary. They used two separate surgical techniques; first the needle was introduced into the uterine tubal junction, and secondly, into the oviduct. Both methods were equally effective in infecting the OSE cells. They found that Cre-mediated activation of oncogenic *K-ras* in the uterine tubal junction and the oviduct induced endometriosis but in order to induce invasive and metastatic endometrioid adenocarcinoma, they had to activate *K-ras* and inactivate *PTEN* in the mouse OSE (Dinulescu *et al.*, 2005). This is the first reported model for endometriosis, indicating it can arise from the epithelial cells in the uterus or fallopian tubes, while endometrioid ovarian carcinoma may arise from the OSE induced by the expression of oncogenic K-*ras* and deletion of *PTEN*.

Another interesting avenue to gain insight in ovarian cancer pathogenesis, in particular familial ovarian cancer, is using mouse models to knock out expression of *BRCA1* since it plays such an important role in hereditary breast and ovarian cancer pathogenesis. Several different *BRCA1* and *BRCA2* knockout mouse lines have been developed with mutations in different portions in the genes. Unfortunately, none showed a strong tumor predisposing phenotype in a heterozygous setting as is found in humans (Evers and

Jonkers, 2006). When bred to homozygosity, most *BRCA1* and *BRCA2* mouse mutants displayed severe embryonic lethal phenotypes. When *BRCA1* was inactivated in the murine OSE, using the conditional and site-directed adenoviral delivery of Cre recombinase bearing loxP sites in introns 4 and 13 of the *BRCA1* gene, preneoplastic changes such as hyperplasia, epithelial invaginations, and inclusion cysts arose earlier than in control ovaries (Clark-Knowles *et al.*, 2007). Interestingly, these changes resemble premalignant lesions reported in human prophylactic oophorectomy specimens from women with germline mutations in *BRCA1* (Clark-Knowles *et al.*, 2007).

Furthermore, Connolly et al. developed the first transgenic model of epithelial ovarian cancer using the promoter region of the Müllerian inhibitory substance type II receptor (*MISRII*) gene to drive tissue-specific expression (Connolly *et al.*, 2003a). MISRII is a single transmembrane serine/threonine kinase in the TGF-β receptor superfamily (di Clemente *et al.*, 1994). Connolly et al. used the 5' upstream regulatory sequences of the mouse *MISRII* gene to target expression of the SV40 *TAg* specifically to the epithelium of the female mouse reproductive tract including the OSE (Connolly *et al.*, 2003b). The transgenic mice developed bilateral ovarian tumors in approximately 50% cases and resembled poorly differentiated carcinomas with occasional cysts and papillary structures present at the surface of the ovary. These tumors invaded the omentum and formed ascites similar to human ovarian tumors. This model demonstrated the successful application of the *MISRII* promoter to induce ovarian carcinoma in a transgenic mouse model and may serve as a useful tool for detection and treatment strategies (Connolly *et al.*, 2003b).

1.2.6 Serum biomarkers

1.2.6.1 CA125

Presently, there is no effective screening test for ovarian cancer, such as the PAP smear for cervical cancer or the mammogram for breast cancer. Currently, cancer antigen 125 (CA125) is the only validated marker for use in ovarian carcinoma. CA125 is a large glycoprotein of unknown function that is normally expressed in several reproductive tissues such as the endometrium and endocervix. CA125 was first identified by Bast et al. (Bast, Jr. *et al.*, 1981), by isolating a monoclonal antibody (OC125) developed by immunizing mice with an ovarian cancer cell line. The unique antigen expressed by the cells was identified as CA125. Unfortunately, CA125 lacks the sensitivity and specificity to detect early disease in ovarian cancer. Forty to fifty percent of Stage I and Stage II ovarian cancer patients are CA125 negative (Jacobs and Bast, Jr., 1989). High serum CA125 is also seen in many cases of benign gynaecologic disease (Buamah and Skillen, 1994) and other types of non-ovarian cancer. Elevated serum CA125 levels have been seen during menstration (Kafali *et al.*, 2004;Kan *et al.*, 1992;Koninckx *et al.*, 1996;Nonogaki *et al.*, 1991) and early pregnancy (Brumsted *et al.*, 1990). CA125 is currently used to monitor recurrent ovarian cancer post chemotherapy.

1.2.6.2 Other potential serum markers

Many potential serum markers for ovarian cancer have been evaluated. Ovarian cancer is relatively uncommon, thus any useful screening method must be highly specific. In the

United States, the annual incidence of epithelial ovarian cancer in postmenopausal women is 40-50 per 100,000. Therefore, for a screening strategy, in order to achieve a positive predictive value of 10%, i.e. 10 operations for each case of ovarian cancer detected a sensitivity of 75% and a specificity of 99.6% would be required (Bast, Jr. *et al.*, 1998a).

Serum inhibin levels have been evaluated either alone or in combination with CA125. Inhibin is produced by the gonads and inhibits the secretion of follicle-stimulating hormone (FSH) by the anterior pituitary gland. Inhibin has been found to be elevated in 7-41% of non-ovarian cancers (Healy *et al.*, 1993;Robertson *et al.*, 1999) and 28% of benign gynaecological disease (Burger *et al.*, 1996;Healy *et al.*, 1993). Elevated levels have also been found in the serum of all patients with granulosa cell tumors of the ovary, 70-87% of ovarian mucinous tumors, and between 15-35% of other ovarian epithelial tumor types (Lambert-Messerlian, 2000). Unfortunately, inhibin is not reliable to test all types of tumors, which precludes its use as an effective prognostic marker for ovarian cancer.

OVX-1 and macrophage-colony stimulating factor (M-CSF) have also been evaluated as ovarian cancer serum tumor markers. The OVX-1 assay measures a modified Lewis X determinant on mucin recognized by a murine monoclonal antibody (Xu *et al.*, 1991). Only 22% of patients with invasive Stage I carcinoma have elevated OVX-1 levels, while M-CSF is elevated in only 31% of the same patients (van Haaften-Day *et al.*, 2001). The sensitivity of detection of these markers is too low to be accepted as a screening tool.

Other tumor-associated antigens such as carbohydrate antigen 19-9 (CA19-9) have also been studied for their potential use as ovarian cancer biomarkers. CA19-9 is a carbohydrate determinant that was originally developed against a human colon carcinoma cell line (Koprowski *et al.*, 1979). Elevated CA19-9 levels have been found in 17-36% of ovarian cancer patients with a false positive rate of 18.9% (Bast, Jr. *et al.*, 1984;Gadducci *et al.*, 1992), potentially creating a high number of unnecessary surgical procedures. Interestingly, when the mucinous ovarian cancers of this group were analyzed, 83% patients had high CA19-9 expression. The combination of CA19-9 and CA125 is now being explored as a biomarker for mucinous ovarian cancer (Dong *et al.*, 2008).

A promising family of proteins that have been implicated in ovarian carcinogenesis and are currently being studied for their potential use as ovarian cancer biomarkers is the human kallikrein family. The kallikrein family consists of 15 genes, named *KLK1* through to *KLK15*. Of these genes, 12 are upregulated in ovarian cancer (Borgono and Diamandis, 2004). The human kallikrein family will be discussed in more detail in a later section.

1.2.6.3 Urinary biomarkers

Recently, biomarkers in the urine have been evaluated for ovarian cancer diagnostics. Ye et al. found two potential markers in the urine, a specific glycosylated form of eosinophil-derived neurotoxin (EDN) and several COOH-terminal osteopontin fragments (Ye *et al.*, 2006). Both of these markers were elevated in ovarian cancer. When used in combination, the sensitivity was 72% and the specificity was 95% compared to 47% and

63% specificity for osteopontin fragments or glycosylated EDN, respectively, when each was tested alone. EDN has also been shown to be elevated in various eosinophilic conditions (Inamura *et al.*, 2003), allergic reactions (Tsunoda *et al.*, 2003) and can fluctuate with pregnancy (Matsumoto *et al.*, 2003). Osteopontin, has also been found to be associated with inflammatory conditions and has been elevated in other tumor types such as metastatic breast cancer (Singhal *et al.*, 1997) and bladder cancer (Ang *et al.*, 2005).

1.2.6.4 MicroRNA

The discovery of microRNAs (miRNAs) has evoked interest as their potential use as possible biomarkers for ovarian cancer. MiRNAs are an abundant class of RNA regulatory genes and have been found to be involved in a novel mechanism of genetic regulation. Active, mature miRNAs are highly conserved RNAs that silence gene expression by binding to target mRNA. They are ~22 nucleotides long and their 5' end binds to target complementary sequences in the 3' untranslated region of mRNAs. Depending on the degree of complementarity, miRNA binding appears to result in translational repression, or in some cases, cleavage of mRNA, causing partial or full silencing of the respective protein coding genes (Lee *et al.*, 1993). MiRNAs have been shown to play a role in various biological processes including, cell differentiation, cell proliferation, apoptosis, stress resistance, and fat metabolism (Ambros, 2003). In human cancer, miRNAs can function as oncogenes or tumor suppressor genes depending on their target. Increasing evidence indicates miRNAs are dysregulated in human cancer

(Esquela-Kerscher and Slack, 2006). Unique miRNA expression profiles indicate some miRNAs may be used as diagnostic and prognostic markers. They have been found in several cancers, including chronic lymphocytic leukemia (Calin *et al.*, 2005), breast cancer (Iorio *et al.*, 2005), pancreatic cancer (Roldo *et al.*, 2006), lung cancer (Yanaihara *et al.*, 2006), and kidney cancer (White *et al.*, 2010a). The miRNA expression profile in serous ovarian cancer has recently been evaluated (Nam *et al.*, 2008). This study found a number of miRNAs were differentially expressed in serous ovarian carcinomas when compared to normal ovarian tissue. High expression of miR-200, miR-141, miR-18a, miR-93, and miR-429, and low expression of miR-199a and let-7b were significantly associated with poor prognosis (Nam et al., 2008). Also, ectopic expression of let-7f, miR-224, and miR-516a have been shown to decrease kallikrein 10 expression and cell proliferation in ovarian cancer cells (White *et al.*, 2010b).

Other groups have also found differential expression of miRNAs in ovarian cancer when compared to normal ovaries and suggest the usefulness of miRNAs as potential ovarian carcinoma biomarkers (Laios *et al.*, 2008;Zhang *et al.*, 2008).

1.3 Kallikrein-related peptidases

1.3.1 Definition and nomenclature

Kallikrein-related peptidases (KLKs) are a family of secreted serine proteases that were first described for their kininogenase activity and ability to generate the vasoactive peptide bradykinin from kininogens. Werle first described this enzymatic activity in the

pancreas and then named the enzyme responsible for the activity "kallikrein," which originates from the Greek term for pancreas, *kallikreas* (Werle, 1934).

There are two classes of kallikrein-related peptidases in humans; plasma kallikrein and tissue kallikrein. Plasma kallikrein, *KLKB1*, is a single gene located on chromosome 4q35. It is a complex serine protease and has other functional domains other than the proteolytic catalytic domain. Plasma kallikrein is exclusively expressed by liver cells and functions in blood clotting, fibrinolysis (Asakai *et al.*, 1987), the regulation of vascular tone and inflammatory reactions (Bhoola *et al.*, 1992). The remainder of this dissertation will focus on the other class of kallikreins, the tissue kallikrein-related peptidases.

The human kallikrein-related peptidase gene family is a group of 15 serine protease genes located on chromosome 19q13. This is the largest continuous cluster of serine proteases in the human genome and accounts for 32% of all serine proteases in the human genome (Paliouras *et al.*, 2007). All genes in the family are transcribed telomere to centromere with the exception of *KLK2* and *KLK3* that are transcribed centromere to telomere (Yousef *et al.*, 2000b). The gene family spans approximately 300 kb and includes a pseudogene, *ΨKLK1* (Yousef et al., 2004a). As is characteristic of a pseudogene, *ΨKLK1*, is structurally similar to other members in the gene family, but is defective in its function as a serine protease. All possible reading frames encode predicted truncated proteins that lack the aspartate and serine of the catalytic triad.

Until recently, the kallikrein genes were named *KLK1* thorough *KLK15* while the kallikrein proteins were named hK1 through hK15. Because this nomenclature was

created for kallikrein expression in humans and not all kallikrein proteins have been shown to display kininogenase activity, the kallikrein family was re-named based on their homology to tissue kallikreins (Lundwall *et al.*, 2006). There is no name change for kallikrein 1 *(KLK1).* Because *KLK1* displays kininogenase activity, it will continue to be called human kallikrein 1. The other kallikreins have not been shown to display kininogenase activity and therefore calling them kallikreins may be misleading. The rest of the kallikrein family has been re-named kallikrein-related peptidase 2 through kallikrein-related peptidase 15 [*KLK2-15;*(Lundwall *et al.*, 2006)]. In order to distinguish between the gene and the protein, the name of the gene is italicized (eg. *KLK2),* while the protein name is written in standard font (eg. KLK2). The previous and current nomenclature for the KLK family is shown in Table 1.1.

1.3.2 Characteristics

All genes in the human kallikrein-related peptidase family share significant homology at both the gene and protein level. All the human kallikrein-related peptidase genes range in size from approximately 4-10Kb, with most differences attributed to intron length. All genes in the family have five coding exons and most have one or more 5' untranslated exons. Also, the methionine start codon is always in the first exon, 37-88 basepairs from the 3' end of the exon. The stop codon is always between 150 and 156 basepairs from the 5' end of the last exon, with the exception of *KLK13* which is 189 basepairs from the start of the codon. As well, the sizes of the exons are very similar or identical and the intron phases are conserved in all genes. The pattern of the intron phases is I-II-I-0.

Table 1.1 The official and additional gene and protein names for the human kallikrein-related peptidase family.

Official Gene	Official Protein	Other Names/Symbols	References
KLK1	KLK1	Pancreatic/renal kallikrein, hPRK	(Fukushima *et al.*, 1985;Schedlich *et al.*, 1987)
KLK2	KLK2	Human glandular kallikrein 1, hGK-1	(Lovgren *et al.*, 1999b)
KLK3	KLK3	Prostate-specific antigen, PSA, APS	(Lundwall, 1989;Riegman *et al.*, 1988;Riegman *et al.*, 1989;Sutherland *et al.*, 1988)
KLK4	KLK4	Prostase, KLK-L1, EMSP1, PRSS17, ARM1	(Hu *et al.*, 2000;Korkmaz *et al.*, 2001;Nelson *et al.*, 1999;Stephenson *et al.*, 1999;Yousef *et al.*, 1999b)
KLK5	KLK5	KLK-L2, HSCTE	(Brattsand and Egelrud, 1999;Yousef and Diamandis, 1999)
KLK6	KLK6	Zyme, Protease M, Neurosin, PRSS9	(Anisowicz *et al.*, 1996;Little *et al.*, 1997;Yamashiro *et al.*, 1997;Yousef *et al.*, 1999a)
KLK7	KLK7	HSCCE, PRSS6	(Hansson *et al.*, 1994;Yousef *et al.*, 2000e)
KLK8	KLK8	Neuropsin; Ovasin; TADG-14, PRSS19, HNP	(Underwood *et al.*, 1999;Yoshida *et al.*, 1998a)
KLK9	KLK9	KLK-L3 protein	(Yousef and Diamandis, 2000)
KLK10	KLK10	NES1, PSSSL1	(Goyal *et al.*, 1998;Liu *et al.*, 1996a;Luo *et al.*, 1998)
KLK11	KLK11	TLSP/Hippostasin, PRSS20	(Mitsui *et al.*, 2000;Yoshida *et al.*, 1998b;Yousef *et al.*, 2000d)
KLK12	KLK12	KLK-L5 protein	(Yousef *et al.*, 2000c)
KLK13	KLK13	KLK-L4 protein	(Yousef *et al.*, 2000a)
KLK14	KLK14	KLK-L6 protein	(Yousef *et al.*, 2001a)
KLK15	KLK15	Prostinogen, HSRNASPH	(Takayama *et al.*, 2001a;Yousef *et al.*, 2001b)

Among the 15 genes, the relative position of the residues of the catalytic triad unique to serine proteases, histidine, aspartate, and serine, are conserved. The histidine is always located near the 5' end of the second coding exon, while the aspartate is constantly in the middle of the third exon and the serine is near the start of the last exon.

Kallikrein-related peptidases are produced as pre-pro-peptides with a signal peptide of approximately 17-20 amino acids at the amino terminus. The signal peptide allows for secretion of the KLK as an inactive protein. KLKs are activated from the secreted zymogen form to the enzymatically active form by cleavage of the activation peptide which is 4-9 amino acids in length. The mature KLK protein is 223 to 252 amino acids in length (Yousef and Diamandis, 2003). A schematic of the activation of KLKs can be seen in Figure 1.1. Eleven of the kallikrein-related peptidases (KLK1, 2, 4-6, 8, 10-14), have an aspartic acid in their substrate-binding pocket, indicating trypsin-like specificity. The four remaining enzymes, KLK3 (has serine), KLK7 (has asparagine), KLK9 (has glycine), and KLK15 (has glutamic acid), are expected to have chymotrypsin-like or some other specific enzymatic activity. All KLK proteins have 10-12 cysteine residues that form 5, (in the case of KLK1, KLK2, KLK3 and KLK13), or 6 (in all other KLK proteins) disulphide bonds. Finally, classical or variant polyadenylation signals have been found 10-20 bases from the poly-A tail of all kallikrein-related peptidase mRNAs (Borgono *et al.*, 2004;Yousef and Diamandis, 2003;Yousef and Diamandis, 2001;Yousef *et al.*, 2005).

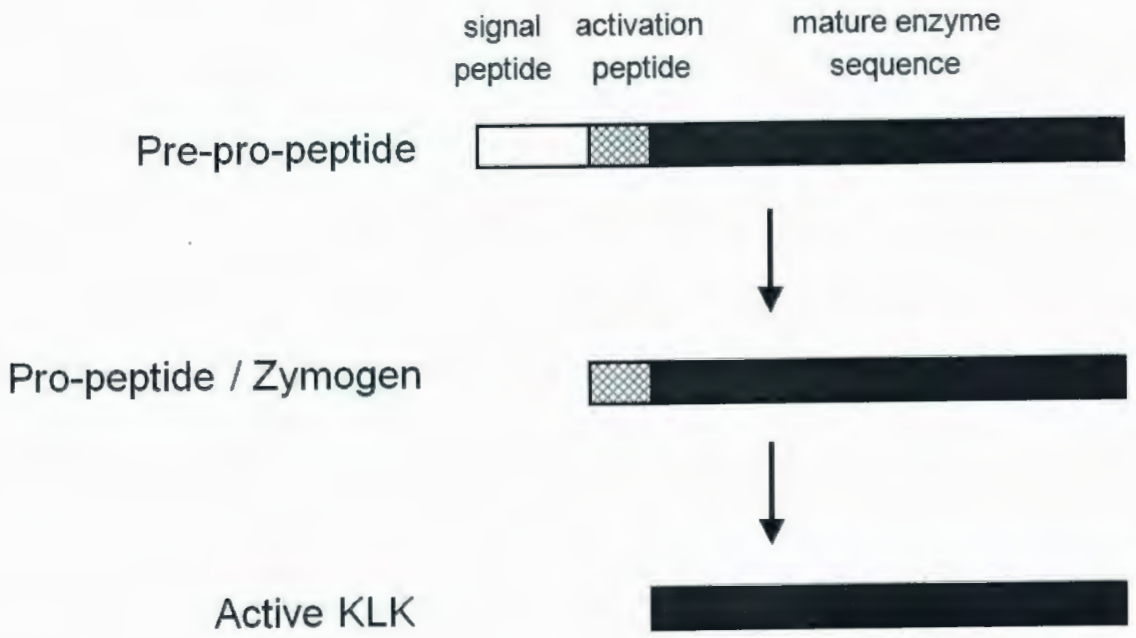


Figure 1.1. A schematic KLK processing from a pre-pro-peptide to an active KLK. KLKs are synthesized as pre-pro-peptides. Cleavage of the signal peptide (white box) allows for secretion outside the cell. The KLK now exists as an inactive, zymogen form. Once the activation peptide (hatched box) is cleaved, the KLK is enzymatically active (black box).

1.3.3 Regulation

1.3.3.1 Transcriptional regulation

Kallikrein-related peptidases are regulated at the transcriptional and post-translational levels. Transcriptional control of many kallikrein-related peptidases has been shown to be responsive to sex-steroid hormones. For example, *KLK2* and *KLK3* are up regulated in response to androgen (Riegman *et al.*, 1991). Androgen response elements (AREs) have been identified in the proximal promoter and enhancer regions of both genes. Likewise, *KLK4* (Myers and Clements, 2001), *KLK5* (Yousef and Diamandis, 1999), and *KLK6* (Yousef *et al.*, 1999a), have been shown to be upregulated by estrogen. Many kallikrein-related peptidases are under sex steroid hormone control and have tissue-specific co-expression, suggesting there is a *cis*-acting locus control region. Locus control regions, as in this case, are operationally defined by their ability to enhance the expression of linked genes in a tissue-specific manner at ectopic chromatin sites (Li *et al.*, 2002).

Although steroid hormone regulation is thought to be the primary regulatory mechanism for kallikreins, alternative splicing may also be an important mechanism. Alternative pre-mRNA, transcriptional start sites and polyadenylation signals are common among members of the kallikrein-related peptidase gene family (Borgono *et al.*, 2004). All kallikrein-related peptidases, with the exception of *KLK14*, have at least one alternative transcript (Kurlender *et al.*, 2005). Many of these variants encode for truncated proteins that have not been proven to be functional. *KLK13* has the most alternative transcripts

among the gene family with eight splice variants. Interestingly, five *KLK13* splice variants were found to be expressed exclusively in the normal, but not in cancerous testis (Chang *et al.*, 2001). The physiological relevance of this differential expression is not yet known. Alternative splicing has been implicated in many physiological processes and 15% of mutations in the mammalian genome that cause disease are associated with problems in RNA splicing signals (Krawczak *et al.*, 1992). Alternative splicing among kallikrein-related peptidases may prove to be important in other cancers as this phenomenon is studied further.

KLK expression has also been shown to be regulated epigenetically by DNA methylation. In particular, *KLK10* has been shown to be down regulated due to CpG hypermethylation in breast, prostate, ovarian, and gastric cancer (Huang *et al.*, 2007;Li *et al.*, 2001;Sidiropoulos *et al.*, 2005). More recently, *KLK6* expression has been proven to be under transcriptional control by DNA methylation as protein expression was restored in breast cancer cells when treated with the DNA demethylating agent 5-aza-2'-deoxycytidine (Lundwall and Brattsand, 2008).

1.3.3.2 Post-translation regulation

Since protease digestion is irreversible, kallikrein-related peptidases are tightly regulated post-translationally. There are several post-translational mechanisms controlling KLK activity including zymogen activation, endogenous inhibition, inactivation through internal cleavage, and allosteric regulation. KLKs are all secreted as zymogens (pro-enzymes), or inactive enzymes. They require cleavage at a specific site to be activated

either through the action of another KLK, or another protease. Zymogens are then activated by cleavage of the 4-9 amino acid propeptide. This is thought to induce a conformational change in both the active site and substrate-specificity pocket, rendering the KLK active (Borgono and Diamandis, 2004).

Once activated, KLK activity is controlled by endogenous inhibitors including serpins and α_2-macrogloblin (α_2M), as many KLKs form complexes with these plasma proteins. Serpins are a superfamily of serine protease inhibitors that fold into a conserved structure and employ a unique substrate-like inhibitory mechanism (Silverman *et al.*, 2001). KLKs interact with serpins through one of two pathways; the inhibitory pathway, or the substrate pathway. In the inhibitory pathway, KLKs form a covalent complex with the serpin, resulting in the deformation and irreversible inactivation of the protease, as has been shown with KLK3 and α_1-antichymotrypsin [αACT, (Christensson *et al.*, 1990)], and KLK5 with α_2-antiplasmin (α_2AP) and α_1-antithrombin [ATIII, (Michael *et al.*, 2005)]. Alternatively, KLKs are inhibited through the substrate pathway in which the serpin is cleaved by the protease, but does not leave the binding pocket, leaving the KLK enzymatically active, but unavailable. This regulatory mechanism is seen with KLK6 and antithrombin III or α_2-antiplasmin (Magklara *et al.*, 2003), as well as KLK2 and α_2M (Heeb and Espana, 1998), but this same interaction with KLK5 only partially inhibits activity (Michael *et al.*, 2005). In general, KLKs bind to an exposed peptide in the "bait" region of α_2M and cleave it, resulting in a conformational change and non-covalent complex which prevents the KLK from interacting with other substrates (Borgono and Diamandis, 2004).

KLKs can also become inactivated through internal cleavage, followed by degradation that may be autolytic or initiated by another protease. Interestingly, there is evidence that both KLK6 and KLK13 can be autolytic (Magklara *et al.*, 2003;Sotiropoulou *et al.*, 2003). Although the enzyme responsible for the inactivation of KLK3 is unknown, fragmented forms of the protein found in prostate tissue have determined KLK3 has a major cleavage site between K^{145}-K^{146} and two minor cleavage sites at R^{85}-F^{86} and K^{182}-S^{183} (Borgono *et al.*, 2004).

Allosteric regulation has also been noted to inhibit KLK activity. Zinc has been shown to reversibly inhibit the activity of both KLK2 and KLK3 in the prostate (Lovgren *et al.*, 1999a;Malm *et al.*, 2000). Interestingly, the prostate has high levels of zinc, while in the seminal plasma, where KLK2 and KLK3 are known to be actively involved in seminal liquefaction, zinc levels are markedly lower (Kavanagh, 1985).

1.3.4 Normal physiological kallikrein-related peptidase cascades

1.3.4.1 Semen liquefaction

Human semen is a mixture of secretions from the seminal vesicle, accounting for almost two thirds of the total ejaculated volume, while the remaining third comes from the prostate, spermatozoa and epididymal fluid [5%; (Lundwall and Brattsand, 2008)]. Seminogelin I (SgI) and semenogelin II (SgII) represent the major proteins secreted by the seminal vesicles. After ejaculation, SgI and SgII aggregate with fibronectin to form a gelatinous mass trapping the spermatozoa within the vaginal canal. In 5-20 minutes, the

mass is liquefied, resulting from SgI and SgII degradation, releasing trapped spermatozoa (Pampalakis and Sotiropoulou, 2007). Liquefaction is a proteolytic cascade that is highly regulated. Before ejaculation, the prostate fluid contains several KLKs (Yousef and Diamandis, 2001) and a high concentration of zinc (Zn^{2+}) ions. These KLKs, including KLK2 (Lovgren *et al.*, 1999a), KLK3 (Malm *et al.*, 2000), KLK4 (Debela *et al.*, 2006), KLK5 (Michael *et al.*, 2006), KLK8 (Kishi *et al.*, 2006), KLK12 (Memari *et al.*, 2007), and KLK14 (Borgono *et al.*, 2007), are allosterically inhibited by reversible binding of Zn^{2+}. After ejaculation, the Zn^{2+} is redistributed to the Sgs and KLKs are reactivated, initiating a proteolytic cascade resulting in semen liquefaction.

A major player in the semen liquefaction process is kallikrein-related peptidase 3 (KLK3), also known as prostate specific antigen (PSA), and is mainly responsible for the degradation of Sgs (Robert *et al.*, 1997). There are a number of KLKs capable of activating pro-KLK3 to the active KLK3, including KLK2 (Lovgren *et al.*, 1997), KLK4 (Takayama *et al.*, 2001b), KLK5 (Michael *et al.*, 2006), KLK15 (Takayama *et al.*, 2001a), and more recently, KLK14 (Emami *et al.*, 2008) has also been identified. KLK5 plays a major role in semen liquefaction as it can not only activate KLK3, but also autoactivates and can degrade SgI, SgII, and fibronectin (Brattsand *et al.*, 2005).

Semen liquefaction is also regulated by internal cleavage of the participant proteases resulting in the complete inhibition of their enzymatic activity (Pampalakis and Sotiropoulou, 2007). KLKs are inactivated by autolysis, in the case of KLK5 and KLK14 (Borgono *et al.*, 2007), or by other KLKs. For example, KLK5 can cleave and

inactivate both KLK3 and KLK2 (Michael *et al.*, 2006). Interestingly, KLK5 has been the only protease identified to inactivate KLK3 by cleavage in semen. The biological roles of KLK8, KLK11, and KLK12 in seminal liquefaction are still under investigation.

1.3.4.2 Skin desquamation

Skin desquamation is the process whereby the outermost corneocytes are shed from the epidermal surface as a result of proteolytic degradation of corneodesmosomes by epidermal proteases (Kishibe *et al.*, 2007). Corneodesmosomes are cellular junctions located within the stratum corneum that provide strong inter-corneocyte cohesion (Chapman *et al.*, 1991). Several stratum corneum proteases are involved in desquamation as they provide proteolytic cleavage of the extracellular part of these cell-cell adhesive structures (Zeeuwen, 2004). Three proteins identified as components of the extracellular part of the corneodesmosomes are two desmosomal cadherins, desmoglein 1 (DSG1) and desmocolin 1 (DSC1), which associate in a calcium dependant manner, and corneodesmosin (CDSN), a glycoprotein secreted by granular keratinocytes, then incorporated into desmosomes (Serre *et al.*, 1991). KLK5, previously known as stratum corneum tryptic enzyme, has been shown to degrade DSG1, DSC1, and CDSN. KLK7, previously known as human corneum chymotryptic enzyme, has also been shown to be able to cleave DSC1 and CDSN (Caubet *et al.*, 2004). More recently, KLK14, expression in the skin has been identified and is also thought to participate in the skin desquamation proteolytic cascade with KLK5 and KLK7 (Brattsand *et al.*, 2005).

The proteolytic cascade begins when pro-KLK5 is autoactivated. This in turn activates both pro-KLK7 and pro-KLK14 (Brattsand *et al.*, 2005). Active KLK14 can further activate pro-KLK5, increasing its total enzymatic activity. Active KLK5 and KLK7 can cleave components of the extracellular domain of corneodesmosomes. KLK8 has also been recently implicated in skin desquamation as hyperkeratosis was observed in a KLK8(-/-) mouse, suggesting a role for KLK8 in skin shedding (Kishibe *et al.*, 2007).

1.3.5 Kallikrein-related peptidases in cancer

Emerging evidence indicates kallikrein-related peptidases are involved in a number of different cancers. All 15 KLKs have been reported to have dysregulated expression at the mRNA and/or protein levels in a number of cancers including ovarian, prostate, breast, testicular, and lung (Chang *et al.*, 2001;Petraki *et al.*, 2003a;Planque *et al.*, 2005;Yousef *et al.*, 2003d;Yousef *et al.*, 2002c;Yousef *et al.*, 2003f). There is increasing evidence these KLKs may serve as potential biomarkers for these cancers.

1.3.5.1 Prostate cancer

Prostate cancer is the most frequently occurring cancer in American males, with a lifetime risk of one in six (Jemal *et al.*, 2005). KLK3, also known as prostate specific antigen (PSA), is currently the most widely used marker for prostate cancer diagnosis and disease monitoring. PSA is a major protein in seminal fluid and its physiological substrates are SgI and SgII (Lilja *et al.*, 1987). PSA levels are high in the circulation of early prostate cancer patients due to a characteristic disruption of the basal cell layer

(Bostwick, 1994). This loss of normal glandular architecture allows PSA direct access to the peripheral circulation. Although it is the best screening tool used today, the PSA test is not without problems, particularly with regard to its sensitivity and specificity (Abrahamsson *et al.*, 1997). Because PSA is expressed in normal, cancerous and hyperplastic tissue, it suffers from poor specificity in discriminating cancer from benign prostate hyperplasia, which also results in increased release of PSA. The specificity of PSA is most problematic in the 2-15ng/mL range resulting in a negative biopsy rate of 70-80% (Hugosson *et al.*, 2004). There have been many different diagnostic parameters explored with regards to prostate cancer screening including age specific PSA, PSA velocity, volume adjusted PSA density, free-to-total PSA ratio, αACT bound PSA, α_2-M bound PSA, α_1-protease inhibitor bound PSA and KLK2 (Karazanashvili and Abrahamsson, 2003). KLK2 can also cleave SgI, SgII, and fibronectin (Deperthes *et al.*, 1996) and has been shown to cleave pro-PSA to generate enzymatically active PSA, suggesting KLK2 may play a physiological role in the biological activity of PSA (Takayama *et al.*, 1997). In addition to PSA, KLK2 appears able to discriminate between benign and malignant prostate disease, as well as organ-confined disease (Haese *et al.*, 2001).

More recently, KLK4 has been related to the progression of prostate cancer. When KLK2, KLK3, and KLK4 were separately transfected into the PC-3 prostate cancer cell line, KLK3 and KLK4, but not KLK2, induced a 2.4 fold and 1.7 fold, respectively, increase in cellular migration, but not invasion, through Matrigel, a synthetic extracellular matrix (Veveris-Lowe *et al.*, 2005). When expression levels of E-cadherin and vimentin

were examined, E-cadherin protein was lost and mRNA levels were significantly decreased in the PC-3 cells expressing KLK4 and KLK3, while the expression of vimentin was increased in these same cells. The loss of E-cadherin and the increase of vimentin are indicative of epithelial to mesenchymal transition, a crucial event in the progression of cancer to an invasive phenotype.

1.3.5.2 Other cancers

Besides ovarian and prostate cancer, dysregulated kallikrein-related peptidase expression has been noted in other steroid hormone related cancers including breast and testicular cancer. More recently, differential expression of KLKs has been detected in steroid hormone-independent cancers such as lung cancer.

Interestingly, while most KLKs show increased expression in ovarian cancer, many reports indicate KLKs are downregulated in breast cancer. KLK3 (Yu *et al.*, 1996), KLK5 (Yousef *et al.*, 2004b), KLK6 (Yousef *et al.*, 2004b), KLK8 (Yousef *et al.*, 2004b), KLK10 (Liu *et al.*, 1996b), KLK12 (Yousef *et al.*, 2000c), KLK13 (Yousef *et al.*, 2000a), and KLK14 (Yousef *et al.*, 2001a), have all been reported to have decreased expression in malignant breast tumors when compared to normal counterparts. *KLK12* was found to be downregulated in breast cancer tissues when compared to normal breast tissue by quantitative real time polymerase chain reaction (qRT-PCR) (Yousef *et al.*, 2000c). KLK5 expression in breast cancer has also been explored. One study examined *KLK5* expression by quantitative RT-PCR and found that high *KLK5* expression is associated with node-positive and estrogen receptor-negative breast cancer patients

(Yousef *et al.*, 2002b). Also, high *KLK5* expression is associated with a shorter RFS and OS in the subgroup of patients with grade I and II tumors.

A few KLKs have been shown to be associated with testicular cancer, including KLK5, KLK10, and KLK13. In normal tissue, KLK5 shows highest expression in the mammary gland and testis, but is decreased when compared to testicular cancers (Yousef *et al.*, 2002a). This study found low KLK5 expression associated with seminoma tumor type, and late stage (II/III) cancer. When 14 normal testis were compared to cancerous counterparts, KLK10 expression was also found to be decreased (Luo *et al.*, 2001c). Interestingly, one group discovered five new KLK13 alternative transcripts expressed exclusively in the testis, with all being decreased in testicular cancer (Chang *et al.*, 2001).

Recently, KLK expression has been examined in lung cancer. When analyzed by qRT-PCR, *KLK5* expression is significantly higher in squamous cell carcinomas, while *KLK7* expression was decreased in lung adenocarcinomas, when compared to normal tissue (Planque *et al.*, 2005). When KLK5, KLK6, KLK7, and KLK8 protein expression was examined in lung cancers by immunohistochemistry, 40-90% of malignant cells showed positive cytoplasmic labelling in squamous cell carcinoma, small cell carcinoma, and carcinoid tumors (Singh *et al.*, 2008). Interestingly, although the lung adenocarcinoma samples lacked cytoplasmic staining, 20% of the tumor nuclei were positive for KLK5, KLK7, and KLK8.

1.3.5.3 Ovarian cancer

Interestingly, 12 of the 15 kallikrein-related peptidases, specifically KLK2, KLK3 (PSA), KLK4, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, KLK14, and KLK15, have increased expression of mRNA and/or protein in ovarian cancer (Borgono *et al.*, 2004). Although KLK2 and KLK3 have been shown to be increased at the mRNA level in ovarian cancer by microarray (Adib *et al.*, 2004), the clinical relevance has yet to be examined. Increased expression of several kallikrein-related peptidases has been associated with unfavourable patient prognosis. Although detection of KLK5 in normal serum is quite low, higher concentrations were found in 69% of ovarian cancer patients (Yousef *et al.*, 2003c). High KLK5 expression has also been associated with more aggressive forms of ovarian cancer as there is a strong correlation between high KLK5 expression and late stage (Stage III/IV) and Grade 3 tumors (Diamandis *et al.*, 2003). Also, patients who were classified as having high KLK5 expression have a significantly shorter regression free survival (RFS) and overall survival (OS) (Diamandis *et al.*, 2003). Increased KLK6 expression is also associated with a poor patient prognosis and has drawn attention for its potential as a new biomarker for ovarian cancer (Diamandis *et al.*, 2000b;Hoffman *et al.*, 2002). In a recent study, increased KLK6 expression was associated with an increased risk of recurrence in ovarian cancer patients [Chapter 3, (White *et al.*, 2009)]. KLK7 has been shown to be increased in ovarian cancer patients at the mRNA level and is associated with advanced stage disease, high tumor grade, suboptimal debulking, and serous type tumor (Yousef *et al.*, 2003a). High KLK7 serum

protein levels, as assessed by enzyme-linked immunoabsorbent assay (ELISA), significantly predicted a shorter RFS and OS (Shan *et al.*, 2006).

The expression of KLK10 has also been assessed in ovarian cancer. Ovarian cancer patients had elevated KLK10 serum concentrations (Luo *et al.*, 2001a) and mRNA levels (Shvartsman *et al.*, 2003) when compared to normal counterparts. High KLK10 expression was significantly associated with advanced stage disease, serous type cancer, suboptimal debulking, and a large residual tumor (Luo *et al.*, 2001b). When the subgroup of ovarian cancer patients with Stage III and IV disease was examined, patients with high KLK10 expression were more likely to have a shorter RFS and OS than patients with low KLK10 expression (Luo *et al.*, 2003;Luo *et al.*, 2001b). KLK15 has also been shown to be an independent predictor of poor prognosis in ovarian cancer patients (Yousef *et al.*, 2003e). When 168 tumors were assessed by quantitative RT-PCR, KLK15 overexpression was significantly associated with a decreased RFS and OS. The overexpression of so many kallikrein-related peptidases and the fact they are predictors of poor prognosis in ovarian cancer patients, suggests they play a role in some enzymatic cascade in which they may contribute to ovarian cancer progression.

Conversely, increased expression of some kallikrein-related peptidases predicts a favourable prognosis for ovarian cancer patients. For example, KLK8 has been shown to be overexpressed at both the gene (Underwood *et al.*, 1999) and protein (Borgono *et al.*, 2006) levels in some ovarian cancer patients. Interestingly, high KLK8 expression has been associated with early clinical stage (Shigemasa *et al.*, 2004), and a longer RFS and

OS, than patients with KLK8 negative tumors (Borgono *et al.*, 2006). Also, KLK11 has been shown to be elevated in serum of 70% of ovarian cancer patients when compared to normal serum (Diamandis *et al.*, 2002). High KLK11 expression is associated with early stage (Stage I/II) disease (Borgono *et al.*, 2003a;Diamandis *et al.*, 2002). When the prognostic significance of KLK11 expression in ovarian cancer was assessed, patients with KLK11 positive tumors have a significantly decreased risk of relapse and death, and a longer RFS and OS (Diamandis *et al.*, 2002). These results are not surprising as early stage ovarian cancer is associated with good patient prognosis. KLK14 has also been shown to be a new potential marker for ovarian cancer (Borgono *et al.*, 2003b). Elevated KLK14 levels were found in 40% of ovarian cancer tissues and in the serum of 65% ovarian cancer patients when compared to normal counterparts.

There are also conflicting reports concerning KLK expression in ovarian cancer. For example, Scorilas et al. showed that KLK13 positive ovarian cancer tumors were most often associated with early stage disease, no residual tumor after surgery and optimal debulking (Scorilas *et al.*, 2004). They also concluded that patients with KLK13 positive tumors had a longer RFS and OS than patients with KLK13 negative tumors. Contrary to this report, White et al. and Chapter 3 of this dissertation, recently showed high KLK13 expression was associated with a significant increased risk of recurrence in ovarian cancer patients (White *et al.*, 2009). Contradiction of these reports may lie in the design of the study. Scorilas et al. studied protein expression in ovarian tumor cytosols and found high KLK13 expression was associated with early stage disease, no residual tumor after surgery and optimal debulking success which are all indicators of good prognosis.

White et al. studied mRNA expression in ovarian tumors and found that high KLK13 expression was associated with invasive ovarian cancer.

Although the expression of some kallikrein-related peptidases indicates a poor prognosis and others tend to point toward a favourable prognosis, the essential characteristic is that they are all overexpressed and may be functioning in some pathway in a variety of potential combinations to contribute to the pathogenesis of ovarian cancer. If this is the case, the discovery of regulatory mechanisms controlling the proposed kallikrein-related peptidase enzymatic cascade pathway and any other key players may lead to new therapeutic treatments for ovarian cancer patients.

The work of this thesis focuses mainly on KLK6 and KLK13. The final sections of this introduction chapter will describe these KLKs in detail.

1.3.6 Kallikrein-related peptidase 6

Kallikrein-related peptidase 6 (*KLK6)* was originally identified by its downregulation in metastatic breast and ovarian tumors when compared to corresponding primary tumors (Anisowicz *et al.*, 1996). The full-length cDNA encoding a 244 amino acid protein was named protease M. Two other groups discovered the same gene independently. Little et al. used polymerase chain reaction (PCR) of Alzheimer disease brain tissue to clone the gene and named it zyme (Little *et al.*, 1997), while Yamashiro et al. cloned cDNA prepared from a colon adenocarcinoma cell line, calling it neurosin (Yamashiro *et al.*, 1997). With the discovery of the extended kallikrein family, protease M/zyme/neurosin

was renamed KLK6. KLK6 is a trypsin-like serine protease with high levels of expression in normal breast, kidney, spinal cord, and brain (Yousef *et al.*, 1999a). Sandwich-type enzyme-linked immunosorbent assay (ELISA) assays have also confirmed KLK6 expression in biological fluids including milk, nipple aspirate fluid, normal male and female serum, cerebrospinal fluid, and aminotic fluid (Diamandis *et al.*, 2000a).

KLK6 has been found to be expressed mainly as a proenzyme in milk and cerebrospinal fluid, while it has been recovered in two forms in milk and ascites; a free form with a molecular mass of ~25KDa and another form with a higher molecular mass in which KLK6 is bound to the serine protease inhibitor αACT (Hutchinson *et al.*, 2003). The physiological mechanism of activation of pro-KLK6 to active KLK6 is still in unknown. Magklara et al. first suggested KLK6 was able to both autoactivate and autoinactivate itself while enzymatic activity can be inhibited by serpins, such as ATIII (Magklara *et al.*, 2003). Bayés et al. later created site-directed mutants of KLK6 and suggested autoactivation occurs through a two-step mechanism. Initially is a proteolytic cut after Q^{19}, however, the enzyme is not activated until the protein is cleaved a second time at K^{21}(Bayes *et al.*, 2004). Recent publications have suggested that it is unlikely KLK6 can autoactivate. Angelo et al. found no evidence of the KLK6 maturation site, K^{21} being hydrolyzed (Angelo *et al.*, 2006). Also, Blaber et al. suggested the KLK6 autolytic mechanism is a negative feedback inhibition as the protein can cleave internally resulting in protein inactivation (Blaber *et al.*, 2007). KLK6 can autoinactivate *in vitro* by autolysis between residues R^{76}-R^{77} (Magklara *et al.*, 2003). The ability of KLK6 to

autoactivate is improbable. Activation of pro-KLK6 requires hydrolysis after a lysine residue and the fact that mature KLK6 exhibits two orders of magnitude reduced affinity for hydrolysis after a lysine versus an arginine, makes autoactivation of KLK6 an unlikely event. The activation of pro-KLK6 is likely accomplished through the action of a separate protease (Blaber *et al.*, 2007). In order to examine regulation of KLK6, Shan et al. examined *KLK6* transcript expression and found it in concordance with KLK6 protein expression, suggesting KLK6 protein levels are under transcriptional control (Shan *et al.*, 2007).

KLK6 has increased expression in ovarian cancer at both the gene and protein levels. KLK6 is increased in the serum of ovarian cancer patients when compared to normal patients and has been identified as a potential biomarker for ovarian cancer (Diamandis *et al.*, 2000c;Ni *et al.*, 2004). KLK6 protein levels were elevated in ascites fluid of ovarian cancer patients (Luo *et al.*, 2006), while mRNA (*KLK6)* has been found to be overexpressed in ovarian cancer tumors (Tanimoto *et al.*, 2001). Hoffman et al. found tumor KLK6 was significantly associated with late stage disease, serous type ovarian cancer, residual tumor > 1cm, and suboptimal debulking (Hoffman *et al.*, 2002). This study also analyzed survival data and concluded that KLK6 is an unfavourable prognostic marker in ovarian cancer.

In order to elucidate the role of KLK6 in ovarian cancer, its function in proliferation, migration, and invasion, has been examined. KLK6 can degrade components of the extracellular matrix, including fibronectin, fibrinogen, laminin, vitronectin, and collagen

(Ghosh *et al.*, 2004;Magklara *et al.*, 2003). Also, the use of a neutralizing anti-KLK6 antibody decreased migration of ovarian cancer cells in a Boyden chamber assay (Ghosh *et al.*, 2004). Together, these data highly suggest KLK6 is involved in the migration and/or invasion of cancer cells and may offer potential future therapeutic target.

KLK6 has been reported as being overexpressed in uterine serous papillary cancer (Santin *et al.*, 2005) and lung carcinoma (Singh *et al.*, 2008). Elevated *KLK6* expression was significantly associated with lymphatic invasion and a poor survival rate in a set of human gastric cancer patients (Nagahara *et al.*, 2005). In colorectal cancer, *KLK6* is overexpressed when compared to normal tissue and is significantly associated with serosal invasion, liver metastasis, advanced stage, and poor prognosis (Ogawa *et al.*, 2005). Interestingly, KLK6 is down regulated in salivary gland tumors when compared to normal glands (Darling *et al.*, 2006). Overall, KLK6 has been shown to be differentially expressed in a number of different cancers and its role as a potential biomarker for these malignancies warrants further investigation. *KLK6* is also presently being investigated for its role in Alzheimer's disease (Yousef *et al.*, 2003b).

1.3.7 Kallikrein-related peptidase 13

Like *KLK6*, the normal function of kallikrein-related peptidase 13 (*KLK13*) remains unknown. Kallikrein-related peptidase 13 (*KLK13*) was cloned using the positional candidate gene approach and was found down regulated in breast cancer cell lines and tissues when compared to normal counterparts (Yousef *et al.*, 2000a). KLK13 is a glycosylated, secreted serine protease with trypsin-like activity.

Although the normal function of KLK13 is unknown, extensive studies regarding the normal pattern of expression and regulation have been carried out. KLK13 has highest expression in normal tissue extracts of the esophagus and tonsil, followed by salivary gland, prostate, kidney, skin, trachea, testis, breast, lung, and thyroid (Kapadia *et al.*, 2003). Protein expression was also assessed by immunohistochemistry, and similar to KLK6, KLK13 was expressed in a variety of tissues, mainly the glandular epithelial (Petraki *et al.*, 2003b). KLK13 had intense immunohistochemical expression in the thyroid gland and the pancreatic endocrine cells. An ELISA detected KLK13 in the seminal plasma, amniotic fluid, breast milk, and follicular fluid, but levels were below the detectable limit in both healthy male and female serum (Kapadia *et al.*, 2003). Low detection levels result from the interaction between KLK13 and serum protease inhibitors, including α_2M, αACT, and α_2AP (Kapadia *et al.*, 2004b). *KLK13* expression is up regulated by progestins and androgens and to a lesser extent by estrogens in the hormonally regulated breast cancer cell line BT-474 (Yousef *et al.*, 2000a).

KLK13 has been shown to have considerable posttranslational glycosylation (Sotiropoulou *et al.*, 2003). Sotiropoulou et al. produced recombinant KLK13 in *Pichis pastoris* yeast expression system and when analyzed by denaturing SDS-PAGE gel, it was detected at a higher molecular mass than what is expected from the primary sequence [28.8 kDa, (Sotiropoulou *et al.*, 2003)]. In order to determine if the increase in molecular mass was due to glycosylation, the recombinant KLK13 was incubated with

peptide: N-glycosidase F (PNGaseF) resulting in a band on the gel at the expected molecular mass predicted from the primary sequence (Sotiropoulou *et al.*, 2003). PNGaseF is an amidase that removes N-linked oligosaccharides from glycoproteins by cleaving the *N*-glycosidic bond between Asn and the first hexosamine. The resulting decrease in apparent molecular mass confirmed KLK13 was *N*-linked glycosylated in this organism. Kapadia et al. found similar results as they also saw a decrease in molecular mass when native KLK13 was treated with PNGaseF (Kapadia *et al.*, 2003). Although the function of KLK13 glycosylation is unknown, like some other proteins, its level of glycosylation may be altered in cancers. The function of KLK13 glycosylation warrants further investigation.

Pre-pro-KLK13 consists of 277 amino acids containing a predicted signal peptide, M^{1}-S^{16}, and an activation peptide of 5 amino acids, Q^{21}-K^{25} (Yousef *et al.*, 2000a). There is a predicted cleavage site between K^{25}-V^{26} which was confirmed by Sotiropoulou et al. (Sotiropoulou *et al.*, 2003) This group also purified KLK13 and detected another peptide of KLK13 displaying an internal fragment verified by N-terminal sequencing to be S^{115}-Q^{277}, suggesting KLK13 is able to autoinactivate *in vitro* between residues R^{114}-S^{115} (Sotiropoulou *et al.*, 2003). This process is thought to be autoinactivation because none of the resulting fragments retain all residues of the catalytic traid.

Recently, the use of *KLK13* as a potential ovarian cancer biomarker has been evaluated. KLK13 is expressed at low to undetectable levels in normal ovaries, but has been shown to have increased expression in 50% of malignant ovaries when compared to normal

counterparts (Kapadia *et al.*, 2003). My recent study found KLK13 is a marker of poor prognosis for ovarian cancer (White *et al.*, 2009). Patients with high KLK13 expression were significantly predicted to have a shorter RFS than patient with low KLK13 expressing tumors. Furthermore, the potential function of KLK13 in tumor progression and metastasis has been examined. KLK13 can degrade major components of the extracellular matrix (ECM) including collagen, fibronectin, and laminin (Kapadia *et al.*, 2004a). Also, cells secreting KLK13 treated with a KLK13 neutralizing antibody migrated less than untreated cells. These data suggest KLK13 may function in tissue remodelling and/or tumor invasion and metastasis.

1.4 Summary

Ovarian cancer is the most lethal of all the gynaecological malignancies. Approximately 90% of ovarian cancers diagnosed are of the epithelial type. There is currently no biomarker for the diagnosis of ovarian cancer. The lack of signs and symptoms associated with the malignancy in addition to the anatomical location, result in late diagnosis in which patients have a poor survival rate. Recently, a family of serine proteases, the human kallikrein-related peptidase (KLK) family, have been implicated in the pathogenesis of ovarian cancer and are currently being explored as potential biomarkers for this malignancy. Many KLKs have shown to have increased expression in ovarian cancer and have shown to be prognostic marker for this malignancy. Also, some KLKs have been shown to be involved in cellular processes that contribute to cancer. In particular, KLK6 and KLK13 have been shown to be able to degrade

components of the extracellular matrix and play a role in ovarian cancer cell migration. This thesis examines the role of KLK13 in ovarian cancer cell migration and invasion and investigates the clinical utility of KLK6 and KLK13 as diagnostic and prognostic makers for ovarian cancer patients.

1.5 Hypotheses

Given that (1) KLKs have been shown to be overexpressed in ovarian cancer; (2) KLK6 and KLK13 have been shown to be involved in extracellular matrix degradation and cellular migration; (3) KLKs have been shown to have prognostic value for ovarian cancer patients: I hypothesize that KLK6 and KLK13 are involved in ovarian cancer pathogenesis and may be potential diagnostic and prognostic markers for ovarian cancer patients.

The specific hypotheses examined in this thesis include:

1. KLK13 plays a role in cellular migration and invasion in ovarian cancer cells
2. KLK6 and KLK13 can be used as prognostic markers for ovarian cancer patients
3. A combination of markers including KLK6, KLK13 and CA125, is a more sensitive test for ovarian cancer than one marker alone

1.6 References

Aarnio,M., Sankila,R., Pukkala,E., Salovaara,R., Aaltonen,L.A., de la Chapelle,A., Peltomaki,P., Mecklin,J.P., and Jarvinen,H.J. (1999). Cancer risk in mutation carriers of DNA-mismatch-repair genes. Int. J. Cancer. *81*, 214-218.

Abrahamsson,P.A., Lilja,H., and Oesterling,J.E. (1997). Molecular forms of serum prostate-specific antigen. The clinical value of percent free prostate-specific antigen. Urol. Clin. North Am. *24*, 353-365.

Adib,T.R., Henderson,S., Perrett,C., Hewitt,D., Bourmpoulia,D., Ledermann,J., and Boshoff,C. (2004). Predicting biomarkers for ovarian cancer using gene-expression microarrays. Br. J. Cancer *90*, 686-692.

Ambros,V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell *113*, 673-676.

Ang,C., Chambers,A.F., Tuck,A.B., Winquist,E., and Izawa,J.I. (2005). Plasma osteopontin levels are predictive of disease stage in patients with transitional cell carcinoma of the bladder. BJU. Int. *96*, 803-805.

Angelo,P.F., Lima,A.R., Alves,F.M., Blaber,S.I., Scarisbrick,I.A., Blaber,M., Juliano,L., and Juliano,M.A. (2006). Substrate specificity of human kallikrein 6: salt and glycosaminoglycan activation effects. J. Biol. Chem. *281*, 3116-3126.

Anisowicz,A., Sotiropoulou,G., Stenman,G., Mok,S.C., and Sager,R. (1996). A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med. *2*, 624-636.

Antoniou,A.C., Gayther,S.A., Stratton,J.F., Ponder,B.A.J., and Easton,D.F. (2000). Risk models for familial ovarian and breast cancer. Genet. Epidemiol. *18*, 173-190.

Asakai,R., Davie,E.W., and Chung,D.W. (1987). Organization of the gene for human factor XI. Biochemistry *26*, 7221-7228.

Auersperg,N., Siemens,C.H., and Myrdal,S.E. (1984). Human ovarian surface epithelium in primary culture. In Vitro *20*, 743-755.

Auersperg,N., Wong,A.S., Choi,K.C., Kang,S.K., and Leung,P.C. (2001). Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr. Rev. *22*, 255-288.

Bai,W., Oliveros-Saunders,B., Wang,Q., Acevedo-Duncan,M.E., and Nicosia,S.V. (2000). Estrogen stimulation of ovarian surface epithelial cell proliferation. In Vitro Cell Dev. Biol. Anim. *36*, 657-666.

Bal,A., Mohan,H., Singh,S.B., and Sehgal,A. (2007). Malignant transformation in mature cystic teratoma of the ovary: report of five cases and review of the literature. Arch. Gynecol. Obstet. *275*, 179-182.

Barua,A., Abramowicz,J.S., Bahr,J.M., Bitterman,P., Dirks,A., Holub,K.A., Sheiner,E., Bradaric,M.J., Edassery,S.L., and Luborsky,J.L. (2007). Detection of ovarian tumors in chicken by sonography: a step toward early diagnosis in humans? J. Ultrasound Med. *26*, 909-919.

Bast,R.C., Jr., Feeney,M., Lazarus,H., Nadler,L.M., Colvin,R.B., and Knapp,R.C. (1981). Reactivity of a monoclonal antibody with human ovarian carcinoma. J. Clin. Invest *68*, 1331-1337.

Bast,R.C., Jr., Klug,T.L., Schaetzl,E., Lavin,P., Niloff,J.M., Greber,T.F., Zurawski,V.R., Jr., and Knapp,R.C. (1984). Monitoring human ovarian carcinoma with a combination of CA 125, CA 19-9, and carcinoembryonic antigen. Am. J. Obstet. Gynecol. *149*, 553-559.

Bast,R.C., Jr., Xu,F.J., Yu,Y.H., Barnhill,S., Zhang,Z., and Mills,G.B. (1998a). CA 125: the past and the future. Int. J. Biol. Markers *13*, 179-187.

Bast,R., Jr., Xu,F., Yu,Y., Fang XJ, Wiener J, and Mills,G. (1998b). Overview, the Molecular Biology of Ovarian Cancer. In: Ovarian Cancer 5, ed. Sharp F, Bakett T, Berek J, and Bast R. Oxford, UK: Isis Medical Media Publishers, 87-97.

Bayes,A., Tsetsenis,T., Ventura,S., Vendrell,J., Aviles,F.X., and Sotiropoulou,G. (2004). Human kallikrein 6 activity is regulated via an autoproteolytic mechanism of activation/inactivation. Biol. Chem. *385*, 517-524.

Beiner,M.E., Rosen,B., Fyles,A., Harley,I., Pal,T., Siminovitch,K., Zhang,S., Sun,P., and Narod,S.A. (2006). Endometrial cancer risk is associated with variants of the mismatch repair genes MLH1 and MSH2. Cancer Epidemiol. Biomarkers Prev. *15*, 1636-1640.

Bell,D.A. (2005). Origins and molecular pathology of ovarian cancer. Mod. Pathol. *18*, S19-S32.

Bell,K.A. and Kurman,R.J. (2000). A clinicopathologic analysis of atypical proliferative (borderline) tumors and well-differentiated endometrioid adenocarcinomas of the ovary. Am. J. Surg. Pathol. *24*, 1465-1479.

Bhoola,K.D., Figueroa,C.D., and Worthy,K. (1992). Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol. Rev. *44*, 1-80.

Blaber,S.I., Yoon,H., Scarisbrick,I.A., Juliano,M.A., and Blaber,M. (2007). The autolytic regulation of human kallikrein-related peptidase 6. Biochemistry *46*, 5209-5217.

Blagosklonny,M.V., Prabhu,N.S., and El-Deiry,W.S. (1997). Defects in p21WAF1/CIP1, Rb, and c-myc signaling in phorbol ester-resistant cancer cells. Cancer Res. *57*, 320-325.

Booth,M., Beral,V., and Smith,P. (1989). Risk-factors for ovarian-cancer - a case control study. Br. J. Cancer *60*, 592-598.

Borgono,C.A. and Diamandis,E.P. (2004). The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer *4*, 876-890.

Borgono,C.A., Fracchioli,S., Yousef,G.M., Rigault De La Longrais IA, Luo,L.Y., Soosaipillai,A., Puopolo,M., Grass,L., Scorilas,A., Diamandis,E.P., and Katsaros,D. (2003a). Favorable prognostic value of tissue human kallikrein 11 (hK11) in patients with ovarian carcinoma. Int. J. Cancer *106*, 605-610.

Borgono,C.A., Grass,L., Soosaipillai,A., Yousef,G.M., Petraki,C.D., Howarth,D.H., Fracchioli,S., Katsaros,D., and Diamandis,E.P. (2003b). Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. Cancer Res. *63*, 9032-41.

Borgono,C.A., Kishi,T., Scorilas,A., Harbeck,N., Dorn,J., Schmalfeldt,B., Schmitt,M., and Diamandis,E.P. (2006). Human kallikrein 8 protein is a favorable prognostic marker in ovarian cancer. Clin. Cancer Res. *12*, 1487-1493.

Borgono,C.A., Michael,I.P., and Diamandis,E.P. (2004). Human tissue kallikreins: physiologic roles and applications in cancer. Mol. Cancer Res. *2*, 257-280.

Borgono,C.A., Michael,I.P., Shaw,J.L., Luo,L.Y., Ghosh,M.C., Soosaipillai,A., Grass,L., Katsaros,D., and Diamandis,E.P. (2007). Expression and functional characterization of the cancer-related serine protease, human tissue kallikrein 14. J. Biol. Chem. *282*, 2405-2422.

Bostwick,D.G. (1994). Prostate-specific antigen. Current role in diagnostic pathology of prostate cancer. Am. J. Clin. Pathol. *102*, S31-S37.

Boyd,J. and Rubin,S.C. (1997). Hereditary ovarian cancer: molecular genetics and clinical implications. Gynecol. Oncol. *64*, 196-206.

Brattsand,M. and Egelrud,T. (1999). Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. J. Biol. Chem. *274*, 30033-40.

Brattsand,M., Stefansson,K., Lundh,C., Haasum,Y., and Egelrud,T. (2005). A proteolytic cascade of kallikreins in the stratum corneum. J. Invest. Dermatol. *124*, 198-203.

Brumsted,J.R., Nakajima,S.T., Badger,G., Riddick,D.H., and Gibson,M. (1990). Serum concentration of CA-125 during the first trimester of normal and abnormal pregnancies. J. Reprod. Med. *35*, 499-502.

Buamah,P.K. and Skillen,A.W. (1994). Serum CA 125 concentrations in patients with benign ovarian tumours. J. Surg. Oncol. *56*, 71-74.

Buck,M. (2008). A novel domain of BRCA1 interacts with p53 in breast cancer cells. Cancer Lett. *268*, 137-145.

Burger,H.G., Robertson,D.M., Cahir,N., Mamers,P., Healy,D.L., Jobling,T., and Groome,N. (1996). Characterization of inhibin immunoreactivity in post-menopausal women with ovarian tumours. Clin. Endocrinol. (Oxf) *44*, 413-418.

Calin,G.A., Ferracin,M., Cimmino,A., Di Leva,G., Shimizu,M., Wojcik,S.E., Iorio,M.V., Visone,R., Sever,N.I., Fabbri,M., Iuliano,R., Palumbo,T., Pichiorri,F., Roldo,C., Garzon,R.,

Sevignani,C., Rassenti,L., Alder,H., Volinia,S., Liu,C.G., Kipps,T.J., Negrini,M., and Croce,C.M. (2005). A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N. Engl. J. Med. *353*, 1793-1801.

Canadian Cancer Society's Steering Committee (2009). Canadian Cancer Statistics 2009, Toronto: Canadian Cancer Society.

Caubet,C., Jonca,N., Brattsand,M., Guerrin,M., Bernard,D., Schmidt,R., Egelrud,T., Simon,M., and Serre,G. (2004). Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J. Invest. Dermatol. *122*, 1235-1244.

Chan,W.Y., Cheung,K.K., Schorge,J.O., Huang,L.W., Welch,W.R., Bell,D.A., Berkowitz,R.S., and Mok,S.C. (2000). Bcl-2 and p53 protein expression, apoptosis, and p53 mutation in human epithelial ovarian cancers. Am. J. Pathol. *156*, 409-417.

Chang,A., Yousef,G.M., Jung,K., Rajpert-De Meyts,E., and Diamandis,E.P. (2001). Identification and molecular characterization of five novel kallikrein gene 13 (KLK13; KLK-L4) splice variants: differential expression in the human testis and testicular cancer. Anticancer Res. *21*, 3147-3152.

Chang,E.T., Lee,V.S., Canchola,A.J., Clarke,C.A., Purdie,D.M., Reynolds,P., Anton-Culver,H., Bernstein,L., Deapen,D., Peel,D., Pinder,R., Ross,R.K., Stram,D.O., West,D.W., Wright,W., Ziogas,A., and Horn-Ross,P.L. (2007). Diet and risk of ovarian cancer in the California Teachers Study cohort. Am. J. Epidemiol. *165*, 802-813.

Chang,E.T., Lee,V.S., Canchola,A.J., Dalvi,T.B., Clarke,C.A., Reynolds,P., Purdie,D.M., Stram,D.O., West,D.W., Ziogas,A., Bernstein,L., and Horn-Ross,P.L. (2008). Dietary patterns and risk of ovarian cancer in the California Teachers Study cohort. Nutr. Cancer *60*, 285-291.

Chapman,S.J., Walsh,A., Jackson,S.M., and Friedmann,P.S. (1991). Lipids, proteins and corneocyte adhesion. Arch. Dermatol. Res. *283*, 167-173.

Choi,K.C. and Auersperg,N. (2003). The ovarian surface epithelium: simple source of a complex disease. Minerva Ginecol. *55*, 297-314.

Christensson,A., Laurell,C.B., and Lilja,H. (1990). Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. Eur. J. Biochem. *194*, 755-763.

Christie,M. and Oehler,M.K. (2006). Molecular pathology of epithelial ovarian cancer. J. Br. Menopause. Soc. *12*, 57-63.

Clark-Knowles,K.V., Garson,K., Jonkers,J., and Vanderhyden,B.C. (2007). Conditional inactivation of Brca1 in the mouse ovarian surface epithelium results in an increase in preneoplastic changes. Exp. Cell Res. *313*, 133-145.

Connolly,D.C., Bao,R., Nikitin,A.Y., Stephens,K.C., Poole,T.W., Hua,X., Harris,S.S., Vanderhyden,B.C., and Hamilton,T.C. (2003a). Female mice chimeric for expression of the

simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res. *63*, 1389-1397.

Connolly,D.C., Bao,R., Nikitin,A.Y., Stephens,K.C., Poole,T.W., Hua,X., Harris,S.S., Vanderhyden,B.C., and Hamilton,T.C. (2003b). Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res. *63*, 1389-1397.

Cortez,D., Wang,Y., Qin,J., and Elledge,S.J. (1999). Requirement of ATM-dependent phosphorylation of BRCA1 in the DNA damage response to double-strand breaks. Science *286*, 1162-1166.

Cuatrecasas,M., Villanueva,A., Matias-Guiu,X., and Prat,J. (1997). K-ras mutations in mucinous ovarian tumors: a clinicopathologic and molecular study of 95 cases. Cancer *79*, 1581-1586.

Darling,M.R., Jackson-Boeters,L., Daley,T.D., and Diamandis,E.P. (2006). Human kallikrein 6 expression in salivary gland tumors. J. Histochem. Cytochem. *54*, 337-342.

Debela,M., Magdolen,V., Grimminger,V., Sommerhoff,C., Messerschmidt,A., Huber,R., Friedrich,R., Bode,W., and Goettig,P. (2006). Crystal structures of human tissue kallikrein 4: activity modulation by a specific zinc binding site. J. Mol. Biol. *362*, 1094-1107.

Deperthes,D., Frenette,G., Brillard-Bourdet,M., Bourgeois,L., Gauthier,F., Tremblay,R.R., and Dube,J.Y. (1996). Potential involvement of kallikrein hK2 in the hydrolysis of the human seminal vesicle proteins after ejaculation. J. Androl. *17*, 659-665.

Devoize,L., Collangettes,D., Le Bouedec,G., Mishellany,F., Orliaguet,T., Dallel,R., and Baudet-Pommel,M. (2008). Giant mature ovarian cystic teratoma including more than 300 teeth. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. *105*, e76-e79.

di Clemente,N., Wilson,C., Faure,E., Boussin,L., Carmillo,P., Tizard,R., Picard,J.Y., Vigier,B., Josso,N., and Cate,R. (1994). Cloning, expression, and alternative splicing of the receptor for anti-Mullerian hormone. Mol. Endocrinol. *8*, 1006-1020.

Diamandis,E.P., Borgono,C.A., Scorilas,A., Yousef,G.M., Harbeck,N., Dorn,J., Schmalfeldt,B., and Schmitt,M. (2003). Immunofluorometric quantification of human kallikrein 5 expression in ovarian cancer cytosols and its association with unfavorable patient prognosis. Tumour Biol. *24*, 299-309.

Diamandis,E.P., Okui,A., Mitsui,S., Luo,L.Y., Soosaipillai,A., Grass,L., Nakamura,T., Howarth,D.J., and Yamaguchi,N. (2002). Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. Cancer Res. *62*, 295-300.

Diamandis,E.P., Yousef,G.M., Petraki,C., and Soosaipillai,A.R. (2000a). Human kallikrein 6 as a biomarker of alzheimer's disease. Clin. Biochem. *33*, 663-7.

Diamandis,E.P., Yousef,G.M., Soosaipillai,A.R., and Bunting,P. (2000b). Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin. Biochem. *33*, 579-583.

Diamandis,E.P., Yousef,G.M., Soosaipillai,A.R., Grass,L., Porter,A., Little,S., and Sotiropoulou,G. (2000c). Immunofluorometric assay of human kallikrein 6 (zyme/protease M/neurosin) and preliminary clinical applications. Clin. Biochem. *33*, 369-375.

Dinulescu,D.M., Ince,T.A., Quade,B.J., Shafer,S.A., Crowley,D., and Jacks,T. (2005). Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. Nat. Med. *11*, 63-70.

Dong,L., Cui,H., Li,X.P., Sun,L.F., Chang,X.H., Liang,X.D., and Zhu,H.L. (2008). [Clinical value of serum CA19-9, CA125 and CP2 in mucinous ovarian tumor: a retrospective study of 273 patients]. Zhonghua Fu Chan Ke. Za Zhi. *43*, 5-8.

Einhorn,N., Nilsson,B., and Sjovall,K. (1985). Factors influencing survival in carcinoma of the ovary. Study from a well-defined Swedish population. Cancer *55*, 2019-2025.

Emami,N., Deperthes,D., Malm,J., and Diamandis,E.P. (2008). Major role of human KLK14 in seminal clot liquefaction. J. Biol. Chem. *283*, 19561-19569.

Esquela-Kerscher,A. and Slack,F.J. (2006). Oncomirs - microRNAs with a role in cancer. Nat. Rev. Cancer *6*, 259-269.

Evers,B. and Jonkers,J. (2006). Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. Oncogene *25*, 5885-5897.

Fathalla,M.F. (1971). Incessant Ovulation - Factor in Ovarian Neoplasia. Lancet *2*, 163-&.

Fitzgibbons,R.J., Lynch,H.T., Stanislav,G.V., Watson,P.A., Lanspa,S.J., Marcus,J.N., Smyrk,T., Kriegler,M.D., and Lynch,J.F. (1987). Recognition and treatment of patients with hereditary nonpolyposis colon cancer (Lynch Syndrome-I and Syndrome-II). Ann. Surg. *206*, 289-295.

Fleming,J.S., Beaugie,C.R., Haviv,I., Chenevix-Trench,G., and Tan,O.L. (2006). Incessant ovulation, inflammation and epithelial ovarian carcinogenesis: revisiting old hypotheses. Mol. Cell Endocrinol. *247*, 4-21.

Flesken-Nikitin,A., Choi,K.C., Eng,J.P., Shmidt,E.N., and Nikitin,A.Y. (2003). Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. Cancer Res. *63*, 3459-3463.

Franceschi,S., La Vecchia,C., Booth,M., Tzonou,A., Negri,E., Parazzini,F., Trichopoulos,D., and Beral,V. (1991). Pooled analysis of 3 European case-control studies of ovarian cancer: II. Age at menarche and at menopause. Int. J. Cancer *49*, 57-60.

Francis-Thickpenny,K.M., Richardson,D.M., van Ee,C.C., Love,D.R., Winship,I.M., Baguley,B.C., Chenevix-Trench,G., and Shelling,A.N. (2001). Analysis of the TGF beta functional pathway in epithelial ovarian carcinoma. Br. J. Cancer *85*, 687-691.

Fredrickson,T.N. (1987). Ovarian tumors of the hen. Environ. Health Perspect. *73*, 35-51.

Froggatt,N.J., Green,J., Brassett,C., Evens,D.G.R., Bishop,D.T., Kolodner,R., and Maher,E.R. (1999). A common MSH2 mutation in English and North American HNPCC families: origin, phenotypic expression, and sex specific differences in colorectal cancer. J. Med. Gent. *36*, 97-102.

Fukushima,D., Kitamura,N., and Nakanishi,S. (1985). Nucleotide sequence of cloned cDNA for human pancreatic kallikrein. Biochemistry *24*, 8037-8043.

Gadducci,A., Ferdeghini,M., Prontera,C., Moretti,L., Mariani,G., Bianchi,R., and Fioretti,P. (1992). The concomitant determination of different tumor markers in patients with epithelial ovarian cancer and benign ovarian masses: relevance for differential diagnosis. Gynecol. Oncol. *44*, 147-54.

Garson,K., Shaw,T.J., Clark,K.V., Yao,D.S., and Vanderhyden,B.C. (2005). Models of ovarian cancer--are we there yet? Mol. Cell Endocrinol. *239*, 15-26.

Gemignani,M.L., Schlaerth,A.C., Bogomolniy,F., Barakat,R.R., Lin,O., Soslow,R., Venkatraman,E., and Boyd,J. (2003). Role of KRAS and BRAF gene mutations in mucinous ovarian carcinoma. Gynecol. Oncol. *90*, 378-381.

Ghosh,M.C., Grass,L., Soosaipillai,A., Sotiropoulou,G., and Diamandis,E.P. (2004). Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol. *25*, 193-199.

Goyal,J., Smith,K.M., Cowan,J.M., Wazer,D.E., Lee,S.W., and Band,V. (1998). The role for NES1 serine protease as a novel tumor suppressor. Cancer Res. *58*, 4782-6.

Gras,E., Catasus,L., Arguelles,R., Moreno-Bueno,G., Palacios,J., Gamallo,C., Matias-Guiu,X., and Prat,J. (2001). Microsatellite instability, MLH-1 promoter hypermethylation, and frameshift mutations at coding mononucleotide repeat microsatellites in ovarian tumors. Cancer *92*, 2829-2836.

Haese,A., Graefen,M., Steuber,T., Becker,C., Pettersson,K., Piironen,T., Noldus,J., Huland,H., and Lilja,H. (2001). Human glandular kallikrein 2 levels in serum for discrimination of pathologically organ-confined from locally-advanced prostate cancer in total PSA-levels below 10 ng/ml. Prostate *49*, 101-109.

Hahn,W.C. and Weinberg,R.A. (2002). Modelling the molecular circuitry of cancer. Nat. Rev. Cancer *2*, 331-341.

Hamilton,T.C., Young,R.C., McKoy,W.M., Grotzinger,K.R., Green,J.A., Chu,E.W., Whang-Peng,J., Rogan,A.M., Green,W.R., and Ozols,R.F. (1983). Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. Cancer Res. *43*, 5379-5389.

Hansson,L., Stromqvist,M., Backman,A., Wallbrandt,P., Carlstein,A., and Egelrud,T. (1994). Cloning, expression, and characterization of stratum corneum chymotryptic enzyme. A skin-specific human serine proteinase. J. Biol. Chem. *269*, 19420-6.

Harley,I., Rosen,B., Risch,H.A., Siminovitch,K., Beiner,M.E., McLaughlin,J., Sun,P., and Narod,S.A. (2008). Ovarian cancer risk is associated with a common variant in the promoter sequence of the mismatch repair gene MLH1. Gynecol. Oncol. *109*, 384-387.

Harris,R., Whittemore,A.S., and Itnyre,J. (1992). Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. III. Epithelial tumors of low malignant potential in white women. Collaborative Ovarian Cancer Group. Am. J. Epidemiol. *136*, 1204-1211.

Healy,D.L., Burger,H.G., Mamers,P., Jobling,T., Bangah,M., Quinn,M., Grant,P., Day,A.J., Rome,R., and Campbell,J.J. (1993). Elevated serum inhibin concentrations in postmenopausal women with ovarian tumors. N. Engl. J. Med. *329*, 1539-42.

Heeb,M.J. and Espana,F. (1998). alpha2-macroglobulin and C1-inactivator are plasma inhibitors of human glandular kallikrein. Blood Cells Mol. Dis. *24*, 412-419.

Heintz,A.P., Odicino,F., Maisonneuve,P., Beller,U., Benedet,J.L., Creasman,W.T., Ngan,H.Y., and Pecorelli,S. (2003). Carcinoma of the ovary. Int. J. Gynaecol. Obstet. *83 Suppl 1*, 135-166.

Ho,S.M. (2003). Estrogen, progesterone and epithelial ovarian cancer. Reprod. Biol. Endocrinol. *1*, 73-80.

Hoffman,B.R., Katsaros,D., Scorilas,A., Diamandis,P., Fracchioli,S., Rigault de la Longrais,I.A., Colgan,T., Puopolo,M., Giardina,G., Massobrio,M., and Diamandis,E.P. (2002). Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new independent unfavourable prognostic biomarker. Br. J. Cancer *87*, 763-771.

Hu,J.C., Zhang,C., Sun,X., Yang,Y., Cao,X., Ryu,O., and Simmer,J.P. (2000). Characterization of the mouse and human PRSS17 genes, their relationship to other serine proteases, and the expression of PRSS17 in developing mouse incisors. Gene *251*, 1-8.

Huang,K.C., Park,D.C., Ng,S.K., Lee,J.Y., Ni,X., Ng,W.C., Bandera,C.A., Welch,W.R., Berkowitz,R.S., Mok,S.C., and Ng,S.W. (2006). Selenium binding protein 1 in ovarian cancer. Int. J. Cancer *118*, 2433-2440.

Huang,W., Zhong,J., Wu,L.Y., Yu,L.F., Tian,X.L., Zhang,Y.F., and Li,B. (2007). Downregulation and CpG island hypermethylation of NES1/hK10 gene in the pathogenesis of human gastric cancer. Cancer Lett. *251*, 78-85.

Hugosson,J., Aus,G., Lilja,H., Lodding,P., and Pihl,C.G. (2004). Results of a randomized, population-based study of biennial screening using serum prostate-specific antigen measurement to detect prostate carcinoma. Cancer *100*, 1397-1405.

Hutchinson,S., Luo,L.Y., Yousef,G.M., Soosaipillai,A., and Diamandis,E.P. (2003). Purification of human kallikrein 6 from biological fluids and identification of its complex with alpha(1)-antichymotrypsin. Clin. Chem. *49*, 746-51.

Inamura,H., Tomita,M., Okano,A., and Kurosawa,M. (2003). Serial blood and urine levels of EDN and ECP in eosinophilic colitis. Allergy *58*, 959-960.

International Federation of Gynecology and Oncology (1986). The new FIGO stage grouping for primary carcinoma of the ovary. Gynecol. Oncol. *25*, 385.

Iorio,M.V., Ferracin,M., Liu,C.G., Veronese,A., Spizzo,R., Sabbioni,S., Magri,E., Pedriali,M., Fabbri,M., Campiglio,M., Menard,S., Palazzo,J.P., Rosenberg,A., Musiani,P., Volinia,S., Nenci,I., Calin,G.A., Querzoli,P., Negrini,M., and Croce,C.M. (2005). MicroRNA gene expression deregulation in human breast cancer. Cancer Res. *65*, 7065-7070.

Jackson,E., Anderson,K., Ashwell,C., Petitte,J., and Mozdziak,P.E. (2007). CA125 expression in spontaneous ovarian adenocarcinomas from laying hens. Gynecol. Oncol. *104*, 192-198.

Jacobs,I. and Bast,R.C., Jr. (1989). The CA125 tumor associated antigen: a review of the literature. Hum. Reprod. *4*, 1-12.

Jemal,A., Murray,T., Ward,E., Samuels,A., Tiwari,R.C., Ghafoor,A., Feuer,E.J., and Thun,M.J. (2005). Cancer statistics, 2005. CA Cancer J. Clin. *55*, 10-30.

Kabawat,S.E., Bast,R.C., Jr., Bhan,A.K., Welch,W.R., Knapp,R.C., and Colvin,R.B. (1983). Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125. Int. J. Gynecol. Pathol. *2*, 275-285.

Kafali,H., Artuc,H., and Demir,N. (2004). Use of CA125 fluctuation during the menstrual cycle as a tool in the clinical diagnosis of endometriosis; a preliminary report. Eur. J. Obstet. Gynecol. Reprod. Biol. *116*, 85-88.

Kaku,T., Ogawa,S., Kawano,Y., Ohishi,Y., Kobayashi,H., Hirakawa,T., and Nakano,H. (2003). Histological classification of ovarian cancer. Med. Electron Microsc. *36*, 9-17.

Kan,Y.Y., Yeh,S.H., Ng,H.T., and Lou,C.M. (1992). Effect of menstruation on serum CA125 levels. Asia Oceania J. Obstet. Gynaecol. *18*, 339-43.

Kapadia,C., Chang,A., Sotiropoulou,G., Yousef,G.M., Grass,L., Soosaipillai,A., Xing,X., Howarth,D.H., and Diamandis,E.P. (2003). Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin. Chem. *49*, 77-86.

Kapadia,C., Ghosh,M.C., Grass,L., and Diamandis,E.P. (2004a). Human kallikrein 13 involvement in extracellular matrix degradation. Biochem. Biophys. Res. Commun. *323*, 1084-1090.

Kapadia,C., Yousef,G.M., Mellati,A.A., Magklara,A., Wasney,G.A., and Diamandis,E.P. (2004b). Complex formation between human kallikrein 13 and serum protease inhibitors. Clin. Chim. Acta *339*, 157-67.

Karazanashvili,G. and Abrahamsson,P.A. (2003). Prostate specific antigen and human glandular kallikrein 2 in early detection of prostate cancer. J. Urol. *169*, 445-457.

Karlan,B.Y., Jones,J., Greenwald,M., and Lagasse,L.D. (1995). Steroid hormone effects on the proliferation of human ovarian surface epithelium in vitro. Am. J. Obstet. Gynecol. *173*, 97-104.

Katabuchi,H. and Okamura,H. (2003). Cell biology of human ovarian surface epithelial cells and ovarian carcinogenesis. Med. Electron Microsc. *36*, 74-86.

Kato,N., Sasou,S., and Motoyama,T. (2006). Expression of hepatocyte nuclear factor-1beta (HNF-1beta) in clear cell tumors and endometriosis of the ovary. Mod. Pathol. *19*, 83-89.

Kavanagh,J.P. (1985). Sodium, potassium, calcium, magnesium, zinc, citrate and chloride content of human prostatic and seminal fluid. J. Reprod. Fertil. *75*, 35-41.

Kido,M. and Shibuya,M. (1998). Isolation and characterization of mouse ovarian surface epithelial cell lines. Pathol. Res. Pract. *194*, 725-730.

Kishi,T., Cloutier,S.M., Kundig,C., Deperthes,D., and Diamandis,E.P. (2006). Activation and enzymatic characterization of recombinant human kallikrein 8. Biol. Chem. *387*, 723-731.

Kishibe,M., Bando,Y., Terayama,R., Namikawa,K., Takahashi,H., Hashimoto,Y., Ishida-Yamamoto,A., Jiang,Y.P., Mitrovic,B., Perez,D., Iizuka,H., and Yoshida,S. (2007). Kallikrein 8 is involved in skin desquamation in cooperation with other kallikreins. J. Biol. Chem. *282*, 5834-5841.

Koninckx,P.R., Meuleman,C., Oosterlynck,D., and Cornillie,F.J. (1996). Diagnosis of deep endometriosis by clinical examination during menstruation and plasma CA-125 concentration. Fertil. Steril. *65*, 280-287.

Koonings,P., Campbell K, Mishell DR Jr, and Grimes DA (1989). Relative frequency of primary ovarian neoplasms: a 10-year review. Obstet. Gynecol. *74*, 921-926.

Koprowski,H., Steplewski,Z., Mitchell,K., Herlyn,M., Herlyn,D., and Fuhrer,P. (1979). Colorectal carcinoma antigens detected by hybridoma antibodies. Somatic. Cell Genet. *5*, 957-971.

Korkmaz,K.S., Korkmaz,C.G., Pretlow,T.G., and Saatcioglu,F. (2001). Distinctly different gene structure of KLK4/KLK-L1/prostase/ARM1 compared with other members of the kallikrein family: intracellular localization, alternative cDNA forms, and Regulation by multiple hormones. DNA Cell Biol. *20*, 435-445.

Krawczak,M., Reiss,J., and Cooper,D.N. (1992). The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum. Genet. *90*, 41-54.

Kurlender,L., Borgono,C., Michael,I.P., Obiezu,C., Elliott,M.B., Yousef,G.M., and Diamandis,E.P. (2005). A survey of alternative transcripts of human tissue kallikrein genes. Biochim. Biophys. Acta. *1755*, 1-14.

Lacey,J.V., Jr., Mink,P.J., Lubin,J.H., Sherman,M.E., Troisi,R., Hartge,P., Schatzkin,A., and Schairer,C. (2002). Menopausal hormone replacement therapy and risk of ovarian cancer. JAMA. *288*, 334-341.

Laios,A., O'Toole,S., Flavin,R., Martin,C., Kelly,L., Ring,M., Finn,S.P., Barrett,C., Loda,M., Gleeson,N., D'Arcy,T., McGuinness,E., Sheils,O., Sheppard,B., and O' Leary,J. (2008). Potential role of miR-9 and miR-223 in recurrent ovarian cancer. Mol. Cancer *7*, 35.

Lambert-Messerlian,G.M. (2000). Is inhibin a serum marker for ovarian cancer? Eur. J. Endocrinol. *142*, 331-3.

Lee,R.C., Feinbaum,R.L., and Ambros,V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Leitao,M.M., Jr., Boyd,J., Hummer,A., Olvera,N., Arroyo,C.D., Venkatraman,E., Baergen,R.N., Dizon,D.S., Barakat,R.R., and Soslow,R.A. (2004). Clinicopathologic analysis of early-stage sporadic ovarian carcinoma. Am. J. Surg. Pathol. *28*, 147-159.

Lewis,P.D. and Long,S.E. (1992). Incidence of non-laying in domestic hens. Br. Poult. Sci. *33*, 289-295.

Li,B., Goyal,J., Dhar,S., Dimri,G., Evron,E., Sukumar,S., Wazer,D.E., and Band,V. (2001). CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. Cancer Res. *61*, 8014-8021.

Li,Q., Peterson,K.R., Fang,X., and Stamatoyannopoulos,G. (2002). Locus control regions. Blood *100*, 3077-3086.

Lilja,H., Oldbring,J., Rannevik,G., and Laurell,C.B. (1987). Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. J. Clin. Invest *80*, 281-285.

Lindgren,P.R., Backstrom,T., Cajander,S., Damber,M.G., Mahlck,C.G., Zhu,D., and Olofsson,J.I. (2002). The pattern of estradiol and progesterone differs in serum and tissue of benign and malignant ovarian tumors. Int. J. Oncol. *21*, 583-9.

Little,S.P., Dixon,E.P., Norris,F., Buckley,W., Becker,G.W., Johnson,M., Dobbins,J.R., Wyrick,T., Miller,J.R., MacKellar,W., Hepburn,D., Corvalan,J., McClure,D., Liu,X., Stephenson,D., Clemens,J., and Johnstone,E.M. (1997). Zyme, a novel and potentially amyloidogenic enzyme cDNA isolated from Alzheimer's disease brain. J. Biol. Chem. *272*, 25135-25142.

Liu,X.L., Wazer,D.E., Watanabe,K., and Band,V. (1996a). Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res. *56*, 3371-9.

Liu,X.L., Wazer,D.E., Watanabe,K., and Band,V. (1996b). Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res. *56*, 3371-3379.

Lovgren,J., Airas,K., and Lilja,H. (1999a). Enzymatic action of human glandular kallikrein 2 (hK2). Substrate specificity and regulation by Zn2+ and extracellular protease inhibitors. Eur. J. Biochem. *262*, 781-789.

Lovgren,J., Rajakoski,K., Karp,M., Lundwall a, and Lilja,H. (1997). Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. Biochem. Biophys. Res. Commun. *238*, 549-555.

Lovgren,J., Valtonen-Andre,C., Marsal,K., Lilja,H., and Lundwall,A. (1999b). Measurement of prostate-specific antigen and human glandular kallikrein 2 in different body fluids. J. Androl *20*, 348-355.

Lundwall,A. (1989). Characterization of the gene for prostate-specific antigen, a human glandular kallikrein. Biochem Biophys. Res. Commun. *161*, 1151-1159.

Lundwall,A., Band,V., Blaber,M., Clements,J.A., Courty,Y., Diamandis,E.P., Fritz,H., Lilja,H., Malm,J., Maltais,L.J., Olsson,A.Y., Petraki,C., Scorilas,A., Sotiropoulou,G., Stenman,U.H., Stephan,C., Talieri,M., and Yousef,G.M. (2006). A comprehensive nomenclature for serine proteases with homology to tissue kallikreins. Biol. Chem. *387*, 637-641.

Lundwall,A. and Brattsand,M. (2008). Kallikrein-related peptidases. Cell Mol. Life Sci. *65*, 2019-2038.

Luo,L., Herbrick,J.A., Scherer,S.W., Beatty,B., Squire,J., and Diamandis,E.P. (1998). Structural characterization and mapping of the normal epithelial cell-specific 1 gene. Biochem. Biophys. Res. Commun. *247*, 580-6.

Luo,L.Y., Bunting,P., Scorilas,A., and Diamandis,E.P. (2001a). Human kallikrein 10: a novel tumor marker for ovarian carcinoma? Clin. Chim. Acta *306*, 111-8.

Luo,L.Y., Katsaros,D., Scorilas,A., Fracchioli,S., Bellino,R., Van Gramberen,M., De Bruijn,H., Henrik,A., Stenman,U.H., Massobrio,M., Van Der Zee,A.G., Vergote,I., and Diamandis,E.P. (2003). The serum concentration of human kallikrein 10 represents a novel biomarker for ovarian cancer diagnosis and prognosis. Cancer Res. *63*, 807-11.

Luo,L.Y., Katsaros,D., Scorilas,A., Fracchioli,S., Piccinno,R., Rigault de la Longrais,I.A., Howarth,D.J., and Diamandis,E.P. (2001b). Prognostic value of human kallikrein 10 expression in epithelial ovarian carcinoma. Clin. Cancer Res. *7*, 2372-9.

Luo,L.Y., Rajpert-De Meyts,E.R., Jung,K., and Diamandis,E.P. (2001c). Expression of the normal epithelial cell-specific 1 (NES1; KLK10) candidate tumour suppressor gene in normal and malignant testicular tissue. Br. J. Cancer *85*, 220-4.

Luo,L.Y., Soosaipillai,A., Grass,L., and Diamandis,E.P. (2006). Characterization of human kallikreins 6 and 10 in ascites fluid from ovarian cancer patients. Tumour Biol. *27*, 227-234.

Lynch,H.T., Casey,M.J., Shaw,T.G., and Lynch,J.F. (1998). Hereditary factors in gynecologic cancer. Oncologist. *3*, 319-338.

Lynch,H.T. and de la Chapelle,A. (1999). Genetic susceptibility to non-polyposis colorectal cancer. J. Med. Gent. *36*, 801-818.

Lynch,H.T. and de la Chapelle,A. (2003). Hereditary colorectal cancer. N. Engl. J. Med. *348*, 919-932.

Lynch,H.T. and Lynch,J.F. (1992). Hereditary ovarian carcinoma. Hematol. Oncol. Clin. North Am. *6*, 783-811.

Madigan,M.P., Ziegler,R.G., Benichou,J., Byrne,C., and Hoover,R.N. (1995). Proportion of breast-cancer cases in the United-States explained by well-established risk-factors. J. Natl. Cancer Inst. *87*, 1681-1685.

Magklara,A., Mellati,A.A., Wasney,G.A., Little,S.P., Sotiropoulou,G., Becker,G.W., and Diamandis,E.P. (2003). Characterization of the enzymatic activity of human kallikrein 6: Autoactivation, substrate specificity, and regulation by inhibitors. Biochem Biophys Res Commun *307*, 948-55.

Maines-Bandiera,S.L. and Auersperg,N. (1997). Increased E-cadherin expression in ovarian surface epithelium: an early step in metaplasia and dysplasia? Int. J. Gynecol. Pathol. *16*, 250-255.

Malm,J., Hellman,J., Hogg,P., and Lilja,H. (2000). Enzymatic action of prostate-specific antigen (PSA or hK3): substrate specificity and regulation by Zn(2+), a tight-binding inhibitor. Prostate *45*, 132-139.

Marrs,J.A. and Nelson,W.J. (1996). Cadherin cell adhesion molecules in differentiation and embryogenesis. Int. Rev. Cytol. *165*, 159-205.

Marshall,M. and Solomon,S. (2007). Hereditary breast-ovarian cancer: clinical findings and medical management. Plast. Surg. Nurs. *27*, 124-127.

Matsumoto,K., Ogasawara,T., Kato,A., Homma,T., Iida,M., Akasawa,A., Wakiguchi,H., and Saito,H. (2003). Eosinophil degranulation during pregnancy and after delivery by cesarean section. Int. Arch. Allergy Immunol. *131 Suppl 1*, 34-39.

McNatty,K.P., Makris,A., DeGrazia,C., Osathanondh,R., and Ryan,K.J. (1979). The production of progesterone, androgens, and estrogens by granulosa cells, thecal tissue, and stromal tissue from human ovaries in vitro. J. Clin. Endocrinol. Metab *49*, 687-699.

Memari,N., Jiang,W., Diamandis,E.P., and Luo,L.Y. (2007). Enzymatic properties of human kallikrein-related peptidase 12 (KLK12). Biol. Chem. *388*, 427-435.

Michael,I.P., Pampalakis,G., Mikolajczyk,S.D., Malm,J., Sotiropoulou,G., and Diamandis,E.P. (2006). Human tissue kallikrein 5 (HK5) is a member of a proteolytic cascade pathway involved in seminal clot liquefaction and potentially in prostate cancer progression. J. Biol. Chem. *281*, 12743-12750.

Michael,I.P., Sotiropoulou,G., Pampalakis,G., Magklara,A., Ghosh,M., Wasney,G., and Diamandis,E.P. (2005). Biochemical and enzymatic characterization of human kallikrein 5 (hK5), a novel serine protease potentially involved in cancer progression. J. Biol. Chem. *280*, 14628-14635.

Miki,Y., Swensen,J., Shattuckeidens,D., Futreal,P.A., Harshman,K., Tavtigian,S., Liu,Q.Y., Cochran,C., Bennett,L.M., Ding,W., Bell,R., Rosenthal,J., Hussey,C., Tran,T., Mcclure,M., Frye,C., Hattier,T., Phelps,R., Haugenstrano,A., Katcher,H., Yakumo,K., Gholami,Z., Shaffer,D., Stone,S., Bayer,S., Wray,C., Bogden,R., Dayananth,P., Ward,J., Tonin,P., Narod,S., Bristow,P.K., Norris,F.H., Helvering,L., Morrison,P., Rosteck,P., Lai,M., Barrett,J.C., Lewis,C., Neuhausen,S., Cannonalbright,L., Goldgar,D., Wiseman,R., Kamb,A., and Skolnick,M.H. (1994). A strong candidate for the Breast and Ovarian-Cancer Susceptibility Gene BRCA1. Science *266*, 66-71.

Mitin,N., Rossman,K.L., and Der,C.J. (2005). Signaling interplay in Ras superfamily function. Curr. Biol. *15*, R563-R574.

Mitsui,S., Yamada,T., Okui,A., Kominami,K., Uemura,H., and Yamaguchi,N. (2000). A novel isoform of a kallikrein-like protease, TLSP/hippostasin, (PRSS20), is expressed in the human brain and prostate. Biochem Biophys. Res. Commun. *272*, 205-211.

Mohle,J., Whittemore,A., Pike,M., and Darby,S. (1985). Gonadotrophins and ovarian cancer risk. J. Natl. Cancer Inst. *75*, 178-180.

Moore,C.M., Hubbard,G.B., Leland,M.M., Dunn,B.G., and Best,R.G. (2003). Spontaneous ovarian tumors in twelve baboons: a review of ovarian neoplasms in non-human primates. J. Med. Primatol. *32*, 48-56.

Moreno-Bueno,G., Gamallo,C., Perez-Gallego,L., de Mora,J.C., Suarez,A., and Palacios,J. (2001). beta-Catenin expression pattern, beta-catenin gene mutations, and microsatellite instability in endometrioid ovarian carcinomas and synchronous endometrial carcinomas. Diagn. Mol. Pathol. *10*, 116-122.

Myers,S.A. and Clements,J.A. (2001). Kallikrein 4 (KLK4), a new member of the human kallikrein gene family is up-regulated by estrogen and progesterone in the human endometrial cancer cell line, KLE. J. Clin. Endocrinol. Metab. *86*, 2323-2326.

Nagahara,H., Mimori,K., Utsunomiya,T., Barnard,G.F., Ohira,M., Hirakawa,K., and Mori,M. (2005). Clinicopathologic and biological significance of kallikrein 6 overexpression in human gastric cancer. Clin. Cancer Res. *11*, 6800-6806.

Nam,E.J., Yoon,H., Kim,S.W., Kim,H., Kim,Y.T., Kim,J.H., Kim,J.W., and Kim,S. (2008). MicroRNA Expression Profiles in Serous Ovarian Carcinoma. Clin. Cancer Res. *14*, 2690-2695.

Narod,S.A., Risch,H., Moslehi,R., Dorum,A., Neuhausen,S., Olsson,H., Provencher,D., Radice,P., Evans,G., Bishop,S., Brunet,J.S., and Ponder,B.A. (1998). Oral contraceptives and the risk of hereditary ovarian cancer. Hereditary Ovarian Cancer Clinical Study Group. N. Engl. J. Med. *339*, 424-428.

Nelson,P.S., Gan,L., Ferguson,C., Moss,P., Gelinas,R., Hood,L., and Wang,K. (1999). Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. Proc. Natl. Acad. Sci. U S A *96*, 3114-3119.

Ness,R.B. and Cottreau,C. (1999). Possible role of ovarian epithelial inflammation in ovarian cancer. J. Natl. Cancer Inst. *91*, 1459-1467.

Ni,X., Zhang,W., Huang,K.C., Wang,Y., Ng,S.K., Mok,S.C., Berkowitz,R.S., and Ng,S.W. (2004). Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br. J. Cancer. *91*, 725-731.

Nicosia,SV., Ku Ni Ni K, Oliveros-Saunders B, Giacominio G, Pierro E, and Mayer J (1997). Ovarian Mesothelium (Surface Epithelium) in Normal, Pathological, and Experimental Conditions. In: Recent Advances in microscopy of cells, tissues and organs., ed. Motta PM. Rome: Ant Delfino Editoreonio, 509-517.

Nonogaki,H., Fujii,S., Konishi,I., Nanbu,Y., Kobayashi,F., and Mori,T. (1991). Serial changes of serum CA125 levels during menstrual cycles. Asia Oceania J. Obstet. Gynaecol. *17*, 369-378.

Obata,K., Morland,S.J., Watson,R.H., Hitchcock,A., Chenevix-Trench,G., Thomas,E.J., and Campbell,I.G. (1998). Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. Cancer Res. *58*, 2095-2097.

Ogawa,K., Utsunomiya,T., Mimori,K., Tanaka,F., Inoue,H., Nagahara,H., Murayama,S., and Mori,M. (2005). Clinical significance of human kallikrein gene 6 messenger RNA expression in colorectal cancer. Clin. Cancer Res. *11*, 2889-2893.

Okamura,H.K.H.a.N.R. (1997). Ultrastructure of Human Ovulation: Histofunctional Parameters. In: Microscopy of Reproduction and Development: A Dynamic Approach, ed. P.M.Motta. Rome: Antonio Delfino Editore, 155-161.

Palacios,J. and Gamallo,C. (1998). Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. Cancer Res. *58*, 1344-1347.

Paliouras,M., Borgono,C., and Diamandis,E.P. (2007). Human tissue kallikreins: the cancer biomarker family. Cancer Lett. *249*, 61-79.

Pampalakis,G. and Sotiropoulou,G. (2007). Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer. Biochim. Biophys. Acta *1776*, 22-31.

Petraki,C.D., Gregorakis,A.K., Papanastasiou,P.A., Karavana,V.N., Luo,L.Y., and Diamandis,E.P. (2003a). Immunohistochemical localization of human kallikreins 6, 10 and 13 in benign and malignant prostatic tissues. Prostate Cancer Prostatic. Dis. *6*, 223-227.

Petraki,C.D., Karavana,V.N., and Diamandis,E.P. (2003b). Human kallikrein 13 expression in normal tissues: an immunohistochemical study. J. Histochem. Cytochem. *51*, 493-501.

Pharoah,P.D.P. and Ponder,B.A.J. (2002). The genetics of ovarian cancer. BestPract. Res. Clin. Obstet. Gynaecol. *16*, 449-468.

Planque,C., de Monte,M., Guyetant,S., Rollin,J., Desmazes,C., Panel,V., Lemarie,E., and Courty,Y. (2005). KLK5 and KLK7, two members of the human tissue kallikrein family, are differentially expressed in lung cancer. Biochem. Biophys. Res. Commun. *329*, 1260-1266.

Press,J.Z., De Luca,A., Boyd,N., Young,S., Troussard,A., Ridge,Y., Kaurah,P., Kalloger,S.E., Blood,K.A., Smith,M., Spellman,P.T., Wang,Y., Miller,D.M., Horsman,D., Faham,M., Gilks,C.B., Gray,J., and Huntsman,D.G. (2008). Ovarian carcinomas with genetic and epigenetic BRCA1 loss have distinct molecular abnormalities. BMC Cancer *8*, 17.

Public Health Agency of Canada. Cancer Surveillance On-Line. 2010.
Ref Type: Online Source

Raptis,S., Mrkonjic,M., Green,R.C., Pethe,V.V., Monga,N., Chan,Y.M., Daftary,D., Dicks,E., Younghusband,B.H., Parfrey,P.S., Gallinger,S.S., McLaughlin,J.R., Knight,J.A., and Bapat,B. (2007). MLH1 -93G>A promoter polymorphism and the risk of microsatellite-unstable colorectal cancer. J. Natl. Cancer Inst. *99*, 463-474.

Riegman,P.H., Klaassen,P., van der Korput,J.A., Romijn,J.C., and Trapman,J. (1988). Molecular cloning and characterization of novel prostate antigen cDNA's. Biochem Biophys. Res. Commun. *155*, 181-188.

Riegman,P.H., Vlietstra,R.J., van der Korput,J.A., Brinkmann,A.O., and Trapman,J. (1991). The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol. Endocrinol. *5*, 1921-1930.

Riegman,P.H., Vlietstra,R.J., van der Korput,J.A., Romijn,J.C., and Trapman,J. (1989). Characterization of the prostate-specific antigen gene: a novel human kallikrein-like gene. Biochem Biophys. Res. Commun. *159*, 95-102.

Riman,T., Dickman,P.W., Nilsson,S., Correia,N., Nordlinder,H., Magnusson,C.M., and Persson,I.R. (2002). Risk factors for invasive epithelial ovarian cancer: results from a Swedish case-control study. Am. J. Epidemiol. *156*, 363-373.

Riman,T., Persson,I., and Nilsson,S. (1998). Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence. Clin. Endocrinol. (Oxf) *49*, 695-707.

Riopel,M.A., Ronnett,B.M., and Kurman,R.J. (1999). Evaluation of diagnostic criteria and behavior of ovarian intestinal-type mucinous tumors: atypical proliferative (borderline) tumors and intraepithelial, microinvasive, invasive, and metastatic carcinomas. Am. J. Surg. Pathol. *23*, 617-635.

Risch,H.A. (1998). Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J. Natl. Cancer Inst. *90*, 1774-86.

Risch,H.A., Marrett,L.D., and Howe,G.R. (1994). Parity, contraception, infertility, and the risk of epithelial ovarian cancer. Am. J. Epidemiol. *140*, 585-97.

Roa,B.B., Boyd,A.A., Volcik,K., and Richards,C.S. (1996). Ashkenazi Jewish population frequencies for common mutations in BRCA1 and BRCA2. Nat. Genet. *14*, 185-187.

Robert,M., Gibbs,B.F., Jacobson,E., and Gagnon,C. (1997). Characterization of prostate-specific antigen proteolytic activity on its major physiological substrate, the sperm motility inhibitor precursor/semenogelin I. Biochemistry *36*, 3811-3819.

Robertson,D.M., Cahir,N., Burger,H.G., Mamers,P., and Groome,N. (1999). Inhibin forms in serum from postmenopausal women with ovarian cancers. Clin. Endocrinol. (Oxf) *50*, 381-6.

Rodriguez,C., Tatham,L.M., Calle,E.E., Thun,M.J., Jacobs,E.J., and Heath,C.W., Jr. (1998). Infertility and risk of fatal ovarian cancer in a prospective cohort of US women. Cancer Causes Control *9*, 645-651.

Rodriguez-Burford,C., Barnes,M.N., Berry,W., Partridge,E.E., and Grizzle,W.E. (2001). Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. Gynecol. Oncol. *81*, 373-379.

Roldo,C., Missiaglia,E., Hagan,J.P., Falconi,M., Capelli,P., Bersani,S., Calin,G.A., Volinia,S., Liu,C.G., Scarpa,A., and Croce,C.M. (2006). MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J. Clin. Oncol. *24*, 4677-4684.

Rosenberg,L., Palmer,J.R., Zauber,A.G., Warshauer,M.E., Lewis,J.L., Jr., Strom,B.L., Harlap,S., and Shapiro,S. (1994). A case-control study of oral contraceptive use and invasive epithelial ovarian cancer. Am. J. Epidemiol. *139*, 654-661.

Sainz,d.l.C., Eichhorn,J.H., Rice,L.W., Fuller,A.F., Jr., Nikrui,N., and Goff,B.A. (1996). Histologic transformation of benign endometriosis to early epithelial ovarian cancer. Gynecol. Oncol. *60*, 238-244.

Santin,A.D., Diamandis,E.P., Bellone,S., Soosaipillai,A., Cane,S., Palmieri,M., Burnett,A., Roman,J.J., and Pecorelli,S. (2005). Human kallikrein 6: a new potential serum biomarker for uterine serous papillary cancer. Clin. Cancer Res. *11*, 3320-3325.

Savage,P., Constenla,D., Fisher,C., Shepherd,J.H., Barton,D.P., Blake,P., and Gore,M.E. (1998). Granulosa cell tumours of the ovary: demographics, survival and the management of advanced disease. Clin. Oncol. (R. Coll. Radiol.) *10*, 242-245.

Schedlich,L.J., Bennetts,B.H., and Morris,B.J. (1987). Primary structure of a human glandular kallikrein gene. DNA *6*, 429-437.

Schildkraut,J.M., Schwingl,P.J., Bastos,E., Evanoff,A., and Hughes,C. (1996). Epithelial ovarian cancer risk among women with polycystic ovary syndrome. Obstet. Gynecol. *88*, 554-559.

Schouten,L.J., Goldbohm,R.A., and van den Brandt,P.A. (2003). Height, weight, weight change, and ovarian cancer risk in the Netherlands cohort study on diet and cancer. Am. J. Epidemiol. *157*, 424-433.

Schumer,S.T. and Cannistra,S.A. (2003). Granulosa cell tumor of the ovary. Journal of Clinical Oncology *21*, 1180-1189.

Scorilas,A., Borgono,C.A., Harbeck,N., Dorn,J., Schmalfeldt,B., Schmitt,M., and Diamandis,E.P. (2004). Human kallikrein 13 protein in ovarian cancer cytosols: a new favorable prognostic marker. J. Clin. Oncol. *22*, 678-685.

Scully,R. (2000). Role of BRCA gene dysfunction in breast and ovarian cancer predisposition. Breast Cancer Res. *2*, 324-330.

Scully,R., Anderson,S.F., Chao,D.M., Wei,W.J., Ye,L.Y., Young,R.A., Livingston,D.M., and Parvin,J.D. (1997a). BRCA1 is a component of the RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. U. S A *94*, 5605-5610.

Scully,R., Chen,J.J., Plug,A., Xiao,Y.H., Weaver,D., Feunteun,J., Ashley,T., and Livingston,D.M. (1997b). Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell *88*, 265-275.

Scully,R.E. (1987). Classification of human ovarian tumors. Environ. Health Perspect. *73*, 15-24.

Scully,R.E. (1988). Juvenile granulosa cell tumor. Pediatr. Pathol. *8*, 423-427.

Scully,R.E. (1995). Pathology of ovarian cancer precursors. J. Cell Biochem. Suppl *23*, 208-218.

Seidman,J.D., Horkayne-Szakaly,I., Haiba,M., Boice,C.R., Kurman,R.J., and Ronnett,B.M. (2004). The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. Int. J. Gynecol. Pathol. *23*, 41-44.

Serre,G., Mils,V., Haftek,M., Vincent,C., Croute,F., Reano,A., Ouhayoun,J.P., Bettinger,S., and Soleilhavoup,J.P. (1991). Identification of late differentiation antigens of human cornified epithelia, expressed in re-organized desmosomes and bound to cross-linked envelope. J. Invest. Dermatol. *97*, 1061-1072.

Shan,S.J., Scorilas,A., Katsaros,D., and Diamandis,E.P. (2007). Transcriptional upregulation of human tissue kallikrein 6 in ovarian cancer: clinical and mechanistic aspects. Br. J. Cancer. *96*, 362-372.

Shan,S.J., Scorilas,A., Katsaros,D., Rigault,d.l.L., I, Massobrio,M., and Diamandis,E.P. (2006). Unfavorable prognostic value of human kallikrein 7 quantified by ELISA in ovarian cancer cytosols. Clin. Chem. *52*, 1879-1886.

Shaw,T.J., Senterman,M.K., Dawson,K., Crane,C.A., and Vanderhyden,B.C. (2004). Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer. Mol. Ther. *10*, 1032-1042.

Shigemasa,K., Tian,X., Gu,L., Tanimoto,H., Underwood,L.J., O'Brien,T.J., and Ohama,K. (2004). Human kallikrein 8 (hK8/TADG-14) expression is associated with an early clinical stage and favorable prognosis in ovarian cancer. Oncol. Rep. *11*, 1153-9.

Shvartsman,H.S., Lu,K.H., Lee,J., Lillie,J., Deavers,M.T., Clifford,S., Wolf,J.K., Mills,G.B., Bast,R.C., Jr., Gershenson,D.M., and Schmandt,R. (2003). Overexpression of kallikrein 10 in epithelial ovarian carcinomas. Gynecol. Oncol. *90*, 44-50.

Sidiropoulos,M., Pampalakis,G., Sotiropoulou,G., Katsaros,D., and Diamandis,E.P. (2005). Downregulation of human kallikrein 10 (KLK10/NES1) by CpG island hypermethylation in breast, ovarian and prostate cancers. Tumour Biol. *26*, 324-336.

Sieben,N.L., Macropoulos,P., Roemen,G.M., Kolkman-Uljee,S.M., Jan,F.G., Houmadi,R., Diss,T., Warren,B., Al Adnani,M., De Goeij,A.P., Krausz,T., and Flanagan,A.M. (2004). In ovarian neoplasms, BRAF, but not KRAS, mutations are restricted to low-grade serous tumours. J. Pathol. *202*, 336-340.

Silva,E.G., Tornos,C., Fritsche,H.A., Jr., el Naggar,A., Gray,K., Ordonez,N.G., Luna,M., and Gershenson,D. (1997). The induction of benign epithelial neoplasms of the ovaries of guinea pigs by testosterone stimulation: a potential animal model. Mod. Pathol. *10*, 879-883.

Silverberg,S.G. (1999). Toward the development of a universal grading system for ovarian epithelial carcinoma. Gynecol. Oncol. *73*, 170-171.

Silverman,G.A., Bird,P.I., Carrell,R.W., Church,F.C., Coughlin,P.B., Gettins,P.G., Irving,J.A., Lomas,D.A., Luke,C.J., Moyer,R.W., Pemberton,P.A., Remold-O'Donnell,E., Salvesen,G.S., Travis,J., and Whisstock,J.C. (2001). The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J. Biol. Chem. *276*, 33293-33296.

Singh,J., Naran,A., Misso,N.L., Rigby,P.J., Thompson,P.J., and Bhoola,K.D. (2008). Expression of kallikrein-related peptidases (KRP/hK5, 7, 6, 8) in subtypes of human lung carcinoma. Int. Immunopharmacol. *8*, 300-306.

Singhal,H., Bautista,D.S., Tonkin,K.S., O'Malley,F.P., Tuck,A.B., Chambers,A.F., and Harris,J.F. (1997). Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. Clin. Cancer Res. *3*, 605-611.

Soslow,R.A. (2008). Histologic subtypes of ovarian carcinoma: an overview. Int. J. Gynecol. Pathol. *27*, 161-174.

Sotiropoulou,G., Rogakos,V., Tsetsenis,T., Pampalakis,G., Zafiropoulos,N., Simillides,G., Yiotakis,A., and Diamandis,E.P. (2003). Emerging interest in the kallikrein gene family for understanding and diagnosing cancer. Oncol. Res. *13*, 381-91.

Stadel,B.V. (1975). Letter: The etiology and prevention of ovarian cancer. Am. J. Obstet. Gynecol. *123*, 772-774.

Stambolic,V., Suzuki,A., de la Pompa,J.L., Brothers,G.M., Mirtsos,C., Sasaki,T., Ruland,J., Penninger,J.M., Siderovski,D.P., and Mak,T.W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29-39.

Stammer,K., Edassery,S.L., Barua,A., Bitterman,P., Bahr,J.M., Hales,D.B., and Luborsky,J.L. (2008). Selenium-Binding Protein 1 expression in ovaries and ovarian tumors in the laying hen, a spontaneous model of human ovarian cancer. Gynecol. Oncol. *109*, 115-121.

Steichen-Gersdorf,E., Gallion,H.H., Ford,D., Girodet,C., Easton,D.F., DiCioccio,R.A., Evans,G., Ponder,M.A., Pye,C., Mazoyer,S., and . (1994). Familial site-specific ovarian cancer is linked to BRCA1 on 17q12-21. Am. J. Hum. Genet. *55*, 870-875.

Stephenson,S.A., Verity,K., Ashworth,L.K., and Clements,J.A. (1999). Localization of a new prostate-specific antigen-related serine protease gene, KLK4, is evidence for an expanded human kallikrein gene family cluster on chromosome 19q13.3-13.4. J. Biol. Chem. *274*, 23210-23214.

Stern,R.C., Dash,R., Bentley,R.C., Snyder,M.J., Haney,A.F., and Robboy,S.J. (2001). Malignancy in endometriosis: frequency and comparison of ovarian and extraovarian types. Int. J. Gynecol. Pathol. *20*, 133-139.

Storey,D.J., Rush,R., Stewart,M., Rye,T., Al Nafussi,A., Williams,A.R., Smyth,J.F., and Gabra,H. (2008). Endometrioid epithelial ovarian cancer : 20 years of prospectively collected data from a single center. Cancer *112*, 2211-2220.

Stouffer,R.L., Chandrasekher,Y.A., Slayden,O.D., and Zelinski-Wooten,M.B. (1993). Gonadotrophic and local control of the developing corpus luteum in rhesus monkeys. Hum. Reprod. *8 Suppl 2*, 107-111.

Sutherland,G.R., Baker,E., Hyland,V.J., Callen,D.F., Close,J.A., Tregear,G.W., Evans,B.A., and Richards,R.I. (1988). Human prostate-specific antigen (APS) is a member of the glandular kallikrein gene family at 19q13. Cytogenet. Cell Genet. *48*, 205-207.

Syed,V., Ulinski,G., Mok,S.C., Yiu,G.K., and Ho,S.M. (2001). Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. Cancer Res. *61*, 6768-6776.

Takayama,T.K., Carter,C.A., and Deng,T. (2001a). Activation of prostate-specific antigen precursor (pro-PSA) by prostin, a novel human prostatic serine protease identified by degenerate PCR. Biochemistry *40*, 1679-1687.

Takayama,T.K., Fujikawa,K., and Davie,E.W. (1997). Characterization of the precursor of prostate-specific antigen. Activation by trypsin and by human glandular kallikrein. J. Biol. Chem. *272*, 21582-21588.

Takayama,T.K., McMullen,B.A., Nelson,P.S., Matsumura,M., and Fujikawa,K. (2001b). Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. Biochemistry *40*, 15341-15348.

Tanimoto,H., Underwood,L.J., Shigemasa,K., Parmley,T.H., and O'Brien,T.J. (2001). Increased expression of protease M in ovarian tumors. Tumour Biol. *22*, 11-18.

Teneriello,M.G., Ebina,M., Linnoila,R.I., Henry,M., Nash,J.D., Park,R.C., and Birrer,M.J. (1993). p53 and Ki-ras gene mutations in epithelial ovarian neoplasms. Cancer Res. *53*, 3103-3108.

Tornos,C., Silva,E.G., Khorana,S.M., and Burke,T.W. (1994). High-stage endometrioid carcinoma of the ovary. Prognostic significance of pure versus mixed histologic types. Am. J. Surg. Pathol. *18*, 687-693.

Tsuchiya,A., Sakamoto,M., Yasuda,J., Chuma,M., Ohta,T., Ohki,M., Yasugi,T., Taketani,Y., and Hirohashi,S. (2003). Expression profiling in ovarian clear cell carcinoma: identification of hepatocyte nuclear factor-1 beta as a molecular marker and a possible molecular target for therapy of ovarian clear cell carcinoma. Am. J. Pathol. *163*, 2503-2512.

Tsukuda,K., Tanino,M., Soga,H., Shimizu,N., and Shimizu,K. (2000). A novel activating mutation of the K-ras gene in human primary colon adenocarcinoma. Biochem Biophys. Res. Commun. *278*, 653-658.

Tsunoda,K., Ninomiya,K., Hozaki,F., and Kaga,K. (2003). Anaphylaxis in a child playing in tall grass. Allergy *58*, 955-956.

Tworoger,S.S., Gertig,D.M., Gates,M.A., Hecht,J.L., and Hankinson,S.E. (2008). Caffeine, alcohol, smoking, and the risk of incident epithelial ovarian cancer. Cancer *112*, 1169-1177.

Underwood,L.J., Tanimoto,H., Wang,Y., Shigemasa,K., Parmley,T.H., and O'Brien,T.J. (1999). Cloning of tumor-associated differentially expressed gene-14, a novel serine protease overexpressed by ovarian carcinoma. Cancer Res. *59*, 4435-9.

Urick,M.E. and Johnson,P.A. (2006). Cyclooxygenase 1 and 2 mRNA and protein expression in the Gallus domesticus model of ovarian cancer. Gynecol. Oncol. *103*, 673-678.

van Haaften-Day,C., Shen,Y., Xu,F., Yu,Y., Berchuck,A., Havrilesky,L.J., de Bruijn,H.W., Van Der Zee,A.G., Bast,R.C., Jr., and Hacker,N.F. (2001). OVX1, macrophage-colony stimulating factor, and CA-125-II as tumor markers for epithelial ovarian carcinoma: a critical appraisal. Cancer *92*, 2837-44.

van Niekerk,C.C., Boerman,O.C., Ramaekers,F.C., and Poels,L.G. (1991). Marker profile of different phases in the transition of normal human ovarian epithelium to ovarian carcinomas. Am. J. Pathol. *138*, 455-463.

Vang,R., Shih,I., and Kurman,R.J. (2009). Ovarian low-grade and high-grade serous carcinoma: pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems. Adv. Anat. Pathol. *16*, 267-282.

Veveris-Lowe,T.L., Lawrence,M.G., Collard,R.L., Bui,L., Herington,A.C., Nicol,D.L., and Clements,J.A. (2005). Kallikrein 4 (hK4) and prostate-specific antigen (PSA) are associated with the loss of E-cadherin and an epithelial-mesenchymal transition (EMT)-like effect in prostate cancer cells. Endocr. Relat Cancer. *12*, 631-643.

Werle,E. (1934). Zur Kenntnis des haushalts des Kallikreins. Biochem Z *269*, 415-134.

Werness,B.A. and Eltabbakh,G.H. (2001). Familial ovarian cancer and early ovarian cancer: Biologic, pathologic, and clinical features. Int. J. Gynecol. Pathol. *20*, 48-63.

White,N.M., Bui,A., Mejia-Guerrero,S., Chao,J., Soosaipillai,A., Youssef,Y., Mankaruos,M., Honey,R.J., Stewart,R., Pace,K.T., Sugar,L., Diamandis,E.P., Dore,J., and Yousef,G.M. (2010a). Dysregulation of kallikrein-related peptidases in renal cell carcinoma: potential targets of miRNAs. Biol. Chem. *391*, 411-423.

White,N.M., Chow,T.F., Mejia-Guerrero,S., Diamandis,M., Rofael,Y., Faragalla,H., Mankaruous,M., Gabril,M., Girgis,A., and Yousef,G.M. (2010b). Three dysregulated miRNAs control kallikrein 10 expression and cell proliferation in ovarian cancer. Br. J. Cancer *102*, 1244-1253.

White,N.M., Mathews,M., Yousef,G.M., Prizada,A., Popadiuk,C., and Dore,J.J. (2009). KLK6 and KLK13 predict tumor recurrence in epithelial ovarian carcinoma. Br. J. Cancer *101*, 1107-1113.

Whitley,B.R., Beaulieu,L.M., Carter,J.C., and Church,F.C. (2007). Phosphatidylinositol 3-kinase/Akt regulates the balance between plasminogen activator inhibitor-1 and urokinase to promote migration of SKOV-3 ovarian cancer cells. Gynecol. Oncol. *104*, 470-479.

Whittemore,A.S., Harris,R., and Itnyre,J. (1992). Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. IV. The pathogenesis of epithelial ovarian cancer. Collaborative Ovarian Cancer Group. Am. J. Epidemiol. *136*, 1212-1220.

Wong,A.S. and Auersperg,N. (2002). Normal Ovarian Surface Epithelium. 161-183.

Wong,A.S., Maines-Bandiera,S.L., Rosen,B., Wheelock,M.J., Johnson,K.R., Leung,P.C., Roskelley,C.D., and Auersperg,N. (1999). Constitutive and conditional cadherin expression in cultured human ovarian surface epithelium: influence of family history of ovarian cancer. Int. J. Cancer *81*, 180-188.

Wooster,R., Bignell,G., Lancaster,J., Swift,S., Seal,S., Mangion,J., Collins,N., Gregory,S., Gumbs,C., Micklem,G., Barfoot,R., Hamoudi,R., Patel,S., Rice,C., Biggs,P., Hashim,Y., Smith,A., Connor,F., Arason,A., Gudmundsson,J., Ficenec,D., Kelsell,D., Ford,D., Tonin,P., Bishop,D.T., Spurr,N.K., Ponder,B.A.J., Eeles,R., Peto,J., Devilee,P., Cornelisse,C., Lynch,H., Narod,S., Lenoir,G., Egilsson,V., Barkadottir,R.B., Easton,D.F., Bentley,D.R., Futreal,P.A.,

Ashworth,A., and Stratton,M.R. (1995). Identification of the Breast-Cancer Susceptibility Gene BRCA2. Nature *378*, 789-792.

Wright,J.W., Pejovic,T., Fanton,J., and Stouffer,R.L. (2008). Induction of proliferation in the primate ovarian surface epithelium in vivo. Hum. Reprod. *23*, 129-138.

Xu,F.J., Yu,Y.H., Li,B.Y., Moradi,M., Elg,S., Lane,C., Carson,L., and Ramakrishnan,S. (1991). Development of two new monoclonal antibodies reactive to a surface antigen present on human ovarian epithelial cancer cells. Cancer Res. *51*, 4012-4019.

Yager,J.D. (2000). Endogenous estrogens as carcinogens through metabolic activation. J. Natl. Cancer Inst. Monogr. 67-73.

Yamashiro,K., Tsuruoka,N., Kodama,S., Tsujimoto,M., Yamamura,Y., Tanaka,T., Nakazato,H., and Yamaguchi,N. (1997). Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. Biochim. Biophys. Acta *1350*, 11-14.

Yanaihara,N., Caplen,N., Bowman,E., Seike,M., Kumamoto,K., Yi,M., Stephens,R.M., Okamoto,A., Yokota,J., Tanaka,T., Calin,G.A., Liu,C.G., Croce,C.M., and Harris,C.C. (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell *9*, 189-198.

Yang,L., Klint,A., Lambe,M., Bellocco,R., Riman,T., Bergfeldt,K., Persson,I., and Weiderpass,E. (2008). Predictors of ovarian cancer survival: A population-based prospective study in Sweden. Int. J. Cancer *123*, 672-679.

Ye,B., Skates,S., Mok,S.C., Horick,N.K., Rosenberg,H.F., Vitonis,A., Edwards,D., Sluss,P., Han,W.K., Berkowitz,R.S., and Cramer,D.W. (2006). Proteomic-based discovery and characterization of glycosylated eosinophil-derived neurotoxin and COOH-terminal osteopontin fragments for ovarian cancer in urine. Clin. Cancer Res. *12*, 432-441.

Yoshida,K. and Miki,Y. (2004). Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. Cancer Sci. *95*, 866-871.

Yoshida,S., Taniguchi,M., Hirata,A., and Shiosaka,S. (1998a). Sequence analysis and expression of human neuropsin cDNA and gene. Gene *213*, 9-16.

Yoshida,S., Taniguchi,M., Suemoto,T., Oka,T., He,X., and Shiosaka,S. (1998b). cDNA cloning and expression of a novel serine protease, TLSP. Biochim. Biophys. Acta. *%20;1399*, 225-228.

Young,R.H. and Scully,R.E. (1985). Ovarian Sertoli-Leydig cell tumors. A clinicopathological analysis of 207 cases. Am. J. Surg. Pathol. *9*, 543-569.

Young,R.H. and Scully,R.E. (1988). Ovarian sex cord-stromal tumors. Problems in differential diagnosis. Pathol. Annu. *23 Pt 1*, 237-296.

Yousef,G. and Diamandis,E.P. (2003). Human kallikreins: Common structural features, sequence analysis and evolution. Current Genomics *4*, 147-165.

Yousef,G.M., Borgono,C.A., Michael,I.P., and Diamandis,E.P. (2004a). Cloning of a kallikrein pseudogene. Clin. Biochem. *37*, 961-967.

Yousef,G.M., Chang,A., and Diamandis,E.P. (2000a). Identification and characterization of KLK-L4, a new kallikrein-like gene that appears to be down-regulated in breast cancer tissues. J. Biol. Chem. *275*, 11891-11898.

Yousef,G.M., Chang,A., Scorilas,A., and Diamandis,E.P. (2000b). Genomic organization of the human kallikrein gene family on chromosome 19q13.3-q13.4. Biochem. Biophys. Res. Commun. *276*, 125-133.

Yousef,G.M. and Diamandis,E.P. (1999). The new kallikrein-like gene, KLK-L2. Molecular characterization, mapping, tissue expression, and hormonal regulation. J. Biol. Chem. *274*, 37511-6.

Yousef,G.M. and Diamandis,E.P. (2000). The expanded human kallikrein gene family: locus characterization and molecular cloning of a new member, KLK-L3 (KLK9). Genomics *65*, 184-194.

Yousef,G.M. and Diamandis,E.P. (2001). The new human tissue kallikrein gene family: structure, function, and association to disease. Endocr. Rev. *22*, 184-204.

Yousef,G.M., Kapadia,C., Polymeris,M.E., Borgono,C., Hutchinson,S., Wasney,G.A., Soosaipillai,A., and Diamandis,E.P. (2003a). The human kallikrein protein 5 (hK5) is enzymatically active, glycosylated and forms complexes with two protease inhibitors in ovarian cancer fluids. Biochim. Biophys. Acta *1628*, 88-96.

Yousef,G.M., Kishi,T., and Diamandis,E.P. (2003b). Role of kallikrein enzymes in the central nervous system. Clin. Chim. Acta. *329*, 1-8.

Yousef,G.M., Luo,L.Y., Scherer,S.W., Sotiropoulou,G., and Diamandis,E.P. (1999a). Molecular characterization of zyme/protease M/neurosin (PRSS9), a hormonally regulated kallikrein-like serine protease. Genomics *62*, 251-9.

Yousef,G.M., Magklara,A., Chang,A., Jung,K., Katsaros,D., and Diamandis,E.P. (2001a). Cloning of a new member of the human kallikrein gene family, KLK14, which is down-regulated in different malignancies. Cancer Res. *61*, 3425-3431.

Yousef,G.M., Magklara,A., and Diamandis,E.P. (2000c). KLK12 is a novel serine protease and a new member of the human kallikrein gene family-differential expression in breast cancer. Genomics. *69*, 331-341.

Yousef,G.M., Obiezu,C.V., Jung,K., Stephan,C., Scorilas,A., and Diamandis,E.P. (2002a). Differential expression of Kallikrein gene 5 in cancerous and normal testicular tissues. Urology *60*, 714-8.

Yousef,G.M., Obiezu,C.V., Luo,L.Y., Black,M.H., and Diamandis,E.P. (1999b). Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. Cancer Res. *59*, 4252-4256.

Yousef,G.M., Obiezu,C.V., Luo,L.Y., Magklara,A., Borgono,C.A., Kishi,T., Memari,N., Michael,P., Sidiropoulos,M., Kurlender,L., Economopolou,K., Kapadia,C., Komatsu,N., Petraki,C., Elliott,M., Scorilas,A., Katsaros,D., Levesque,M.A., and Diamandis,E.P. (2005). Human tissue kallikreins: from gene structure to function and clinical applications. Adv. Clin. Chem. *39:11-79.*, 11-79.

Yousef,G.M., Polymeris,M.E., Grass,L., Soosaipillai,A., Chan,P.C., Scorilas,A., Borgono,C., Harbeck,N., Schmalfeldt,B., Dorn,J., Schmitt,M., and Diamandis,E.P. (2003c). Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res. *63*, 3958-65.

Yousef,G.M., Polymeris,M.E., Yacoub,G.M., Scorilas,A., Soosaipillai,A., Popalis,C., Fracchioli,S., Katsaros,D., and Diamandis,E.P. (2003d). Parallel overexpression of seven kallikrein genes in ovarian cancer. Cancer Res. *63*, 2223-7.

Yousef,G.M., Scorilas,A., and Diamandis,E.P. (2000d). Genomic organization, mapping, tissue expression, and hormonal regulation of trypsin-like serine protease (TLSP PRSS20), a new member of the human kallikrein gene family. Genomics *63*, 88-96.

Yousef,G.M., Scorilas,A., Jung,K., Ashworth,L.K., and Diamandis,E.P. (2001b). Molecular cloning of the human kallikrein 15 gene (KLK15). Up-regulation in prostate cancer. J. Biol. Chem. *276*, 53-61.

Yousef,G.M., Scorilas,A., Katsaros,D., Fracchioli,S., Iskander,L., Borgono,C., Rigault De La Longrais IA, Puopolo,M., Massobrio,M., and Diamandis,E.P. (2003e). Prognostic value of the human kallikrein gene 15 expression in ovarian cancer. J. Clin. Oncol. *21*, 3119-3126.

Yousef,G.M., Scorilas,A., Kyriakopoulou,L.G., Rendl,L., Diamandis,M., Ponzone,R., Biglia,N., Giai,M., Roagna,R., Sismondi,P., and Diamandis,E.P. (2002b). Human kallikrein gene 5 (KLK5) expression by quantitative PCR: an independent indicator of poor prognosis in breast cancer. Clin. Chem. *48*, 1241-50.

Yousef,G.M., Scorilas,A., Magklara,A., Memari,N., Ponzone,R., Sismondi,P., Biglia,N., Abd,E.M., and Diamandis,E.P. (2002c). The androgen-regulated gene human kallikrein 15 (KLK15) is an independent and favourable prognostic marker for breast cancer. Br. J. Cancer. *87*, 1294-1300.

Yousef,G.M., Scorilas,A., Magklara,A., Soosaipillai,A., and Diamandis,E.P. (2000e). The KLK7 (PRSS6) gene, encoding for the stratum corneum chymotryptic enzyme is a new member of the human kallikrein gene family - genomic characterization, mapping, tissue expression and hormonal regulation. Gene *254*, 119-28.

Yousef,G.M., Scorilas,A., Nakamura,T., Ellatif,M.A., Ponzone,R., Biglia,N., Maggiorotto,F., Roagna,R., Sismondi,P., and Diamandis,E.P. (2003f). The prognostic value of the human kallikrein gene 9 (KLK9) in breast cancer. Breast Cancer Res. Treat. *78*, 149-58.

Yousef,G.M., Yacoub,G.M., Polymeris,M.E., Popalis,C., Soosaipillai,A., and Diamandis,E.P. (2004b). Kallikrein gene downregulation in breast cancer. Br. J. Cancer *90*, 167-72.

Yu,H., Diamandis,E.P., Levesque,M., Giai,M., Roagna,R., Ponzone,R., Sismondi,P., Monne,M., and Croce,C.M. (1996). Prostate specific antigen in breast cancer, benign breast disease and normal breast tissue. Breast Cancer Res. Treat. *40*, 171-178.

Zeeuwen,P.L. (2004). Epidermal differentiation: the role of proteases and their inhibitors. Eur. J. Cell Biol. *83*, 761-773.

Zhang,L., Volinia,S., Bonome,T., Calin,G.A., Greshock,J., Yang,N., Liu,C.G., Giannakakis,A., Alexiou,P., Hasegawa,K., Johnstone,C.N., Megraw,M.S., Adams,S., Lassus,H., Huang,J., Kaur,S., Liang,S., Sethupathy,P., Leminen,A., Simossis,V.A., Sandaltzopoulos,R., Naomoto,Y., Katsaros,D., Gimotty,P.A., DeMichele,A., Huang,Q., Butzow,R., Rustgi,A.K., Weber,B.L., Birrer,M.J., Hatzigeorgiou,A.G., Croce,C.M., and Coukos,G. (2008). Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. Proc. Natl. Acad. Sci. U. S A *105*, 7004-7009.

Chapter 2 Human Kallikrein-Related Peptidase 13 (KLK13) is Involved in Cellular Migration and Invasion

A version of this chapter is currently being prepared as a manuscript.

2.1 Introduction

The lethality of ovarian cancer is partially credited to it being relatively asymptomatic in the early stages and its rapid metastatic spread intraperitoneally (Scarberry *et al.*, 2010). The presence of malignant cells in ascites is often an indicator of poor prognosis for the majority of patients (Curtin *et al.*, 1997;Griffiths *et al.*, 1979). Malignant cells with metastatic potential can metastasize to distant sites and form new tumors. Survival rates for women with metastatic ovarian cancer are estimated at 15-20% over five years (Schink, 1999).

A current working model for the metastatic process of ovarian carcinoma suggests that cancer cells are shed from the ovarian tumor into the peritoneal cavity and attach to the layer of mesothelial cells that line the inner surface of the peritoneum (Sawada *et al.*, 2007). Cancer cells invade the superficial layers of abdominal organs and may then spread to retroperitoneal lymph nodes and the pleural cavity (Auersperg *et al.*, 2001;Cannistra *et al.*, 1993). The invasiveness of cells depends in part on their ability to degrade basement membrane extracellular matrix (ECM), which presumably depends on the cellular production of proteolytic enzymes.

Proteolytic enzymes are involved in tumorigenesis as they can facilitate migratory, invasive and tissue remodelling events (Pepper, 2001;Tarui *et al.*, 2002). Serine proteases of the plasminogen/plasmin family, for example, have been shown to play an active role in these processes (Mignatti and Rifkin, 1993). Identifying the key proteases and their roles in the metastatic process may contribute to a better understanding of the

mechanisms involved and the development of novel diagnostic and therapeutic approaches (Xu *et al.*, 2009).

Recently, a family of serine proteases, the human kallikrein-related peptidase (KLK) family, has been implicated in tumorigenesis and metastasis of ovarian cancer (Emami and Diamandis, 2008). This family of genes consists of 15 members, of which prostate specific antigen, (PSA/KLK3), is best known, as it is widely used as a marker for prostate cancer (Stephan *et al.*, 2007). Recent data point to an important role of specific KLKs in several other malignancies, including those of the gastrointestinal tract, lung, brain, and head and neck cancers (Emami and Diamandis, 2008). In addition to their role as potential biomarkers for malignancy, KLKs have been shown to be involved in carcinogenesis on a molecular level. KLK3 and KLK4 have been shown to promote cell migration of a prostate cancer cell line while inducing epithelial to mesenchymal transition (EMT)-like effects such as loss of E-cadherin expression and increased vimentin expression (Veveris-Lowe *et al.*, 2005). Additionally, KLK6 has also been shown to be involved in the migration and invasion of skin and colon cancer cells as well as degrade components of the ECM (Ghosh *et al.*, 2004;Henkhaus *et al.*, 2008;Klucky *et al.*, 2007).

Another member of the family, KLK13, has recently been implicated in carcinogenesis (Yousef *et al.*, 2000), in addition to its increased expression in breast (Chang *et al.*, 2002), lung (Planque *et al.*, 2008), and salivary gland cancers (Darling *et al.*, 2006). KLK13 shows increased expression in ovarian cancer patients (Kapadia *et al.*,

2003;Scorilas *et al.*, 2004) and is capable of degrading components of the extracellular matrix *in vitro*, including collagen, fibronectin, and laminin (Kapadia *et al.*, 2004). Also, migration of cells through a Boyden chamber assay was decreased when cells were treated with an anti-KLK13 antibody. These data together, like KLK6, suggests KLK13 may be involved in tumor cell migration and invasion.

Interestingly, the mature KLK13 protein has considerable *N*-linked glycosylation. When treated with peptide:*N*-glycosidase-F (PNGaseF), an amidase that removes *N*-linked oligosaccharides, a decrease in KLK13 molecular weight was observed following sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE; (Sotiropoulou *et al.*, 2003)]. Many tumor markers used in clinics are serum glycoproteins and detection of altered glycans in serum tumor glycoproteins may be useful to increase specificity in tumor detection (Peracaula *et al.*, 2008). Aberrant glycosylation has been detected in the secreted proteins present in serum, including PSA/KLK3 (Peracaula *et al.*, 2003), although there has been no reported biological significance related to KLK13 glycosylation.

The current study hypothesized the involvement of KLK13 in ovarian epithelial cell cellular migration and invasion. Using an ovarian cell model that has low endogenous KLK13 expression, mutant forms of KLK13 were introduced to elucidate the role of KLK13 on cellular migration and invasion. Infection of the immortalized ovarian surface epithelial cell (IOSE) model with a KLK13-wild type virus increased both cellular migration and invasion. A similar effect was seen in another epithelial cell line, Mv1Lu.

Conversely, when the expression of KLK13 was decreased in a cell line with high endogenous KLK13 expression, the SKOV-3 cell line, cellular migration was decreased. These data together implicate KLK13 may play a role in ovarian cancer pathogenesis.

2.2 Materials and methods

2.2.1. Cell culture

Immortalized ovarian surface epithelial cells (IOSE-398) were a kind gift from Dr. Nelly Auersperg (Canadian Ovarian Tissue Bank, Vancouver, BC) and were maintained in 1:1 of 199:MCDB105 media with 5% fetal calf serum (FCS) and 50µg/mL gentamicin. Ovarian cancer cell lines, CAOV-3, OVCAR-3, and SKOV-3, as well as the Mv1Lu cell line were purchased from the American Type Culture Collection (ATCC; Manassas, VA). CAOV-3, OVCAR-3, and SKOV-3 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, RPMI 1640 supplemented with 20% FCS and 0.01 mg/mL bovine insulin, or McCoy's 5a medium supplemented with 10% FCS, respectively. The Mv1Lu cell line was maintained in DMEM supplemented with 10% FCS and IX non-essential amino acids (NEAA). The GP2-293 packaging cell line (Clontech, Mountain View, CA) was maintained in DMEM supplemented with 10% FCS in DMEM. All cells were incubated in 5% CO_2 at 37°C.

2.2.2. Northern blot

Total RNA was extracted from IOSE-398, CAOV-3, OVCAR-3 and SKOV-3 ovarian cancer cell lines using Trizol (Invitrogen, Carlsband, CA), according to the manufacture's protocol. Total RNA concentration was determined spectrophotometrically at 260nm and samples were stored at -80°C. Total RNA (50µg) was precipitated by adding two

equivalent volumes of ethanol and 1.5M potassium acetate to 2%. Samples were centrifuged for 15 minutes at 12,000g at 4°C. Samples were then re-suspended in sample buffer [50% (v/v) formamide, 10% (v/v) formaldehyde, 1X 3-(N-morpholino) propanesulfonic acid (MOPS), pH 8.0] and separated on a denaturing formaldehyde gel, transferred to a nylon membrane (Hybond-XL, Amersham Biosciences, Piscataway, NJ) and crosslinked by ultra violet irradiation (Ultraviolet Crosslinker, UltraLum Claremont, CA). The membrane was washed in a pre-hybridization solution [10% formamide, 1X SSPE (150mM NaCl, 10mM NaH_2PO_4-H_20, and 10mM Na_2EDTA), 0.1% (w/v) sodium dodecyl sulphate (SDS), 5X Dernhardt's, 1μg/μL single stranded, sheared, salmon sperm DNA] for 4 hours at 42°C.

A 128bp probe, specific to KLK13, recognizing all eight KLK13 splice variants, was synthesized by polymerase chain reaction (PCR). The reaction was set as follows: 1X buffer, 200μM each of dNTP, 25μM of KLK13-Forward primer 5'- act tcc cat gtg ggc aac ct – 3', 25μM of KLK13-Reverse primer 5`- tta ttg tgg gcc ctt caa cc – 3', and 2.5U HotStar Taq (Qiagen, Mississauga, ON), with 100ng KLK13 cDNA in the pcDNA™3.1D/V5-His-TOPO® cloning vector. The PCR cycling conditions were as follows: 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute, followed by a final 10 minute extension step at 72°C. In order to confirm the KLK13 generated probe was the KLK13 sequence, it was run on an agarose gel. The only template DNA in the reaction was KLK13 and the displayed band was the expected fragment size of 128bp, confirming the probe was in fact KLK13.

Reaction products were separated on a 1% Nuseive (Lonza, Rockland, ME) agarose gel. The gel portion containing the DNA band was excised and ethanol precipitated.

This purified PCR product was then used to create the radioactive-labelled probe in a separate PCR reaction. The reaction was set up as follows: 1X buffer, 200μM each of dATP, dTTP, dGTP, 200μM of α-^{32}P-dCTP, 25μM of KLK13-forward primer, 25μM of KLK13-reverse primer, and 2.5U HotStar Taq (Qiagen, Valencia, CA), with 200ng of template DNA. The PCR cycling conditions were as follows: 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for one minute, followed by a final 10 minute extension step at 72°C. The radioactive-labeled probe was then purified through a Micro Bio-Spin 6 Exclusion Column (Bio-Rad, Hercules, CA). The amount of radioactivity incorporated into the probe was determined using the Beckman-Coulter LS 6500 Multi-Purpose Scintillation Counter (Beckman-Coulter, Fullerton, CA) and 6 x 10^6 cpm/mL of probe was added to hybridization solution (same as pre-hybridization) and hybridized to the membrane in a rotisserie overnight at 42°C.

The next day, the membrane was subjected to washes with increasing stringency; washed twice in 5X SSPE/0.1% SDS at 42°C for 5 min, followed by one wash in 1X SSPE/0.1% SDS at 42°C, and 2 washes in 0.2X SSPE/0.1% SDS at 65°C. Autoradiographs were obtained by exposing Kodak AR5 Film (GE Healthcare, Baie d'Urfe, Quebec) at -80°C using a Cronex intensifying screen.

2.2.3. Quantitative real time PCR

Total RNA was extracted and quantified as described previously. CDNA was synthesized using 6μg total RNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using random hexamers. Quantitative polymerase chain reaction was performed using 1μl of cDNA with the ABI Prism 7000 (Applied Biosystems, Foster City, CA). Primer/probe sets were purchased as pre-made TaqMan Assays on Demand for *KLK13* and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase). Target gene expression was normalized to endogenous *GAPDH* and the IOSE cell line. Thermal cycling conditions were according to the manufacture's protocol and all samples were analyzed in triplicate. Relative quantification, the amount of target, normalized to an endogenous control, was determined using the ΔΔ threshold cycle (C_T) method (Pfaffl, 2001).

2.2.4. Western blot

Cells were lysed using RIPA Buffer [1X PBS (phosphate-buffered saline), 1% w/v Triton X-100, 0.1% (w/v) SDS, 50mM sodium fluoride, 50mM glycerol phosphate, 50mM Tris-HCL (pH 7.6), 0.5% (w/v) deoxycholate, 1X protease inhibitor (complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail tablets (Roche Applied Science, Laval, QC), 5mM EDTA (pH 7.6), 50μM orthovanadate, and 50 ng/mL phenylmethylsulphonyl fluoride (PMSF)] and cleared by centrifugation at 21,000 x g for 10 minutes at 4°C. Protein concentration was determined with the BCA (bicinchoninic acid) Protein Assay Reagent (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as a standard. 5 X sample buffer (5% SDS, 10% glycerol, 5% β-

mercaptoethanol, 0.15M Tris-HCl pH6.8, and 0.01% bromophenol blue) was diluted in total protein to a final concentration of 1X and then incubated 100°C for five minutes.

Total protein was separated on a 10% SDS-PAGE gel and transferred to a polyvinylidine difluoride (PVDF) membrane for western blot analysis of KLK13, or nitrocellulose membrane for the detection of V5 (Millipore Corporation, Bedford, MA). Fifty micrograms per lane were used to determine KLK13 expression, while 75µg total protein per gel lane were used to determine V5 expression. The membranes were then blocked with 5% non-fat milk in TBST [150mM NaCl, 10mM Tris-HCl pH 8.0, and 0.1% (v/v) Tween 20] and incubated with anti-KLK13 primary antibody (1 µg/mL, R&D Systems, Minneapolis, MN), or anti-V5 antibody (1:5000, Invitrogen, Carlsband, CA) diluted in blocking solution and incubated overnight at 4°C with shaking. Membranes were then washed with four changes of TBST for 10 minutes each followed by incubating with anti-mouse horseradish peroxidase-conjugated secondary antibody diluted in blocking solution for one hour, and washed with four changes of TBST for 10 minutes. Immune complexes were visualized using enhanced chemiluminescence (ECL; Pierce Company, Rockford, IL) and exposing to Hyper Film (GE Healthcare, Piscataway, NJ) for 10 minutes and film development in an automatic film processor (Mini Medical Series, AFP Imaging, Elmsford, NY). In order to re-probe the membranes, they were incubated in stripping buffer [6mM Tris-HCl, pH 6.8, 2% (v/v) SDS, and 0.01% (v/v) β-mercaptoethanol] at 50°C for 10 minutes. Membranes were then blocked and washed as described above and re-probed for α-tubulin (1:1000, Cell Signalling Technology Inc, Danvers, MA) as a loading control.

2.2.5. De-glycosylation of KLK13

In order to confirm KLK13 was in fact glycosylated, we incubated 100μg of total cell lysate with PNGaseF (New England Biolabs, Ipswich, MA) as per the manufacture's instructions. PNGase F is an amidase that cleaves between the innermost *N*-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins. Briefly, total protein was incubated in 1X denaturing buffer at 100°C for 10 minutes. Once denatured, the protein was then incubated with 1X G7 reaction buffer and 10% NP-40 at 37°C for one hour. In order to see a decrease in molecular weight and to confirm KLK13 is glycosylated, we loaded 100μg total protein on an SDS-PAGE gel and western blotted for KLK13.

2.2.6. Vector constructs and site-directed mutagenesis

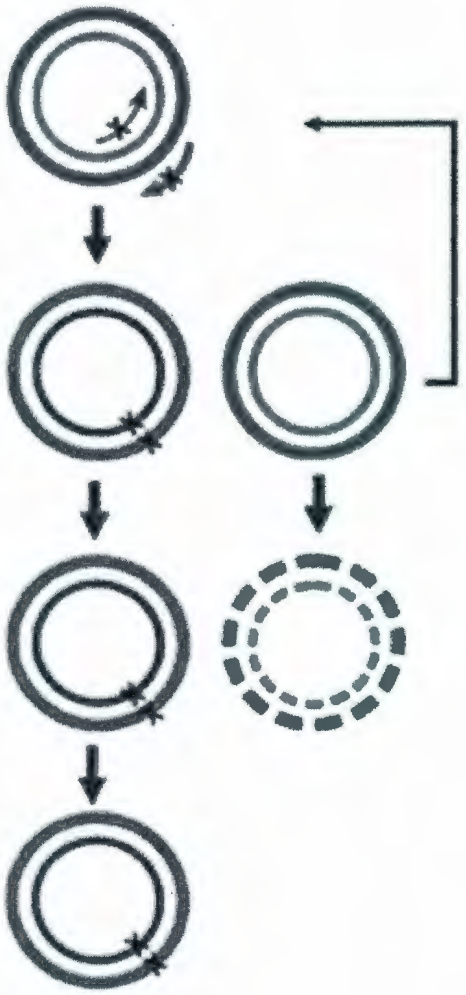
In order to look more closely at the specific role KLK13 plays in ovarian cancer, we created several site-specific mutant KLK13 proteins. The KLK13 gene was in the pcDNA™3.1D/V5-His-TOPO® cloning vector with a V5 tag. The five described mutant KLK13 genes were created using site-directed mutagenesis. In a 50μL site-directed mutagenesis reaction was; 1U Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland), 25μM forward primer, 25μM reverse primer, 1X HF Buffer (Finnzymes, Espoo, Finland), 100ng pcKLK13 template DNA. Site-directed mutagenesis primer sequences are shown in Table 2.1 and a schematic of the site-directed mutagenesis protocol is seen in Figure 2.1. In order to create the double glycosylation mutant, KLK13-N30Q+N225Q, we took the pc-KLK13-N30Q as the template and mutated the second glycosylation site. Reactions were cycled through 98°C for one minute, 55°C for

Table 2.1 Primer sequences for KLK13 site-directed mutagenesis.

Mutation	Primer	Sequence
S218A[1]	Forward	5' – ggt gac **gct** ggg ggc ccc ctg gtc tgt aac aga a – 3'
	Reverse	5' – gcc ccc **agc** gtc acc ctc aca gga gtc ttt gcc – 3'
K25Q[2]	Forward	5' – tct tcc **cag** gtt ctc aac acc aat ggg acc agt g – 3'
	Reverse	5' – cct cca cag agg **gtc** ctc aga agg gtc caa gag – 3'
N30Q[3]	Forward	5' – aac acc **caa** ggg acc agt ggg ttt ctc cca – 3'
	Reverse	5' – ggt ccc **ttg** ggt gtt gag aac ctt gga aga ct – 3'
N225Q[4]	Forward	5' – gtc tgt **caa** aga aca ctg tat ggc atc gtc t – 3'
	Reverse	5' – tgt tct **ttg** aca gac cag ggg gcc ccc –3`

Site-directed mutations are bolded.

1. Serine (tct) was mutated to alanine (gct)
2. Lysine (aag) was mutated to glutamine (cag)
3. Asparagine (aat) was mutated to glutamine (caa)
4. Asparagine (aac) was mutated to glutamine (caa)



1. Mutant strand synthesis.
   Thermal cycling will:
   - denature DNA template
   - anneal metagenic primers containing the desired mutation
   - extend and incorporate primers with high fidelity polymerase

2. DpnI digestion of template
   Parental methylated DNA will be digested

3. Transformation
   Transform mutated plasmid into competent cells

Figure 2.1 Schematic of the site-directed mutagenesis protocol. The mutant plasmid is created by thermal cycling with mutagenic primers. After the PCR, parental methylated and hemimethylated DNA is digested with DpnI. The plasmid harbouring the desired mutation is then transformed into competent cells for nick repair and amplification.

one minute, and ten minutes at 72°C, for 18 cycles. Reactions were then incubated for one hour at 37°C with Dpn1. Dpn1 is a nuclease that cleaves methylated DNA, ensuring that the parental template DNA produced in bacteria was digested and only the PCR product, the mutant DNA, was still intact. These mutants were cloned into the viral vector pLNCX2 and corresponding virons were produced using the GP2-293 packaging cell line and the VSV-G coat protein.

Figure 2.2A is a schematic of the KLK13 protein. The first domain, separated by a black line, is the signal/secretion peptide, followed by the activation peptide and finally the mature enzyme. The S denotes the active serine of the catalytic triad at position 218 [S218; (Yousef *et al.*, 2000)]. The red triangles denotes the two putative *N-linked* glycosylation sites at asparagine 30 (N30) and asparagine 225 (N225). These glycosylation sites were determined by *in-silico* analysis using the KLK13 Ref Seq NM_015596.1 and the Center for Biological Sequence Analysis NetNGlyc Prediction software (Gupta *et al.*, 2004;Gupta *et al.*, 2010). The results of this analysis can be seen in Appendix A.

Figure 2.2B describes the KLK13 site-directed mutants. The KLK13 wild type protein is first displayed with the amino acid sequence (tct) of S218 highlighted. The first mutant was KLK13-S218A, in which the active serine at position 218 was replaced with an alanine by mutating the tct (S) to gct (A), creating a potential enzymatically dead KLK13. We also mutated the activation site of the serine protease, the lysine (aag) at position 25 (K25), to a glutamine (cag, Q25), creating a pro-enzyme that potentially cannot be

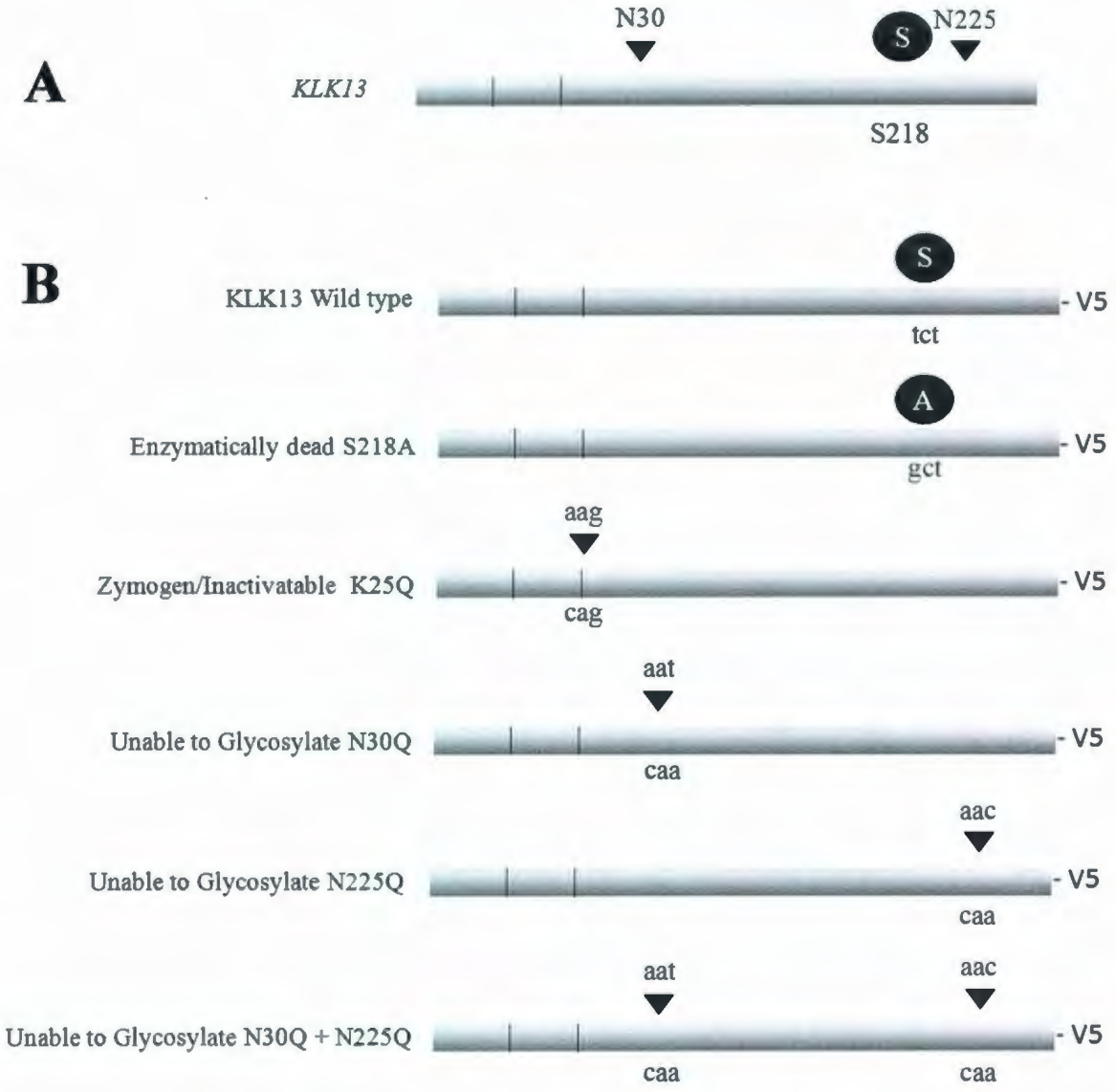


Figure 2.2 Schematic of KLK13 wild type and mutants. (A) Schematic of the KLK13 protein. KLK13 is produced as a pre-propeptide. The signal peptide allows for secretion outside the cell, and once cleaved off, leaves only the propeptide/zymogen form of the protein. Cleavage of the activation peptide at K25 is required for enzyme activation. The active serine of this serine protease is S218. There are two putative glycosylation sites at N30 and N225. (B) KLK13-WT and mutants created by site-directed mutagenesis. The enzymatically active serine at position 218 was mutated to an alanine to create an enzymatically dead KLK13, KLK13-S218A. The lysine at the cleavage site of the activation peptide was mutated to a glutamine to create a KLK13 mutant that cannot be activated, KLK13-K25Q. We also created three glycosylation mutants by mutating asparagine to glutamine at the first (N30Q), second (N225Q), and both (N30Q + N225Q), putative glycosylation sites to create KLK13-N30Q, KLK13-N225Q, and KLK13-N30Q+N225Q, respectively.

activated beyond its zymogen state, KLK13-K25Q. In order to further explore the role of glycosylation on the biological function of KLK13, the two putative glycosylation sites, N30 and N225 we modified to create three mutants with differential *N*-linked glycosylation. The first was KLK13-N30Q, in which we mutated aat (N30) to caa (Q30) in which the protein could possibly only be glycosylated on the second site, N225. The second was KLK13-N225Q, where the aac (N225) was mutated to caa (Q225) in which the protein could only be glycosylated on the first site, N30. The final mutant was KLK13-N30Q+N225Q, in which we mutated both the N30 and N225 to Q30 and Q225, respectively, in which there could be no *N*-linked glycosylation at either of these sites.

2.2.7. Site-directed mutant validation

In order to prepare DNA for sequencing, we transformed 5μg of each plasmid, pc-KLK13-S218A, pc-KLK13-K25Q, pc-KLK13-N30Q, pc-KLK13-N225Q, and pc-KLK13-N30Q+N225Q DNA into 50μL of DH5α Competent E.Coli Cells (New England Biolabs, Ipswich, MA) in separate reactions. DNA and competent cells were gently mixed and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for exactly 50 seconds and placed on ice for two minutes. Cells were allowed to recover in 900μL SOC (New England Biolabs, Ipswich, MA) growth media at 37°C for 60 minutes with shaking (250 rpm). One hundred microliters of the mixture were cells were spread on Luria-Bertani (LB)/Ampicillin (100μg/mL) bacterial plates and allowed to grow at 37°C overnight. The next morning, clones were chosen for DNA extraction and sequencing.

Clones selected from overnight plates were grown up in 2mL LB/Amp broth (100μg/mL ampicillin) overnight at 37°C with shaking (250 rpm). The following morning, plasmid

DNA was extracted using the Qiagen MiniPrep Kit as described by the manufacturer (Qiagen, Valencia, CA). DNA concentration was determined spectrophotometrically at 260nm and was stored at 4°C until required for DNA sequencing.

In order to ensure we had the desired mutations, all clones were sequenced. Sequencing was performed on the ABI Prism 3730 (Applied Biosystems, Foster City, CA) using the following reaction mix: 13.5μL H_2O, 1X sequencing buffer, 0.5μL sequencing mix, 3.8μM forward primer, 3.8μM reverse primer, and 500ng DNA. Sequencing primers are detailed in Table 2.2. The reaction was heated to 96°C for 6 minutes, then 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. DNA was then precipitated overnight at 4°C with 125nM ethylene diaminetetraacetic acid (EDTA) and 95% ethanol. The following day, Hi-Di formamide was added to each sample and the samples were denatured at 96°C for 4 minutes. Samples were then loaded on the sequencer and clones with the desired confirmed mutations were selected. A representative sample of DNA sequencing results can be seen in Appendix B.

2.2.8. Plasmid construction

The wild-type and mutated KLK13 clones were sub-cloned into the pLNCX2 retroviral vector (Clontech, Mountain View, CA) in order to create virus. We chose the pLNCX2 retroviral vector due to its ability to produce retrovirus when in the presence of the coat protein VSV-G, its cytomegalovirus (CMV) promoter, and its eukaryotic antibiotic selection gene, neomycin.

Table 2.2.Primer sequences used to sequence KLK13 mutants.

Mutation	Primer	Sequence
S218A	Forward	5' – cag ccc cca ggt gaa tta– 3'
	Reverse	5' – gga ttg ttt cac gga tcc ac – 3'
K25Q	Forward	5' – gct ggt tta gtg aac cgt cag – 3'
	Reverse	5' – ttg agc ccc tcc ttt aga ca – 3'
N30Q, N225Q, and N30Q+N225Q	Forward	5' – caa cat cca act tcg ctc ag – 3'
	Reverse	5' - ttc aac cat ttt tgc tgc tg –3`

KLK13 was cut from pc-KLK13 at the HindIII and Pme1 sites. The pLNCX2 vector was prepared to insert KLK13 mutants by cutting at HindIII and NotI. All vector and KLK13 insert products were separated on a 1% Nusieve low melting point agarose gel and precipitated with ethanol. The following equation was used to determine the KLK13 insert mass for ligation reactions:

Insert mass (ng) = 3 x (Insert Length/Vector Length) x Vector Mass (ng)

We used 30ng of vector for each reaction and the appropriate amount of insert required was calculated. The empty pLNCX2 vector and KLK13, along with each KLK13 mutant were ligated in separate reactions using 400 units T4 ligase, 1X ligase buffer, and 30ng of cut pLNCX2 overnight at 4°C. We isolated and designated the resultant sub-clones as; pLN-KLK13, pLN-KLK13-S218A, pLN-KLK13-L25Q, pLN-KLK13-N30Q, pLN-KLK13-N225Q, pLN-KLK13-N30Q+N225Q. The empty vector control was the pLNCX2 containing no KLK13 gene.

The GP2-293 packaging cell line was transfected using Lipofectamine 2000 (Invitrogen, Carlsband, CA) with one of each KLK13 retroviral plasmids and the plasmid containing the VSV-G coat protein gene to produce retrovirus as follows. GP2-293 cells were plated at 2.5 x 10^5 cells/well in a 6-well plate. The following day, the media was replaced with serum-free DMEM and cells were co-transfected with 0.75μg of pLN-KLK13-X DNA and 0.75μg of VSV-G DNA using 4.5μL of Lipofectamine in 100μL 1X Opti-MEM® Reduced Serum Medium. The following day, the media was replaced with normal growth media. The cells were allowed to recover for 24 hours and were then allowed to

produce virus for two days. The media containing virus was then collected and filtered through a 0.45μm syringe filter (Pall, Mississauga, ON).

Viral titre was determined using the Mv1Lu cell line. We prepared six ten-fold serial dilutions by first adding 150μL virus to 1.35mL media. We then transferred 150μL of the viral stock to the next tube with 1.35mL media and continued the serial dilutions in this manner. We infected the cells with 1mL virus containing media and a final concentration of 5μg/mL polybrene (hexadimethrine bromide, Sigma-Aldrich, Oakville, ON) in DMEM. Cells were infected overnight and the media was replaced the following day to allow cells to recover for 24 hours. The next ten days, the cells were grown in 900μg/mL G418 sulfate (geneticin, Invitrogen Carlsband, CA) in order to select for infected cells. After antibiotic selection, the viral titre was calculated as the number of colonies present at the highest dilution, multiplied by the dilution factor. For example, the presence of 8 colonies at the 10^5 dilution factor would represent a titre of 8 colony forming units (cfu) x $10^5 = 8 \times 10^5$ cfu/mL.

2.2.9. Viral infection

Both IOSE and Mv1Lu cell lines were plated at 5.0×10^4 cells/well in each well of a 6-well plate and allowed to attach overnight. The next day, cells were infected at a multiplicity of infection (MOI) of 10:1, or 5.0×10^5 cfu/cell, with a final concentration of 5μg/mL polybrene in DMEM and left to infect overnight. The next day, the virus containing media was replaced with normal growth media allowing cells to recover. IOSE cells were then re-plated for migration and invasion assays. Mv1Lu cells on the other hand, were subjected to antibiotic selection in 900μg/mL G418 sulfate (Invitrogen,

Carslband, CA) in DMEM supplemented with 10% FCS for ten days, resulting in a "pool" of selected cells, in which all cells stably carry the selected KLK13 mutant gene.

2.2.10. Wound healing assay

The ability of cells to migrate, or their migratory capacity, was measured by a wound healing assay. Cellular migration of the IOSE infected cells and the Mv1Lu cells that expressed KLK13 wild type or mutant proteins, was measured. The IOSE cells were plated in 6-well plates at 5.0 x 10^4 cells/well and infected as described above. The following day, cells were trypsinized, diluted 2 fold and re-plated in 12-well plates. They were serum depleted with 2% FCS in IOSE media (the lowest FCS concentration that the cells could survive in) in order to reduce proliferation effects on the assay although cell proliferation was not tested. The monolayer of cells was wounded with a 200μL pipette tip, photomicrographs were taken. The rate of migration is displayed as the percent of cell covered area. The percent of cell area covered was determined as (100 - percent cell-free area) where percent cell-free area is defined as [(cell-free $area_{24hrs}$/cell-free $area_{0hrs}$) x 100]. Area was measured using the Image J Software (National Institutes of Health, Bethesda, ME, http://rsbweb.nih.gov/ij/). The cell-free area was selected with the free-hand tool and area was determined by selecting the option "analyze." Cells were infected with each virus in three separate infections and each was analyzed in triplicate. There were six images taken per "wound." Cell-free area was measured at time "0" (time of the wound) and 24 hours later. Mv1Lu cells were plated at 2.0 x 10^5 cells/well in a 12-well plate. Cells were serum depleted with 0.5% FCS in DMEM and the same procedure

was carried out. To ensure photomicrographs were being taken at the exact same place each time, a gridded coverslip was attached to the bottom of each well.

2.2.11. Antisense KLK13 vector construct

In order to assess if a decrease in KLK13 expression would inhibit migration, we created an expression vector to knock down KLK13 expression. In order to create a KLK13-WT Antisense vector, we first digested the pc-KLK13 clone with SpeI and blunted the ends with DNA polymerase I, Large Klenow (New England Biolabs, Ipswich, MA). The plasmid was then cut with HindIII. This created a 200bp KLK13 fragment, with one sticky and one blunt end, which included approximately 30bp of the multiple cloning site of the original cloning vector and the unique KLK13 sequence, including the atg start site.

The KLK13 fragment was then cloned into the pLNCX2 retroviral vector. In order to ensure the KLK13 insert would be inserted in the antisense direction, we first cut the pLNCX2 vector with XhoI and blunted the ends with DNA polymerase I, Large Klenow (New England Biolabs, Ipswich, MA). The DNA was then precipitated, digested with HindIII, and incubated with phosphatase (New England Biolabs, Ipswich, MA), removing 5' phosphate groups, ensuring the ends of the vector didn't re-ligate. This vector, similar to the KLK13 insert, has a sticky and blunt end, so that the KLK13 insert would be ligated in the reverse direction, creating an anti-sense KLK13.

In order to create a control sense KLK13 control vector, we designed the vector so the sticky and blunt ends would ligate in the sense orientation. We digested the pLNCX2

vector with NotI and blunted the ends with Klenow fragment. The DNA product was precipitated and then digested with HindIII and the 5' phosphate groups were removed with phosphatase.

Products were ligated as described previously and the orientations of the KLK13 inserts confirmed by restriction endonuclease digestion. SKOV-3 cells were transfected with 1μg KLK13 Antisense or Sense DNA with 3μL Lipofectamine 2000 in 100μL 1X Opti-MEM® Reduced Serum Medium (Invitrogen, Carlsband, CA). Cells were co-transfected with the pLNCX2 vector containing the green fluorescent protein sequence to ensure suitable transfection efficiency. To ensure KLK13 expression was in fact decreased, cells were lysed and immunoblotted for KLK13. In order to ensure there was no antiviral response, cells were lysed and immunoblotted for oligoadenylate synthase (OAS).

2.2.12. Invasion assay

IOSE cells were infected as described previously for the wound assays with KLK13 wild type (KLK13-WT), KLK13-S218A, KLK13-K25Q, KLK13-N30Q, KLK13-N225Q, KLK13-N30Q+N225Q, and pLNCX2 virus containing media. Ten thousand cells were plated in triplicate in the upper chamber of the Cytoselect™ 96-well Cell Invasion Assay plate (Cell Biolabs, San Diego, CA) in 2% FCS IOSE media. Normal IOSE media, 5% FCS, was placed in the bottom chamber as a chemoattractant. Cells were allowed to invade through the basement membrane for 24 hours at 37°C in 5% CO_2. Following this incubation, cells were dissociated from the underside of the membrane into a clean harvesting tray, by incubating in 150μL of cell detachment buffer at 37°C for 30 minutes. Cells were then lyzed in a 1:75 mixture of CyQuant®GR Dye: 4X Lysis Buffer for 20

minutes at room temperature. 150µL of the mixture was transferred to a 96-well plate suitable for fluorescence measurement and fluorescence was read at an excitation of 480nm and emission of 520nm. The extent of cellular invasion was measured in relative fluorescence units (RFU). This assay was performed in triplicate using three separate viral infections.

2.2.13. Statistical analysis

All statistical analyses were performed with GraphPad Prism 4 Software (La Jolla, CA). One-way ANOVA and post-hoc multiple comparisons (Tukey's) were used to compare differences in mRNA expression, wound healing, and invasion assays. A p-value less than 0.05 was considered significant.

2.3 Results

2.3.1. *KLK13* mRNA is increased in ovarian cancer cell lines

Since there had been several KLK13 transcripts described via *in-silico* analysis and transcripts for KLK13 had been shown to differ between testicular cancer and normal tissue (Chang *et al.*, 2001), the *KLK13* mRNA transcript was examined in ovarian cancer. *KLK13* mRNA expression was examined in the IOSE cell line, as well as three ovarian cancer cell lines, CAOV-3, OVCAR-3, and CAOV-3 by northern blot analysis (Figure 2.3A). A 128bp KLK13-specific probe that could detect all eight splice variants of KLK13 while excluding other KLKs was used for detection. There was one major *KLK13* transcript identified at 1.8Kb. The IOSE cells appeared to have less *KLK13* mRNA expression when compared to the ovarian cancer cell lines. The ovarian cancer cell lines, CAOV-3, OVCAR-3, and SKOV-3, had comparable levels of *KLK13*

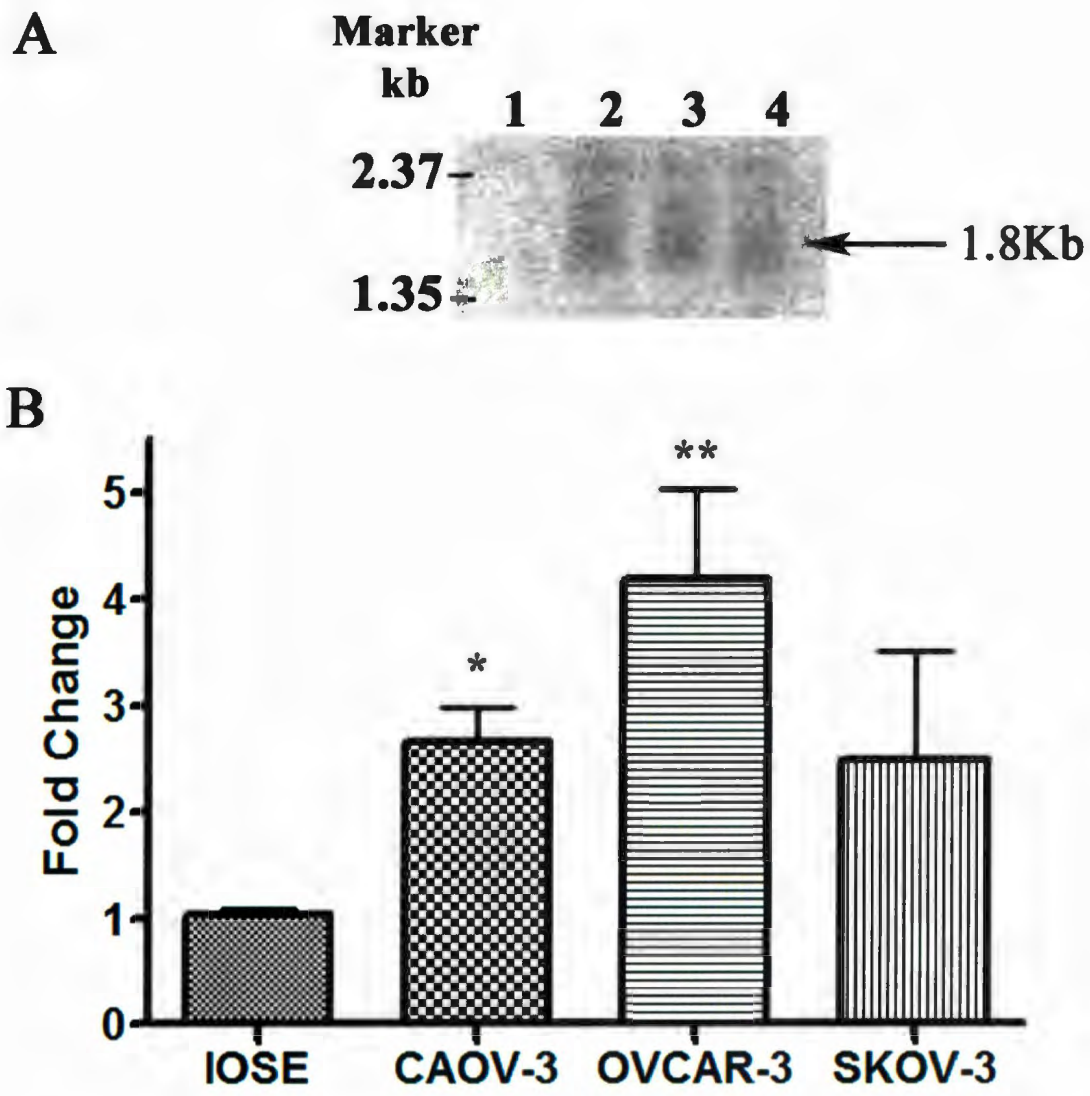


Figure 2.3 *KLK13* mRNA expression in IOSE and ovarian cancer cell lines. (A) Northern blot of *KLK13* mRNA expression in IOSE and ovarian cancer cell lines. 50μg total RNA in Lane 1 (IOSE), Lane 2 (CAOV-3), lane 3 (OVCAR-3), and lane 4 (SKOV-3) was run on an agarose gel and transferred to a nylon membrane. The blot was probed with a KLK13-specific 128bp radioactively labelled probe. The *KLK13* mRNA transcript was located at approximately 1.8Kb (arrow). (B) Relative *KLK13* mRNA expression in IOSE and ovarian cancer cell lines measured by qRT-PCR. CAOV-3 and SKOV-3 cell lines had a 2-fold increase, while OVCAR-3 had a 4-fold increase in *KLK13* mRNA expression when compared to IOSE cells. *, $p<0.01$; **, $p<0.05$.

expression. Differential expression of KLK13 mRNA was confirmed by qRT-PCR (Figure 2.3B). When analyzed by qRT-PCR, the IOSE cells did express the least amount of *KLK13* transcript, while the CAOV-3 ($p<0.01$) and SKOV-3 cell lines had a 2-fold increase in expression, and the OVCAR-3 cell line ($p<0.05$) had a 4-fold increase in *KLK13* mRNA expression when compared to IOSE cells.

2.3.2. KLK13 exhibits differential glycosylation between ovarian cancer cell lines

KLK13 protein expression in total cell lysates of IOSE, CAOV-3, OVCAR-3, and SKOV-3 cells was analyzed by immunoblotting (Figure 2.4A). KLK13 showed differential expression patterns among the cell lines. There were two observed bands that migrated to approximately 48K and 36K for KLK13 in the CAOV-3 and OVCAR-3 cell lysate. Interestingly, there were three KLK13 bands in the SKOV-3 cell lysate and two were similar to those found in CAOV-3 and OVCAR-3. The two common KLK13 bands were at approximately 48K and 36K, while the unique protein was found at 28K. This likely represents an unglycosylated form of KLK13. The IOSE cells also had two forms of KLK13 present at 48K and 36K. The predicted molecular weight, based on amino acid sequence, of KLK13 is 28K. It is unlikely any of these bands are indicative of a product that results from auto-inactivation as the molecular weight would be less than 28K (Sotiropoulou *et al.*, 2003). It is also unlikely that these bands represent different transcripts of KLK13 as the protein product is predicted to be less than 28K in all cases. All four cell lines examined had KLK13 expression that was larger than 28K. In order to determine if the high molecular weight band was due to protein glycosylation, the

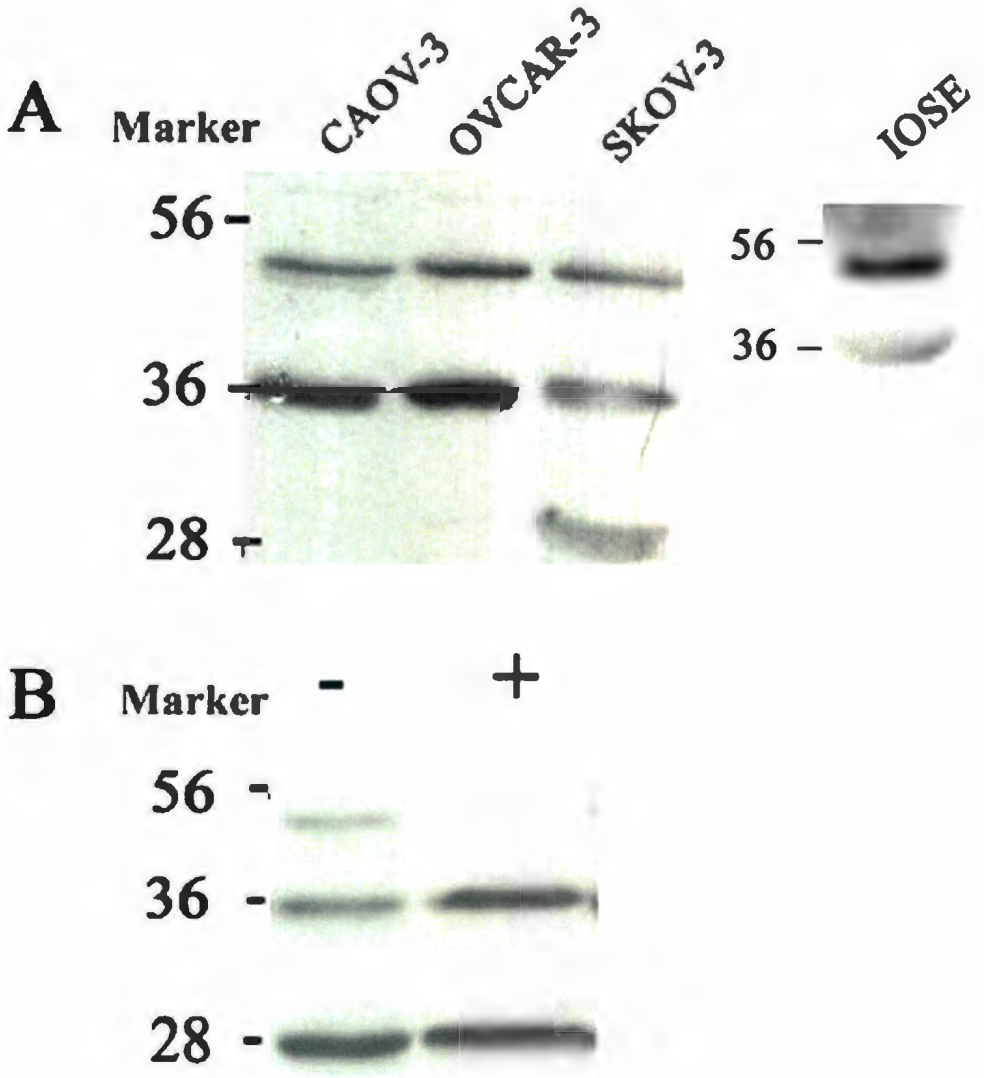


Figure 2.4 Differential expression of KLK13 between ovarian cancer cell lines. (A) The relative mobility of the molecular weight marker (in kilodaltons) is indicated in the first lane, on the left side of the blot (Marker). KLK13 expression was observed in 50μg total protein from ovarian cancer cell lines. Both CAOV-3 and OVCAR-3 showed two bands expressing KLK13, while SKOV-3 had three bands with KLK13 expression. (B) 100μg of SKOV-3 total protein was untreated (-) or treated (+) with PNGaseF. A decrease in the high molecular weight band at 48K and an increase in the low molecular weight band at 36K was seen after treatment.

SKOV-3 total cell lysate was incubated with PNGaseF (Figure 2.4B). After the amidase treatment, a clear decrease in the intensity of the KLK13 band at 48K and an increase in band intensity at approximately 36K was seen. There was also a slight increase in the intensity of the band at 28K. To ensure this was not an effect of partial protein digestion, the protein was treated with PNGaseF for an additional one and two hours and the same results were obtained (data not shown). These results confirmed that the KLK13 protein is at least partially *N*-linked glycosylated with high mannose, hybrid, or complex oligosaccharides. Since there was not a complete decrease in the band at 36K, KLK13 may be partially resistant to PNGaseF by being protected in some way which limits the action of the deglycosidase. It is also important to note that KLK13 may have other post-translational modifications that are resistant to PNGaseF, including O-linked glycosylation, which was not examined in this study.

2.3.3. KLK mutant production

Viral infection and production of the mutant KLK13 proteins in Mv1Lu cells was confirmed by immunoblotting for the V5 tag (Figure 2.5). A schematic of the expected KLK13 protein structures are depicted in Figure 2.5A. When total cell lysates of infected cells were immunoblotted for V5, the KLK13-WT, KLK13-S218A, and KLK13-K25Q proteins all had a signal covering a range of polypeptides at approximately 36K (Figure 2.5B). The KLK13-WT and KLK13-S218A lanes also have a slight band at approximately 48K. These bands likely represent variations in glycosylation of the KLK13 protein in the Mv1Lu cells, similar to what was observed in the ovarian cancer cell lines.

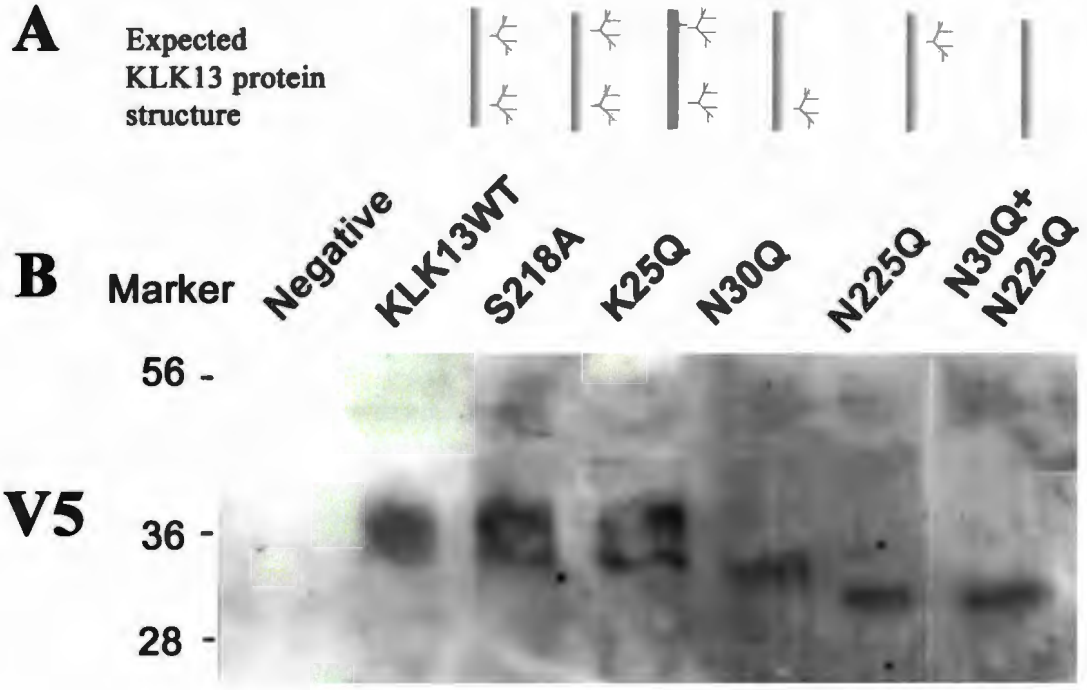


Figure 2.5 Western blot confirmation of the expression of KLK13-WT and KLK13 mutants in Mv1Lu cells with a V5 immunoblot in total cell lysate. (A) A schematic of the expected KLK13 protein produced in the cell. (B) There was no expression in the uninfected cells (Negative). The KLK13-WT, KLK13-S218A, and KLK13-K25Q, all migrated approximately to 36K. The KLK13-N30Q and KLK13-N225Q migrated to approximately 32k and 31, respectively. The double glycosylation mutant, KLK13-N30Q+N225Q, migrated to approximately 31K.

The KLK13-N30Q migrated to approximately 32K and also had a slight band at approximately 36K. The KLK13-N225Q migrated to approximately 31K. The KLK13-N30Q+N225Q, with no available *N*-linked glycosylation sites, also had a molecular weight of approximately 31K, similar to what was seen in the KLK13-N225Q. The fact that there is no band at 36K in the KLK13-N225Q and that KLK13-N30Q is a lower molecular mass than the KLK13-WT, yet higher than the double glycosylation mutant, suggests that there may be a sequential order in which KLK13 is glycosylated. In this case, the N30 would have to be glycosylated before the N225. These data together confirm first; that the KLK13 constructs are being produced, as seen with expression of the V5 tag, and secondly, these data further support the fact the predicted N30 and N225 glycosylation sites are in fact true KLK13 *N*-linked glycosylation sites. A further confirmation of KLK13 glycosylation would have been to deglycosylate the KLK13 mutants with PNGaseF, but this was not performed in this study.

2.3.4. Increased KLK13 expression increased migratory capacity

Each virus-containing media, KLK13-WT, KLK13-S218A, KLK13-K25Q, KLK13-N30Q, KLK13-N225Q, KLK13-N30Q+N225Q, and the empty vector virion, pLNCX2, was used to infect IOSE cells and cellular migration was assayed (Figure 2.6). The IOSE cells were chosen because of the low KLK13 expression and non-migratory characteristic (Auersperg *et al.*, 1984) Cellular migration was measured by creating a wound in the monolayer of cells and measuring the percent of cell covered areas described in the Methods (Section 2.2.10) at 0hrs and 24hrs post-wounding. A representative sample of the assay is displayed in Figure 2.6A. Cells infected with the empty vector control,

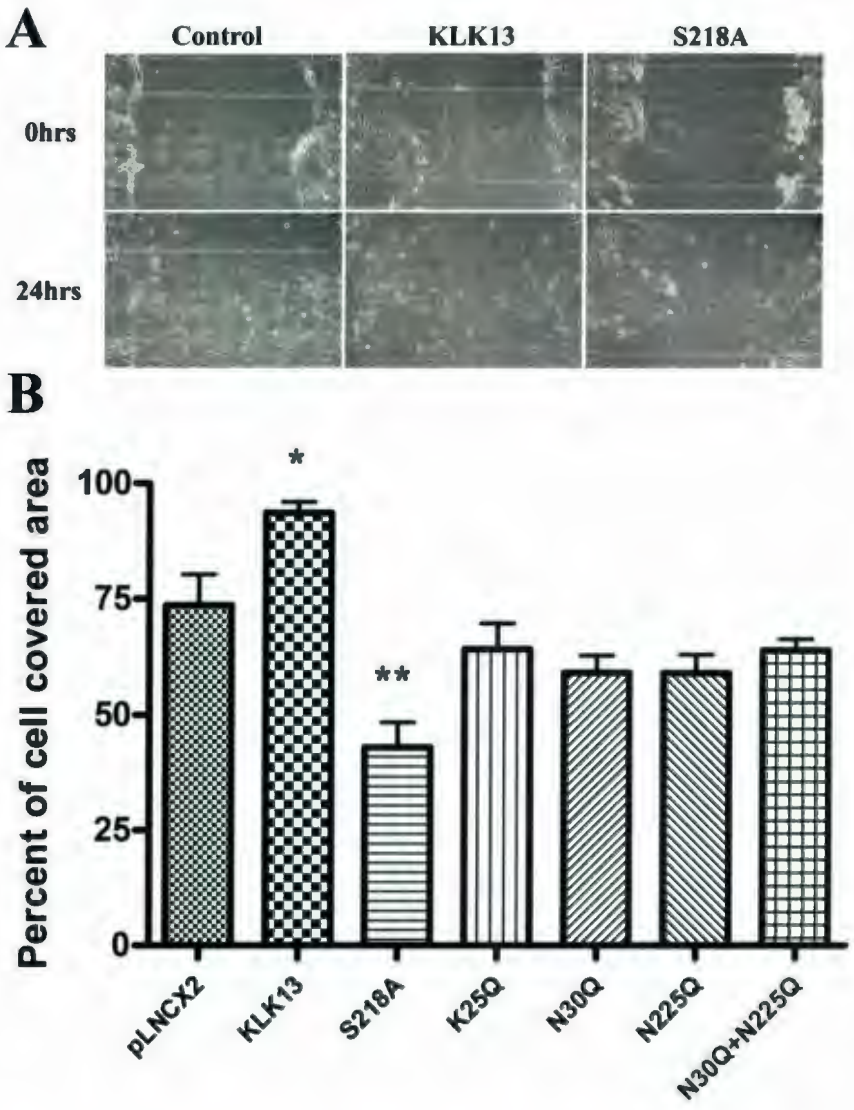


Figure 2.6 IOSE cells infected with KLK13-WT and KLK13 mutants show differential rates of cellular migration. Cellular migration of infected IOSE cells was measured with a wound healing assay. (A) Representative micrographs of the uninfected control IOSE cells and IOSE cells infected with the KLK13-WT and KLK13-S218A virus. The migration of cells was assayed by measuring the cell covered area at the time of wounding (0hrs) and 24 hours later (24hrs). (B) IOSE cells infected with KLK13-WT migrated significantly faster than the uninfected control and empty vector control, pLNCX2. IOSE cells were infected with each virus in three separate infections and each was analyzed in triplicate. *, $p<0.05$; **, $p<0.05$.

pLNCX2, and uninfected cells showed no significant difference in cellular migration after 24 hours (data not shown). Cells infected with the KLK13-WT virus migrated faster than cells infected with the empty vector pLNCX2 (94% vs. 74% cell covered area, respectively, $p<0.05$). IOSE cells infected with the enzymatically dead protein, KLK13-S218A, on the other hand, migrated significantly slower than the control cells (43% vs. 74% cell covered area, $p<0.01$), suggesting KLK13 enzymatic activity is required for cellular migration. The migration rate of the cells infected with the KLK13-K25Q, KLK13-N30Q, KLK13-N225Q, and KLK13-N30Q+N225Q, showed no significant change in the rate of migration from the control cells (64%, 60%, 59%, and 64% vs. 74% cell covered area, respectively). The KLK13-K25Q mutant is constructed so that it will never progress beyond its zymogen to its active state and therefore should have no effect on the level of active KLK13 in the cell model. The cells infected with the glycosylation mutants, KLK13-N30Q, KLK13-N225Q, and KLK13-N30Q+N225Q, showed no significant change in rate of migration when compared to the control cells. Proper glycosylation is required for the proper folding and hence proper function of KLKs. Mutations that interfered with KLK13 protein glycosylation sites were not able to increase cellular migration similar to the KLK13-WT. The absence of KLK13 glycosylation in the glycosylation mutants appears to have no effect on cellular migration (either in a positive or negative manner), and suggests effective KLK13 glycosylation is not required for cellular migration.

2.3.5. Decreased KLK13 expression decreased migratory capacity

IOSE cells, cells that have low endogenous KLK13, infected with KLK13-WT were shown to migrate faster than control cells (Figure 2.6). Based on these findings, when KLK13 expression is decreased in a cell line that has high KLK13 expression, a decrease in cellular migration should be observed. In order to select an appropriate cell model, the CAOV-3, OVCAR-3 and SKOV-3 cell lines, which have high KLK13 expression (Figure 2.3), were tested for migratory capacity by a wound healing assay. When compared to the OVCAR-3 and CAOV-3 cells, SKOV-3 cells had the fastest rate of migration. OVCAR-3 and CAOV-3 cells needed more than 48 hours to close a wound while the SKOV-3 cells took approximately 12 hours (data not shown). This agreed with another study that found SKOV-3 cells to be migratory (Whitley *et al.*, 2007). SKOV-3 cells were transfected with vectors containing the antisense KLK13, sense KLK13, and empty vector control (pLNCX2), and immunoblotted for KLK13 (Figure 2.7A). The cells transfected with the antisense KLK13 vector showed a marked decrease in KLK13 protein expression of all three forms of KLK13 at 48K, 36K, and 28K. The cells transfected with the KLK13 sense vector also showed a slight decrease in KLK13 expression but there was also a corresponding decrease in total protein loaded as shown by the α-tubulin immunoblot. The western blot also shows an immunoblot for oligoadenylate synthase (OAS). OAS is increased in the cell upon anti-viral response. These cells were immunoblotted for OAS in order to account for any non-specific effects of the anti-sense RNA. There was no increased OAS expression which confirmed the transfection had not initiated an anti-viral response in the cells. The cells were then

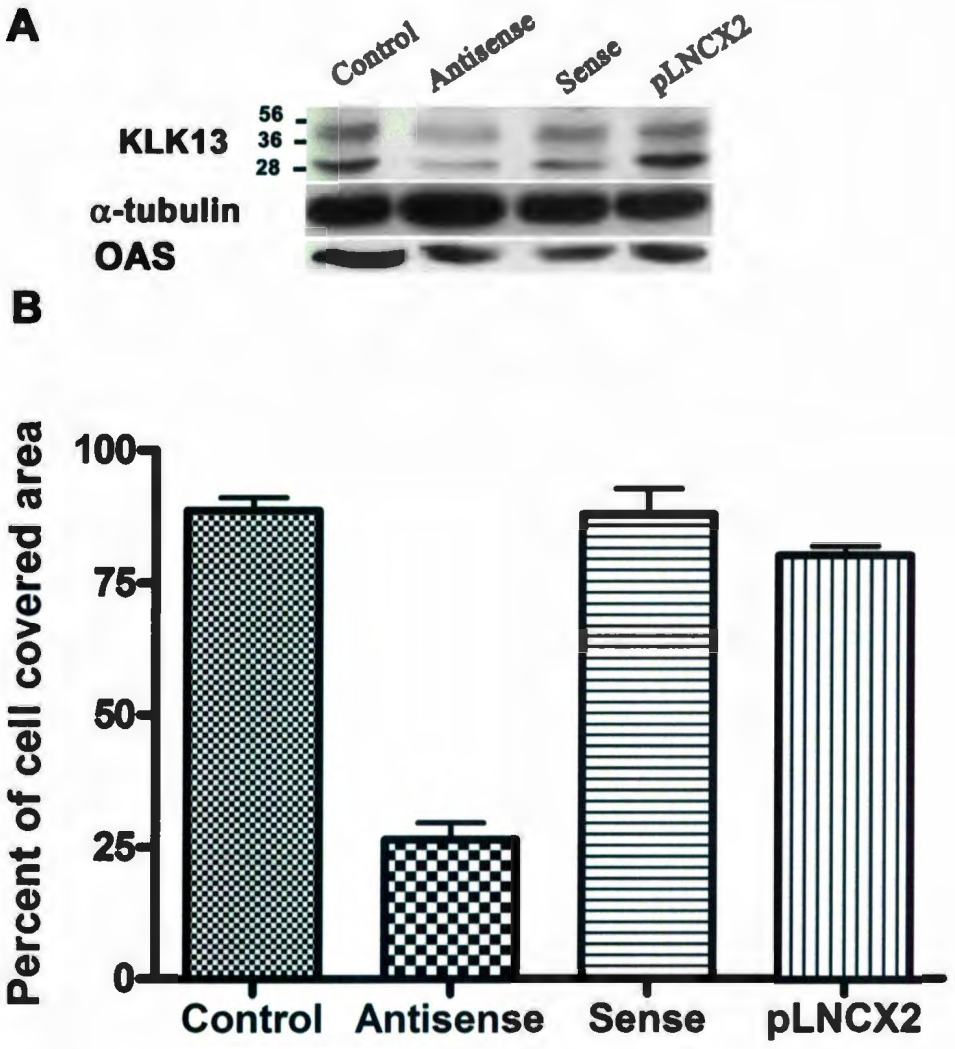


Figure 2.7 Decreased expression of KLK13 in SKOV-3 cells decreased cellular migration. (A) Decreased KLK13 expression in SKOV-3 cells was confirmed by immunoblot and α-tubulin was used as a loading control. To account for non-specific effects of the Antisense RNA, cellular lysates were assayed for oligoadenylate synthase (OAS). (B) SKOV-3 cells were transfected with an Antisense or Sense KLK13. Cells were grown to a confluent monolayer and wounded with a pipette tip. 12hrs later, cellular migration was assessed by measuring the percent of cell covered area. SKOV-3 cells transfected with the antisense KLK13 migrated slower than the uninfected and empty vector control, pLNCX2, cells. There was no significant difference in the rate of cellular migration of cells transfected with the Sense KLK13 and control cells. SKOV-3 cells were infected with each vector in three separate transfections and each was analyzed in triplicate. *, $p<0.001$.

subjected to a wound healing assay. SKOV-3 cells transfected with the KLK13 antisense vector showed a significant decreased in cellular migration when compared to the untransfected control ($p<0.001$), the sense KLK13 ($p<0.001$), and empty vector ($p<0.001$; 26% vs. 89% vs. 89% vs. 80% cell covered area, respectively; Figure 2.7B). There was no significant difference in cellular migration between the cells transfected with the sense KLK13 and controls. A decrease in KLK13 protein expression in the SKOV-3 cell line had a negative effect on cellular migration, supporting previous results suggesting KLK13 is required for ovarian cellular migration. These results also suggest the use of an anti-KLK13 therapy may potentially be used as therapeutic treatment to decrease tumor metastasis.

2.3.6. Increased KLK13 expression increased cell migration of Mv1Lu Cells

In order to investigate if the involvement of KLK13 expression in cellular migration was a true phenomena or a cell-specific effect, the effect of KLK13 on cellular migration was examined in another epithelial cell line, the Mv1Lu cell line (Figure 2.8). Although the endogenous KLK13 expression in Mv1Lu cells was not measured, a wound healing assay showed KLK13 was involved in cellular migration in this epithelial cell line. Similar to what was seen with the IOSE cells, the Mv1Lu cells that were stably infected with the KLK13-WT migrated faster than the empty vector control cells infected with the pLNCX2 virus (95% vs. 66% cell covered area; $p<0.001$). Also, the cells infected with the enzymatically dead, KLK13-S218A, virus migrated slower than the control cells (39% vs. 66% cell covered area; $p<0.001$). These results are similar to what was observed when IOSE cells were infected with KLK13-WT and KLK13-S218A. Mv1Lu

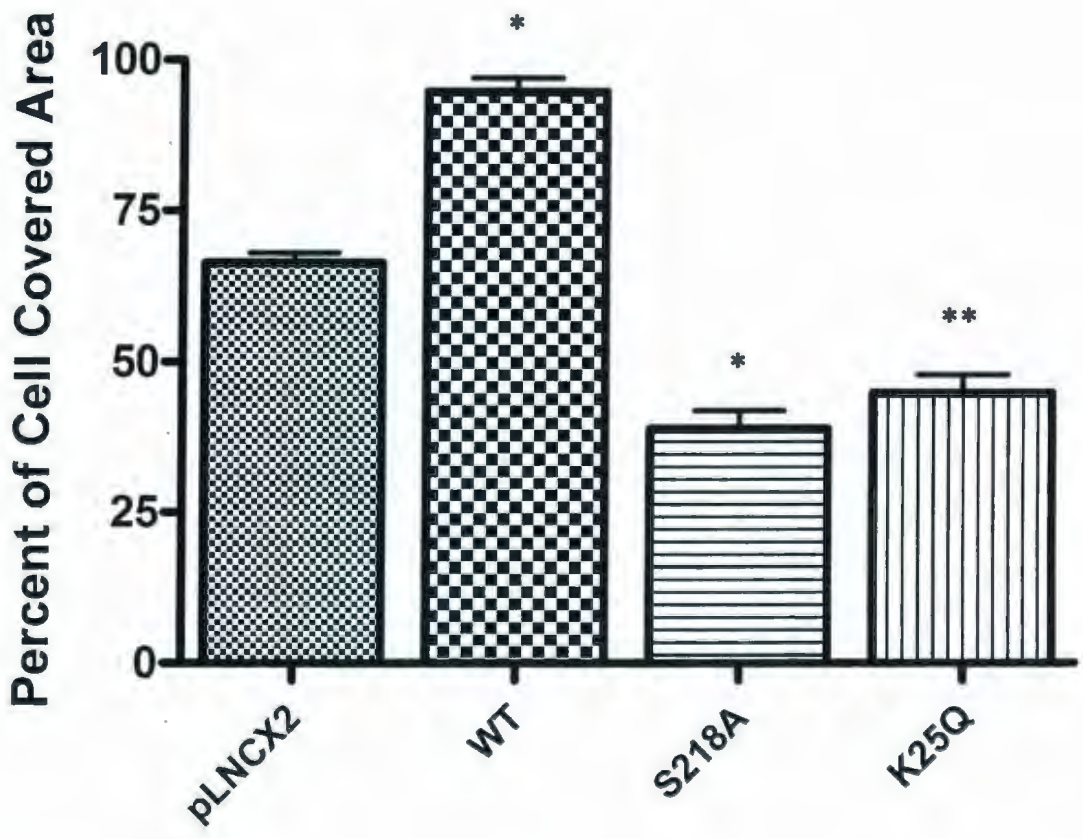


Figure 2.8 Mv1Lu cells infected with KLK13-WT and KLK13 mutants show differential rates of cellular migration. Mv1Lu cells infected with the KLK13-WT, KLK13-S218A, and KLK13-K25Q virus and were subjected to the wound healing assay. Cells infected with the KLK13-WT virus migrated faster than the empty vector control (pLNCX2) cells. Also, the cells infected with the KLK13-S218A virus migrated slower than the cells infected with pLNCX2. Cells infected with KLK13-K25Q migrated slower than the control cells. Mv1Lu cells were infected with each virus in three separate infections and each was analyzed in triplicate. *, $p<0.001$; **, $p<0.01$.

cells infected with the KLK13-K25Q virus also migrated slower than control cell (45% vs. 66% cell covered area, respectively). This effect on migration was not observed in the IOSE cells. It would be interesting to further investigate this differential effect. The results regarding the three glycosylation mutants were not consistent between replicates and were not included in the graph.

2.3.7. Increased KLK13 expression increased the invasiveness of IOSE cells

Since KLK13 was clearly shown to have a significant effect on cellular migration, its effect on cellular invasion was assayed. IOSE cells were infected with KLK13-WT, KLK13-S218A, KLK13-K25Q, KLK13-N30Q, KLK13-N225Q, KLK13-N30Q+N225Q, or the empty vector, pLNCX2 virus. Cells were plated in the upper chamber of a well-insert in 2% FCS in IOSE media on a matri-gel basement membrane. The bottom chamber of the well had 5% FCS in IOSE media as a chemoattractant. Invasions assays were conducted through the matri-gel basement membrane for 24 hours. After 24 hours, the cells that had invaded through the basement membrane were dissociated from the bottom of the membrane and lysed in lysis buffer containing CyQuant®GR Dye (see Materials and Methods section for more detail). Invasion was determined by measuring the relative fluorescent units (RFU) in each cell lysate as the CyQuant®GR fluorescent dye binds to nucleic acids of the cells that have invaded thorough the basement membrane. The IOSE cells infected with the KLK13-WT virus invaded significantly more than the cells infected with the empty vector control (20,000 RFU vs. 16,000 RFU, respectively, $p<0.05$), suggesting KLK13 is involved in cellular invasion (Figure 2.9). The IOSE cells infected with the enzymatically dead KLK13-S218A

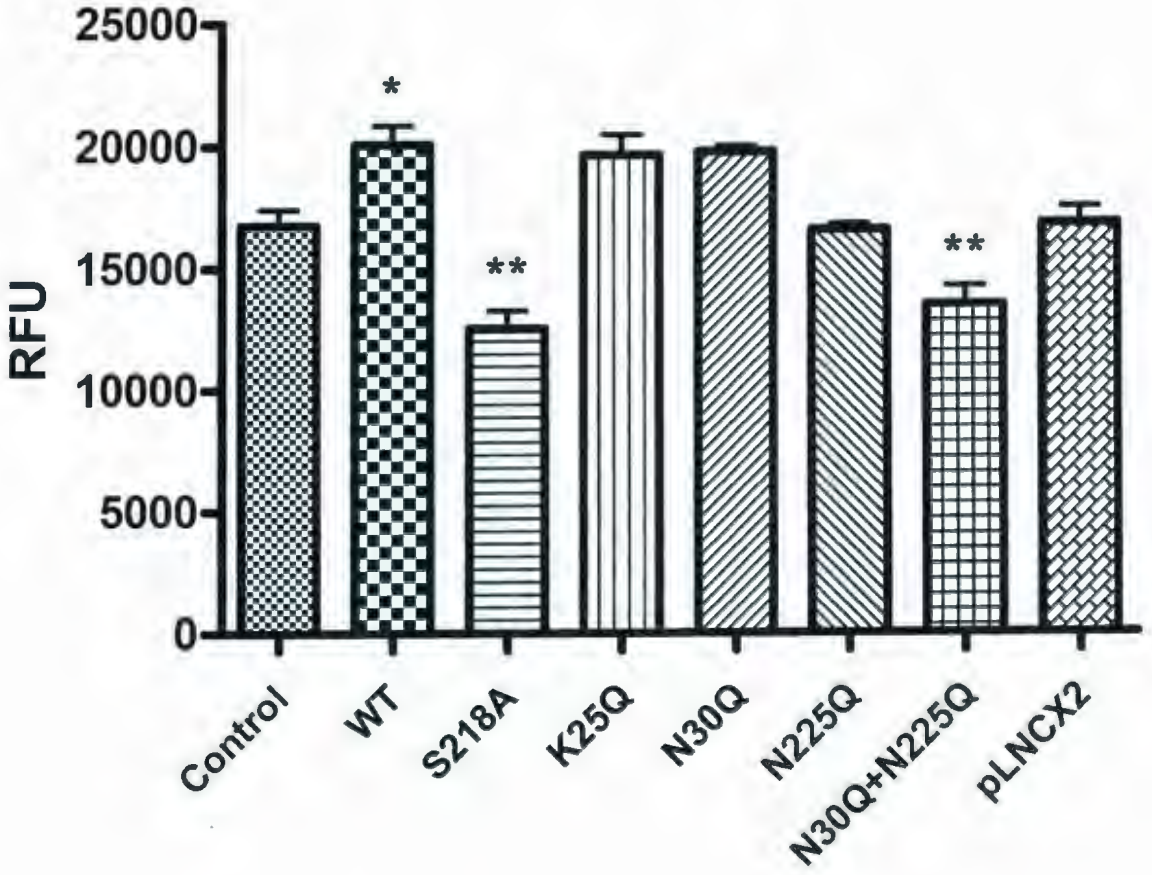


Figure 2.9 IOSE cells infected with KLK13-WT and KLK13 mutants show differential cellular invasion. IOSE cells were infected with the KLK13-WT and each KLK13 mutant virus and subjected to invasion assays for 24 hours and cellular invasion was measured by relative fluorescence units (RFU). The cells infected with the KLK13-WT virus invaded faster than the cells infected with the empty vector control, pLNCX2. Also the cells infected with the KLK13-S218A virus invaded slower than empty vector control cells (pLNCX2). The cells infected with the double glycosylation mutant, KLK13-N30Q+N225Q, invaded slower than the cells infected with the empty vector control (pLNCX2). IOSE cells were infected with each virus in three separate infections and each was analyzed in triplicate. *, $p<0.05$; **, $p<0.01$.

(12,500RFU), invaded significantly less than the cells infected with the empty vector control ($p<0.01$), supporting the previous finding that KLK13 plays a role in cellular migration. There was no significant difference in cellular invasion between the cells infected with the inactivatable KLK13-K25Q (19,500RFU), and the cells infected with the empty vector. Also, there was no significant difference detected between cells infected with the single glycosylation mutants (KLK13-N30Q and KLK13-N225Q; 19,500RFU and 15,500RFU, respectively) and cells infected with the empty vector control cells. Conversely, cells infected with the double glycosylation mutant, KLK13-N30Q+N225Q (13,000RFU) invaded significantly less than the cells infected with the empty vector control ($p<0.01$), suggesting that a KLK13 glycosylation on both sites is required for cellular invasion, but not migration.

2.4 Discussion

Metastasis is a complex process involving degradation of the basement membrane, invasion of the stroma, adhesion, angiogenesis, cell proliferation, and migration (Yoshida *et al.*, 2000). Considerable evidence supports the concept that each discrete step of metastasis is regulated by different proteins (Fidler and Radinsky, 1990). Extracellular proteolytic enzymes, including serine proteases, such as urokinase plasminogen activator (uPA) have been reported to be associated with destruction of the ECM and the basement membrane, both involved in the process of invasion and metastasis of cancer cells (Iwamoto *et al.*, 2005). UPA is secreted as a single chain zymogen, pro-uPA that must be activated to its two-chain serine protease active form. UPA activates plasminogen from an inactive state to active plasmin, which has been shown to accelerate localized

degradation of ECM, which in turn facilitates tumor cell migration and invasion (Sharma and Sharma, 2007). Another group of serine proteases known to be involved in cancer is the KLK family. Interestingly, KLK2 (Frenette *et al.*, 1997), KLK4 (Takayama *et al.*, 2001b), and KLK15 (Takayama *et al.*, 2001a) have been shown to activate pro-uPA to uPA, resulting in plasmin activation and ECM degradation, suggesting these KLKs may also play a role in invasion and metastasis. The current study set out to determine if KLK13, a KLK which has been documented as being upregulated in ovarian cancer (Scorilas *et al.*, 2004), contributes to cellular migration and invasion in ovarian epithelial cells.

In order to examine the role of KLK13 in cellular migration and invasion, a suitable cell model had to be chosen. This required a cell line that had low endogenous levels of KLK13 and was not known to be highly migratory was required. The IOSE cell line was the closest to a normal ovarian surface epithelial that was available at the time of the study. To determine the level of KLK13 expression in this cell line, the expression of *KLK13* mRNA by was examined by qRT-PCR. *KLK13* mRNA was increased in ovarian cancer cell lines when compared to the IOSE cell line (Fig. 2.2B). These findings agreed with previous studies that found increased expression of KLK13 in ovarian cancer (Scorilas *et al.*, 2004;White *et al.*, 2009) and low KLK13 in normal ovaries (Kapadia *et al.*, 2003;White *et al.*, 2009).

When KLK13 protein expression was examined in the ovarian cancer cell lines CAOV-3, OVCAR-3, and SKOV-3, different expression patterns were found (Figure 2.3). While

the first two cell lines, CAOV-3 and OVCAR-3, had two KLK13 bands at 48K and 36K, the SKOV-3 cell line showed an additional band at 28K. This 28K band is suggestive of the pre-glycosylated form of KLK13. It is interesting to note that this band does not appear in the other ovarian cancer cell lines studied, suggesting there may be different KLK13 protein processing and production in different forms of ovarian cancer. Another contributing factor may be the cell line origin. The CAOV-3 and OVCAR-3 cell lines were derived from ovarian adenocarcinomas, while the SKOV-3 cell line was derived from the ascites of a metastatic ovarian adenocarcinoma, which contributes to its metastatic phenotype.

The difference in molecular weight may be explained as a result of *N*-linked glycosylation. *N*-linked glycosylation is required for proper protein folding and is especially important for kallikrein-related peptidases as they require the formation of the substrate binding pocket for activity. In order to confirm KLK13 glycosylation, SKOV-3 total cell lysate was incubated with PNGaseF. There was a decrease in the protein at 48K (shown as a less intense band), and an increase in the lower molecular weight protein at 36K (increased band intensity; Figure 2.4B), but no increase at the band at 28K (data not shown). This suggests that there may be other post-translational modifications other than *N*-linked glycosylation that increases the molecular weight of KLK13 in ovarian cancer.

The role of KLK13 in cellular migration and invasion was also examined. IOSE cells infected with the KLK13-WT virus migrated significantly faster than control cells (Figure 2.6). Interestingly, when IOSE cells were infected with the putative

enzymatically dead protein, KLK13-S218A, the opposite effect on migration over the same period of time was observed, as these cells migrated much slower than the control cells.

In order to verify if the involvement of KLK13 with migration was a cell-specific or common effect, migration assays were performed on mink lung epithelial cells, Mv1Lu cells. These cells were chosen assuming epithelial cells should behave similarly to the IOSE cells when overexpressing KLK13. Additionally, KLK13 has also been shown to be overexpressed in lung cancer (Planque *et al.*, 2008), suggesting a similarity in metastatic mechanism. Similar to what was observed in the IOSE cells, the Mv1Lu cells infected with the KLK13-WT virus showed increased migration while the cells infected with the enzymatically dead KLK13, KLK13-S218A, showed decreased cellular migration when compared to control cells (95% vs. 39% vs. 66% cell covered area, respectively; Figure 2.8).

The effect of increased cellular migration with increasing KLK13 expression may be explained in terms of a KLK enzymatic cascade, as KLKs have been shown to be involved in tumor promoting enzymatic cascades (Borgono and Diamandis, 2004). Besides KLK2, KLK4, and KLK15 being involved in the uPA activation cascade, KLK1, KLK2, and KLK3 can degrade insulin-like growth factor binding proteins (IGFBP 2, 3, 4, and 5) in prostate cancer, increasing insulin-like growth factor (IGF) availability and subsequently inducing cell proliferation and preventing apoptosis (Cohen *et al.*, 1992). There may be a tumor promoting enzymatic cascade in which KLK13 is

involved in this cell model. The enzymatically active wild type KLK13 can bind, cleave, and release its cleavage products. On the other hand, the enzymatically dead protein, KLK13-S218A, can effectively sequester its substrates, but because it has no protease activity, the substrate stays bound to the inactive KLK13. This has a negative effect on KLK13 targets such as ECM proteins, including fibronectin and laminin, as they cannot be cleaved and therefore may inhibit cellular migration, suggesting an anti-KLK13 therapy may be a useful therapeutic option.

The potential of KLK13 as a therapeutic target for ovarian cancer was further supported when a decreased rate of migration in SKOV-3 cells upon a decrease in KLK13 expression was observed (Fig. 2.7). This may be a direct result of decreased protease activity, resulting in a decreased in ECM degradation. Kapadia et al. found similar results as they observed a decrease in the number of cells that migrated through a Boyden chamber assay when treated with a neutralizing KLK13 antibody (Kapadia *et al.*, 2004). These data suggest KLK13 expression levels are directly related to cellular migration, a critical step in cancer metastasis. Potential anti-metastatic therapy may target proteins involved in cellular migration such as KLK13, thereby decreasing the chances of tumor metastasis.

The involvement of KLK13 in cellular migration also supports the finding that increased KLK13 expression increases the invasive capacity of IOSE cells (Figure 2.9). Metastatic tumor cells invade host tissues through a series of steps, one or more of which requires proteolytic enzymes for invasion (Mignatti and Rifkin, 1993). KLK13 has been

previously shown to be able to degrade collagen, fibronectin and laminin (Kapadia *et al.*, 2004), thereby giving cancer cells the means to invade through the basement membrane. Interestingly, similar to what was seen in the migration assay, the cells infected with the KLK13-WT virus invaded significantly more than the control cells while the cells infected with the enzymatically dead KLK13, KLK13-S218A, showed significantly less cellular invasion, suggesting KLK13 may also be involved in tumor metastasis (Figure 2.9). Increased KLK13 expression may increase the degradation of ECM proteins, which is critical for cellular invasion, and thereby aiding the cells in this process. Also, a reduction in migratory capacity of IOSE cells was seen when the cells were infected with the enzymatically dead KLK13, suggesting the inhibition of KLK13 activity decreases cellular invasion and that KLK13 is directly involved in tumor invasion. Interestingly, the cells infected with the double glycosylation mutant, KLK13-N30Q+N225Q, showed decreased cellular invasion (Figure 2.9) but no significant change in cellular migration. This may be explained by the activation of specific molecular pathways required for each biological process. In order for cells to migrate, they must activate pathways that are involved in cell-cell adhesion and cell movement. On the other hand, cell invasion largely depends on the degradation of components of the ECM. KLK13 has been previously shown to be able to degrade collagen, laminin, and fibronectin (Kapadia *et al.*, 2004) and may contribute directly to cellular invasion by its action on these components of the ECM. Therefore, KLK13 may play a more direct role in cellular invasion than migration. These results suggest proper KLK13 glycosylation is required for cellular invasion but not migration.

These data show that KLK13 is involved in cellular migration and invasion. Although much of these data analyzes cellular migration and invasion based on the potential enzymatic activity of the KLK13 mutants, enzymatic activity assays on these mutant proteins was not performed. KLK13 is a secreted protein and the purification of KLK13 in cell media proved to be quite problematic. The concentration of KLK13 in the media was low and below the limits of detection by immunoblotting. Although enzyme-linked immunosorbent assay (ELISA) offers a more sensitive detection method, there were no commercially available tests for KLK13. Due to these limitations, enzymatic assays were not performed for KLK13 and the mutant proteins. The immunoblot analysis for the V5 tag, confirms the KLK13 mutants were being processed as expected (Figure 2.5) and based on the findings regarding cellular migration and invasion, it appears that the KLK13 mutants are displaying the expected enzymatic activity, but the exact mechanism in which KLK13 has this effect is unknown. Other KLKs that have been shown to be involved in cellular migration are KLK6, where the presence of an anti-KLK6 antibody resulted in decreased cell migration and ECM degradation *in-vitro* (Ghosh *et al.*, 2004). Furthermore, KLK6 has been shown to be involved with colon cancer cell migration in a K-RAS dependant manner (Henkhaus *et al.*, 2008). The involvement of KLKs in prostate cancer migration and invasion is also evident. Currently, the most widely used marker for prostate cancer is KLK3/PSA. When studied immunohistochemically, PSA staining is decreased in prostate carcinoma, while serum levels of the protein are increased. The elevation of serum PSA is due to the disruption of the basal cell layer and basement membrane, and this loss of the normal glandular architecture appears to allow

PSA increased access to the circulation (Bostwick, 1994). This leakage likely increases the exposure of the basement membrane and ECM to the proteolytic effects of PSA, contributing to invasion and cancer progression (Whitbread *et al.*, 2006).

These data show *in-vitro* that KLK13 is directly involved in cellular migration and invasion. Increased KLK13 expression in the poorly-migratory cell line, IOSE, increased both cellular migration and invasion. Supporting these findings, when KLK13 expression was decreased in a cancer cell line that is known to be migratory, SKOV-3, cellular migration was significantly decreased. These findings support a role of KLK13 in tumor metastasis and may have important implications in the development of novel diagnostic and therapeutic approaches for metastatic ovarian cancer.

2.5 Acknowledgements

I would like thank Dr. Nelly Auersperg for access to the Canadian Ovarian Tumor Bank.

2.6 References

Auersperg,N., Siemens,C.H., and Myrdal,S.E. (1984). Human ovarian surface epithelium in primary culture. In Vitro *20*, 743-755.

Auersperg,N., Wong,A.S., Choi,K.C., Kang,S.K., and Leung,P.C. (2001). Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr. Rev. *22*, 255-288.

Borgono,C.A. and Diamandis,E.P. (2004). The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer *4*, 876-890.

Bostwick,D.G. (1994). Prostate-specific antigen. Current role in diagnostic pathology of prostate cancer. Am. J. Clin. Pathol. *102*, S31-S37.

Cannistra,S.A., Kansas,G.S., Niloff,J., DeFranzo,B., Kim,Y., and Ottensmeier,C. (1993). Binding of ovarian cancer cells to peritoneal mesothelium in vitro is partly mediated by CD44H. Cancer Res. *53*, 3830-3838.

Chang,A., Yousef,G.M., Jung,K., Rajpert-De Meyts,E., and Diamandis,E.P. (2001). Identification and molecular characterization of five novel kallikrein gene 13 (KLK13; KLK-L4) splice variants: differential expression in the human testis and testicular cancer. Anticancer Res. *21*, 3147-3152.

Chang,A., Yousef,G.M., Scorilas,A., Grass,L., Sismondi,P., Ponzone,R., and Diamandis,E.P. (2002). Human kallikrein gene 13 (KLK13) expression by quantitative RT-PCR: an independent indicator of favourable prognosis in breast cancer. Br. J. Cancer *86*, 1457-1464.

Cohen,P., Graves,H.C., Peehl,D.M., Kamarei,M., Giudice,L.C., and Rosenfeld,R.G. (1992). Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab. *75*, 1046-1053.

Curtin,J.P., Malik,R., Venkatraman,E.S., Barakat,R.R., and Hoskins,W.J. (1997). Stage IV ovarian cancer: impact of surgical debulking. Gynecol. Oncol. *64*, 9-12.

Darling,M.R., Jackson-Boeters,L., Daley,T.D., and Diamandis,E.P. (2006). Human kallikrein 13 expression in salivary gland tumors. Int. J. Biol. Markers *21*, 106-110.

Emami,N. and Diamandis,E.P. (2008). Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers. Clin. Chem. *54*, 1600-1607.

Fidler,I.J. and Radinsky,R. (1990). Genetic control of cancer metastasis. J Natl. Cancer Inst. *82*, 166-168.

Frenette,G., Tremblay,R.R., Lazure,C., and Dube,J.Y. (1997). Prostatic kallikrein hK2, but not prostate-specific antigen (hK3), activates single-chain urokinase-type plasminogen activator. Int. J. Cancer *71*, 897-899.

Ghosh,M.C., Grass,L., Soosaipillai,A., Sotiropoulou,G., and Diamandis,E.P. (2004). Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol. *25*, 193-199.

Griffiths,C.T., Parker,L.M., and Fuller,A.F., Jr. (1979). Role of cytoreductive surgical treatment in the management of advanced ovarian cancer. Cancer Treat. Rep. *63*, 235-240.

Gupta,N., Jung,E., and Brunak S (2004). Prediction of N-glycosylation sites in human proteins. In preparation.

Gupta,N., Jung,E., and Brunak S. Predicition of N-glycosylation sites in human proteins. 2010. Ref Type: Online Source

Henkhaus,R.S., Gerner,E.W., and Ignatenko,N.A. (2008). Kallikrein 6 is a mediator of K-RAS-dependent migration of colon carcinoma cells. Biol. Chem. *389*, 757-764.

Iwamoto,J., Mizokami,Y., Takahashi,K., Nakajima,K., Ohtsubo,T., Miura,S., Narasaka,T., Takeyama,H., Omata,T., Shimokobe,K., Ito,M., Takehara,H., and Matsuoka,T. (2005). Expressions of urokinase-type plasminogen activator, its receptor and plasminogen activator inhibitor-1 in gastric cancer cells and effects of Helicobacter pylori. Scand. J. Gastroenterol. *40*, 783-793.

Kapadia,C., Chang,A., Sotiropoulou,G., Yousef,G.M., Grass,L., Soosaipillai,A., Xing,X., Howarth,D.H., and Diamandis,E.P. (2003). Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin. Chem. *49*, 77-86.

Kapadia,C., Ghosh,M.C., Grass,L., and Diamandis,E.P. (2004). Human kallikrein 13 involvement in extracellular matrix degradation. Biochem. Biophys. Res. Commun. *323*, 1084-1090.

Klucky,B., Mueller,R., Vogt,I., Teurich,S., Hartenstein,B., Breuhahn,K., Flechtenmacher,C., Angel,P., and Hess,J. (2007). Kallikrein 6 induces E-cadherin shedding and promotes cell proliferation, migration, and invasion. Cancer Res. *67*, 8198-8206.

Mignatti,P. and Rifkin,D.B. (1993). Biology and biochemistry of proteinases in tumor invasion. Physiol. Rev. *73*, 161-195.

Pepper,M.S. (2001). Extracellular proteolysis and angiogenesis. Thromb. Haemost. *86*, 346-355.

Peracaula,R., Barrabes,S., Sarrats,A., Rudd,P.M., and de Llorens,R. (2008). Altered glycosylation in tumours focused to cancer diagnosis. Dis. Markers *25*, 207-218.

Peracaula,R., Tabares,G., Royle,L., Harvey,D.J., Dwek,R.A., Rudd,P.M., and de Llorens,R. (2003). Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. Glycobiology *13*, 457-470.

Pfaffl,M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.

Planque,C., Blechet,C., Ayadi-Kaddour,A., Heuze-Vourc'h,N., Dumont,P., Guyetant,S., Diamandis,E.P., El Mezni,F., and Courty,Y. (2008). Quantitative RT-PCR analysis and immunohistochemical localization of the kallikrein-related peptidases 13 and 14 in lung. Biol. Chem. *389*, 781-786.

Sawada,K., Radjabi,A.R., Shinomiya,N., Kistner,E., Kenny,H., Becker,A.R., Turkyilmaz,M.A., Salgia,R., Yamada,S.D., Vande Woude,G.F., Tretiakova,M.S., and Lengyel,E. (2007). c-Met overexpression is a prognostic factor in ovarian cancer and an effective target for inhibition of peritoneal dissemination and invasion. Cancer Res. *67*, 1670-1679.

Scarberry,K.E., Dickerson,E.B., Zhang,Z.J., Benigno,B.B., and McDonald,J.F. (2010). Selective removal of ovarian cancer cells from human ascites fluid using magnetic nanoparticles. Nanomedicine *6*, 399-408.

Schink,J.C. (1999). Current initial therapy of stage III and IV ovarian cancer: challenges for managed care. Semin. Oncol. *26*, 2-7.

Scorilas,A., Borgono,C.A., Harbeck,N., Dorn,J., Schmalfeldt,B., Schmitt,M., and Diamandis,E.P. (2004). Human kallikrein 13 protein in ovarian cancer cytosols: a new favorable prognostic marker. J. Clin. Oncol. *22*, 678-685.

Sharma,M.C. and Sharma,M. (2007). The role of annexin II in angiogenesis and tumor progression: a potential therapeutic target. Curr. Pharm. Des. *13*, 3568-3575.

Sotiropoulou,G., Rogakos,V., Tsetsenis,T., Pampalakis,G., Zafiropoulos,N., Simillides,G., Yiotakis,A., and Diamandis,E.P. (2003). Emerging interest in the kallikrein gene family for understanding and diagnosing cancer. Oncol. Res. *13*, 381-391.

Stephan,C., Jung,K., Lein,M., and Diamandis,E.P. (2007). PSA and other tissue kallikreins for prostate cancer detection. Eur. J. Cancer *43*, 1918-1926.

Takayama,T.K., Carter,C.A., and Deng,T. (2001a). Activation of prostate-specific antigen precursor (pro-PSA) by prostin, a novel human prostatic serine protease identified by degenerate PCR. Biochemistry *40*, 1679-1687.

Takayama,T.K., McMullen,B.A., Nelson,P.S., Matsumura,M., and Fujikawa,K. (2001b). Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. Biochemistry *40*, 15341-15348.

Tarui,T., Majumdar,M., Miles,L.A., Ruf,W., and Takada,Y. (2002). Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. J Biol. Chem. *277*, 33564-33570.

Veveris-Lowe,T.L., Lawrence,M.G., Collard,R.L., Bui,L., Herington,A.C., Nicol,D.L., and Clements,J.A. (2005). Kallikrein 4 (hK4) and prostate-specific antigen (PSA) are associated with the loss of E-cadherin and an epithelial-mesenchymal transition (EMT)-like effect in prostate cancer cells. Endocr. Relat. Cancer *12*, 631-643.

Whitbread,A.K., Veveris-Lowe,T.L., Lawrence,M.G., Nicol,D.L., and Clements,J.A. (2006). The role of kallikrein-related peptidases in prostate cancer: potential involvement in an epithelial to mesenchymal transition. Biol. Chem. *387*, 707-714.

White,N.M., Mathews,M., Yousef,G.M., Prizada,A., Popadiuk,C., and Dore,J.J. (2009). KLK6 and KLK13 predict tumor recurrence in epithelial ovarian carcinoma. Br. J. Cancer *101*, 1107-1113.

Whitley,B.R., Beaulieu,L.M., Carter,J.C., and Church,F.C. (2007). Phosphatidylinositol 3-kinase/Akt regulates the balance between plasminogen activator inhibitor-1 and urokinase to promote migration of SKOV-3 ovarian cancer cells. Gynecol. Oncol. *104*, 470-479.

Xu,A., Hao,J., Zhang,Z., Tian,T., Jiang,S., Hao,J., Liu,C., Huang,L., Xiao,X., and He,D. (2009). 14-kDa phosphohistidine phosphatase and its role in human lung cancer cell migration and invasion. Lung Cancer *67*, 48-56.

Yoshida,B.A., Sokoloff,M.M., Welch,D.R., and Rinker-Schaeffer,C.W. (2000). Metastasis-suppressor genes: a review and perspective on an emerging field. J Natl. Cancer Inst. *92*, 1717-1730.

Yousef,G.M., Chang,A., and Diamandis,E.P. (2000). Identification and characterization of KLK-L4, a new kallikrein-like gene that appears to be down-regulated in breast cancer tissues. J. Biol. Chem. *275*, 11891-11898.

Chapter 3 Human Kallikrein-Related Peptidases 6 and 13 Predict Tumor Recurrence in Ovarian Cancer Patients

A version of this chapter has been published in the *British Journal of Cancer 2009:101 (7):1107-13.*

3.1 Introduction

Ovarian carcinoma is the most lethal of all the gynaecological malignancies and is considered a great clinical challenge as it is often diagnosed in the late stages due to its anatomical location and relative asymptomatic occurrence (Holschneider and Berek, 2000). Approximately 75% of patients are diagnosed in late stage disease (Stage III/IV) and have a five year survival rate of only 15-20%, compared to a 80-90% five year survival rate when diagnosed in the early stages [Stage I/II; (Schink, 1999)]. The disease is treatable and in most cases curable if diagnosed in the early stages.

Recently, a family of serine proteases has been identified on human chromosome 19q13 and named the human kallikrein-related peptidase (KLK) family (Yousef and Diamandis, 2003). The family consists of 15 genes, of which 12 *(KLK2, KLK3, KLK4, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, KLK14*, and *KLK15*) appear to be overexpressed in ovarian cancer. As been shown for prostate specific antigen (PSA/*KLK3*) in prostate cancer, these proteins may represent potential novel biomarkers for ovarian carcinoma (Borgono and Diamandis, 2004). This study examines the expression and prognostic significance of *KLK6* and *KLK13* in ovarian cancer.

Kallikrein-related peptidase 6 (*KLK6*) was initially identified by three different groups who named it protease M in breast cancer (Anisowicz *et al.*, 1996), zyme in Alzheimer's disease (Little *et al.*, 1997) and neurosin in colon adenocarcinoma (Yamashiro *et al.*, 1997). *KLK6 is* reported to have differential expression in ovarian, breast, uterine, and colon cancers (Anisowicz *et al.*, 1996;Hoffman *et al.*, 2002;Ogawa *et al.*, 2005;Santin *et*

al., 2005;Tanimoto *et al.*, 2001). Overexpression of *KLK6* has been reported at both the gene and protein levels in ovarian cancer and has been associated with poor patient prognosis (Diamandis *et al.*, 2000a;Kountourakis *et al.*, 2008;Tanimoto *et al.*, 2001). Recently, *KLK6* has been implicated in the loss of cell-cell contact and promotion of cell proliferation, migration and invasion in keratinocytes (Klucky *et al.*, 2007). With the involvement in these biological functions, the overexpression of *KLK6* in ovarian cancer suggests it may play a role promoting cancer invasion and metastasis. *In vitro* assays have shown recombinant KLK6 proteins are capable of extracellular matrix (ECM) protein digestion and neutralizing KLK6 antibodies can decrease the rate of migration of ovarian cancer cell lines, further supporting this hypothesis (Ghosh *et al.*, 2004).

Kallikrein-related peptidase 13 *(KLK13)* was first identified as being downregulated in breast cancer tissues and cell lines (Yousef *et al.*, 2000). However, 50% of malignant ovarian tissues had increased KLK13 expression relative to nearly undetectable levels in normal or benign tissue (Kapadia *et al.*, 2003). Additionally, Scorilas et al., found high levels of KLK13 in early stage cancers and consequently, associated high KLK13 expression with a better prognosis (Scorilas *et al.*, 2004). Similar to KLK6, KLK13 can degrade major components of the ECM and when treated with an anti-KLK13 antibody, an ovarian cancer cell line demonstrated decreased migratory capacity (Kapadia *et al.*, 2004). When KLK13 was overexpressed in the immortalized ovarian surface epithelial (IOSE) cell line, cellular migration (Figure 2.4) and invasive capacity (Figure 2.6) was increased. Also, when KLK13 expression was decreased in the SKOV-3 ovarian cancer

cell line, cellular migration was decreased (Figure 2.5), suggesting KLK13 plays a direct role in ovarian cellular migration.

Based on the effects *KLK6* and *KLK13* have on ovarian cancer cells, the aim of this study was to evaluate the prognostic significance of *KLK6* and *KLK13* in epithelial ovarian cancer by quantifying gene expression levels and correlating them with clinical variables and patient survival data.

3.2 Materials and methods

3.2.1. Ovarian cancer samples

The study analyzed formalin fixed, paraffin embedded (FFPE) tissues from 106 cases of sporadic ovarian carcinoma diagnosed in the Province of Newfoundland and Labrador, Canada, between 1983 and 2002. Eight normal ovary samples were also obtained for comparison. Tissues were collected from pathology archives and selected based on haematoxylin and eosin stains reviewed by a pathologist. Samples containing at least 80% tumor cells were selected for the study. Clinical staging was performed using the standard International Federation of Gynaecology and Obstetrics (FIGO) staging, with tumors graded as borderline, well differentiated (Grade I), moderately differentiated (Grade II), or poorly differentiated (Grade III). Clinical history was obtained by review of patients' medical records in accordance with Memorial University's Human Investigation Committee protocol.

3.2.2. Immunohistochemistry

Sections were cut 4μm thick and dried on glass slides overnight. Sections were deparaffinized in two changes of xylene (five minutes each) and rehydrated through decreasing graded alcohols of 100%, 95%, and 70% ethanol. The sections were then rinsed in running water for five minutes. Endogenous peroxidises were inhibited by incubating in 3% hydrogen peroxidase for ten minutes and rinsed in running water for five minutes. Antigen retrieval utilized a pressure cooker for 10 minutes in citrate buffer (pH 6.0). Slides were then rinsed in phosphate buffered saline (PBS, pH 7.4) and blocked with normal goat serum for 20 minutes. Slides were then incubated overnight in primary antibody. The antibody dilution for the KLK6 rabbit polyclonal antibody (Diamandis *et al.*, 2000b) was 1:500 and the KLK13 mouse monoclonal antibody, clone 33.1 (Kapadia *et al.*, 2003) was 1:1000. The following day, slides were then washed twice with PBS (pH 7.4). Detection of KLK6 immunocomplex utilized Envision (Dako, Mississauga, ON) for 30 minutes, while localization of KLK13 immunocomplex utilized the LSAB+, Link and Strepavadin reagents (Dako, Mississauga, ON) for 15 minutes each. Slides were then washed twice with PBS (pH 7.4). Immune complexes were visualized by incubating with diaminobenzidine (DAB) for one minute. Slides were then rinsed in PBS (pH 7.4) and water. Sections were counterstained with hemotoxylin for three minutes and rinsed in water. Sections were blued in Scott's tap water (1:1 mix of 83mM sodium bicarbonate and 332mM magnesium sulphate) for one minute and rinsed in water. Sections were then dehydrated though decreasing alcohols, 70%, 95%, and 100% ethanol. Finally, sections were cleared in two changes of xylene and mounted.

3.2.3. RNA extraction

Five 10μm sections were cut from each FFPE tissue for nucleic acid isolation. Total RNA was extracted using the High Pure RNA Paraffin Kit (Roche, Indianapolis, IN) which has been optimized for nucleic acid extraction from paraffin embedded tissue. Samples were first deparaffinized by mixing 800μL xylene to each sample. Next, 400μL ethanol was added followed by a two minute centrifugation at 17,000 x g. The supernatant was discarded and 1mL of ethanol was added to the sample and vortexed. Again, the samples were spun for two minutes at 17,000 x g, the supernatant was discarded, and the tissue pellet was dried for 10 minutes at 55°C.

From each ovarian tumor sample, RNA was isolated by first adding 100μL tissue lysis buffer (supplied by the manufacturer), 1.2% sodium dodecyl sulphate (SDS) and 2mg/mL Proteinase K. Samples were vortexed briefly and incubated overnight at 55°C. The following day, 325μL binding buffer (supplied by the manufacturer) and 325μL ethanol was added to the samples, mixed, and pipetted into the upper chamber of High Pure filter tube. Samples were spun for 30 seconds at 10,000 x g and the flow-through was discarded. The spin was repeated in order to dry the filter fleece completely. Samples were then washed three times with wash buffer (supplied by the manufacturer) and spun each time to discard the flow-through. The High Pure Filter was then spun for two minutes at maximum speed before eluting nucleic acid in 90μL elution buffer. One unit of DNase in DNase Incubation Buffer was added to the eluate and incubated for 45 minutes at 37°C. Tissue lysis buffer with a final concentration of 3.6% SDS and 2mg/mL Proteinase K was added to the samples and incubated for one hour at 55°C.

After the incubation, 325µL Binding Buffer (supplied by the manufacturer) and 325µL 100% ethanol was added to the samples and pipetted into a fresh High Pure filter tube. Samples were spun for 30 seconds at 10,000 x g and the flow through was discarded, repeated by a second spin to dry the filter fleece completely. Samples were washed three times with wash buffer and spun each time to discard the flow through. The High Pure Filter was then spun for two minutes at maximum speed before finally eluting the RNA in 50µL elution buffer.

Total RNA concentration was determined spectrophotometrically at 260 nm (NanoDrop 1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, DE) and samples were stored at -80°C. The presence of high molecular weight total RNA was determined using a bioanalyzer (Agilent Technologies, Bio-Rad, Hercules, CA) and ethidium bromide staining of samples using formaldehyde gel electrophoresis. Samples that did not have an A_{260}/A_{280} ratio greater than or equal to 1.8 were not included in the study.

3.2.4. Reverse transcription

CDNA was synthesized using 2µg total RNA and Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carslband, CA) with random hexamers to ensure representation of all mRNA independent of polyadenylated tail. We first mixed 2µg total RNA, 50ng random hexamers, 1µL dNTP mix (10mM each of dCTP, dATP, dTTP, dGTP), and added RNAse-free water up to 10µL. The contents of the tube were heated for five minutes at 65°C followed by a chill on ice. The products were collected by centrifugation, and to a final volume of 20µL, 1X RT Buffer (10X stock), 20mM dithiothreitol (DTT), 10mM $MgCl_2$, and 40 units of RNAse™ Out was added. The

reaction mix was incubated for two minutes at 25°C before adding 200 units Superscript™ RT, followed by a ten minute incubation at 25°C and a 50 minute incubation at 42°C. The enzyme was inactivated by incubating the reaction mix at 70°C for 15 minutes.

3.2.5. Quantitative real time PCR

Five µL cDNA was used for quantitative real time polymerase chain reaction (qRT-PCR). Reactions were performed using the ABI Prism 7000 (Applied Biosystems, Foster City, CA) with 96-well optical reaction plates (Applied Biosystems, Foster City, CA). Primer/probe sets were purchased as pre-made TaqMan Assays on Demand for *KLK13, KLK6*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The details for each probe are described in Table 3.1. 500ng cDNA was added to TaqMan PCR Reaction Mix, TaqMan Probe, and water to 20µL total reaction volume. Thermal cycling was carried out as detailed by the manufacturer in the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for one minute. All samples were analyzed in triplicate for each target gene.

The expression of each probe was measured by relative quantification, defined as the amount of the specific mRNA normalized to normal ovary as determined using the ΔΔ cycle threshold (C_T) method (Pfaffl, 2001). In order to effectively use the $\Delta\Delta C_T$ method, it is recommended by the manufacture that a validation experiment of the target and control genes' amplification efficiency is required. This test ensures that the efficiency of the target gene and the reference gene amplification are approximately equal. This validation experiment is carried out by examining how ΔC_T varies with template dilution.

Table 3.1 TaqMan Probes used for qRT-PCR analysis.

Probe	Target	Accession Number	Transcript Variants Detected	mRNAs Detected	Amplicon Length
Hs00160519_m1	KLK6[1]	NM_002774.3 NM_001012964.1 NM_001012965.1	Transcript Variant A Transcript Variant B Transcript Variant C	13	119bp
Hs01087307_m1	KLK13[2]	NM_015596.1	Splice Variant 1 Splice Variant 2 Splice Variant 3 Splice Variant 4 Splice Variant 5 Splice Variant 6 Splice Variant 7 Splice Variant 8	10	82bp
Hs99999905_m1	GAPDH[3]	NM_002046.3	N/A	121	122bp

1. Human kallikrein-related peptidase 6
2. Human kallikrein-related peptidase 13
3. Glyceraldehyde 3-phosphate dehydrogenase

The absolute value of the slope of log input amount vs. ΔC_T should be less than 0.1. The absolute slopes of our validation experiments were 0.076 and 0.024 for *KLK6* and *KLK13*, respectively. A summary of the validation experiment for *KLK6* can been seen in Figure 3.1.

The relative target gene expression was defined as $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\ \text{normal ovary}} - \Delta C_{T\ \text{ovarian cancer}}$. A representative normal ovarian sample having the median level of expression was chosen as the calibrator sample (ie., target gene expression = 1). ΔC_T is defined as $C_{T\ \text{target}} - C_{T\ GAPDH}$, where the target genes were *KLK13* and *KLK6*. Relative to the calibrator sample, target gene relative expression (RE) was classified as being low or high kallikrein expression. The expression level for determining high expressing samples was defined as one standard deviation above the mean value for normal ovarian RE, for a target gene. Samples with a RE equal to or above this value were classified as high expression, while samples below the cut-off were classified as having low expression. One standard deviation above the mean was chosen as the cut-off in order to account for inherent variation of each target gene. The cut-off for *KLK6* was determined to be 5.211 RE and *KLK13* was 0.981 RE.

3.2.6. Statistical analysis

All statistical analysis was performed with the Statistical Package for Social Sciences (SPSS; version 13.0, Chicago, IL, USA). The relationships between *KLK6* and *KLK13* mRNA expression and patient clinical characteristics were analyzed with the χ^2 test. For survival analysis, two endpoints were examined; cancer recurrence (defined as either a

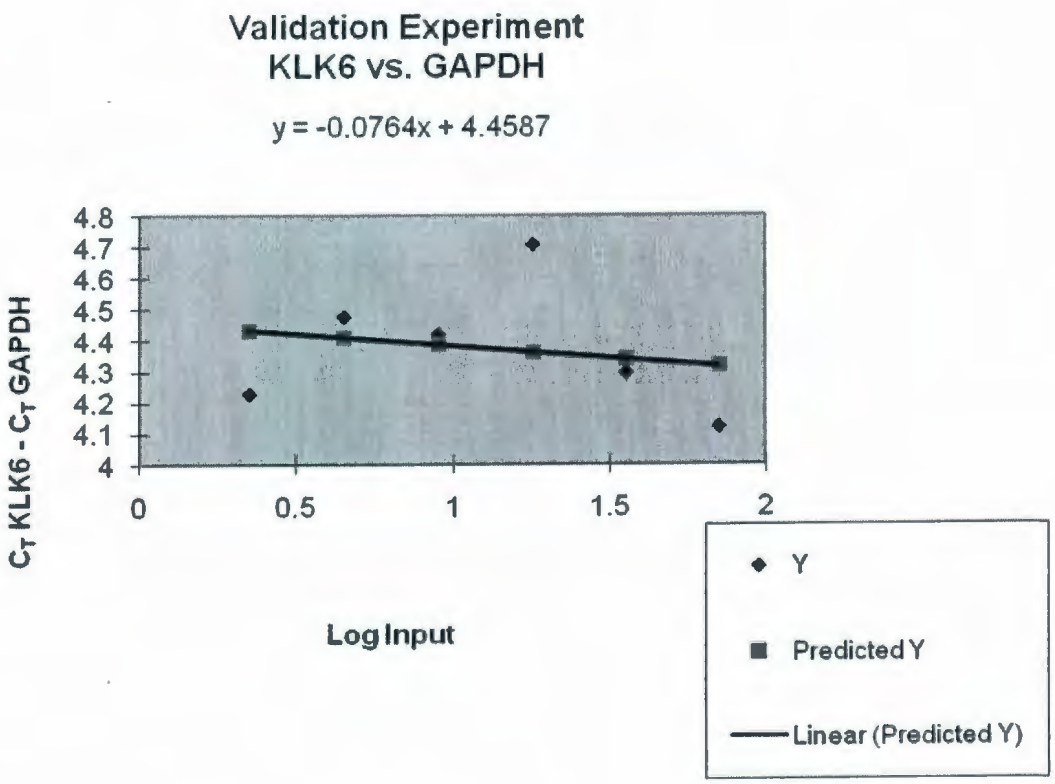


Figure 3.1 Validation experiment for the amplification of *KLK6* and *GAPDH* by qRT-PCR. The validity of the efficiency of *KLK6* and *GAPDH* amplification was assessed by plotting log input amount versus ΔC_T. The absolute slope is 0.0764, indicating the $\Delta\Delta C_T$ calculation is valid.

local recurrence or metastasis) and death. These endpoints were used to calculate the recurrence-free survival (RFS) and overall survival (OS), respectively. RFS is defined as the time from first diagnosis to the time of first detected recurrence or metastasis. OS is defined as the time from initial diagnosis to the time of death. Two survival models, the Kaplan-Meier and the Cox Hazard Regression Model, were used for analysis. The Kaplan-Meier model was used to examine survival between the patients expressing kallikreins at low or high levels, while significance was measured with the log-rank test. The Cox Hazard Regression Model, using both univariate and multivariate models, was used to determine the hazard ratio (HR).

3.3 Results

3.3.1. Immunohistochemical localization of KLK6 and KLK13 in ovarian carcinoma

Figure 3.2 illustrates KLK6 and KLK13 localization in ovarian surface epithelium (OSE) and epithelial ovarian carcinoma. Both KLK6 and KLK13 were localized in the cytoplasm of the normal ovarian surface epithelium (Figure 3.2 A & B). Negative control sections were stained with non-immune rabbit serum and there was no positive KLK6 or KLK13 staining (data not shown). Also, both KLK6 and KLK13 showed staining in all types of ovarian adenocarcinoma. Both KLK6 and KLK13 displayed strong staining in serous adenocarcinoma (Figure 3.2 C & D), while KLK6 was expressed in a mucinous adenocarcinoma (Figure 3.2 E), and KLK13 was expressed in clear cell tumor (Figure 3.2 F). In all cases, staining was most prominent in epithelial

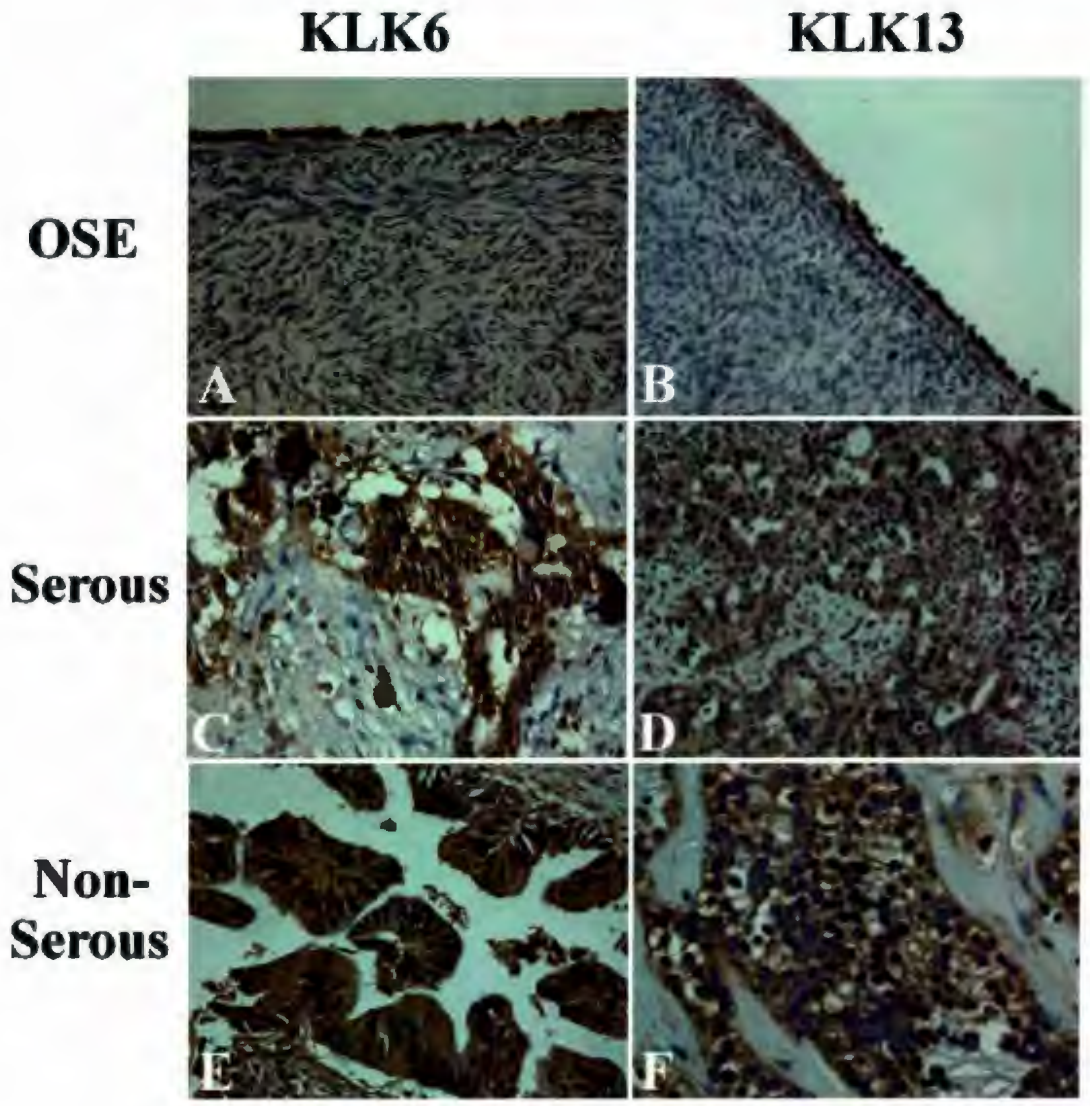


Figure 3.2 KLK6 and KLK13 expression in normal ovarian surface epithelium (OSE) and epithelial ovarian cancer as determined by immunohistochemistry. The normal OSE stained positive for both KLK6 (A) and KLK13 (B) in the cytoplasm. Serous epithelial cancers expressed KLK6 (C) and KLK13 (D) in the cytoplasm. In a mucinous ovarian tumor, KLK6 showed strong cytoplasmic and some nuclear expression (E). KLK13 is positively expressed in a clear cell ovarian tumor (F). All photomicrographs were taken at 400X magnification.

cells, whether they were normal surface epithelium or carcinoma. Immunohistochemical evaluation of protein levels resulted in no significant associations with clinical characteristics (data not shown). This may be due to the fact that the intensity of staining in each section may not be a true representation of kallikrein expression levels in the sample, but may be confounded by variations in the fixing and embedding procedures used during the processing of tissues. Therefore, in this case, immunohistochemistry analysis specified KLK protein localization in normal ovary and ovarian cancer. KLK gene expression in ovarian cancer was assessed by qRT-PCR.

3.3.2. Association between kallikrein-related peptidase gene expression and clinical variables

The relationship between *KLK6* and *KLK13* expression levels and clinical characteristics is summarized in Table 3.2. Patient ages range from 20 to 89 years old with a mean age of 60 years. Compared to patients with low *KLK6* expressing tumors, a significantly larger proportion of patients with high *KLK6* expression had invasive (p=0.002) and late stage cancers (p=0.001). Compared to patients with low *KLK13* expression, a larger proportion of patients with high *KLK13* expression had invasive cancer (p=0.039). Unlike *KLK6, KLK13* was not associated with clinical stage. At diagnosis, 65% of all patient samples had serous ovarian cancer. In supplementary analysis, when we compared serous to non-serous ovarian cancers (mucinous, endometrioid, clear cell and unknown), high *KLK6* expression was associated with serous carcinomas (p=0.001; data not shown). Interestingly, all endometrioid ovarian cancers had high *KLK13* expression

Table 3.2 Association between *KLK6* and *KLK13* mRNA expression with clinical data for 106 ovarian carcinoma patients and 8 normal ovaries.

Variable	n	No. of Patients (%)			No. of Patients (%)		
		KLK6 **Low**	***KLK6*** **High**	***p-*** **value**	***KLK13*** **Low**	***KLK13*** **High**	***p-*** **value**
Age							
<50	24	8 (33.3)	16 (19.5)	0.155	9 (18.8)	15 (25.9)	0.384
≥ 50	82	16 (66.7)	66 (80.5)		39 (81.2)	43 (74.1)	
Status							
Normal	8	6 (20.0)	2 (2.4)	0.002	7 (12.7)	1 (1.7)	0.039
Borderline	6	3 (10.0)	3 (3.6)		4 (7.3)	2 (3.4)	
Invasive	100	21 (70.0)	79 (94.0)		44 (80.0)	56 (94.9)	
Histological							
Serous	69	10 (41.6)	59 (72.0)	0.073	33 (68.8)	36 (62.1)	0.082
Mucinous	15	6 (25.0)	9 (11.0)		5 (10.4)	10 (17.2)	
Endometrioid	6	3 (12.5)	3 (3.6)		0	6 (10.3)	
Clear Cell	4	1 (4.2)	3 (3.6)		2 (4.2)	2 (3.4)	
Unknown	12	4 (16.7)	8 (9.8)		8 (16.6)	4 (7.0)	
Clinical Stage							
Early (I/II)	32	14 (58.3)	18 (22.0)	0.001	16 (33.3)	16 (27.6)	0.521
Late (III/IV)	74	10 (41.7)	64 (78.0)		32 (66.7)	42 (72.4)	
Tumor Grade							
GB/G1	21	6 (25.0)	15 (18.3)	0.468	12 (25.0)	9 (15.5)	0.223
G2/G3	85	18 (75.0)	67 (81.7)		36 (75.0)	49 (84.5)	

relative to normal ovaries, but the small sample size was unable to provide sufficient power for a conclusive association.

3.3.3. High *KLK6* expression is associated with recurrence and patient survival

Kaplan-Meier survival curves indicated patients with high *KLK6* expression were more likely to have a shorter RFS (p=0.002, Figure 3.3A), and OS (p=0.011, Figure 3.3B), when compared to patients who had low *KLK6* expressing tumors. These data are further supported by the Cox Regression analysis presented in Table 3.3. In univariate analysis, patients with high *KLK6* expression had a greater risk of recurrence (p=0.004) than patients with low *KLK6* expressing tumors (Table 3.3). As expected, clinical stage (p<0.001), tumor grade (p=0.012), and histological type (p=0.024), were all significant predictors of recurrence. In the multivariate model (Table 3.4), high *KLK6* expression remains a significant predictor of recurrence (p=0.040), indicating these patients were approximately three times more likely to have a recurrence than patients with low *KLK6* expression. Overall, late clinical stage (Stage III/IV) was the strongest predictor of recurrence (p=0.001).

When OS was examined in a Cox univariate model (Table 3.3), high *KLK6* expression was significantly associated with a shorter OS (p=0.013). As expected, clinical stage (p<0.001), tumor grade (p<0.001), and histological type (p=0.037), were all associated with a shorter OS. Interestingly, patients 50 years of age or older, at the time of diagnosis, also had a significantly shorter OS (p=0.045) than patients under the age of 50. When these factors were included in a multivariate analysis (Table 3.4), clinical stage was the strongest predictor of OS as patients with late stage (Stage III/IV) cancers had a

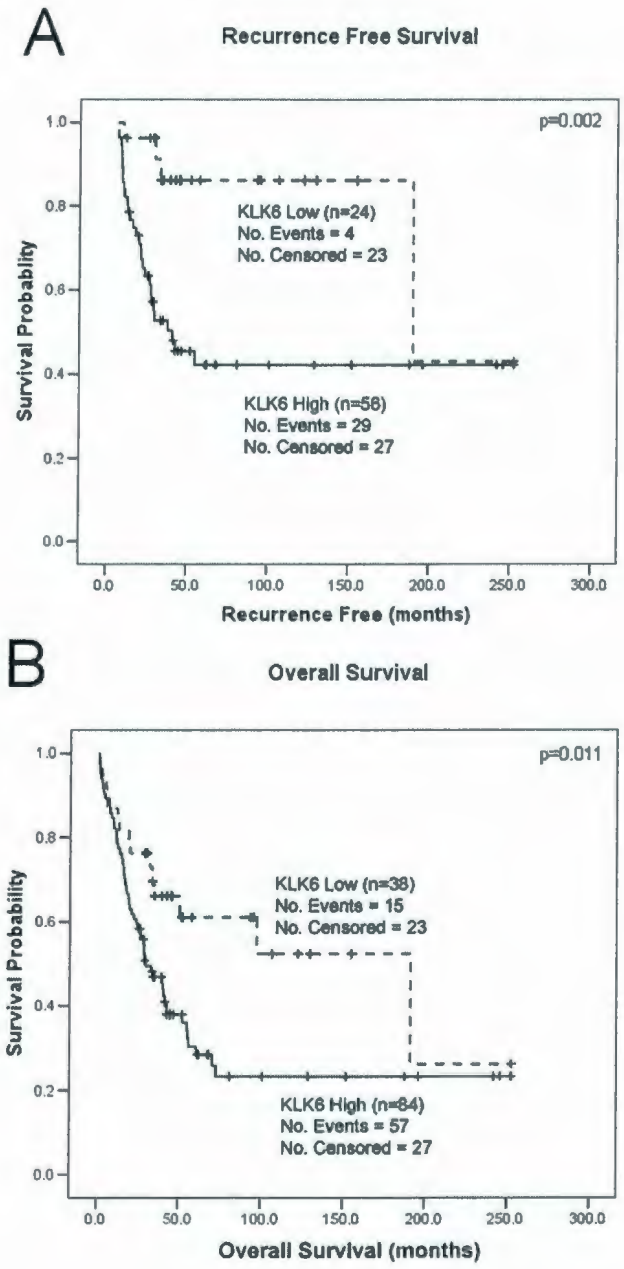


Figure 3.3 Kaplan-Meier survival curves show the prognostic significance of *KLK6* mRNA expression in terms of RFS and OS in ovarian cancer patients. (A) Patients who have high *KLK6* expressing tumors have a shorter recurrence free survival (RFS) than patients with low *KLK6* expressing tumors (p=0.002). (B) Patients who have high *KLK6* expressing tumors have a shorter overall survival (OS) than patients with low *KLK6* expressing tumors (p=0.011). Patients whose tumors have low *KLK6* expression are represented with the broken line, while patients with high *KLK6* expression are represented by the solid line. n, number of patients

Table 3.3 Univariate Cox Regression Analysis of *KLK6* and *KLK13* expression in ovarian cancer patients.

Variable	RFS			OS		
	HR[1]	95% CI[2]	*p*-value	HR[1]	95% CI[2]	*p*-value
Univariate Analysis						
KLK6						
Low	1.00			1.00		
High	***4.59***	***1.61-13.08***	***0.004***	***2.06***	***1.16-3.63***	***0.013***
KLK13						
Low	1.00			1.00		
High	***2.19***	***1.08-4.46***	***0.030***	1.33	0.83-2.19	0.231
Clinical Stage[3]	11.89	3.61-39.15	<0.001	5.91	2.82-12.35	<0.001
Tumor Grade[4]	3.42	1.31-8.94	0.012	3.83	1.82-8.07	<0.001
Histological Type[5]	2.31	1.12-4.78	0.024	1.67	1.03-2.71	0.037
Age[6] (ordinal)	2.76	0.97-7.87	0.057	1.98	1.02-3.87	0.045

1. HR, Hazard Ratio;
2. 95% CI, 95% Confidence Interval
3. Clinical Stage, late vs. early Stage
4. Tumor Grade, poor vs. well differentiated
5. Histological Type, serous vs. non-serous
6. Age, ≥50 vs. <50

Table 3.4 Multivariate Cox Regression Analysis of *KLK6* expression in ovarian cancer patients..

Variable	RFS			OS		
	HR[1]	95% CI[2]	*p*-value	HR[1]	95% CI[2]	*p*-value
Multivariate						
KLK6						
Low	1.00			1.00		
High	***3.03***	***1.05-8.74***	***0.040***	1.45	0.81-2.60	0.215
Clinical Stage[3] (ordinal)	8.57	2.45-30.05	0.001	3.73	1.70-8.18	0.001
Tumor Grade[4] (ordinal)	1.48	0.52-4.16	0.457	2.08	0.94-4.63	0.073
Histological Type[5]	1.92	0.91-4.04	0.087	1.25	0.77-2.04	0.367
Age[6] (ordinal)	2.19	0.72-6.67	0.167	1.47	0.74-2.94	0.271

1. HR, Hazard Ratio;
2. 95% CI, 95% Confidence Interval
3. Clinical Stage, late vs. early Stage
4. Tumor Grade, poor vs. well differentiated
5. Histological Type, serous vs. non-serous
6. Age, ≥50 vs. <50

four fold increased likelihood of a shorter OS (p=0.001) than patients having early stage (Stage I/II) cancer. Other clinical characteristics lost their prognostic ability to predict OS when subjected to multivariate analysis, including high *KLK6* expression (p=0.215).

3.3.4. High *KLK13* expression in ovarian tumors is associated with poor prognosis

When *KLK13* expression was analyzed with the Kaplan–Meier model, patients with high *KLK13* expression had a shorter RFS than patients with low *KLK13* expression (p=0.027, Figure 3.4A). The strength of association between *KLK13* high expressing tumors and survival outcome is presented in both univariate and multivariate Cox regression models (Table 3.3 & 3.5, respectively). Univariate analysis showed high *KLK13* was a significant predictor of recurrence (p=0.030), indicating a 2.2 fold increased probability of recurrence compared to patients with low *KLK13* expression (Table 3.3). When *KLK13* expression was examined in a multivariate model, it retained the ability to significantly predict a shorter RFS (p=0.047; Table 3.4). Late clinical stage and serous type cancer also significantly predicted a shorter RFS (p=0.001 and p=0.024, respectively). When we examined *KLK13* expression along with other clinical parameters in a multivariate model, late clinical stage was the strongest predictor of OS (p<0.001).

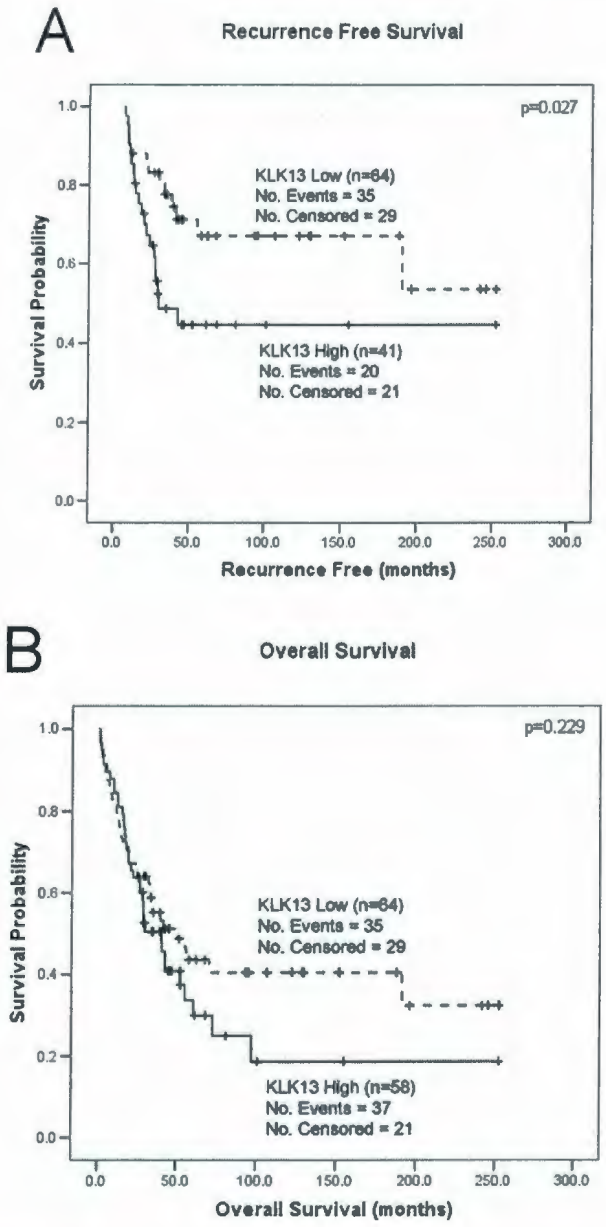


Figure 3.4 Kaplan-Meier survival curves show the prognostic significance of *KLK13* mRNA expression in terms of RFS and OS in ovarian cancer patients. (A) Patients who have high *KLK13* expressing tumors have a shorter recurrence free survival (RFS) than patients with low *KLK13* expressing tumors (p=0.027). (B) Although there is a trend suggesting patients who have high *KLK13* expressing tumors have a shorter overall survival (OS) than patients with low *KLK13* expressing tumors, the finding was not significant (p=0.229). Patients whose tumors have low *KLK13* expression are represented with the broken line, while patients with high *KLK13* expression are represented by the solid line. n, number of patients.

Table 3.5 Multivariate Cox Regression Analysis of *KLK13* expression in ovarian cancer patients.

Variable	RFS			OS		
	HR[1]	95% CI[2]	*p*-value	HR[1]	95% CI[2]	*p*-value
Multivariate Analysis						
KLK13						
Low	1.00			1.00		
High	***2.20***	***1.01-4.78***	***0.047***	1.00	0.61-1.61	0.988
Clinical Stage[3] (ordinal)	9.08	2.61-31.65	0.001	4.00	1.84-8.70	<0.001
Tumor Grade[4] (ordinal)	1.22	0.42-3.55	0.717	2.14	0.95-4.81	0.065
Histological Type[5] (ordinal)	2.43	1.13-5.24	0.024	1.29	0.79-2.10	0.308
Age[6] (ordinal)	2.49	0.82-7.54	0.108	1.46	0.73-2.91	0.286

1. HR, Hazard Ratio;
2. 95% CI, 95% Confidence Interval
3. Clinical Stage, late vs. early Stage
4. Tumor Grade, poor vs. well differentiated
5. Histological Type, serous vs. non-serous
6. Age, ≥50 vs. <50

3.4 Discussion

Unlike other reproductive malignancies, such as prostate cancer, ovarian cancer lacks a biomarker that may be used for general population screening. Currently, CA125, the only marker used in ovarian cancer patients, is reliable for monitoring response to treatment and disease recurrence (Rustin *et al.*, 2004). The identification of early and novel biomarkers for the diagnosis and prognosis of ovarian cancer may lead to novel therapeutic applications and potential screening tests, thus decreasing the mortality of this deadly malignancy. The aim of this study was to examine the expression of *KLK6* and *KLK13* in ovarian cancer to determine their diagnostic and/or prognostic value. This is the first report to examine *KLK13* mRNA expression in ovarian cancer and the largest set of specimens in which *KLK6* mRNA expression has been analyzed.

QRT-PCR was used to assess *KLK6* and *KLK13* mRNA expression in FFPE ovarian cancer tissues. Recent advances in biotechnology have lead to studies that have yielded high quality data from FFPE tissues, data that is reproducible, precise, and comparable to data obtained from frozen specimens (Specht *et al.*, 2001). These advances can be attributed to the determination of optimized reagent conditions. In order to extract total RNA from archival tissues, the High Pure RNA Paraffin Kit (Roche, Indianapolis, IN) was used. This kit is specifically optimized for the isolation of RNA from FFPE tissues. In order to ensure RNA was suitable to use for qRT-PCR, RNA concentration and purity was measured on a spectrophotometer and bioanaylzer. Also, RNA samples were run on a denaturing formaldehyde gel and stained with ethidium bromide to ensure high molecular weight RNA. Recently, the expression of a number of genes has been

examined by extracting mRNA from FFPE tissues in esophageal (Tanaka *et al.*, 2009), pancreatic (Ikenaga *et al.*, 2009), and breast and ovarian cancer (Berger *et al.*, 2010) as well as Barrett's esophagus (Botelho *et al.*, 2010).

The quality of RNA in FFPE samples was considered when performing the reverse transcription reactions. Rather than the traditional oligo(dT) used to prime RNA, random hexamers were used. In some cases, due to the increased susceptibility of RNA degradation, the poly-A tail may be lost and the use of random hexamers facilitates increased successful cDNA production from multiple points along an RNA molecule. Another consequence of RNA degradation is the lack of ability to produce long amplicons via PCR. In order to avoid a misrepresentation of gene expression, a short amplicon of less than 200bp is recommended for qRT-PCR. The amplicon lengths were 119bp, 82bp, and 122 bp, for *KLK13*, *KLK6*, and *GAPDH*, respectively. Together, the use of random hexamers to prime the reverse transcription and the short amplicon length allowed a high tolerance for obtaining quality data. Also, when choosing probes for the successful detection of mRNA, it is recommended that the probe targets more than two exons of the mRNA (Bustin *et al.*, 2009). The TaqMan probe used for *KLK13* targeted all five coding exons and detected all eight splice variants, while the *KLK6* probe targeted four of the five coding exons, detecting all splice variants. The probe for *GAPDH* targeted three of the nine coding exons and detected 121 reported mRNA sequences.

One limitation that comes with the use of FFPE tissues that must be acknowledged is the fact that the detection of mRNA in this tissue source is dependent on the state of

degradation of the mRNA. In order to determine the maximum length of mRNA, a series of primers, with increasing amplicon lengths, can be designed in order to identify the largest detectable RNA. In the absence of this test, one must be aware that variations in gene expression may be due to sample variation and detection. In order to account for these concerns, RNA samples were subjected to a bioanalyzer, which measured RNA concentration, 260/280 ratio, and RNA integrity, as well as agarose gels to ensure high molecular weight RNA. Samples that were not suitable were not used in the study. Supporting these findings is the fact that the current results agree with previously reported findings. KLK6 is well documented to be a poor prognostic indicator in ovarian cancer (Anisowicz *et al.*, 1996;Diamandis *et al.*, 2000a;Kountourakis *et al.*, 2008;Luo *et al.*, 2006;Ni *et al.*, 2004;Oikonomopoulou *et al.*, 2006;Shan *et al.*, 2007;Tanimoto *et al.*, 2001;Yousef *et al.*, 2004). As well, previously published findings support results that KLK13 has increased expression in ovarian cancer (Kapadia *et al.*, 2003) and is a poor prognostic indicator for this malignancy.

Although there are many considerations when using FFPE specimens, the advances in technology and careful considerations made by investigators when using this tissue source ensure reliable results. FFPE tissue also comes with a major advantage. Because the tissue is usually stored for long periods of time, patient clinical outcome is known. This greatly facilitates retrospective studies, especially those examining prognostic variables, as we have the information available to correlate gene expression with tumor and patient characteristics, as well as clinical outcome.

When tissues were examined for *KLK* expression, high *KLK6* mRNA expression was associated with serous ovarian cancer and late stage disease (Table 3.2). These results are similar to previous studies that found increased *KLK6* expression in ovarian cancers when compared to normal ovarian tissue (Anisowicz *et al.*, 1996;Ni *et al.*, 2004;Tanimoto *et al.*, 2001). Consistent with the current study (Table 3.2), previous studies have also associated high KLK6 levels with advanced ovarian cancer (Stage III-IV) and serous tumor histology (Hoffman *et al.*, 2002;Shan *et al.*, 2007). Ovarian serous tumors of borderline and low grade are thought to arise from a step-wise progression from adenoma to borderline tumor to carcinoma via the *Ras-Raf* signalling pathway (Bell, 2005). Recently, KLK6 expression and secretion has been shown to be Ras-dependant in a colon carcinoma cell line (Henkhaus *et al.*, 2008). Constitutively active mutant K-Ras resulted in enhanced colon cancer cell invasion through both laminin and Matrigel matrixes. Together, these data suggest that *Ras-Raf* mutations may increase the invasive potential of these borderline tumors through increased expression of KLK6. This may also hold true for ovarian cancer as high KLK6 expression is associated with later stage, more invasive, cancers.

This study is the first to report *KLK13* mRNA expression in normal ovary and ovarian cancer patients. The expression of *KLK13* mRNA in normal OSE was extremely low, while 55% of ovarian cancers examined had high *KLK13* expression (Table 3.2). These findings support Kapadia et al., who found serum levels of KLK13 were below levels of detection in healthy individuals, yet 50% of ovarian cancer patients were positive for KLK13 (Kapadia *et al.*, 2003). It also corroborates previous data as the IOSE cell line as

an appropriate cell model as it also had low *KLK13* expression (Figure 2.1). Interestingly, 100% of ovarian endometrioid cancer samples were high grade and expressed *KLK13* at a high level (Table 3.2). Although the sample size is small (6 cases), this expression pattern warrants further investigation. Low-grade endometrioid carcinomas have been suggested to arise from endometriosis or borderline endometrioid tumors (Obata *et al.*, 1998), while high-grade endometrioid carcinomas have changes similar to high-grade serous carcinomas (Bell, 2005;Giordano *et al.*, 2008;Press *et al.*, 2008) suggesting endometrioid cancers may represent two separate malignancies. This characteristic expression pattern of KLK13 may represent not only a novel marker to distinguish between high and low grade endometrioid cancers, but a unique pathway in which KLK13 may be involved in ovarian carcinogenesis.

When the clinical utility of *KLK6* and *KLK13* was assessed in terms of RFS and OS, both KLKs were associated with poor prognosis in ovarian cancer patients (White *et al.*, 2009). Previous studies have reported similar results (Anisowicz *et al.*, 1996;Diamandis *et al.*, 2000a;Hoffman *et al.*, 2002;Kountourakis *et al.*, 2008;Ni *et al.*, 2004;Prezas *et al.*, 2006;Scorilas *et al.*, 2004). Additionally, *KLK6* has been shown to have increased expression in uterine and pancreatic cancers (Ruckert *et al.*, 2008;Santin *et al.*, 2005) and has been shown to be a poor prognostic marker in colon, gastric, and lung cancers (Nagahara *et al.*, 2005;Nathalie *et al.*, 2009;Ogawa *et al.*, 2005). KLK13 has been shown to have increased expression in lung and salivary gland tumors (Darling *et al.*, 2006a;Planque *et al.*, 2008). The increased expression of both KLK6 and KLK13 over a number of different cancers, in addition to their ability to cleave ECM (Ghosh *et al.*,

2004;Kapadia *et al.*, 2004) strongly suggests they may play a role in cancer pathogenesis and may have use as potential diagnostic and prognostic markers.

Kallikrein protein expression was examined immunohistochemically in order to determine protein localization (Figure 3.2). When the prognostic significance of these sections was analyzed, no significant associations were determined. These findings are similar to previous reports examining the expression of KLK6 in primary pancreatic ductal adenocarcinoma (Ruckert *et al.*, 2008) and salivary gland tumors (Darling *et al.*, 2006b), finding no significant associations with patient survival. Although the OSE showed positive KLK protein expression, KLK mRNA expression in the ovary was very low or had no expression. This discordance may be explained by the fact that kallikreins take extended time to be secreted. Although the mRNA signal is low in the cell, there is a build up of protein while they are held in the cytoplasm and this may be reflected in the immunostains. There may also be variations in fixing and embedding procedures used during the processing of tissues which may be reflected in the staining. The use of negative controls for OSE sections indicated KLK protein expression was true and not an artifact that could be caused by the edge effect of immunohistochemistry.

Among both the normal ovary and ovarian cancers, there was cytoplasmic expression of both human KLK6 and KLK13 (Figure 3.4). There was also some nuclear KLK6 expression seen in a mucinous adenocarcinoma of the ovary. Both KLK6 and KLK13 are expressed in normal epithelium (Petraki *et al.*, 2003;Petraki *et al.*, 2001), including the OSE as shown here. Ovarian cancer is thought to arise from either OSE or OSE cells

bordering inclusion cysts (Kaku *et al.*, 2003), therefore it is expected that ovarian cancer cells would express a basal level of kallikrein expression. Given the semi-quantitative nature of immunohistochemistry and the fact that KLKs are secreted proteins, to see a significant difference in the cellular levels between normal OSE and ovarian cancer would require not only changes in KLK protein production, but also a significant change in the rate of kallikrein exocytosis.

Epithelial ovarian cancer has the worst prognosis among the gynaecological malignancies, mainly due to the fact that most women are diagnosed in the later stages. Thus, there is an essential need for diagnostic and prognostic markers for this malignancy. Although there have been many candidates, none can fulfill the sensitivity and specificity requirements for a general population screening. Expression of the KLK family in ovarian cancer has been studied extensively and many KLKs have proven to be potential diagnostic and prognostic markers (Borgono *et al.*, 2003a;Borgono *et al.*, 2003b;Borgono *et al.*, 2006;Diamandis *et al.*, 2003;Diamandis *et al.*, 2000a;Dong *et al.*, 2003;Dong *et al.*, 2001;Kim *et al.*, 2001;Kountourakis *et al.*, 2008;Kurlender *et al.*, 2004;Kyriakopoulou *et al.*, 2003;Luo *et al.*, 2001;Magklara *et al.*, 2001;Shigemasa *et al.*, 2004a;Shigemasa *et al.*, 2004b;Shvartsman *et al.*, 2003;Sidiropoulos *et al.*, 2005;Yousef *et al.*, 2003).

Our study has confirmed that both KLK6 and KLK13 are overexpressed in ovarian cancer and are independent predictors of poor prognosis in ovarian cancer patients (White *et al.*, 2009). We have shown that patients with high expression of KLK6 (Table 3.4) or

KLK13 (Table 3.5) are more likely to have a tumor recurrence than patients with low kallikrein expressing tumors. This study is the first to report increased expression of *KLK13* mRNA in ovarian cancer patients and indicated that *KLK13* may represent a specific biomarker for endometrioid carcinoma. These findings support the potential role of *KLK6* and *KLK13* as novel ovarian cancer biomarkers and may, in the future, offer targets for therapeutic applications.

3.5 Authors Contributions

A version of the chapter was published in the *British Journal of Cancer* as:

KLK6 and KLK13 predict tumor recurrence in epithelial ovarian carcinoma

British Journal of Cancer 2009:101 (7):1107-13.

The following outlines the authors' contributions to this study:

Nicole White

- Conception and design of the study, collection, analysis and interpretation of data, collection and assembly of data, writing the manuscript

Maria Mathews

- Assistance in analysis and interpretation of data

George Yousef

- Consultation for the conception and design of the study, selection of patient samples

Amrah Prizada

- Assistance in the collection of the data

Cathy Popadiuk

- Recruitment of patient samples and editorial input during manuscript preparation

Jules Doré

- Consultation for the conception and design of the study, interpretation of data, editorial revision of the article manuscript

3.6 References

Anisowicz,A., Sotiropoulou,G., Stenman,G., Mok,S.C., and Sager,R. (1996). A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med. *2*, 624-636.

Bell,D.A. (2005). Origins and molecular pathology of ovarian cancer. Mod. Pathol. *18*, S19-S32.

Berger,R., Fiegl,H., Goebel,G., Obexer,P., Ausserlechner,M., Doppler,W., Hauser-Kronberger,C., Reitsamer,R., Egle,D., Reimer,D., Muller-Holzner,E., Jones,A., and Widschwendter,M. (2010). Toll-like receptor 9 expression in breast and ovarian cancer is associated with poorly differentiated tumors. Cancer Sci. *101*, 1059-1066.

Borgono,C.A. and Diamandis,E.P. (2004). The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer *4*, 876-890.

Borgono,C.A., Fracchioli,S., Yousef,G.M., Rigault De La Longrais IA, Luo,L.Y., Soosaipillai,A., Puopolo,M., Grass,L., Scorilas,A., Diamandis,E.P., and Katsaros,D. (2003a). Favorable prognostic value of tissue human kallikrein 11 (hK11) in patients with ovarian carcinoma. Int. J. Cancer *106*, 605-610.

Borgono,C.A., Grass,L., Soosaipillai,A., Yousef,G.M., Petraki,C.D., Howarth,D.H., Fracchioli,S., Katsaros,D., and Diamandis,E.P. (2003b). Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. Cancer Res. *63*, 9032-41.

Borgono,C.A., Kishi,T., Scorilas,A., Harbeck,N., Dorn,J., Schmalfeldt,B., Schmitt,M., and Diamandis,E.P. (2006). Human kallikrein 8 protein is a favorable prognostic marker in ovarian cancer. Clin. Cancer Res. *12*, 1487-1493.

Botelho,N.K., Schneiders,F.I., Lord,S.J., Freeman,A.K., Tyagi,S., Nancarrow,D.J., Hayward,N.K., Whiteman,D.C., and Lord,R.V. (2010). Gene expression alterations in formalin-fixed, paraffin-embedded Barrett's esophagus and esophageal adenocarcinoma tissues. Cancer Biol. Ther. *10*.

Bustin,S.A., Benes,V., Garson,J.A., Hellemans,J., Huggett,J., Kubista,M., Mueller,R., Nolan,T., Pfaffl,M.W., Shipley,G.L., Vandesompele,J., and Wittwer,C.T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. *55*, 611-622.

Darling,M.R., Jackson-Boeters,L., Daley,T.D., and Diamandis,E.P. (2006a). Human kallikrein 13 expression in salivary gland tumors. Int. J. Biol. Markers *21*, 106-110.

Darling,M.R., Jackson-Boeters,L., Daley,T.D., and Diamandis,E.P. (2006b). Human kallikrein 6 expression in salivary gland tumors. J. Histochem. Cytochem. *54*, 337-342.

Diamandis,E.P., Borgono,C.A., Scorilas,A., Yousef,G.M., Harbeck,N., Dorn,J., Schmalfeldt,B., and Schmitt,M. (2003). Immunofluorometric quantification of human kallikrein 5 expression in ovarian cancer cytosols and its association with unfavorable patient prognosis. Tumour Biol. *24*, 299-309.

Diamandis,E.P., Yousef,G.M., Soosaipillai,A.R., and Bunting,P. (2000a). Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin. Biochem. *33*, 579-583.

Diamandis,E.P., Yousef,G.M., Soosaipillai,A.R., Grass,L., Porter,A., Little,S., and Sotiropoulou,G. (2000b). Immunofluorometric assay of human kallikrein 6 (zyme/protease M/neurosin) and preliminary clinical applications. Clin. Biochem. *33*, 369-375.

Dong,Y., Kaushal,A., Brattsand,M., Nicklin,J., and Clements,J.A. (2003). Differential splicing of KLK5 and KLK7 in epithelial ovarian cancer produces novel variants with potential as cancer biomarkers. Clin. Cancer Res. *9*, 1710-20.

Dong,Y., Kaushal,A., Bui,L., Chu,S., Fuller,P.J., Nicklin,J., Samaratunga,H., and Clements,J.A. (2001). Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. Clin. Cancer Res. *7*, 2363-2371.

Ghosh,M.C., Grass,L., Soosaipillai,A., Sotiropoulou,G., and Diamandis,E.P. (2004). Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol. *25*, 193-199.

Giordano,G., Azzoni,C., D'Adda,T., Rocco,A., Gnetti,L., Froio,E., Merisio,C., and Melpignano,M. (2008). Human papilloma virus (HPV) status, p16(INK4a), and p53 overexpression in epithelial malignant and borderline ovarian neoplasms. Pathol. Res. Pract. *204*, 163-174.

Henkhaus,R.S., Gerner,E.W., and Ignatenko,N.A. (2008). Kallikrein 6 is a mediator of K-RAS-dependent migration of colon carcinoma cells. Biol. Chem. *389*, 757-764.

Hoffman,B.R., Katsaros,D., Scorilas,A., Diamandis,P., Fracchioli,S., Rigault de la Longrais,I.A., Colgan,T., Puopolo,M., Giardina,G., Massobrio,M., and Diamandis,E.P. (2002). Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new independent unfavourable prognostic biomarker. Br. J. Cancer *87*, 763-771.

Holschneider,C.H. and Berek,J.S. (2000). Ovarian cancer: epidemiology, biology, and prognostic factors. Semin. Surg. Oncol. *19*, 3-10.

Ikenaga,N., Ohuchida,K., Mizumoto,K., Yu,J., Fujita,H., Nakata,K., Ueda,J., Sato,N., Nagai,E., and Tanaka,M. (2009). S100A4 mRNA is a diagnostic and prognostic marker in pancreatic carcinoma. J. Gastrointest. Surg. *13*, 1852-1858.

Kaku,T., Ogawa,S., Kawano,Y., Ohishi,Y., Kobayashi,H., Hirakawa,T., and Nakano,H. (2003). Histological classification of ovarian cancer. Med. Electron Microsc. *36*, 9-17.

Kapadia,C., Chang,A., Sotiropoulou,G., Yousef,G.M., Grass,L., Soosaipillai,A., Xing,X., Howarth,D.H., and Diamandis,E.P. (2003). Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin. Chem. *49*, 77-86.

Kapadia,C., Ghosh,M.C., Grass,L., and Diamandis,E.P. (2004). Human kallikrein 13 involvement in extracellular matrix degradation. Biochem. Biophys. Res. Commun. *323*, 1084-1090.

Kim,H., Scorilas,A., Katsaros,D., Yousef,G.M., Massobrio,M., Fracchioli,S., Piccinno,R., Gordini,G., and Diamandis,E.P. (2001). Human kallikrein gene 5 (KLK5) expression is an indicator of poor prognosis in ovarian cancer. Br. J. Cancer *84*, 643-50.

Klucky,B., Mueller,R., Vogt,I., Teurich,S., Hartenstein,B., Breuhahn,K., Flechtenmacher,C., Angel,P., and Hess,J. (2007). Kallikrein 6 induces E-cadherin shedding and promotes cell proliferation, migration, and invasion. Cancer Res. *67*, 8198-8206.

Kountourakis,P., Psyrri,A., Scorilas,A., Camp,R., Markakis,S., Kowalski,D., Diamandis,E.P., and Dimopoulos,M.A. (2008). Prognostic value of kallikrein-related peptidase 6 protein expression levels in advanced ovarian cancer evaluated by automated quantitative analysis (AQUA). Cancer Sci. *99*, 2224-2229.

Kurlender,L., Yousef,G.M., Memari,N., Robb,J.D., Michael,I.P., Borgono,C., Katsaros,D., Stephan,C., Jung,K., and Diamandis,E.P. (2004). Differential expression of a human kallikrein 5 (KLK5) splice variant in ovarian and prostate cancer. Tumour Biol. *25*, 149-56.

Kyriakopoulou,L.G., Yousef,G.M., Scorilas,A., Katsaros,D., Massobrio,M., Fracchioli,S., and Diamandis,E.P. (2003). Prognostic value of quantitatively assessed KLK7 expression in ovarian cancer. Clin. Biochem. *36*, 135-43.

Little,S.P., Dixon,E.P., Norris,F., Buckley,W., Becker,G.W., Johnson,M., Dobbins,J.R., Wyrick,T., Miller,J.R., MacKellar,W., Hepburn,D., Corvalan,J., McClure,D., Liu,X., Stephenson,D., Clemens,J., and Johnstone,E.M. (1997). Zyme, a novel and potentially amyloidogenic enzyme cDNA isolated from Alzheimer's disease brain. J. Biol. Chem. *272*, 25135-25142.

Luo,L.Y., Bunting,P., Scorilas,A., and Diamandis,E.P. (2001). Human kallikrein 10: a novel tumor marker for ovarian carcinoma? Clin. Chim. Acta *306*, 111-8.

Luo,L.Y., Soosaipillai,A., Grass,L., and Diamandis,E.P. (2006). Characterization of human kallikreins 6 and 10 in ascites fluid from ovarian cancer patients. Tumour Biol. *27*, 227-234.

Magklara,A., Scorilas,A., Katsaros,D., Massobrio,M., Yousef,G.M., Fracchioli,S., Danese,S., and Diamandis,E.P. (2001). The human KLK8 (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic value in ovarian cancer. Clin. Cancer Res. *7*, 806-11.

Nagahara,H., Mimori,K., Utsunomiya,T., Barnard,G.F., Ohira,M., Hirakawa,K., and Mori,M. (2005). Clinicopathologic and biological significance of kallikrein 6 overexpression in human gastric cancer. Clin. Cancer Res. *11*, 6800-6806.

Nathalie,H.V., Chris,P., Serge,G., Catherine,C., Benjamin,B., Claire,B., Christelle,P., Briollais,L., Pascale,R., Marie-Lise,J., and Yves,C. (2009). High kallikrein-related peptidase 6 in non-small cell lung cancer cells: an indicator of tumor proliferation and poor prognosis. J. Cell Mol. Med. *13*, 4014-4022.

Ni,X., Zhang,W., Huang,K.C., Wang,Y., Ng,S.K., Mok,S.C., Berkowitz,R.S., and Ng,S.W. (2004). Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br. J. Cancer. *91*, 725-731.

Obata,K., Morland,S.J., Watson,R.H., Hitchcock,A., Chenevix-Trench,G., Thomas,E.J., and Campbell,I.G. (1998). Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. Cancer Res. *58*, 2095-2097.

Ogawa,K., Utsunomiya,T., Mimori,K., Tanaka,F., Inoue,H., Nagahara,H., Murayama,S., and Mori,M. (2005). Clinical significance of human kallikrein gene 6 messenger RNA expression in colorectal cancer. Clin. Cancer Res. *11*, 2889-2893.

Oikonomopoulou,K., Scorilas,A., Michael,I.P., Grass,L., Soosaipillai,A., Rosen,B., Murphy,J., and Diamandis,E.P. (2006). Kallikreins as markers of disseminated tumour cells in ovarian cancer-- a pilot study. Tumour Biol. *27*, 104-114.

Petraki,C.D., Karavana,V.N., and Diamandis,E.P. (2003). Human kallikrein 13 expression in normal tissues: an immunohistochemical study. J. Histochem. Cytochem. *51*, 493-501.

Petraki,C.D., Karavana,V.N., Skoufogiannis,P.T., Little,S.P., Howarth,D.J., Yousef,G.M., and Diamandis,E.P. (2001). The spectrum of human kallikrein 6 (zyme/protease M/neurosin) expression in human tissues as assessed by immunohistochemistry. J. Histochem. Cytochem. *49*, 1431-1441.

Pfaffl,M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45-e50.

Planque,C., Blechet,C., Ayadi-Kaddour,A., Heuze-Vourc'h,N., Dumont,P., Guyetant,S., Diamandis,E.P., El Mezni,F., and Courty,Y. (2008). Quantitative RT-PCR analysis and immunohistochemical localization of the kallikrein-related peptidases 13 and 14 in lung. Biol. Chem. *389*, 781-786.

Press,J.Z., De Luca,A., Boyd,N., Young,S., Troussard,A., Ridge,Y., Kaurah,P., Kalloger,S.E., Blood,K.A., Smith,M., Spellman,P.T., Wang,Y., Miller,D.M., Horsman,D., Faham,M., Gilks,C.B., Gray,J., and Huntsman,D.G. (2008). Ovarian carcinomas with genetic and epigenetic BRCA1 loss have distinct molecular abnormalities. BMC. Cancer *8*, 17-29.

Prezas,P., Arlt,M.J., Viktorov,P., Soosaipillai,A., Holzscheiter,L., Schmitt,M., Talieri,M., Diamandis,E.P., Kruger,A., and Magdolen,V. (2006). Overexpression of the human tissue kallikrein genes KLK4, 5, 6, and 7 increases the malignant phenotype of ovarian cancer cells. Biol. Chem. *387*, 807-811.

Ruckert,F., Hennig,M., Petraki,C.D., Wehrum,D., Distler,M., Denz,A., Schroder,M., Dawelbait,G., Kalthoff,H., Saeger,H.D., Diamandis,E.P., Pilarsky,C., and Grutzmann,R. (2008). Co-expression of KLK6 and KLK10 as prognostic factors for survival in pancreatic ductal adenocarcinoma. Br. J. Cancer *99*, 1484-1492.

Rustin,G.J., Quinn,M., Thigpen,T., du,B.A., Pujade-Lauraine,E., Jakobsen,A., Eisenhauer,E., Sagae,S., Greven,K., Vergote,I., Cervantes,A., and Vermorken,J. (2004). Re: New guidelines to evaluate the response to treatment in solid tumors (ovarian cancer). J. Natl. Cancer Inst. *96*, 487-488.

Santin,A.D., Diamandis,E.P., Bellone,S., Soosaipillai,A., Cane,S., Palmieri,M., Burnett,A., Roman,J.J., and Pecorelli,S. (2005). Human kallikrein 6: a new potential serum biomarker for uterine serous papillary cancer. Clin. Cancer Res. *11*, 3320-3325.

Schink,J.C. (1999). Current initial therapy of stage III and IV ovarian cancer: challenges for managed care. Semin. Oncol. *26*, 2-7.

Scorilas,A., Borgono,C.A., Harbeck,N., Dorn,J., Schmalfeldt,B., Schmitt,M., and Diamandis,E.P. (2004). Human kallikrein 13 protein in ovarian cancer cytosols: a new favorable prognostic marker. J. Clin. Oncol. *22*, 678-685.

Shan,S.J., Scorilas,A., Katsaros,D., and Diamandis,E.P. (2007). Transcriptional upregulation of human tissue kallikrein 6 in ovarian cancer: clinical and mechanistic aspects. Br. J. Cancer. *96*, 362-372.

Shigemasa,K., Gu,L., Tanimoto,H., O'Brien,T.J., and Ohama,K. (2004a). Human kallikrein gene 11 (KLK11) mRNA overexpression is associated with poor prognosis in patients with epithelial ovarian cancer. Clin. Cancer Res. *10*, 2766-2770.

Shigemasa,K., Tian,X., Gu,L., Tanimoto,H., Underwood,L.J., O'Brien,T.J., and Ohama,K. (2004b). Human kallikrein 8 (hK8/TADG-14) expression is associated with an early clinical stage and favorable prognosis in ovarian cancer. Oncol. Rep. *11*, 1153-9.

Shvartsman,H.S., Lu,K.H., Lee,J., Lillie,J., Deavers,M.T., Clifford,S., Wolf,J.K., Mills,G.B., Bast,R.C., Jr., Gershenson,D.M., and Schmandt,R. (2003). Overexpression of kallikrein 10 in epithelial ovarian carcinomas. Gynecol. Oncol. *90*, 44-50.

Sidiropoulos,M., Pampalakis,G., Sotiropoulou,G., Katsaros,D., and Diamandis,E.P. (2005). Downregulation of human kallikrein 10 (KLK10/NES1) by CpG island hypermethylation in breast, ovarian and prostate cancers. Tumour Biol. *26*, 324-336.

Specht,K., Richter,T., Muller,U., Walch,A., Werner,M., and Hofler,H. (2001). Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am. J Pathol. *158*, 419-429.

Tanaka,K., Otake;K., Mohri,Y., Ohi,M., Yokoe,T., Toiyama,Y., Miki,C., Tonouchi,H., and Kusunoki,M. (2009). Clinical significance of the gene expression profile in residual tumor cells after neoadjuvant chemo-radiotherapy for esophageal cancer. Oncol. Rep. *21*, 1489-1494.

Tanimoto,H., Underwood,L.J., Shigemasa,K., Parmley,T.H., and O'Brien,T.J. (2001). Increased expression of protease M in ovarian tumors. Tumour Biol. *22*, 11-18.

White,N.M., Mathews,M., Yousef,G.M., Prizada,A., Popadiuk,C., and Dore,J.J. (2009). KLK6 and KLK13 predict tumor recurrence in epithelial ovarian carcinoma. Br. J. Cancer *101*, 1107-1113.

Yamashiro,K., Tsuruoka,N., Kodama,S., Tsujimoto,M., Yamamura,Y., Tanaka,T., Nakazato,H., and Yamaguchi,N. (1997). Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. Biochim. Biophys. Acta *1350*, 11-14.

Yousef,G. and Diamandis,E.P. (2003). Human Kallikreins: Common Structural features, Sequence Analysis and Evolution. Current Genomics *4*, 147-165.

Yousef,G.M., Borgono,C.A., White,N.M., Robb,J.D., Michael,I.P., Oikonomopoulou,K., Khan,S., and Diamandis,E.P. (2004). In silico analysis of the human kallikrein gene 6. Tumour Biol. *25*, 282-289.

Yousef,G.M., Chang,A., and Diamandis,E.P. (2000). Identification and characterization of KLK-L4, a new kallikrein-like gene that appears to be down-regulated in breast cancer tissues. J. Biol. Chem. *275*, 11891-11898.

Yousef,G.M., Polymeris,M.E., Grass,L., Soosaipillai,A., Chan,P.C., Scorilas,A., Borgono,C., Harbeck,N., Schmalfeldt,B., Dorn,J., Schmitt,M., and Diamandis,E.P. (2003). Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res. *63*, 3958-65.

Chapter 4 The Combination of Kallikrein-Related Peptidase 6 (KLK6), Kallikrein-Related Peptidase 13 (KLK13) and *MUC16* Increases Sensitivity in the Detection of Ovarian Cancer

A version of this chapter is has been published in *Cancer Biomarkers 2009:5(6):279-287.*

4.1 Introduction

Ovarian carcinoma is the most lethal of all the gynaecological malignancies. About 75% of patients are diagnosed in late stage disease (Stage III/IV) with a five year survival rate of only 15-20%, compared to a 80-90% when women are diagnosed in the early stages (Stage I/II) (Schink, 1999). Presently, ovarian cancer is diagnosed using high resolution computed tomography (CT) scans combined with serum tests for elevated CA125 levels (Kim *et al.*, 2009). Although CA125 is used as a marker of recurrence in ovarian cancer patients following primary treatment (Rustin *et al.*, 2004), it is not useful in general population screening as it lacks the sensitivity and specificity required for a diagnostic biomarker. Forty to fifty percent of Stage I/II ovarian cancers are CA125 negative (Jacobs and Bast, Jr., 1989) and almost 6% of women without cancer have elevated serum CA125 levels (Fields and Chevlen, 2006). A number of groups have looked at improving the sensitivity and specificity of CA125 by combining it with other markers. For example, macrophage colony stimulating factor has been shown to have 96-98% specificity in detecting ovarian cancer when used in combination with CA125, but only had 20% sensitivity (Suzuki *et al.*, 1993). More recently, interleukin 18 and fibroblast growth factor 2 combined with CA125 showed similarly poor characteristics (Le Page *et al.*, 2006). Similar to CA125, other single markers such as lysophosphatidic acid (Xu *et al.*, 1998), inhibin (Robertson *et al.*, 2004), and osteopontin (Nakae *et al.*, 2006), have been examined but have all lacked the sensitivity and specificity requirements to be validated as a biomarker for early stage ovarian cancer. Ovarian cancer is relatively uncommon, thus any useful screening method must be highly specific. It is estimated

that a specificity of 99.7% is needed to achieve a positive predictive value of 10%, with a sensitivity of 67% in postmenopausal women (Bast, Jr. *et al.*, 1998).

CA125 was first identified by a monoclonal antibody, OC125, that had been developed in mice immunized with an ovarian cancer cell line (Bast, Jr. *et al.*, 1981). CA125 is a high molecular mass glycoprotein (Lloyd *et al.*, 1997) and despite the current clinical utility of CA125, the normal biological function of the protein remains unknown. The gene for CA125 has been cloned and named *MUCIN16* (*MUC16*), and has been suggested to play a biological role in metastasis of ovarian cancer (Yin and Lloyd, 2001). *MUC16* has been shown to bind specifically to mesothelin in advanced grade ovarian adenocarcinoma (Rump *et al.*, 2004). This novel binding may contribute to the metastasis of cancer from the ovary to the peritoneum by initiating cell attachment to the mesothelial epithelium via binding to mesothelin (Rump *et al.*, 2004). A more complete examination of the biology of *MUC16* is required as it may lead to a more complete understanding of ovarian oncogenesis.

Recently, the kallikrein-related peptidase (KLK) family has shown promise as potential markers for ovarian carcinoma (White *et al.*, 2009;Yousef and Diamandis, 2003). The family consists of 15 secreted serine proteases that have been implicated in a number of different cancers. Interestingly, 12 KLKs have been shown to be overexpressed in ovarian cancer at the mRNA and/or protein level (Borgono and Diamandis, 2004). In particular, kallikrein-related peptidase 6 (KLK6) has been implicated in ovarian carcinogenesis. KLK6 was cloned by three separate groups as protease M (Anisowicz *et*

al., 1996), zyme (Little *et al.*, 1997), and neurosin (Yamashiro *et al.*, 1997). With the discovery of the extended KLK family, this gene was renamed KLK6. KLK6 has been found to have increased expression in ovarian cancer patients at both the gene and protein levels. One study found *KLK6* elevated in 30 of 32 ovarian carcinomas (Tanimoto *et al.*, 2001). Protein expression of KLK6 was also examined in several studies. Using a KLK6-specific immunoflourometric assay, KLK6 was found to be increased in the serum of ovarian cancer patients when compared to normal patients (Diamandis *et al.*, 2000b;Ni *et al.*, 2004), and was also found in the ascites fluid of ovarian cancer patients (Luo *et al.*, 2006). Hoffman et al., concluded KLK6 expression was an unfavourable prognostic marker as KLK6 was significantly associated with late stage disease, serous histological type, residual tumor, and suboptimal debulking in ovarian cancer patients (Hoffman *et al.*, 2002).

Similar to KLK6, kallikrein-related peptidase 13 (KLK13), has been shown to be associated with a number of endocrine related malignancies, including ovarian cancer (Yousef *et al.*, 2000). KLK13 was cloned using the positional candidate gene approach and was found to be downregulated in breast cancer cell lines and tissues when compared to normal counterparts (Yousef *et al.*, 2000). Recently, the use of KLK13 as a potential ovarian cancer biomarker has been evaluated (Kapadia *et al.*, 2003;White *et al.*, 2009). KLK13 is expressed at low to undetectable levels in normal ovaries, but has been shown to have increased expression in 50% of malignant ovaries when compared normal counterparts (Kapadia *et al.*, 2003). Also, *KLK13* has been shown to be a marker of poor prognosis for ovarian cancer patients (White *et al.*, 2009).

Since ovarian cancer has been described as a spectrum of cancers of differing originating mechanisms, it is unlikely a single marker will provide the desired information for diagnosis, prognosis and determination of treatment strategies. However, a simple detection method using a panel of markers in which ovarian cancer has a specific signature may allow for early detection and offer treatment options where none presently exist (Urban, 2003). The current study uses a unique approach to investigate the sensitivity and specificity of three genes, *MUC16, KLK6* and *KLK13*, alone and in combination, to detect ovarian cancer.

4.2 Materials and methods

4.2.1 Patient samples

Formalin fixed paraffin embedded ovarian cancer tissues were collected from 106 cases of sporadic ovarian carcinoma diagnosed in St. John's, Newfoundland, Canada, between 1983 and 2002. Patients varied from 20 to 81 years of age with a mean age of 60 years. Eight normal ovary samples were also collected for comparison. Cases were selected from the pathology archives at the Health Sciences Centre, St. John's, Newfoundland, Canada, based on pathological review of hemotoxylin and eosin stained tissue sections. Samples containing at least 80% tumor cells were selected for the study. Clinical staging was performed using the International Federation of Gynecology and Obstetrics (FIGO) criteria. Tumors were graded as borderline, well differentiated (Grade I), moderately differentiated (Grade II), or poorly differentiated (Grade III). Clinical data was obtained by review of patients' records in accordance with the Memorial University Human Investigation Committee protocol. This sample set was also utilized in Chapter 3.

4.2.2 Total RNA extraction

Five sections of 10μm each were cut from paraffin embedded tissues for nucleic acid isolation. Total RNA was extracted using the High Pure RNA Paraffin Kit (Roche, Indianapolis, IN) according to the manufacture's protocol. A detailed protocol for total RNA extraction can be found in Section 3.2.3. Total RNA concentration was determined spectrophotometrically at 260nm and samples were stored at -80°C. CDNA was synthesized using 2μg total RNA and the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carslband, CA) with random hexamers.

4.2.3 Quantitative real time PCR

QRT-PCR was performed using the ABI Prism 7000 (Applied Biosystems, Foster City, CA) to carry out a retrospective screening of 106 ovarian cancers and 8 normal ovaries. TaqMan Assays on Demand were purchased for *KLK13, KLK6*, *MUC16* and *GAPDH* (Applied Biosystems, Foster City, CA). The *MUC16* probe (Hs01065178_m1) was designed using Accession number NM_024690.2 (Kolwijck *et al.*, 2009), utilized six coding exons to detect mRNA expression, and produced an amplicon of 72bp. The details of the probes used for *KLK6*, *KLK13*, and *GAPDH* can be found in Table 3.1.

Target gene expression was normalized to endogenous *GAPDH* and gene expression in normal ovaries. Thermal cycling conditions were according to the manufacture's protocol and all reactions for each sample were performed in triplicate. A detailed account of the reverse transcription and qRT-PCR protocol can be found in Section 3.2.4.

Relative quantification, the amount of target normalized to endogenous control, was performed with the ΔΔ cycle threshold (C_T) method (Pfaffl, 2001). In order to effectively use the $\Delta\Delta C_T$ method, a validation experiment of the amplification efficiency of the target and control genes is required to ensure the efficiency of the target amplification and the efficiency of the reference amplification are approximately equal. The absolute value of the slope of log input amount vs. ΔC_T should be less than 0.1. The slope of our validation experiment for MUC16 was 0.073 as shown in Figure 4.1. The slopes for *KLK6* and *KLK13* were 0.076 and 0.024, respectively.

Patients were classified as having low or high gene expression based on qRT-PCR results. Patients who had high expression were classified as being "positive" for that gene. One standard deviation above the mean value of the normal ovary gene expression was used as the cut-off point. The cut off values for *KLK6, KLK13,* and *MUC16* were 5.21, 0.98, and 1.19, respectively. In the combined groups, patients having either gene expressed at a high level were classified as "positive" gene expression. These values were used to determine sensitivity, specificity, positive and negative predictive values.

4.2.4 Statistical analysis

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) 13.0 (Chicago, IL, USA). Differences in gene expression was analyzed with a one way ANOVA and a value was considered significant if $p<0.05$.

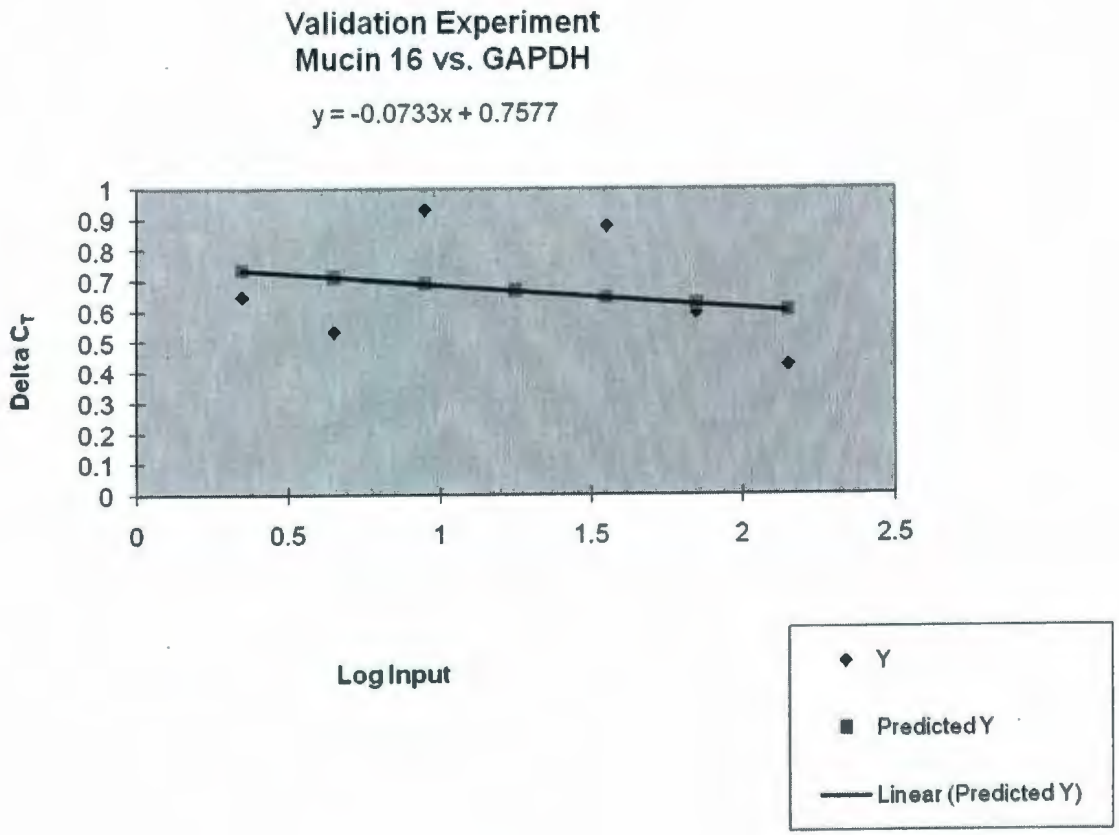


Figure 4.1 Validation experiment for the amplification of *MUCIN16* and *GAPDH* by qRT-PCR. The validity of the *MUCIN16* and *GAPDH* probes were assessed by plotting log input amount versus ΔC_T. The absolute slope of 0.0733 indicates the $\Delta\Delta C_T$ calculation is valid.

4.3 Results

4.3.1 Patient characteristics

The clinical characteristics of 106 ovarian cancer patients are summarized in Table 4.1. The majority of patients were older than 50 years of age (77%). Similar to the frequency seen in a clinical setting, a small number of cases were classified as borderline ovarian cancer (5%), while the majority of cases were diagnosed as late stage cancer (70%; Stage III and IV). Among the 106 ovarian cancers, 65% of cases were serous type ovarian cancers, and 42% tumors were poorly differentiated. Eight normal ovaries were also analyzed.

4.3.2 MRNA expression

When *KLK6* mRNA expression levels were examined in normal ovary and ovarian cancer tissues, 25% of normal ovaries were classified as positive for *KLK6* expression, compared to borderline tumors where 50% had high *KLK6* expression (Figure 4.2). When we examined the subset of invasive ovarian cancers, 79% were classified as having high *KLK6* expression ($p<0.05$; Figure 4.2).

The expression of *KLK13* mRNA was significantly increased in invasive ovarian cancers when compared to that of normal ovary ($p<0.001$; Figure 4.2). Only 12.5% of normal ovaries were positive for *KLK13* expression, while 33.3% of borderline cases and 56% of invasive ovarian cancers tested positive for *KLK13* (Figure 4.2). In four of the eight normal ovaries examined, *KLK13* expression was below the limit of detection.

Table 4.1 Clinical characteristics of 106 ovarian cancer patients and 8 normal ovaries.

Variable	Characteristic	No. of patients (%)	
Age	<50	24	(22.6)
	≥50	82	(77.4)
Sample	Normal	8	(7.0)
	Borderline	6	(5.3)
	Invasive	100	(87.7)
Histological Type	Serous	69	(65.1)
	Mucinous	15	(14.2)
	Endometrioid	6	(5.7)
	Clear Cell	4	(3.8)
	Unknown	12	(11.3)
Clinical Stage	I	24	(22.6)
	II	8	(7.5)
	III	64	(60.4)
	IV	10	(9.4)
Tumor Grade	GB	6	(5.7)
	G1	16	(15.1)
	G2	40	(37.7)
	G3	44	(41.5)

Ovarian cancers are thought to be derived from the ovarian surface epithelium (OSE) and comprise a large proportion of the cell content in an ovarian tumor (Figure 3.2). On the other hand, normal ovaries have only a small fraction of epithelial cells relative to stroma tissue. When KLK13 protein expression was examined in the normal ovary, the OSE was positive for KLK13 expression. This created the possibility that epithelial cell number accounted for the increased levels of *KLK13* mRNA in the cancer tissues rather than a true upregulation of gene expression in each cell. To address this, total RNA from normal OSE and stroma was isolated separately and analyzed for *KLK13* expression. Although *KLK13* expression is quite low in both areas of the ovary, it was approximately two times greater in the epithelium than stromal cells. When the expression of *KLK13* was compared between the normal OSE and cancer epithelium, the ovarian cancer cells had higher KLK13 expression confirming that the cancerous epithelium truly does up regulate KLK13 expression (data not shown).

MUC16 showed a significant increase in mRNA expression in invasive cancer relative to normal expression levels ($p<0.001$; Figure 4.2). Approximately 12.5% of normal ovaries were positive for *MUC16* expression, while 50% of borderline cases and 84% of invasive ovarian carcinoma were *MUC16* positive. It should be noted that these samples were primary tumors and not obtained as recurrences following primary treatment, as CA125 levels are usually used for screening.

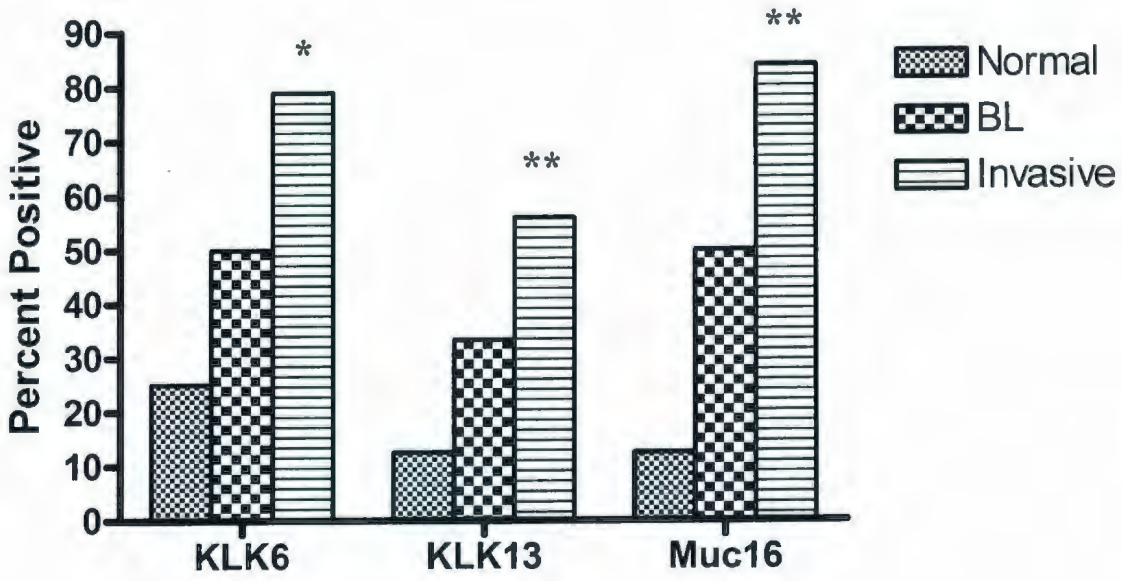


Figure 4.2. Positive *KLK6, KLK13* and *MUC16* expression in 106 ovarian carcinomas and 8 normal ovaries. *KLK6* expression was positive in 25%, 50%, and 79% of normal ovary, borderline (BL) and invasive ovarian cancers, respectively. *KLK13* expression was positive in 12.5%, 33.3%, and 56% of normal ovary, borderline and invasive ovarian cancers. *MUC16* expression was positive in 12.5%, 50%, and 84% of normal ovary, borderline and invasive ovarian cancers, respectively. *, $p<0.05$, **, $p<0.001$.

4.3.3 Sensitivity and specificity

The sensitivity of *KLK13, KLK6* and *MUC16* to detect ovarian cancer is displayed in Figure 4.3. The sensitivity of *KLK13* alone to detect ovarian cancer was 55%, while *KLK6* showed a sensitivity level comparable to *MUC16* (77% vs. 82%, respectively). The sensitivity to detect ovarian cancer improved to 90% when *MUC16* and *KLK6* were used in combination and was further enhanced to 93% when all three genes were examined simultaneously. When analyzed in combination, samples were considered positive if they displayed high expression of either marker.

When only the early stage (Stage I and II) cancers are examined (n=32), a similar trend was found in sensitivity. *MUC16* alone detected 56% of cancers while *KLK6* and *KLK13* detected 56% and 50%, respectively (Figure 4.3). When *KLK6* and *MUC16* were examined concurrently, 72% of the early stage cancers were detected and when *KLK13* was added to the analysis, the sensitivity increased to 84% for detecting early stage ovarian cancers.

Table 4.2 shows the sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) for *KLK13*, *KLK6* and *MUC16* alone and in combination. When all ovarian cancers were analyzed, the specificity of the test remained the same as when *MUC16* was tested alone. The PPV also remained the same, 87.5%, when the tests were combined. On the other hand, the NPV increased from 27% with *MUC16* alone to 50% when *KLK13, KLK6,* and *MUC16* were used in combination.

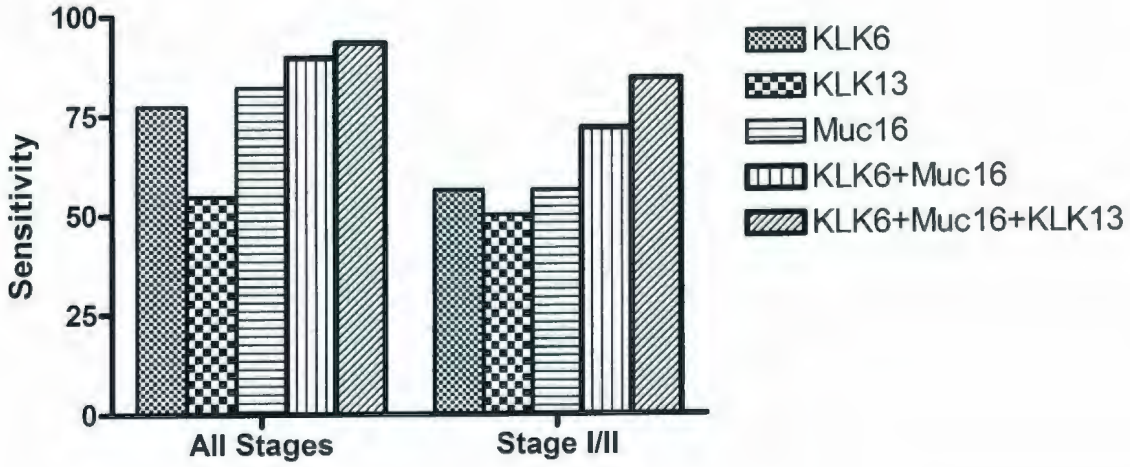


Figure 4.3 Sensitivity of *KLK6*, *KLK13* and *MUC16* for the detection all stage (Stage I-IV) and early stage (Stage I and II) ovarian cancers. The sensitivity of detection in all stages of ovarian cancer was 77%, 55%, 82%, 90%, and 93% for *KLK6, KLK13, MUC16, KLK6* and *MUC16,* and *KLK6* and *MUC16* and *KLK13*, respectively. The sensitivity of detection in early stage cancers was 56%, 50%, 56%, 72%, and 84% with *KLK6, KLK13, MUC16, KLK6* and *MUC16,* and *KLK6* and *MUC16* and *KLK13*, respectively.

Table 4.2 Sensitivity, specificity, positive and negative predictive values of all cancers and early stage cancers.

	All Cancers (n=106)				**Early Stage Cancers** (n=32)			
	Sensitivity	**Specificity**	**PPV[a]**	**NPV[b]**	**Sensitivity**	**Specificity**	**PPV[a]**	**NPV[b]**
KLK6	77.4%	75%	97.6%	20%	56.3%	75%	90%	30%
KLK13	54.7%	87.5%	98.3%	12.7%	50%	87.5%	94.1%	30.4%
MUC16	82.1%	87.5%	98.9%	26.9%	56.3%	87.5%	94.7%	33.3%
MUC16 OR *KLK6*	89.6%	75%	97.9%	33.3%	71.9%	75%	92%	40%
MUC16 OR *KLK6* OR *KLK13*	93.4%	87.5%	99%	50%	84.4%	87.5%	96.4%	58.3%

[a], PPV; positive predictive value
[b], NPV; negative predictive value

More interesting, are the results examining the early stage cancers (n=32; Table 4.2). The specificity of the test remained 87.5% when *MUC16* was tested alone, compared to all three markers in combination. On the other hand, the PPV increased slightly from 95% when *MUC16* was tested alone to 96% when all three markers were used in combination. However, the NPV dramatically increased from 30-33% for each gene individually, to 58% for the combined analysis (Table 4.2).

4.4 Discussion

Ovarian cancer is often diagnosed in late stage disease and the death rates for this malignancy are the highest among all the gynaecological cancers (Badgwell and Bast, Jr., 2007). Currently, there are no biomarkers approved for general population screening for ovarian cancer. It is essential to identify a biomarker for ovarian cancer that is able to detect disease in the early stages. This marker will drastically improve ovarian cancer survival rates as the disease is treatable and often curable when diagnosed in the early stages (Stage I and II).

Kallikrein-related peptidases have been suggested to be novel potential markers for ovarian cancer diagnosis, prognosis, and disease monitoring. This study examines the usefulness of combining expression levels of two members of this family, *KLK6* and *KLK13*, along with the most used clinical marker for ovarian cancer, CA125/*MUC16*, in an attempt to improve sensitivity and specificity. Unlike many previous studies that examined protein expression (Bast, Jr. *et al.*, 1984;Buamah and Skillen, 1994;Diamandis *et al.*, 2000a;Kapadia *et al.*, 2003;Kountourakis *et al.*, 2008;Scorilas *et al.*, 2004), we

examined mRNA expression of *KLK6*, *KLK13*, and *MUC16* in ovarian cancer tumors. Our approach, to quantify expression levels of three genes by qRT-PCR, is much more sensitive than serum protein testing as PCR can detect minute amounts of transcript and is not subject to dilution or metabolic breakdown. A recent study found ovarian cancer cells reach peripheral circulation more often than one would expect (Marth *et al.*, 2002). In early stage ovarian cancer, approximately 23% of patients had detectable levels of ovarian cancer cells in the blood. These data suggest a blood test analyzing gene expression of nucleated cells (such as a buffy coat from a simple centrifugation), may be a novel early diagnostic tool for ovarian cancer that may be superior to current serum protein based clinical diagnostic strategies.

Our results show increased levels of *KLK13* mRNA in over half of the invasive ovarian tumors in the study and very low levels in normal ovaries. These results are similar to Kapadia et al., who using an immunofluorometric assay, showed KLK13 protein was below detectable limits in normal serum, but elevated in 50% of ovarian cancer patients (Kapadia *et al.*, 2003). Although we did detect high *KLK13* mRNA in one of eight normal tissue samples, gene expression overall was extremely low. This may be attributed to our ability to detect minute levels of transcripts and the fact that we directly assayed tumor tissue, rather than the secreted protein diluted into the blood.

Similar to other studies, we found *KLK6* mRNA expression levels were elevated in ovarian cancers when compared to normal ovarian tissue (Anisowicz *et al.*, 1996;Ni *et al.*, 2004;Tanimoto *et al.*, 2001). With high serum levels of KLK6, patients have

previously been shown to be more likely to have advanced disease (Stage III-IV), serous tumor histology and more residual tumor (Hoffman *et al.*, 2002). Similarly, our study shows high *KLK6* mRNA levels in 86% of tumors of patients with late stage disease, with 73% of these patients having serous type tumors. Stage of disease is the most reliable prognostic marker for ovarian cancer and the fact that *KLK6* expression is elevated in the majority of late stage ovarian cancers consistently, over a number of studies, suggests it may be involved in the progression of ovarian cancer. This hypothesis is further supported by the fact that KLK6 has been shown to cleave extracellular matrix proteins and the use of an KLK6 antibody can decrease migration of ovarian cancer cells (Ghosh *et al.*, 2004).

Recently, a multiparametric analysis found the combination of KLK7, KLK10, KLK13, B7-H4, and CA125 was superior to CA125 alone in distinguishing between non-pathological tissues and metastatic tumors (Zheng *et al.*, 2007). B7-H4 has been reported to have increased expression in invasive ovarian cancer when compared to benign lesions (Tringler *et al.*, 2005). Also, in another study, the combination of KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK12, KLK13, and KLK14 were capable of distinguishing primary tumors from normal tissue (Shih *et al.*, 2007). In our study, by combining *KLK6* and *KLK13* with *MUC16,* an additional nine early stage ovarian cancer patients were correctly detected, increasing the sensitivity from 56% to 84% (Table 4.2). Unfortunately, we did not see an increase in the specificity, as it remained at 88%. There was a slight increase in the PPV as it increased from 94% when *MUC16* was tested alone to 96% when the combined test was applied. Interestingly, the combined assay resulted

in a dramatic increase in NPV, from 30-33% with each gene individually, to 58%, demonstrating this test is more accurate in detecting truly negative patients. For example, in a patient cohort of 100 individuals, testing for each individual gene would detect only 33 people as true negatives, while the combined test would detect 58 patients. This drastic increase in the early stage sensitivity and the ability to sort truly negative individuals in our study warrants further examination as using the kallikrein-related peptidases as early markers for ovarian carcinoma. Our study supports the concept that a panel of markers may be a more sensitive test to detect early stage ovarian cancer and adds to the growing evidence that KLKs can be considered potential biomarkers for this malignancy which may provide a novel method of widespread screening for this clinically difficult problem.

4.5 Authors contributions

A version of the chapter was published in the *Cancer Biomarkers* as:

Human kallikrein related-peptidase 6 and 13 in combination with CA125 is a more sensitive test for ovarian cancer than CA125 alone.

Cancer Biomarkers 2009:5 (6):279-287.

The following outlines the authors' contributions to this study:

Nicole White

- Conception and design of the study, collection, analysis and interpretation of data, collection and assembly of data, writing the manuscript

Maria Mathews

- Assistance in analysis and interpretation of data

George Yousef

- Consultation for the conception and design of the study, selection of patient samples

Amrah Prizada

- Assistance in the collection of the data

Daniel Fontaine

- Assistance in the collection of the data

Prafull Ghatage

- Recruitment of patient samples

Catherine Popadiuk

- Recruitment of patient samples and editorial input during manuscript preparation

Lesa Dawson

- Recruitment of patient samples

Jules Doré

- Consultation for the conception and design of the study, interpretation of data, editorial revision of the article manuscript

4.6 References

Anisowicz,A., Sotiropoulou,G., Stenman,G., Mok,S.C., and Sager,R. (1996). A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med. *2*, 624-636.

Badgwell,D. and Bast,R.C., Jr. (2007). Early detection of ovarian cancer. Dis. Markers *23*, 397-410.

Bast,R.C., Jr., Feeney,M., Lazarus,H., Nadler,L.M., Colvin,R.B., and Knapp,R.C. (1981). Reactivity of a monoclonal antibody with human ovarian carcinoma. J. Clin. Invest. *68*, 1331-1337.

Bast,R.C., Jr., Klug,T.L., Schaetzl,E., Lavin,P., Niloff,J.M., Greber,T.F., Zurawski,V.R., Jr., and Knapp,R.C. (1984). Monitoring human ovarian carcinoma with a combination of CA 125, CA 19-9, and carcinoembryonic antigen. Am. J. Obstet. Gynecol. *149*, 553-559.

Bast,R.C., Jr., Xu,F.J., Yu,Y.H., Barnhill,S., Zhang,Z., and Mills,G.B. (1998). CA 125: the past and the future. Int. J. Biol. Markers *13*, 179-187.

Borgono,C.A. and Diamandis,E.P. (2004). The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer *4*, 876-890.

Buamah,P.K. and Skillen,A.W. (1994). Serum CA 125 concentrations in patients with benign ovarian tumours. J. Surg. Oncol. *56*, 71-74.

Diamandis,E.P., Yousef,G.M., Soosaipillai,A.R., and Bunting,P. (2000a). Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin. Biochem. *33*, 579-583.

Diamandis,E.P., Yousef,G.M., Soosaipillai,A.R., Grass,L., Porter,A., Little,S., and Sotiropoulou,G. (2000b). Immunofluorometric assay of human kallikrein 6 (zyme/protease M/neurosin) and preliminary clinical applications. Clin. Biochem. *33*, 369-375.

Fields,M.M. and Chevlen,E. (2006). Screening for disease: making evidence-based choices. Clin. J. Oncol. Nurs. *10*, 73-76.

Ghosh,M.C., Grass,L., Soosaipillai,A., Sotiropoulou,G., and Diamandis,E.P. (2004). Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol. *25*, 193-199.

Hoffman,B.R., Katsaros,D., Scorilas,A., Diamandis,P., Fracchioli,S., Rigault de la Longrais,I.A., Colgan,T., Puopolo,M., Giardina,G., Massobrio,M., and Diamandis,E.P. (2002). Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new independent unfavourable prognostic biomarker. Br. J. Cancer *87*, 763-771.

Jacobs,I. and Bast,R.C., Jr. (1989). The CA125 tumor associated antigen: a review of the literature. Hum. Reprod. *4*, 1-12.

Kapadia,C., Chang,A., Sotiropoulou,G., Yousef,G.M., Grass,L., Soosaipillai,A., Xing,X., Howarth,D.H., and Diamandis,E.P. (2003). Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin. Chem. *49*, 77-86.

Kim,K., Visintin,I., Alvero,A.B., and Mor,G. (2009). Development and validation of a protein-based signature for the detection of ovarian cancer. Clin. Lab. Med. *29*, 47-55.

Kolwijck,E., Thomas,C.M., Bulten,J., and Massuger,L.F. (2009). Preoperative CA-125 levels in 123 patients with borderline ovarian tumors: a retrospective analysis and review of the literature. Int. J. Gynecol. Cancer *19*, 1335-1338.

Kountourakis,P., Psyrri,A., Scorilas,A., Camp,R., Markakis,S., Kowalski,D., Diamandis,E.P., and Dimopoulos,M.A. (2008). Prognostic value of kallikrein-related peptidase 6 protein expression levels in advanced ovarian cancer evaluated by automated quantitative analysis (AQUA). Cancer Sci. *99*, 2224-2229.

Le Page,C., Ouellet,V., Madore,J., Hudson,T.J., Tonin,P.N., Provencher,D.M., and Mes-Masson,A.M. (2006). From gene profiling to diagnostic markers: IL-18 and FGF-2 complement CA125 as serum-based markers in epithelial ovarian cancer. Int. J. Cancer. *118*, 1750-1758.

Little,S.P., Dixon,E.P., Norris,F., Buckley,W., Becker,G.W., Johnson,M., Dobbins,J.R., Wyrick,T., Miller,J.R., MacKellar,W., Hepburn,D., Corvalan,J., McClure,D., Liu,X., Stephenson,D., Clemens,J., and Johnstone,E.M. (1997). Zyme, a novel and potentially amyloidogenic enzyme cDNA isolated from Alzheimer's disease brain. J. Biol. Chem. *272*, 25135-25142.

Lloyd,K.O., Yin,B.W., and Kudryashov,V. (1997). Isolation and characterization of ovarian cancer antigen CA 125 using a new monoclonal antibody (VK-8): identification as a mucin-type molecule. Int. J. Cancer *71*, 842-850.

Luo,L.Y., Soosaipillai,A., Grass,L., and Diamandis,E.P. (2006). Characterization of human kallikreins 6 and 10 in ascites fluid from ovarian cancer patients. Tumour Biol. *27*, 227-234.

Marth,C., Kisic,J., Kaern,J., Trope,C., and Fodstad,O. (2002). Circulating tumor cells in the peripheral blood and bone marrow of patients with ovarian carcinoma do not predict prognosis. Cancer. *94*, 707-712.

Nakae,M., Iwamoto,I., Fujino,T., Maehata,Y., Togami,S., Yoshinaga,M., and Douchi,T. (2006). Preoperative plasma osteopontin level as a biomarker complementary to carbohydrate antigen 125 in predicting ovarian cancer. J. Obstet. Gynaecol. Res. *32*, 309-314.

Ni,X., Zhang,W., Huang,K.C., Wang,Y., Ng,S.K., Mok,S.C., Berkowitz,R.S., and Ng,S.W. (2004). Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br. J. Cancer. *91*, 725-731.

Pfaffl,M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.

Robertson,D.M., Pruysers,E., Burger,H.G., Jobling,T., McNeilage,J., and Healy,D. (2004). Inhibins and ovarian cancer. Mol. Cell Endocrinol. *225*, 65-71.

Rump,A., Morikawa,Y., Tanaka,M., Minami,S., Umesaki,N., Takeuchi,M., and Miyajima,A. (2004). Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. J. Biol. Chem. *279*, 9190-9198.

Rustin,G.J., Quinn,M., Thigpen,T., du,B.A., Pujade-Lauraine,E., Jakobsen,A., Eisenhauer,E., Sagae,S., Greven,K., Vergote,I., Cervantes,A., and Vermorken,J. (2004). Re: New guidelines to evaluate the response to treatment in solid tumors (ovarian cancer). J. Natl. Cancer Inst. *96*, 487-488.

Schink,J.C. (1999). Current initial therapy of stage III and IV ovarian cancer: challenges for managed care. Semin. Oncol. *26*, 2-7.

Scorilas,A., Borgono,C.A., Harbeck,N., Dorn,J., Schmalfeldt,B., Schmitt,M., and Diamandis,E.P. (2004). Human kallikrein 13 protein in ovarian cancer cytosols: a new favorable prognostic marker. J. Clin. Oncol. *22*, 678-685.

Shih,I., Salani,R., Fiegl,M., Wang,T.L., Soosaipillai,A., Marth,C., Muller-Holzner,E., Gastl,G., Zhang,Z., and Diamandis,E.P. (2007). Ovarian cancer specific kallikrein profile in effusions. Gynecol. Oncol. *105*, 501-507.

Suzuki,M., Ohwada,M., Aida,I., Tamada,T., Hanamura,T., and Nagatomo,M. (1993). Macrophage colony-stimulating factor as a tumor marker for epithelial ovarian cancer. Obstet. Gynecol. *82*, 946-950.

Tanimoto,H., Underwood,L.J., Shigemasa,K., Parmley,T.H., and O'Brien,T.J. (2001). Increased expression of protease M in ovarian tumors. Tumour Biol. *22*, 11-18.

Tringler,B., Zhuo,S., Pilkington,G., Torkko,K.C., Singh,M., Lucia,M.S., Heinz,D.E., Papkoff,J., and Shroyer,K.R. (2005). B7-h4 is highly expressed in ductal and lobular breast cancer. Clin. Cancer Res. *11*, 1842-1848.

Urban,N. (2003). Specific keynote: ovarian cancer risk assessment and the potential for early detection. Gynecol. Oncol. *88*, S75-S79.

White,N.M., Mathews,M., Yousef,G.M., Prizada,A., Popadiuk,C., and Dore,J.J. (2009). KLK6 and KLK13 predict tumor recurrence in epithelial ovarian carcinoma. Br. J. Cancer *101*, 1107-1113.

Xu,Y., Shen,Z., Wiper,D.W., Wu,M., Morton,R.E., Elson,P., Kennedy,A.W., Belinson,J., Markman,M., and Casey,G. (1998). Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. JAMA. *280*, 719-723.

Yamashiro,K., Tsuruoka,N., Kodama,S., Tsujimoto,M., Yamamura,Y., Tanaka,T., Nakazato,H., and Yamaguchi,N. (1997). Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. Biochim. Biophys. Acta *1350*, 11-14.

Yin,B.W. and Lloyd,K.O. (2001). Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. J. Biol. Chem. *276*, 27371-27375.

Yousef,G. and Diamandis,E.P. (2003). Human Kallikreins: Common Structural features, Sequence Analysis and Evolution. Current Genomics *4*, 147-165.

Yousef,G.M., Chang,A., and Diamandis,E.P. (2000). Identification and characterization of KLK-L4, a new kallikrein-like gene that appears to be down-regulated in breast cancer tissues. J. Biol. Chem. *275*, 11891-11898.

Zheng,Y., Katsaros,D., Shan,S.J., de,l.L., I, Porpiglia,M., Scorilas,A., Kim,N.W., Wolfert,R.L., Simon,I., Li,L., Feng,Z., and Diamandis,E.P. (2007). A multiparametric panel for ovarian cancer diagnosis, prognosis, and response to chemotherapy. Clin. Cancer Res. *13*, 6984-6992.

Chapter 5 General Conclusions and Future Directions

5.1 KLK13 is involved in cellular motility

Epithelial ovarian cancer is the most deadly gynaecological disease. This year in Canada there are predicted to be approximately 2500 new cases and over 1700 deaths due to the malignancy (Canadian Cancer Society's Steering Committee, 2009). The high mortality rate is due to the fact that approximately 75% of women are diagnosed in late stage (Stage III or IV) disease. The failure to detect localized ovarian tumors when they are in early stage is due to the insidious nature of the disease, the unreliability of clinical examination, and the lack of an effective screening technique (Sherbet and Patil, 2003). Stage I and II ovarian cancer involves tumor that is limited to one or both ovaries with pelvic extension, while Stage III epithelial ovarian cancer is classified as a tumor that involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis (Tavassoli and Devilee, 2004). Patients who have any distant, metastasis outside the peritoneum, are classified as Stage IV (Tavassoli and Devilee, 2004). Ovarian cancer tumor stage, characterized by the level of tumor metastasis, is the most significant prognostic predictor of this malignancy [Table 3.4 and 3.5; (Einhorn *et al.*, 1985)].

The metastatic cascade is simply characterized by a sequence of basic steps – local invasion, intravasation, survival in the circulation, extravastion, and colonization (Nguyen *et al.*, 2009). In order to physically invade into the blood vessels, proteolytic degradation is required. Proteolytic enzymes such as matrix metalloproteinases (Egeblad and Werb, 2002), cysteine proteases (Mohamed and Sloane, 2006), and serine proteases

(Laufs *et al.*, 2006), are often produced by cancer cells. These proteases can promote cancer cell invasion through several mechanisms: cleavage of cell adhesion proteins, such as E-cadherin; processing and activation of cytokines and growth factors; and degradation and turnover of extracellular matrix (ECM) proteins (Joyce and Pollard, 2009).

Tumor cells require interaction with the ECM at several stages during metastasis. First, the cells must breech the underlying basement membrane, followed by traversing into the interstitial connective tissue, and finally gaining access to the circulation by penetrating the vascular basement membrane. In order to create a new metastatic site, this cycle must be repeated as the cells have to leave the circulation by invading through the vascular basement membrane and the ECM.

Invasion of the ECM is an active process that requires detachment of the tumor cells from each other, attachment to the ECM, degradation of the ECM, and the migration of tumor cells. Once the cells are attached to the ECM, tumor cells must create passageways for migration. Invasion of the matrix requires active enzymatic degradation of ECM components, usually by secretion of proteolytic enzymes (Price *et al.*, 1997).

The experiments described in Chapter 2 of this thesis focus on the secreted serine protease, KLK13, and how it promotes cellular migration and invasion, as a model of cancer metastasis. KLK13 was shown to have increased expression in the ovarian cancer cell lines, CAOV-3, OVCAR-3, and SKOV-3, when compared to the IOSE cell line (Figure 2.1). When IOSE cells were infected with KLK13 and migratory capacity was

assessed by a wound healing assay, the cells infected with the KLK13 wild type sequence, which codes for KLK13-WT protein, migrated faster than control cells (Figure 2.4). Also, the cells infected with the KLK13-S218A virus, the putative enzymatically dead KLK13, migrated slower than uninfected control cells. Although KLK13-S218A has an enzymatic pocket in which substrates can bind, it should not be able to perform its proteolytic action on the substrates, as its active site is mutated. This situation decreases the overall effect of KLK13 proteolytic activity as substrates remain bound to KLK13 and it does not perform its proteolytic activity. Further supporting the role of KLK13 in cellular migration is the fact that there was no significant change in cellular migration when the IOSE cells were infected with the inactivatable KLK13. This may be attributed to the fact that increased expression of the inactivatable KLK13 protein increased the pro-KLK13 levels in the cells, but due to a mutation in the activation site, the protein could likely not be processed beyond its zymogen form. Since the protein is inactive in its zymogen form, there was no overall effect of KLK13 activity in the cells. These data together support a role of KLK13 in cellular migration. These results are further supported by a wound healing assay with another epithelial cell type, the Mv1Lu cell line (Figure 2.7). When both the IOSE and Mv1Lu cells were infected with the KLK13-WT virus, cells migrated faster than the control cells, while the cells infected with the enzymatically dead KLK13, KLK13-S218A, migrated significantly slower than the control cells, indicating KLK13 can play a role in the migration of not only IOSE cells, but other epithelial cells.

The role of KLK13 in cellular migration was further assessed in the SKOV-3 cell line which has high KLK13 expression (Figure 2.2). When the expression of KLK13 was decreased in the migratory SKOV-3 cells, there was a significant decrease in the rate of cellular migration (Figure 2.5). This has important potential therapeutic applications for ovarian cancer patients. The inhibition of KLK13 activity in ovarian tumors may potentially inhibit ovarian cancer tumor progression and metastasis in patients.

These data together support a role of KLK13 in epithelial cell migration. The role of KLK13 in cellular invasion was also examined. When IOSE cells were infected with the KLK13-WT virus, there was a significant increase in cellular invasion; while the cells expressing the enzymatically dead KLK13 invaded less that the control cells (Figure 2.6). Increased cellular invasion was due to the increased active KLK13 protein and proteolytic nature of KLK13. Recently, KLK13 has been shown to cleave components of the ECM (Kapadia *et al.*, 2004), a process necessary for cellular invasion. Increased KLK13 expression in IOSE cells increased the invasion of these poorly-invasive cells, supporting the involvement of KLK13 in cellular invasion.

These results indicate KLK13 affects epithelial cellular migration and invasion, two processes critical for ovarian cancer metastasis. Ovarian cancer patients who have metastasis outside the pelvic region are diagnosed with late stage, Stage III/IV, ovarian cancer are a given a poor prognosis (Einhorn *et al.*, 1985). A number of genes known to be involved in the metastatic process have been shown to be significant predictors of survival. Since these results showed KLK13 may be involved in ovarian cancer

metastasis and KLK13 is increased in ovarian cancer patients, the prognostic value of KLK13 and KLK6 was assessed for ovarian cancer patients.

5.2 *KLK6* and *KLK13* are potential biomarkers for ovarian cancer

Numerous reports indicate aberrant protease expression is often associated with a poor prognosis in ovarian cancer patients (Ghosh *et al.*, 2002;Konecny *et al.*, 2001;Lengyel *et al.*, 2001). The prognostic relevance of protease inhibitors has also proved important in the development of new treatments targeted to metastasis (Coussens *et al.*, 2002;Dunbar *et al.*, 2000;Harbeck *et al.*, 2002;Hidalgo and Eckhardt, 2001). The study that comprised Chapter 3 in this thesis, examined the prognostic significance of two serine proteases, *KLK6* and *KLK13,* in ovarian cancer patients by measuring the expression of the genes by qRT-PCR. High *KLK6* and *KLK13* expression were associated with invasive ovarian cancer when compared to normal ovaries ($p<0.05$, Table 3.2). Also, high *KLK6* expression in ovarian cancer patients was associated with late stage (Stage III/IV) ovarian cancers ($p=0.001$).

When the prognostic significance of *KLK6* in ovarian cancer patients was assessed by Kaplan-Meier analysis, patients with high *KLK6* expressing tumors were more likely to have a recurrence ($p=0.002$) and die earlier ($p=0.011$) than patients with low *KLK6* expressing tumors (Figure 3.2). When the prognostic value of *KLK6* expression was assessed by univariate Cox-regression analysis, although clinical stage, tumor grade, and histological type of tumor were strong predictors of recurrence, high *KLK6* expression was still a significant predictor of tumor recurrence and death ($p<0.05$; Table 3.3).

In order to see if *KLK6* expression was still significant in predicting recurrence or death even when all other prognostic factors were taken into consideration, *KLK6* expression was analyzed by multivariate analysis. Although clinical stage, tumor grade, and type of tumor, were considered, *KLK6* was still able to predict recurrence in ovarian cancer patients (Table 3.4). More specifically, patients who have high *KLK6* expressing tumors were three times more likely to have a recurrence than patients who had low *KLK6* expressing tumors (Table 3.4).

A similar analysis was carried out for *KLK13* expression. When *KLK13* expression was assessed by Kaplan-Meier analysis, patients who had high *KLK13* expression were more likely to have a recurrence than patients with low *KLK13* expressing tumors (Figure 3.3). When analyzed by univariate Cox-regression analysis, similar results were found. Patients with high *KLK13* tumors were more likely to have a recurrence than patients who had low *KLK13* expressing tumors (Table 3.3). When stage of disease, histological type of tumor, and tumor grade were taken into consideration, patients who had high *KLK13* expressing tumors were still approximately two times more likely to have a recurrence than patients with low *KLK13* expressing tumors (Table 3.5). These data together indicate *KLK6* and *KLK13* are indicators of poor prognosis in ovarian cancer patients. These data agreed with previously published results (Kapadia *et al.*, 2003;Kountourakis *et al.*, 2008;White *et al.*, 2009).

Many biomarkers are now being examined for their diagnostic ability to detect early disease. Recently, much research has been focused on identifying a panel of biomarkers,

or a "biomarker signature" for early ovarian cancer detection. This was recently been explored by Oikonomopoulou et al. who found the combination of CA125, KLKs 5, 6, 10, 11, B7-H4, and Spondin 2 correlated with a worse progression free survival in ovarian cancer patients (Oikonomopoulou *et al.*, 2008). Also, Zheng et al. found the combination of CA125, B7-H4, KLK7, 10, 11, and 13 were accurate in distinguishing between primary tumors and healthy ovarian tissue (Zheng *et al.*, 2007). Given that KLK6 and KLK13 have been shown to be increased in ovarian cancer and CA125 has been show to have low sensitivity in detecting early stage ovarian cancers, the utility of the combination of CA125, KLK6 and KLK13 was assessed for its increased sensitivity to detect ovarian cancer when compared to either marker alone.

The ability of *KLK6*, *KLK13,* and *MUC16* to detect ovarian cancer was assessed by qRT-PCR. Expression of all three genes significantly increased from the normal ovary to borderline to invasive ovarian cancers (Figure 4.2). Also, when sensitivity, specificity, PPV, and NPV, was analyzed, a combination of these three markers was superior in detecting ovarian cancer relative to either gene alone (Table 4.2).

These findings were especially true for the early stage ovarian cancers. When only the early stage cancers (n=32) were examined, *MUC16* alone detected 56% of cancers. When all three markers were combined, the sensitivity of the test increased to 84%, detecting an additional nine early stage ovarian cancers (Table 4.2). Although there was no significant change in the specificity or PPV, 88% and 96%, respectively, there was a

dramatic increase in the NPV, as it increased from 30-33% for individual genes to 58% when combining the markers.

These findings are important because early detection is the best defence against ovarian cancer. An increase in the sensitivity of the test increases the number of patients that will test positive for ovarian cancer in early stage disease. Since many women are diagnosed in late stage disease, prognosis is dismal. Early detection, through a combination of markers, could drastically improve the survival rate of ovarian cancer.

There was also an increase in the NPV using the panel of markers which resulted in a decrease in the number of false negatives detected. This has important implications to both the patient and the healthcare system. Women who are incorrectly diagnosed deal with much mental stress and burden and have to go on to have further testing to validate the primary screen. This also creates unnecessary financial burden on the healthcare system. This study (Chapter 4) validated that a combination of markers is a more sensitive test for early stage ovarian cancer than either marker alone. These results warrant further investigation potentially using a larger panel of markers.

Another route to early detection is to gain an understanding of ovarian cancer carcinogenesis on a molecular level. The identification of the molecular players in ovarian cancer would enable the development of effective novel therapeutic applications. This thesis has shown that KLK13 is a potential therapeutic target for the management of ovarian cancer metastasis as ovarian cancer cells displayed decreased migration and invasion when KLK13 activity was decreased. This warrants further investigation.

5.3 Future directions

The evidence presented herein strongly suggests that KLK13 plays a direct role in epithelial cellular migration and invasion. These results also suggest the invasive capacity of cells is dependent on KLK13 glycosylation. A detailed analysis of the effect of glycosylation on the enzymatic activity of KLK13 is required to confirm this relationship. In particular, the isolation and purification of KLK13-WT and mutant proteins, KLK13-S218A, KLK13-K25Q, KLK13-N30Q, KLK13-N225Q, and KLK13-N30Q+N225Q, would shed light on how these mutations effect KLK13 activity. *In-vitro* assays measuring the degradation of a suitable trypsin-like synthetic substrate, such as the fluorescent Val-Pro-Arg-AMC substrate, that has been used successfully in a previous report (Kapadia *et al.*, 2004), would provide an accurate assessment of how the introduced mutations effect KLK13 enzymatic activity.

Another important aspect to consider in order to truly appreciate the function of KLK13, is to understand the mechanism of KLK13 interaction with other KLKs. *KLK6* and *KLK13* have increased expression in ovarian cancer when compared to normal ovaries (Table 3.2). Other studies have shown that 12 of the 15 KLKs have increased expression in ovarian cancer patients when compared to normal counterparts (Borgono and Diamandis, 2004). There have been two proposed mechanisms which may control the coordinated expression of kallikreins. Kallikrein expression may be regulated through an enzymatic cascade in which certain KLKs activate other KLKs or through a cis-acting locus control region. These regions can regulate the expression of linked genes over a

distance as long as 100 kb or more in a tissue and copy number specific manner in a wide spectrum of mammalian families (Borgono and Diamandis, 2004). It would be interesting to explore the existence of a KLK cascade in ovarian carcinoma.

The prognostic and/or diagnostic capabilities of certain genes and proteins are very useful when clinicians make patient treatment decisions. *KLK6* and *KLK13* can predict recurrence in ovarian cancer patients (Table 3.4 and 3.5, respectively). This study included 106 ovarian cancer patients of all histological types. It has been shown that subtypes of epithelial ovarian cancer are associated with different molecular events during oncogenesis (Christie and Oehler, 2006). Oncologists have also noted that the subtypes of ovarian carcinoma respond differently to chemotherapy. Taking these facts into consideration, it is unrealistic to expect a single marker will be accurate for the prognostic and diagnostic requirements for each subtype of ovarian cancer. In order to circumvent this issue, it would be useful to analyze each ovarian cancer subtype independently which has recently proven successful (Kobel *et al.*, 2008).

A number of KLKs have been shown to be potential biomarkers for ovarian cancer. It would be interesting to collect serum from ovarian cancer patients, pre- and post-treatment, in order to measure their CA125 and KLK levels and determine the potential biomarker utility of a combination of markers. Through statistical analysis, the ideal combination of a panel of biomarkers, providing a "biomarker signature" for each subtype of ovarian cancer, both pre- and post-treatment could be accurately assessed. This retrospective approach, in which all clinical data on the patient, including survival

data is available, would be helpful in determining the ideal combinations for prognostic markers in ovarian cancer. It would also be interesting to extend this study to other cancers such as breast, prostate, and colon cancers in which KLKs have recently been implicated (Emami and Diamandis, 2008).

5.4 References

Borgono,C.A. and Diamandis,E.P. (2004). The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer *4*, 876-890.

Canadian Cancer Society's Steering Committee (2009). Canadian Cancer Statistics 2009, Toronto: Canadian Cancer Society.

Christie,M. and Oehler,M.K. (2006). Molecular pathology of epithelial ovarian cancer. J. Br. Menopause Soc. *12*, 57-63.

Coussens,L.M., Fingleton,B., and Matrisian,L.M. (2002). Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science *295*, 2387-2392.

Dunbar,S.D., Ornstein,D.L., and Zacharski,L.R. (2000). Cancer treatment with inhibitors of urokinase-type plasminogen activator and plasmin. Expert Opin. Investig. Drugs *9*, 2085-2092.

Egeblad,M. and Werb,Z. (2002). New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer *2*, 161-174.

Einhorn,N., Nilsson,B., and Sjovall,K. (1985). Factors influencing survival in carcinoma of the ovary. Study from a well-defined Swedish population. Cancer *55*, 2019-2025.

Emami,N. and Diamandis,E.P. (2008). Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers. Clin. Chem. *54*, 1600-1607.

Ghosh,S., Wu,Y., and Stack,M.S. (2002). Ovarian cancer-associated proteinases. Cancer Treat. Res. *107*, 331-351.

Harbeck,N., Schmitt,M., Kates,R.E., Kiechle,M., Zemzoum,I., Janicke,F., and Thomssen,C. (2002). Clinical utility of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 determination in primary breast cancer tissue for individualized therapy concepts. Clin. Breast Cancer *3*, 196-200.

Hidalgo,M. and Eckhardt,S.G. (2001). Development of matrix metalloproteinase inhibitors in cancer therapy. J. Natl. Cancer Inst. *93*, 178-193.

Joyce,J.A. and Pollard,J.W. (2009). Microenvironmental regulation of metastasis. Nat. Rev. Cancer *9*, 239-252.

Kapadia,C., Chang,A., Sotiropoulou,G., Yousef,G.M., Grass,L., Soosaipillai,A., Xing,X., Howarth,D.H., and Diamandis,E.P. (2003). Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin. Chem. *49*, 77-86.

Kapadia,C., Ghosh,M.C., Grass,L., and Diamandis,E.P. (2004). Human kallikrein 13 involvement in extracellular matrix degradation. Biochem. Biophys. Res. Commun. *323*, 1084-1090.

Kobel,M., Kalloger,S.E., Boyd,N., McKinney,S., Mehl,E., Palmer,C., Leung,S., Bowen,N.J., Ionescu,D.N., Rajput,A., Prentice,L.M., Miller,D., Santos,J., Swenerton,K., Gilks,C.B., and Huntsman,D. (2008). Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. PLoS. Med. *5*, 1749-1760.

Konecny,G., Untch,M., Pihan,A., Kimmig,R., Gropp,M., Stieber,P., Hepp,H., Slamon,D., and Pegram,M. (2001). Association of urokinase-type plasminogen activator and its inhibitor with disease progression and prognosis in ovarian cancer. Clin. Cancer Res. *7*, 1743-1749.

Kountourakis,P., Psyrri,A., Scorilas,A., Camp,R., Markakis,S., Kowalski,D., Diamandis,E.P., and Dimopoulos,M.A. (2008). Prognostic value of kallikrein-related peptidase 6 protein expression levels in advanced ovarian cancer evaluated by automated quantitative analysis (AQUA). Cancer Sci. *99*, 2224-2229.

Laufs,S., Schumacher,J., and Allgayer,H. (2006). Urokinase-receptor (u-PAR): an essential player in multiple games of cancer: a review on its role in tumor progression, invasion, metastasis, proliferation/dormancy, clinical outcome and minimal residual disease. Cell Cycle *5*, 1760-1771.

Lengyel,E., Schmalfeldt,B., Konik,E., Spathe,K., Harting,K., Fenn,A., Berger,U., Fridman,R., Schmitt,M., Prechtel,D., and Kuhn,W. (2001). Expression of latent matrix metalloproteinase 9 (MMP-9) predicts survival in advanced ovarian cancer. Gynecol. Oncol. *82*, 291-298.

Mohamed,M.M. and Sloane,B.F. (2006). Cysteine cathepsins: multifunctional enzymes in cancer. Nat. Rev. Cancer *6*, 764-775.

Nguyen,D.X., Bos,P.D., and Massague,J. (2009). Metastasis: from dissemination to organ-specific colonization. Nat. Rev. Cancer *9*, 274-284.

Oikonomopoulou,K., Li,L., Zheng,Y., Simon,I., Wolfert,R.L., Valik,D., Nekulova,M., Simickova,M., Frgala,T., and Diamandis,E.P. (2008). Prediction of ovarian cancer prognosis and response to chemotherapy by a serum-based multiparametric biomarker panel. Br. J. Cancer *99*, 1103-1113.

Price,J.T., Bonovich,M.T., and Kohn,E.C. (1997). The biochemistry of cancer dissemination. Crit Rev. Biochem. Mol. Biol. *32*, 175-253.

Sherbet,G.V. and Patil,D. (2003). Genetic abnormalities of cell proliferation, invasion and metastasis, with special reference to gynaecological cancers. Anticancer Res. *23*, 1357-1371.

Tavassoli,F. and Devilee,P. (2004). World Health Organization Classification of Tumour Pathology and Genetics of Tumours of the Breast and Female Genital Organs., ed. Jaffe ES, Harris NL, Stein H, and Vardiman JW. Lyon: IARC Press.

White,N.M., Mathews,M., Yousef,G.M., Prizada,A., Popadiuk,C., and Dore,J.J. (2009). KLK6 and KLK13 predict tumor recurrence in epithelial ovarian carcinoma. Br. J. Cancer *101*, 1107-1113.

Zheng,Y., Katsaros,D., Shan,S.J., de,l.L., I, Porpiglia,M., Scorilas,A., Kim,N.W., Wolfert,R.L., Simon,I., Li,L., Feng,Z., and Diamandis,E.P. (2007). A multiparametric panel for ovarian cancer diagnosis, prognosis, and response to chemotherapy. Clin. Cancer Res. *13*, 6984-6992.

Appendix A

Predicted KLK13 *N*-linked glycosylation sites. Predicted from the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk/services/NetNGlyc/).

CBS

NetNGlyc 1.0 Server - prediction results

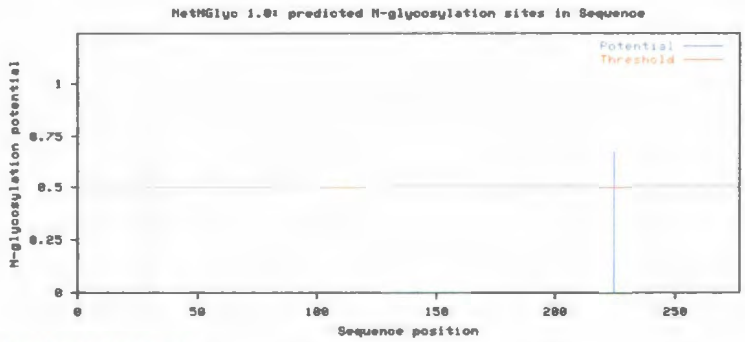
Technical University of Denmark

Asn-Xaa-Ser/Thr sequons in the sequence output below are highlighted in blue.
Asparagines predicted to be N-glycosylated are highlighted in red.

Output for 'Sequence'

```
Name:  Sequence          Length:  277
MWPLALVIASLTLALSGGVSQRSSKVLNTNGTSGFLPGGYTCPPHSQPWQAALLVQGRLLCGGVLVHPKWVLTAAHCLKK      80
GLKVYLGKHALGRVEAGEQVREVVHSIPHPEYRRSPTHLNHDHDIMLLELQSPVQLTGYIQTLPLSHNNRLTPGTTCRVS     160
GWGTTTSPQVNYPKTLQCANIQLRSDEECRQVYPGKITDNMLCAGTKEGGKDSCEGDSGGPLVCNRTLYGIVSWGDFPCG     240
QPDRPGVYTRVSRYVLWIRETIRKYETQQQKWLKGPQ
.............................N.....................................................      80
.....................................................................................     160
.....................................................................N...............     240
...................................                                                  320

(Threshold=0.5)
-------------------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                   agreement result
-------------------------------------------------------------------------------
Sequence       30 NGTS   0.7664     (9/9)   +++
Sequence      225 NRTL   0.6754     (9/9)   ++
-------------------------------------------------------------------------------
```



Graphics in PostScript

Explain the output. Go back.

Appendix B

Representative sample of DNA sequencing results containing the KLK13 S218A mutation.