A STUDY OF THE RELATIONSHIP OF APOPTOSIS AND PROLIFERATION TO LOCAL RECURRENCE IN BREAST CANCER

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A Study of the Relationship of Apoptosis and Proliferation to Local Recurrence in Breast Cancer

by

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A thesis submitted to the
School of Graduate Studies
in partial fulfilment of the
requirements for the degree of
Master of Science

Faculty of Medicine
Memorial University of Newfoundland

January 1999

St John's Newfoundland

I dedicate this thesis to my life-long friend who has been my source of hope, and faith.

Love, Syble

Abstract

This retrospective study on breast cancer investigated the inter-relation of the apoptotic factors, Bcl-2, Bax, and proliferation factors Ki-67, PCNA, together with their potential as predictors of local recurrence of tumour. Tumour specimens of patients with local recurrence (defined as recurrence of tumour within a period of 5 years following diagnosis, at the site of original surgery or adjacent axillary region) were expected to show increased proliferation and decreased apoptotic behavior thereby substantially increasing the risk of local recurrence. In addition, loss of function of the tumour suppressor gene, p53, which normally functions as the inhibitor of the proliferation and an activator of the apoptosis, was expected to play an integral part in promoting tumour growth.

Immunohistochemical analysis of the apoptotic and proliferative markers showed several significant associations between the biological parameters. Analysis of the association between p53 and Bcl-2 expression indicated no statistical significance. Further analysis of the raw data revealed a general trend towards an inverse association between the two markers. Thus, suggesting that p53 in its mutant form behaves in a similar manner as the wild type p53 which functions to inhibit the expression of Bcl-2. Loss of p53 function was also associated with an increase in the proliferative rate as measured by the expression of Ki-67 and PCNA.

The Bcl-2 protein, known to inhibit apoptosis, failed to display an inverse association with the rate of apoptosis (as measured by the TUNEL method) and the pro-apoptotic

protein, Bax. In addition, Bcl-2, also known to promote the retention of cells in the G0/G1 phase of the cell cycle did not exhibit an inverse relationship with expression of Bcl-2 expression and the proliferative markers, Ki-67 and PCNA.

Among the clinicopathological parameters such as tumour grade and lymph node status, only tumour size showed a significant association with local recurrence where tumour size greater than 2 cm indicated an increased likelihood of local recurrence. In an analysis of the inter-relation between the biological parameters and clinicopathological features, tumour grade and not tumour size exhibited significant positive associations with Bcl-2 and p53 expression. As expected, Bcl-2 positive tumours were associated with low tumour grade while loss of p53 function was associated with high tumour grade. The presence of lymph node status indicated a positive association with the proliferative marker, PCNA.

Contrary to our expectations, the expression of Bcl-2 and p53 failed to predict local recurrence of breast cancer. The results obtained from the study confirm the need for further analysis of the inter-relations of the biological markers using more quantitative laboratory techniques namely, quantitative RT-PCR and flow cytometry.

Acknowledgments

At this time, I would like to express my gratitude to those who have helped me during the course of attaining the requirements for my Masters. Dr. Robb, thank-you for the many teaching sessions both in Pathology and in Life. You always said, 'life is hard, Syble' You have challenged me to become a stronger and more persistent individual.

To the supervisory committee members, Dr. George, Dr. Khalifa, Dr. Larsen, Dr. Haegert, and Dr. Gadag, thank-you for your insight and support especially in the development of this manuscript.

Cathy Searle, thank-you for helping me finish this project..and for all the misty stories! To my family, you have always been there for me..Mom, your sacrifice and unconditional love and support has been my foundation. Anna (big brother), thank-you for the years of friendship, guidance, and love. To Marc, you have been my partner throughout this long and sometimes tiresome journey. Thank-you!

Finally I would like to say a special thank-you to Shyam Kottilil. Shyam, thank-you for all those teaching sessions in Immunology I and II.

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BH	**Bcl-2 domain**
DAB	**diaminobenzidine**
ER\PR	**estrogen\progesterone receptor**
FACS	**fluorescent-activated cell sorter**
G0	**gap 0**
IHC	**immunohistochemistry**
LFI	**Li-fraumeni syndrome**
PBS	**phosphate buffered saline**
PCNA	**proliferating cell nuclear antigen**
PCR	**polymerase chain reaction**
PHA	**phytohemagglutinin A**
S-phase	**synthesis of DNA**
SLE	**systemic lupus erythematous**
TM	**transmembrane**
TUNEL	**terminal deoxynucleotidyl transferase (TdT)- mediated deoxyuridine triphosphate (dUTP) nick-end labelling**
WT	**wild type**

Chapter 1

BREAST CANCER

1.0 Epidemiology

Breast cancer is a serious clinical problem in Canada, occurring at a frequency of 1:9 with the expectation that one in 25 women will die of the disease. All the provinces have shown an increased incidence rate in breast cancer since 1987. During 1987, 12 396 cases of breast cancer were recorded by Statistics Canada; in 1997, this figure had risen to 18 400.

In 1995, breast cancer was the most prevalent type of cancer in Newfoundland women, and was a major cause of death. There has been a steady increase in the incidence of breast cancer in the province since 1985. A 10-year study by the Dr. R. H. Bliss Murphy Centre showed that in 1985, 167 women were diagnosed with breast cancer. In 1995, 290 women were diagnosed with the disease. This increase may partly be due to such factors as the recent introduction of regular mammographic screening, self awareness and media attention.

1.1 Risk Factors

Identifying women with the risk of developing the disease allows planning of preventive and diagnostic strategies. The relative risk of development of breast cancer is assessed by comparing the incidence rate of women having a particular characteristic to the rate of individuals in whom that characteristic is not present (Greenfield & Mullholland, 1997). Many risk factors have been identified for breast cancer. These include increasing age,

menstrual and reproductive history, exogenous estrogen stimulation, family history, diet and geography, and fibrocystic changes (Greenfield & Mullholland, 1997; Rosai, 1996).

1.1.1 Increasing Age

In women less than 25 years of age, development of breast cancer is uncommon. The incidence increases steadily after the age of 25 until menopause, followed by a slower rise in the remainder of life (Greenfield & Mullholand, 1997). For instance, statistics from the 1995 incidence of breast cancer recorded by the Bliss Murphy Centre showed that 143 of the 290 women diagnosed were 50 years of age or older (Bliss Murphy Centre, 1995).

1.1.2 Menstrual and Reproductive History

Uninterrupted menstrual cycles (as seen in nulliparous women), early menarche (before the age of 12) or late menopause (after the age of 55), places a woman at higher risk for developing breast cancer (Greenfield & Mullholland, 1997). Also, women who give birth before the age of 30 have a lower risk of developing breast cancer compared to women who give birth to their first child after 30 years of age or never bear children (Rosai, 1996). This age-related effect is further demonstrated by the fact that women who give birth before age of 20 years and have their second child before 25 years of age have an even lower risk of developing breast cancer (Rosai, 1996).

1.1.3 Exogenous Estrogen Stimulation

It is now argued that the basic common denominator in development of breast cancer is strong estrogen stimulation operating on a genetically susceptible background (Moore et al., 1983). Studies have shown that women who use exogenous estrogen to combat

symptoms of menopause have an increased risk (2.5 fold) of developing breast cancer (Ross et al., 1984; Kelsey & Gammon, 1991). However, some studies have stressed that this risk is only significant in patients who have had a previous diagnosis of fibrocystic change (Hoover et al., 1976). Furthermore, various studies have also shown that oral contraceptive agents do not constitute a risk for breast cancer (Rosai, 1996).

1.1.4 Family History (Genetic Influences)

A history of one or more family members (sister, daughter, mother, maternal grandmother or maternal aunt) having breast cancer is strongly associated with a risk of developing breast cancer (Greenfield & Mullholland, 1997). The risk becomes more pronounced if the relative developed bilateral breast cancer or was initially diagnosed while pre-menopausal (Greenfield & Mullholland, 1997). Women with a family history of breast cancer have an absolute lifetime breast cancer incidence of 50% or greater (Greenfield & Mullholland, 1997).

It is now clear that two thirds of familial breast cancer cases, which account for about 12-15% of all cases of breast cancer, have a well defined genetic basis associated with mutational events occurring in the BRCA1 (Futreal et al., 1994) and BRCA2 (Wooster et al., 1994) genes. BRCA1, located on chromosome 17, appears to function normally as a tumour suppressor gene; loss of heterozygosity in a germline mutation carrier results in the development of breast carcinoma (Futreal et al., 1994). BRCA1 germline mutations are also associated with a significant risk for the development of ovarian carcinoma (Miki et al., 1994). As yet, relatively little is known about the normal biological function of BRCA2 (Rosai,

1996). Germline mutational events in BRCA2 are associated with a lower risk than BRCA1 for ovarian carcinoma but are linked with a small but significant risk of prostatic carcinoma in males (Rosai, 1996).

1.1.5 Diet and Country of Birth

The incidences of breast cancer are high in North America and Northern Europe, intermediate in Southern Europe and Latin America and very low in most Asian and African countries (Sondik, 1994). Various studies have demonstrated that individuals emigrating from low incidence countries acquire the risk of incidence of their country of adoption (Greenfield & Mullholland, 1997). This suggests that different cultural customs, dietary factors and other environmental conditions may be linked with the development of breast cancer (Greenfield & Mullholland, 1997). Greenfield & Mullholland (1997), indicate that diets high in saturated fatty acids and obesity increase the risk for breast cancer in women.

1.1.6 Fibrocystic Change of Breast

Many studies have been carried out in an effort to ascertain the role of fibrocystic change in breast cancer tumorigenesis (Rosai, 1996). There appears to be a definite link between breast cancer and some elements of proliferative epithelial lesions (Rosai, 1996). Individuals with simple ductal hyperplasia have a twofold risk over the general population while those with atypical ductal hyperplasia appear to have a five fold risk (Page, 1986; Dupont et al., 1993). It is now a general clinical policy to carefully follow-up patients diagnosed with atypical ductal hyperplasia on a yearly basis (Rosai, 1996).

1.2 Prognosis of Breast Cancer

The clinical outcome of breast cancer is a complex issue which appears to be linked to (Chandrasoma & Taylor, 1995):

1) Effective early screening

2) Type of treatment

3) Various prognostic indicators

1.2.1 Screening

Screening methods for detecting tumours at a very early stage of development utilizing mammograms and self or physician examinations have been in progress for some time (Rubins & Farber, 1994). A review of results from randomized studies in Canada, U.S. and Sweden have shown that the routine use of mammograms in women from the age range 50-69 years reduces breast cancer mortality by about 30% (Love & Parker, 1996).

1.2.2 Treatment

Surgery has been the principal form of treatment in breast cancer (Greenfield & Mullholland, 1997). There are two types of surgery that are effective in removing tumours. *1) **Modified radical mastectomy*** includes removal of the breast but preservation of the pectoralis muscle with dissection of the axilla. *2) **Lumpectomy*** involves the complete excision of tumour with clear margins and axillary lymph node dissection followed by radiation (Greenfield & Mullholland, 1997).

Breast cancer is a moderately radiosensitive tumour (Chandrasoma & Taylor, 1995). Radiotherapy is assigned to patients following breast-conserving surgery (e.g. lumpectomy)

and in patients who suffer a local recurrence in the chest wall. Studies indicate a reduced chance of recurrence following radiation treatment of lumpectomy cases (Rubins & Farbers, 1994; Chandrasoma & Taylor, 1995).

Following surgery, chemotherapy is administered in all but well differentiated axillary lymph node negative cancers with no adverse prognostic indicators (Chandrasoma & Taylor, 1995). This form of treatment targets microscopic foci of tumour cells in the distant metastatic sites. In this situation, chemotherapy has increased disease free survival but is not curative (Chandrasoma & Taylor, 1995).

Since 50% of breast cancers exhibit nuclear estrogen receptor protein, anti-estrogen hormonal therapy has also been effective in prolonging disease free survival (Rosai, 1996). The effect of estrogen in promoting tumour growth can be suppressed by either anti-estrogen drugs such as Tamoxifen, or surgical removal of the ovaries or adrenal glands. Anti-estrogen therapy has been successful (60-80%) in women with estrogen or progesterone receptor positive breast carcinoma (Greenfield & Mullholland, 1997). However it should be stressed that the general clinical outcomes of breast cancer, both in regard to disease free survival and overall survival, appear to be related more to the actual biological characteristics of the tumour than to the type of treatment employed (Chandrasoma & Taylor, 1995).

1.2.3 Prognostic Indicators

The search for prognostic indicators which will predict clinical outcome and serve as a means of selecting patients for proper adjuvant therapy is ongoing (Greenfield & Mullholland, 1997). A reliable independent prognostic factor must satisfy two criteria: 1) it

must discriminate independently of other known factors between groups of patients with a good or poor prognosis; 2) it must be demonstrated through clinical trials that treatment intervention in the poor prognosis group improves clinical outcome (Greenfield & Mullholland, 1997). Multivariate analyses have shown that so far only a small number of these parameters have relatively independent significance (Greenfield & Mullholland, 1997). These include axillary lymph node metastasis, local recurrence, tumour size, tumour grade, cell proliferation, abnormalities in tumour suppressor gene expression, and estrogen receptor status.

1.2.3a Axillary Lymph Node Metastasis

Axillary Lymph node metastasis is one of the most important known prognostic parameters (Alderson et al., 1971). There is a sharp difference in survival rates between patients who are lymph node positive and those who are lymph node negative. Patients with metastasis have a reduced survival rate compared to those without metastasis. In the case of lymph node metastasis, the survival rate also depends on the level of axillary node involved (low, medium or high) (Berg & Robbins, 1966), the absolute number (fewer than four versus four or more) (Fisher et al., 1976; Fisher et al., 1983), the amount of metastatic tumour (Huvos et al., 1971; Rosen et al., 1981), the presence of extranodal spread and the presence of tumour cells in the efferent vessels (Clemente et al., 1992). It appears that patients with axillary lymph node micrometastatic deposits have the same prognosis as patients without metastasis (Pickren, 1961; Fisher et al., 1978). Overall three prognostic groups of lymph node status are identified in breast cancer: (1) negative nodes (2) 1-3 positive nodes (3) 4 or more

positive nodes (Rosai, 1996).

1.2.3b Local Recurrence

Local recurrence, defined as the recurrence of tumour in the region of the ipsilateral chest wall, is an adverse prognostic factor (Rosai, 1996). The vast majority of patients who have local recurrence eventually die of metastasis (Gilliland et al., 1983).

1.2.3c Tumour Size

Recent studies have demonstrated that the size of the primary tumour is a very important prognostic parameter (Carter et al., 1978; Russo et al., 1987; Rosai, 1996). The incidence of nodal metastasis and rate of relapse show a strong correlation with the maximum dimension of the primary tumour (Carter et al., 1978; Russo et al., 1987). It is now agreed that tumour size, potentially quick and easy to measure, represents one of the strongest predictors of dissemination and rate of relapse in node negative breast cancer (Greenfield & Mullholland, 1997). A tumour size of less than 1 cm, defined as minimal breast carcinoma, is associated with a 75% chance of 10-year disease-free survival (Saigo & Rosen, 1980). However, practical difficulties in obtaining an exact measurement of tumour size can reduce its value as an important prognostic indicator (Greenfield & Mullholland, 1997).

1.2.3d Tumour Histological Grade

Tumour grade as represented by the Nottingham Modification of the Bloom Richardson (Elston & Ellis, 1991; Frierson et al., 1995) system, is a strong independent prognostic factor predicting disease free survival and overall survival for patients with breast carcinoma. The grade is derived by adding the numerical scores for tubule formation, nuclear

pleomorphism and mitotic count, each of which is assigned a score of 1, 2, or 3 points (Elston & Ellis, 1991; Frierson et al., 1995). Grade I (good prognosis) tumours have 3-5 points, Grade II tumours have 6-7 points and Grade III (aggressive) tumours have 8 or 9 points. All pathology reports of breast carcinoma include an assessment of microscopic grade (Elston & Ellis, 1991; Frierson et al., 1995).

1.2.3e DNA Ploidy and Cell Proliferation

DNA ploidy status has not been shown to be a significant prognostic factor in breast cancer (Rubins & Farbers, 1994). However, tumour cell proliferation rate measured by Ki-67 expression (immunohistochemistry)(Sahin et al., 1991; Mauri et al., 1994), mitotic count (Biesterfield et al., 1995), or S-phase fraction (flow cytometry) (Witzig et al., 1994) has proved to be a strong independent prognostic factor for disease free survival. Thus, proliferation rate ranks with microscopic grade as a powerful prognostic factor and has the added advantage over microscopic grade, that its measurement is more objective (Rosai, 1996).

1.2.3f Abnormalities in Tumour-Suppressor Gene Expression

Accumulation of p53 protein (which normally restricts entry of cells into the S-phase of the cell cycle), whether as a result of gene mutation or abnormalities in the processes of transcription or translation, is thought to correlate with reduced patient survival (Barnes et al., 1993; Hurlimann, 1993).

1.2.3g Estrogen Receptor Status

Several studies have indicated that estrogen receptor positive breast tumours are associated with a longer disease free survival than estrogen receptor negative tumours (Hilf et al., 1980; Aamdal et al., 1984; Butler et al., 1985). However, the difference between the two types of tumours in terms of overall survival seems to be minimal and not statistically significant (Rosai, 1996).

1.3 Biological Characteristics, Inter-relationship and Prognostic Significance of Parameters Investigated in this Study

During the past few years, a number of studies have been carried out to test the significance of various potential prognostic parameters in breast cancer (McGuire, 1987; Weidner, 1993). Many of the parameters are related to various aspects of tumour biology such as morphology, proliferation characteristics, oncogene status, invasive or metastatic potential and hormone receptors status (Rosai, 1996; Rubins & Farbers, 1994;Greenfield & Mullholland, 1997). A potential biological parameter should correlate with other known prognostic factors such as tumour size, tumour grade, lymph node status and independently predict the clinical outcome.

In the present study of breast cancer, potential prognostic parameters relating to apoptosis, proliferation and morphology and their association to tumour size, tumour grade, lymph node status, and local recurrence have been examined. Local recurrence is defined as the recurrence of tumour within a period of 5 years following diagnosis, at the site of original surgery or in the adjacent axillary region. The following is a brief description of the current

state of knowledge and clinical significance of the biological parameters investigated in this study.

1.3.1 Apoptosis

1.3.1a The Bcl-2 Family

The role played by the Bcl-2 family of apoptotic factors and the tumour suppressor gene p53 has been a subject of particular interest with regard to tumour biological behaviour and clinical outcome. Bcl-2 is a proto-oncogene with the ability to inhibit apoptosis. It encodes a 26 kd protein which can be found on mitochondrial membranes, the nuclear envelope and endoplasmic reticulum (Kroemer, 1997). Expression of Bcl-2 in breast cancer is related to a high degree of histological differentiation (van Slooten et al., 1998) and positive estrogen/progesterone status (Diaz-Cano et al., 1997), both markers for a favourable prognosis. In the case of node positive breast carcinomas, Bcl-2 is an independent indicator of longer disease free survival (Berardo et al., 1998).

Bax, another important member of the Bcl-2 family of proteins commits a cell to apoptosis and antagonizes the anti-apoptotic activity of Bcl-2 (Reed, 1996). All of the members in the Bcl-2 family contain three similar structural motifs in the protein sequence designated BH1, BH2 and BH3 regions (Reed, 1996). An over representation of the Bcl-2/Bax heterodimers leads to decreased apoptosis whereas an over representation of Bax/Bax homodimers leads to apoptosis (Reed, 1996).

1.3.1b p53

Wild type p53 is a protein that acts as a DNA-binding transcriptional regulator that contributes to tumour suppression by inducing cell death or apoptosis and by limiting the entry of tumour cells into the S-phase of the cell cycle (Ko & Prives, 1996). Wild type p53 transcriptionally suppresses Bcl-2 expression while inducing expression of Bax (Ko & Prives, 1996). Loss of p53 function by mutation frequently occurs in breast cancer (Barnes, 1993, Ko & Prives, 1996). This loss of wild type p53 function directly correlates to poor clinical outcome probably as a result of increased cellular proliferation and a reduction in the rate of apoptosis (Bellamy, 1996; Ko & Prives, 1996). In a study of 304 breast cancer cases with a 7 year follow-up, mutant p53 expression was associated with shortened disease free survival (Porter-Jordon & Lippman, 1994). Patients who were lymph node negative but p53 immunopositive (indicating mutant p53 expression) showed an 80% 5 year survival compared with 93% for lymph node negative and p53 negative tumours.

1.3.2 Proliferative Characteristics

1.3.2a Ki-67

Expression of cell proliferation markers as a potential prognostic tool has been studied immunohistochemically using monoclonal antibodies (Bouzabar et al., 1989; Brown et al., 1990; Dervan et al., 1992) . Two markers of interest in this present study are Ki-67, and Proliferating Cell Nuclear Antigen (PCNA). Ki-67 is a nonhistone nuclear protein found in all phases of the cell cycle except the G0 phase (Yu et al., 1992). The percentage of nuclei staining for Ki-67 is a semi-quantitative measure of the proliferative fraction of cells (Yu et

al., 1992). Studies have indicated that high expression of Ki-67 in the nuclei correlates with poor tumour grade. (Pierga et al., 1996). In addition, high levels of Ki-67 are associated with shortened disease free and overall survival (Porter-Jordon & Lippman, 1994). Expression of Ki-67 in breast cancer appears to be independent of age, nodal status, or hormone status (Porter-Jordon & Lippman, 1994).

1.3.2b *PCNA*

PCNA is a 36 kd nonhistone nuclear protein that functions as an accessory protein to DNA polymerase delta (Dietrich, 1993). The level of protein expression is associated with the degree of cellular proliferation, specifically DNA synthesis. The level of PCNA rises during the late G1 phase, just prior to the S-phase DNA synthesis of the cell cycle. Levels peak during the S-phase and decline during the G2 and M phases of the cell cycle (Dietrich, 1993). PCNA appears to function by facilitating the entry of cells into the S-phase of the cell cycle. In breast cancer, expression of PCNA has been associated with increased mitotic activity and tumour grade (Porter-Jordon & Lippman, 1994).

1.4 Bcl-2 and Proliferation

Bcl-2 is a significant inhibitor of cellular proliferation presumably by causing cells to remain in the G0/G1 phase of the cell cycle. It promotes cell viability without promoting cell proliferation (Craig, 1995). The first indication of the effect of Bcl-2 on cell viability came from studies of hematopoietic cell lines (Craig, 1995). These cells die by apoptosis upon removal of various growth factors required for their survival. However the process of death by apoptosis can be prevented if the cells are previously transfected with Bcl-2 with the

transfectants remaining in the quiescent non-proliferating G0 phase of the cell cycle (Craig, 1995). Some studies have indicated that Bcl-2 negative breast cancer is associated with high proliferation rates whereas strong expression of Bcl-2 is related to low proliferation rates (van Slooten et al., 1998). Thus, it seems that increased Bcl-2 expression in breast cancer indicates a favourable prognosis (van Slooten et al., 1998).

1.5 Summary

Relatively few studies have concentrated on the interrelation of pro-apoptotic, anti-apoptotic and proliferative factors and their effect on the clinical outcome of breast cancer. Tumour growth is determined by the rate of cell proliferation and cell death. In this study, we examined apoptosis and proliferation in breast cancer and their potential role as predictors of local recurrent disease. We hypothesize that local recurrence of breast cancer is predicted by low expression of Bcl-2 in association with a high rate of both proliferation and apoptosis. The succeeding chapters describe the role proliferation and apoptosis in tumour growth.

Chapter 2

CELL DEATH

2.0 Apoptosis

Apoptosis, an ancient Greek word for the 'falling off' of petals from flowers was suggested by Kerr et al.(1972) to describe a spontaneous cell death that occurred in different tissues under various conditions. Through the electron microscope, Kerr and his associates (1972) detected apoptosis in untreated malignant neoplasms, in regression of tumours following therapy, and in healthy tissues.

Apoptosis typically affects single cells or small groups of cells in an asynchronous pattern (Wylie, 1993). Structural changes in cells undergoing apoptosis are characterized by two discreet stages: 1) formation of apoptotic bodies and 2) phagocytosis and degradation by other cells (Wylie, 1993).

A cell undergoing apoptosis involves marked condensation of both the nucleus and the cytoplasm (Arends & Wylie, 1991). Contraction of the cytoplasm due to loss in intracellular fluid and ions results in the endoplasmic reticulum dilating and forming vesicles that fuse with the plasma membrane (Arends & Wylie, 1991). Consequently, the cell surface exhibits a pronounced convoluted outline which fragments to provide the membrane surrounding apoptotic bodies. The nucleus is broken into a number of fragments of condensed chromatin (Arends & Wylie, 1991). The apoptotic bodies vary in number, size, and composition; some contain one or more nuclear fragments as well as condensed cytoplasm whereas others contain material solely derived from the cytoplasm (Wylie, 1993). The

apoptotic bodies are engulfed by members of the mononuclear-phagocyte system. Once ingested, the apoptotic bodies undergo rapid degradation via lysosomal enzymes produced by the phagocyte (Wylie, 1993).

The potential exists for the assessment of the extent of apoptosis in tissues by the semi-quantitative or quantitative measurement of pro- and anti-apoptotic protein factors using flow cytometric, immunohistochemical and molecular techniques. Apoptosis may also be detected by microscopic examination but the method is generally thought to be unreliable (Kraupp & Nedecky, 1995). In the present study apoptotic rate was measured by the terminal deoxynucleotidyl transferase (TdT)- mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labelling (TUNEL) in situ hybridization method (this method is discussed in chapter 5).

2.2 The Bcl-2 Family

There are three phases in the process of apoptosis: initiation, effector and degradation. The initiation phase can be triggered by a variety of stimuli whereas the effector and the degradation phases are common to most cells undergoing an apoptotic process (Lewin, 1997). The gene products of the Bcl-2 family are influential regulators of the effector phase in the apoptotic process (Kroemer, 1997).

The Bcl-2 gene was identified adjacent to the breakpoint of a chromosome rearrangement commonly found in follicular B cell lymphoma (Tsujimoto & Croce, 1986). The rearrangement involves the translocation of Bcl-2 from chromosome 18q21 to the long arm of chromosome 14q32, the immunoglobulin heavy chain locus (Craig,1995). The translocation results in an increased expression of Bcl-2 (Craig,1995). This increase in Bcl-2

expression is thought to be due to the presence of enhancers in the immunoglobulin heavy chain gene (Craig,1995).

2.2.1 Death Agonists and Antagonists of the Bcl-2 Family

Since the discovery of Bcl-2, several other genes which share a homology to the Bcl-2 gene, and whose products function as regulators of apoptosis have been adopted into the Bcl-2 family. The members of the Bcl-2 gene family are divided into two groups; (1) death agonists: Bax, Bak, Bad, Bid, Bik, Bcl-Xs (2) death antagonists: Bcl-2, Mcl-1, Bcl-xL, and A1 (Yang & Korsmeyer, 1996). Studies to date indicate that there are four well conserved domains in the members of the Bcl-2 family called BH1,BH2,BH3 and BH4 (BH denotes the Bcl-2 Homology Domain)(Reed et al., 1996). With the exception of Bik and Bad, most members share a homology in at least 2 of the 4 domains. These domains interact with each other, forming homodimers or heterodimers that commit a cell to death or survival (Reed et al., 1996).

2.2.2 Bcl-2 and Bax

Two members of the Bcl-2 family are of interest in this study: the death antagonist Bcl-2 and the death agonist Bax. The Bcl-2 gene encodes for a 25-26 KD protein which contains a stretch of hydrophobic amino acids at the C-terminal end required for insertion into cellular membranes (Brown, 1997). Insertion into the membrane is closely associated with the ability of Bcl-2 to regulate apoptosis (Brown, 1997). Subcellular localization experiments indicate that Bcl-2 is present on the mitochondrial outer membrane, the nuclear membrane, and the endoplasmic reticulum (Kromer,1997).

Bax, a death agonist, encodes a 21 KD protein that shares homology with Bcl-2 at the BH1, BH2 and the BH3 domains (Brown,1997). Bax was identified in immunoprecipitation studies as a Bcl-2 binding protein. It has a 45% homology to Bcl-2 and antagonizes the inhibitory effect of Bcl-2 (Brown, 1997).

Current studies suggest that Bcl-2 and Bax are required to localize to mitochondria in order to regulate apoptosis (Kroemer, 1997). Bcl-2α, a predominant splice variant of Bcl-2, contains a 21 amino acid carboxy-terminal signal anchor sequence that targets the protein to the intracellular membranes. Deletion of this transmembrane (TM) region, results in: (1) inability to suppress cell death, and (2) prevention of targeting of other Bcl-2 family proteins like Bax to the mitochondria (Zha et al., 1996). Adding the TM region from yeast outer mitochondrial membrane protein Mas-p70 which has a similar sequence, restored the inhibitory effect of Bcl-2 (Zha et al., 1996). Similar effects, such as failure to target mitochondria and loss of death promoting effect, have been observed in the Bax protein upon removal of its TM region (Zhu et al., 1996). Fusing the TM domain of Mas-p40 to the truncated Bax protein restores its ability to target mitochondria and perform its cytotoxic effect (Zhu et al., 1996).

Bcl-2 is also active when specifically targeted to the endoplasmic reticulum. This suggests that Bcl-2 exerts some effect on organelles other than mitochondria to prevent pro-apoptotic proteins from targeting the mitochondria (Kromer, 1997). For instance, Bcl-2 has been shown to interact with Bax (which itself must target mitochondria to promote cell death) in the endoplasmic reticulum to prevent cell death (Zha et al., 1996).

The ratio of death agonist (Bax) to death antagonist (Bcl-2) determines whether a cell will respond to an apoptotic signal. Initiation of cell death depends on the competitive dimerizaton of agonists and antagonists. Though amino acid sequences are similar in the 4 domains of the Bcl-2 family, the structural features required for homo or heterodimerization are different (Reed et al., 1996).

Bcl-2 homodimers form an antiparallel head -to -tail association where the BH4 domain of one peptide interacts with the BH1, BH2, and BH3 domain of the other peptide (Sato et al., 1994). Deletion of the BH1, BH2, and BH4 domains prevents the homodimerization of the resultant mutant (Hanada et al., 1995). The mutants are unable to inhibit cell death suggesting that intact Bcl-2 homodimerization, dependent on BH1, BH2 and BH3, is important in this function (Hanada et al., 1995). The wild type Bcl-2 can dimerize with the mutant but has no ability to enhance cell death. Furthermore, dimerization between a single deletion of BH4 in a Bcl-2 protein and wild type Bcl-2 still renders the dimers dysfunctional (Hunter & Parslow, 1996). A deletion of BH1 and BH2 can still result in the mutant binding to the wild type (via the BH4 domain binding to the BH1 and BH2 domain of the mutant) but the inhibitory effect on apoptosis is still absent. Single deletions in segments other than the BH1- BH4 domains also suppress the inhibitory effect of the Bcl-2 gene product (Hunter et al., 1996).

Bax, a death agonist, does not contain the BH4 domain thus suggesting that this domain is expendable in interacting with the Bcl-2 protein. As a result the structural features by which Bcl-2 interacts with Bax are different; BH1, BH2 and the BH3 domains are

necessary for Bcl-2/Bax heterodimerization (Yin et al., 1994). In lymphoid cells, deletions of a single amino acid in the BH1 or the BH2 regions of Bcl-2 have been shown to impair dimerization with Bax and inhibit its anti-apoptotic function (Yin et al., 1994). Consequently, this suggests the necessity of BH1, BH2, and BH3 of Bax to interact with Bcl-2 to suppress cell death. However, the finding that the BH4 domain of the Bcl-2 protein is needed to inhibit cell death but not for dimerization with Bax suggests that Bcl-2/Bax interaction alone is not sufficient to suppress cell death (Reed et al., 1996). Current research indicates that in mammalian cells, a mutant Bcl-2 protein lacking in the BH4 domain is unable to prevent cell death despite binding to Bax (Sato et al., 1994). An explanation for this could be that the BH4 domain may sterically interfere with the binding of Bax to some other death effector proteins. Another explanation could be that the BH4 domain may serve as a effector domain that binds the Bcl-2 protein to other proteins to inhibit cell death whereas dimerizing to the BH1 and BH2 domain serves to target Bcl-2 to Bax (Reed et al., 1996).

Studies indicate that Bax mutant deletions in the BH1 and the BH2 domain do not hinder homodimerization with wild type Bax, or with other Bax mutants (Zha et al., 1996). Bax/Bax homodimerization occurs in a tail to tail interaction (Zha et al., 1996). The BH3 domain however is essential for homodimerizing with Bax and heterodimerizing with Bcl-2. Bax mutants lacking in BH3 domain fail to homodimerize with wild type Bax and are incapable of promoting cell death (Reed et al., 1996).

To summarize from the studies:

1) Bax-Bax homodimers promote cell death.

2) Bcl-2 functions as an inhibitor of Bax by binding to Bax and forming an heterodimeric complex. For this to happen, the BH1, BH2 and BH3 sequences must be intact. Following Bcl-2/Bax dimerization, BH4 may play a fundamental role in inhibiting the function of Bax.

3) Bcl-2/Bcl-2 homodimers inhibit cell death, with Bax working to prevent Bcl-2 homodimerization.

Formation of dimers, Bax/Bax, Bcl-2/Bcl-2 or Bcl-2/Bax results in a ratio of death agonist to antagonist which determines whether a cell will respond to an apoptotic signal. Although dimerization of Bax and Bcl-2 has not been directly examined in the present study, an increase in death agonist (Bax) or death antagonist (Bcl-2) and their influence in apoptosis was examined. The inter-relation of Bax and Bcl-2 and their apoptotic influence in the primary tumour was also investigated.

2.3 Bcl-2 and Cancer

Immunohistochemical techniques indicate the expression of the Bcl-2 family genes in a wide variety of tissues, both in terms of specific cell types and stage of cellular differentiation (Reed, 1995). Expression of Bcl-2 has been reported in hormonal or growth factor regulated tissues (thyroid, breast, prostate), long-lived stem cells (skin or intestine) and long lived postmitotic cells such as neurons (Reed, 1995). Cells of mesodermal origin do not express the Bcl-2 gene (Diaz-Cano et al., 1997). The widespread expression of many Bcl-2 family genes means that many types of cells simultaneously contain several Bcl-2 family

proteins. These proteins, whose relative amounts vary according to cell type and degree of tissue differentiation, are available for physical and functional interactions and therefore provide the unique characteristics of each cell type in terms of lifespan regulation and relative sensitivity or resistance to apoptotic stimuli (Reed, 1995).

High levels and aberrant patterns of Bcl-2 expression have been observed in a number of human carcinomas; prostate, lung, gastric, renal, and colorectal (Reed, 1995). High levels of Bcl-2 have also been found in other cancers such as acute and chronic leukaemia, non-Hodgkin's lymphoma and neuroblastoma (Reed, 1995). Abnormalities in Bcl-2 expression can be observed in early or late stages of tumour progression (Bronner et al., 1995). The causes for the dysregulation of Bcl-2 expression in neoplasms are varied. One explanation for the aberrant Bcl-2 expression is that alterations occur in the transcriptional activators and repressers that regulate the Bcl-2 gene (Miyashita et al., 1994). One such repressor, the p53 gene, will be described in the following chapters.

As discussed earlier, immunohistochemical analysis of Bcl-2 has been performed in many types of human cancers, including breast carcinoma. The precise role of Bcl-2 in breast cancer is still unclear. The ratio of anti-apoptotic to the pro-apoptotic proteins plays an important role in determining the sensitivity to apoptotic stimuli (Reed, 1995). Elevated expression of Bcl-2 protein and reduced expression of pro-apoptotic Bax protein have been observed in women with metastatic breast cancer (Reed, 1995), therby suggesting that a decrease in apoptosis leads to increase in the survival of tumour cells which predisposes to metastases. In contrast to this result, Bargou et al.(1995) found no significant increase in the

expression of Bcl-2 in breast carcinoma. However, the expression of the Bax protein was significantly lower in breast carcinoma when compared to the expression of Bax in normal breast tissue. This finding suggests that perhaps it is the ratio of low Bax expression to the unchanging Bcl-2 protein in breast carcinoma that hinders the process of apoptosis and enhances the survival of tumour cells (Bargou et al.,1995). Similarly, Krajewski et al.(1995) also observed that the reduction of Bax expression in women with metastatic breast cancer was associated with diminished apoptosis.

A recent study by Mustonen (1997) showed a decrease in the Bcl-2 expression in the late stages of invasive breast carcinoma. This decrease was associated with an increase in the rate of apoptosis. A decrease in the expression of Bcl-2 and Bax in the later stage of breast cancer has been demonstrated in a number of studies (Reed et al., 1996). The cause for this decrease in Bcl-2 and Bax expression in the later stages of tumour progression remains unknown. Krajewski et al.(1995) suggest that tumours, after a certain point in their biological progression, may be less dependent on Bcl-2 for survival and that one reason for this non-dependency could be the diminished expression of Bax. In contrast to Mustonen's study which showed a strong inverse correlation between rate of apoptosis and Bcl-2 expression, Berardo et al.(1998) found no association between Bcl-2 expression and apoptotic rate in lymph node positive breast carcinoma. The difference in the two results can probably be explained by considering the fact that other proteins in the Bcl-2 family are known to regulate apoptosis. Neither study analysed the expression of apoptotic proteins such as Bax and Bcl-X_s or anti-apoptotic proteins such as Bcl-X_L or Mcl-1. The interactions of the Bcl-2 family are complex

and interdependent and therefore analysis of multiple components may be needed (Reed, 1995).

Knowledge of the status of Bax and Bcl-2 expression in breast cancer and its association with apoptosis and clinical parameters such as lymph node status, estrogen/progesterone receptor status, disease free survival, and tumour grade could determine the clinical significance of Bcl-2.

2.4 Clinical Significance of Bcl-2 Expression in Breast Cancer

Studies have examined the association of Bcl-2 expression to a number of well known clinicopathological parameters. As described in the previous chapter, only tumour size, axillary lymph node status, presence of metastatic disease and ER/PR status have so far met the rigid specifications required of a good independent prognostic parameter. Bhargava et al.(1994) reported strong positive correlation between Bcl-2 and estrogen/progesterone receptor expression, suggesting the possibility of estrogen/progesterone regulation of the Bcl-2 protein expression in breast carcinoma. Similarly, Berardo et al. (1998), in a study of 979 cases with invasive breast carcinoma showed a strong positive correlation between ER/PR receptor status and Bcl-2 expression. Sierra et al. (1995), in their study of 124 primary tumours from patients with or without lymph node metastasis, showed an inverse correlation between expression of Bcl-2 and histological grade of tumour. In contrast to high Bcl-2 expression in Grades I and II tumours, Bcl-2 was significantly reduced or absent in the majority of the cases of Grade III carcinoma. Sierra's results also indicated that Bcl-2 was more frequently expressed in cases with lymph node involvement (72%) than those without

lymph node involvement (45.7%), again suggesting that the anti-apoptotic effect of Bcl-2 may be conducive to metastatic spread in spite of a relatively high degree of tumour differentiation. Sierra concluded that Bcl-2 is more frequently expressed in T1 (tumours less than 1cm) with lymph node metastasis. Similar results have been demonstrated by Diaz-Cano et al. (1997). He showed a strong positive correlation between expression of Bcl-2, ER/PR status, and low grade tumours. This suggests that Bcl-2 expression is a probable mechanism by which neoplastic cells reach longterm growth in a well differentiated ER positive breast carcinoma (Diaz-Cano et al., 1997). A high grade ER/PR negative tumour which lacks Bcl-2 expression does not rely on such mechanisms possibly because a selective advantage for growth has already been acquired by other mechanisms (Diaz-Cano et al., 1997).

A high expression of Bcl-2 is associated with prognostic factors known to predict disease free survival and overall survival of breast cancer patients (Kobayashi et al., 1997 ; Joensuu et al., 1994). If this is the case, then it is logical to ascertain if Bcl-2 by itself is an independent favourable prognostic factor. Josenuu et al., (1994) showed no independent prognostic value of Bcl-2 expression in node negative and node positive tumours. On the other hand, Hellmans et al. (1995) suggest that the absence of Bcl-2 was an independent marker of poor prognosis in axillary node positive breast carcinoma but not in node negative tumours. Another study by van Slooten et al.(1996), however, showed that even though Bcl-2 was associated with various other prognostic factors, Bcl-2 by itself is not an independent marker of prognosis.

As indicated above, studies determining the prognostic value of Bcl-2 have yielded different results. To investigate the effectiveness of Bcl-2 as a prognostic indicator, the present retrospective study examined the inter-relation of Bcl-2 and Bax to the clinical parameters and the ability of Bcl-2 to predict local recurrence.

Chapter 3

CELLULAR PROLIFERATION

Cell proliferation is a biological process that plays a fundamental role in physiology and pathology. Cell growth occurs in a series of consecutive stages where G0 (G = gap) is the initial phase where synthesis of DNA does not occur. As a result, cells in the G0 phase are not strictly considered to be part of the cell cycle. When stimulated, the cells in the G0 phase progress to the G1 phase where messenger RNAs and proteins are synthesized. The G1 stage is succeeded by the S-phase (S = synthesis of DNA) phase in which DNA is being replicated. During this stage, the total content of DNA increases from the diploid value of 2n to the replicated value of 4n. The S phase is followed by G2 phase where the cell has two complete diploid sets of chromosomes and is ready for mitosis (Lewin, 1997; Yu et al., 1992).

3.0 Cell Cycle and Cancer

The cell cycle is a highly regulated event where dysfunctions in the regulatory proteins represent potential origins for tumourigenesis. Studies have indicated that cell cycle alterations are a common feature of neoplasia. Indeed, cell cycle defects seem necessary for cancer development (Nielsen et al., 1997).

Various techniques have been used in determining cell proliferation in tumour cells. Counting mitoses in tissue sections is an old but well established method (Hall & Levinson, 1990). Though fairly crude, the technique can be rigidly standardized and has been shown to be quite reliable (Hall & Levinson, 1990). A more recent and reliable method to determine proliferation is the use of flow cytometry which measures proliferation as the percentage of

cells present in the S-phase of the cell cycle (Yu et al., 1992). Though reproducible and precise, flow cytometric analysis requires expensive equipment and technical knowledge (Yu et al., 1992). In general, the measurement of proliferation by histochemical methods has the advantage of preserving the spatial orientation of proliferating cells within tissues (Yu et al., 1992). Antibodies to proliferation associated markers such as Proliferating Cell Nuclear Antigen (PCNA) and Ki-67 antigen can be assessed immunohistochemically (Brown & Gatter, 1990). This method, even with a number of limitations, is a useful procedure with regards to the preservation of the cellular tissue architecture, relative simplicity of the method, and the ability to measure proliferation reliably in paraffin embedded tissues (Brown & Gatter, 1990).

The present study includes the investigation of the expression of two proliferative markers, Ki-67 and PCNA immunohistochemically in paraffin embedded tissue sections. The following is a discussion of the markers and their involvement in the cell cycle.

3.2 Proliferating Cell Nuclear Antigen: PCNA

PCNA, described by Miyachi et al. (1978) as a nuclear antigen, was detected in proliferating cells by autoantibodies from patients with systemic lupus erythematosus (SLE). The sera of these patients reacted with proliferating cells in a variety of tissues including mouse, rabbit and human (Yu et al., 1992). Further investigations indicated that PCNA like proteins and homologous genes occur in mammals, birds, and some viruses suggesting that PCNA is an evolutionary conserved nuclear protein (Dietrich, 1993). The PCNA gene product is a 36 kD nuclear nonhistone protein which functions as an accessory protein for DNA polymerase delta synthesis (Dietrich, 1993).

Study of PCNA synthesis in the cell cycle using flow cytometry and immunofluorescence indicates maximum PCNA production during the S-phase and at sites of ongoing replication (Dietrich, 1993). Reports show that the concentration of PCNA increases during the late G1 phase, reaches a maximum level in the S-phase (approximately 7 times the level found in the quiescent cell) and gradually declines during the G2 phase and mitosis (Dietrich, 1993).

Similar results were observed in molecular studies. PCNA mRNA was undetectable in quiescent cells. Increased expression of PCNA mRNA was detected 8-10 hrs after stimulation of the quiescent cells by growth factors and reached a maximum at 18-20 hrs which corresponds to the peak of DNA synthesis (Jaskulski et al., 1988). There are two types of PCNA present in the S-phase; a soluble form not involved in replication and an insoluble form, associated with ongoing DNA synthesis (Bravo & McDonald-Bravo, 1987).

Monoclonal antibodies to PCNA have been used to assess the degree of proliferation of tumour cells. The mouse monoclonal antibody, PC10, recognizes an epitope of human PCNA in tissues fixed in formalin and embedded in paraffin (Yu et al., 1992). The measurement of proliferation using immunohistochemistry has been shown to correlate well with the S-phase measurement using flow cytometry (Visscher et al., 1992). A number of studies have examined the proliferative rate of breast cancer cells using immunohistochemical analysis of PCNA expression.

3.2.1 PCNA and Breast Cancer

In 1993, Cummings examined the relationship of PCNA expression with survival rates of patients with breast cancer. The results indicated no clear correlation between PCNA positivity and survival rates. Overall, PCNA appeared to have little prognostic value in patients with breast cancer. Cummings (1993) also showed no correlation between PCNA positivity and tumour grade or lymph node involvement. In contrast, Tahan et al. (1993) showed that PCNA had a potential value as a prognostic indicator in breast cancer; patients with a high PCNA score had shorter disease free survival. A study by Sheen-Cheng et al.(1997) associated PCNA positivity to histological grade, primary tumour status and S-phase fraction measured by flow cytometry. Sheen-Cheng et al. (1997) concludes that in a multivariate analysis, high PCNA expression in breast cancer cells can be used as a useful adverse prognostic indicator in lymph node positive tumours. Horiguchi et al. (1998) found that patients with breast cancer whose tumours had a high PCNA expression showed poor survival rates. Also, as Sheen-Cheng's results indicated, PCNA was, in a multivariate analysis, an independent prognostic indicator.

The variability in these studies show that PCNA as an individual marker is not completely reliable in predicting survival or rate of recurrence. No doubt some of these discrepancies may be related to the use of different antibodies and technical variations in staining. However, it does seem that PCNA, combined with other markers may prove more useful in predicting clinical outcome. The strength and association of two or more proliferation markers may provide more valuable results in terms of validity and reliability. For example,

expression of the proliferative marker Ki-67 appears to correlate well with that of PCNA (Haerslev et al., 1996). The use of the marker Ki-67, has also brought some insight into tumour proliferation and has provided some useful clinical information.

3.3 Ki-67

In 1983, Ki-67, an IgG_1 mouse monoclonal antibody was obtained in studies directed at producing antibodies to Hodgkins disease (Gerdes et al., 1983). They observed that Ki-67 antibody reacted only with the nuclear antigen of cells undergoing proliferation. To validate this observation, Gerdes stimulated lymphocytes with phytohemagglutinin A (PHA) and found the antigen recognized by Ki-67 appeared only in the nuclei of proliferating cells whereas the antigen disappeared when proliferating cells were induced to the resting stage. He concluded that this nuclear antigen detected by Ki-67 was present in proliferating cells. The following year, Gerdes et al.(1984) investigated whether the presence of this antigen is expressed in all proliferating cells during all phases of cell cycle or if its occurrence is limited to certain phases of the cell cycle. He used two methods. 1) An indirect method which related RNA and DNA production to the expression of Ki-67 in PHA stimulated peripheral mononuclear blood leucocytes. 2) A direct method which separated cells according to the phases of cell cycle using a fluorescent-activated cell sorter (FACS) followed by immunostaining of these cells with Ki-67. Both methods indicated that Ki-67 is present in all phases of the cell cycle except the G0 phase. Gerdes et al. (1984) concluded that immunostaining with Ki-67 would provide a means by which the growth fraction of normal and neoplastic cells could be assessed. Since the initial report, many other studies have confirmed these observations. For example, Scott

et al. (1990) showed a good correlation between the measurement of proliferation rate using a labelled mitosis method and the immunohistochemical detection of Ki-67 expression in a tumour xenograft model.

Analysis of the structure of Ki-67 shows no significant homology to any other known sequence (Schulter et al., 1993). The centre of the cDNA contains a large 6,845-bp exon with 16 tandemly repeated 366-bp elements called Ki-67 repeats (Schulter et al., 1993). Each Ki-67 repeat contains a 66 bp highly conserved region known as the Ki-67 motif, known to encode the epitope recognized by the antibody (Schulter et al., 1993).

As described above, the Ki-67 antigen is expressed in all stages of the cell cycle except the G0 phase (Gerdes et al., 1983). Cells in the resting phase entering the cell cycle lack Ki-67 in the early part of the G1 phase (Gerdes et al., 1983). One study found that in proliferating cells, the actual amount of Ki-67 antigens varies with the different stages of the cell cycle (Sasaki et al., 1987). They observed that Ki-67 expression increases with cell progression, rising during the latter part of the S-phase and reaching a peak in the G2\M phase. The simultaneous increase of Ki-67 expression with cell cycle progression is found in both normal and neoplastic cells (Verheijen et al., 1989).

3.3.1 Ki-67 and Breast Cancer

The proliferative activity of a tumour is determined by the growth fraction which is the proportion of cells within the active part of the cycle (Veroni et al., 1988). Growth fractions measured by flow cytometry and Ki-67 immunohistochemical staining have shown good correlation, confirming that immunohistochemical staining is as reliable a method as flow

cytometry (Veroni et al., 1988; Kamel et al., 1989). Positive correlations have been reported between Ki-67 immunohistochemical staining and mitotic count, histological grade, lymph node involvement, and tumour diameter (Brown & Gatter, 1990). A 1996 study by Pierga also confirmed that Ki-67 expression was positively correlated to prognostic variables such as mitotic index, tumour size, receptor status, histological grading and lymph node status. Various studies from the late 1980s and early 1990s indicate Ki-67 to be an independent prognostic tool for assessing the clinical outcome of individual patients. For instance, Bouzabar et al.(1989), Wintzer et al.(1991) and Pierga et al. (1996) showed significant correlation between increased Ki-67 staining, and decreased disease free and overall survival. Ki-67 staining and flow cytometry are now thought to be reliable techniques for measuring tumour proliferation (Veroni et al., 1988; Kamel et al., 1989). Furthermore, Ki-67 is thought to be a valid prognostic tool in assessing clinical outcome, independent of nodal status, tumour size and grade (Porter-Jordan & Lippman 1994).

3.4 Ki-67 and PCNA

Though Ki-67 can perform as an independent prognostic indicator it has certain limitations. For example, Ki-67 antigen expression is influenced by nutritional supply of the cell and as a result tissue taken from the central area of a large tumour may display lower expression of the antigen (Brown & Gatter, 1990). Also, most tumours contain a heterogenous cell population within which there are different proliferation rates (Brown & Gatter, 1990). Thus a combination of two markers may prove more reliable in assessing the rate of proliferation.

A number of studies have examined the joint association of PCNA and Ki-67 to clinical outcome in breast cancer. Dawson et al.(1990) and Battersby & Anderson (1990) showed a positive correlation between PCNA and Ki-67. Haerslev et al. (1996), investigated the expression of PCNA and Ki-67 in 487 breast cancer patients. They found that high PCNA and Ki-67 scores correlated with clinicopathological features such as large tumour size, high histological grade, and absence of estrogen and progesterone receptors. In a univariate analysis of survival data, Ki-67 and PCNA emerged as parameters predictive of a poor overall survival in both lymph node positive and negative patients. However in a multivariate study, both PCNA and Ki-67 failed to be parameters of independent prognostic significance. However, in another study that examined the two markers, PCNA expression did not correlate with that of Ki-67. PCNA was not associated with any of the clinical markers such as grade, size, and mitotic index whereas Ki-67 showed a positive correlation to these clinical parameters (Leonardi et al., 1992). The differences in the results suggest that more studies are needed to examine the reliability of the proliferative markers.

3.5 Bcl-2 and Proliferation

A number of studies have documented a strong inverse correlation between Bcl-2 expression and proliferation rate. In a study of 441 premenopausal women with breast cancer, van Slooten et al. (1998) showed a strong relationship between low Bcl-2 expression and a high proliferative rate as measured by Ki-67 immunohistochemical staining. Other reports have observed similar results where Bcl-2 positive tumours are characterized by low proliferation rates when compared to Bcl-2 negative tumours (Gorczyca et al., 1995; van Slooten et

al.,1996).

Tumours with high Bcl-2 expression have been associated with low proliferative activity and, as a result, good clinical outcome. This association has been confirmed by a number of studies which show strong association between increased Bcl-2 expression and good clinical prognosis (Joensuu et al., 1994; Krajewski et al.,1995; Kobayashi et al., 1997). Also, tumours that lack Bcl-2 expression are usually high grade and have increased rates of proliferation consistent with a poor prognosis (Sierra et al., 1995). Bcl-2 over expressing cells tend to accumulate in the G0/G1 phase, confirming Craig's (1995) observation that Bcl-2 promotes cell viability but not cell proliferation (Borner et al., 1996). The mechanism by which Bcl-2 is involved in the proliferation of cells is not fully understood. Some researchers propose that deregulation in the growth regulatory pathway may result in one of two events: inability to control cell growth or apoptosis (Ko & Prives, 1996).

In this study expression of the proliferative markers, Ki-67 and PCNA were associated with the expression of tumour suppressor gene, p53, the apoptotic antagonist Bcl-2, and various prognostic factors. The tumour suppressor gene p53 and its association with proliferation and apoptosis will be discussed in the next chapter.

Chapter 4

p53: The Tumour Suppressor Gene

4.0 p53

p53, a tumour suppressor gene, named for its molecular size, was discovered by Linzer and Levine (1979) and Lane and Crawford (1979) as a 53 kd protein that bound in high quantity to the large T antigen of the DNA tumour virus, SV40, in SV40 transformed rodent cells. Since the discovery of p53, inferences regarding its function and role have undergone a number of changes. In 1989, p53 was correctly classified as a tumour suppressor gene, a view which has been confirmed frequently by subsequent studies (Finlay et al., 1989). DNA screening in patients suffering from various types of malignancies showed a high frequency of mutations in the p53 gene (Bellamy, 1997). In fact, carriers of Li-Fraumeni Syndrome, a rare form of hereditary cancer, possess a mutated p53 germline allele; mutation also of the normal allele (second hit) brings about the development of malignancy because of the complete lack of tumour suppression. The importance of p53 as a tumour suppressor is further suggested by studies which show that nullizygous mice, with both germline p53 alleles inactivated, develop normally but are highly susceptible to tumours, whereas p53 heterozygous mice (with one normal allele) display a lower susceptibility to cancer (Harvey et al., 1993). The need to understand the influence of mutant p53 in tumourigenesis, requires the study of wt p53 in normal cells. Research has been ongoing in this study.

Normal cells express low levels of p53 (Ko & Prives, 1996). Activation of wild type p53 causes two types of events: growth arrest and apoptosis (Lewin, 1997). P53 was defined

as a cell cycle checkpoint factor when studies showed that DNA damaging agents induced high expression of wt p53 (Lewin, 1997). p53 appears to act like an 'emergency brake', inducing either cell cycle arrest or apoptosis thus protecting the cell from accumulating excess mutations (Ko & Prives, 1996).

4. 1 Arrest or Apoptosis

The decision of whether p53 will induce apoptosis or growth arrest in various cells has been of interest to many researchers. Cell type seems to be one factor in how the choice is made. Current experiments have shown that it is possible to manipulate cells to undergo either response (Ko & Prives, 1996). Rodent primary cells that generally undergo G1 arrest in response to irradiation undergo apoptosis when these cells express the adenovirus antigen E1A (Debbas & White, 1995). Similarly, haemopoietic cells which are arrested in the G1 phase in response to irradiation, undergo apoptosis upon removal of the growth factor, Il-3 (Canman et al., 1995). Bellamy (1997) suggests that p53 simultaneously signals growth arrest and apoptosis while downstream events such as transcriptional function prevents one or other outcome. He observes that suppression of apoptotic pathways may initiate growth arrest or vice versa. For instance, introduction of Bcl-2 transgene into myeloid leukaemic cells causes delay in p53 dependent apoptosis, with the onset of p53 dependent growth arrest (Guillfouf et al., 1995). On removal of p21 (a p53 target gene that induces growth arrest) from colorectal carcinoma cell lines that normally engaged in p53-dependent growth arrest, p53 dependent apoptosis is initiated (Guillouf et al., 1996). From these studies, Bellamy (1997) concludes that although mechanistically different, the p53 dependent cell arrest pathway and apoptosis

pathway may communicate substantially.

4.1.1 p53 and Apoptosis

p53 induces apoptosis in several cell types. Various forms of stimuli activates p53 dependent apoptosis such as adenovirus E1A expression, withdrawal of growth factors or DNA damage (Ko & Prives, 1994). Evidence that p53 plays a direct role in apoptosis comes from the work of Clarke & Harrison (1993) and Lowe et al. (1993). Mouse thymocytes, normal or p53 deficient in one or both alleles were treated with a number of apoptosis inducing agents. Results showed that thymocytes with wt p53 entered apoptosis after ionizing radiation, whereas mutant p53 cells deficient in both alleles did not enter apoptosis. Similarly, results from of a study by Lee & Berstein (1993) showed that spleen and bone marrow cells from transgenic mice expressing mutant p53 resulted in increased resistance to apoptosis. Moreover, thymocytes from these animals showed decreased levels of apoptosis after irradiation. The study provided strong evidence that p53 activates apoptosis in response to agents that cause DNA strand breakage (Lee & Berstein, 1993).

p53 mediated apoptosis has been shown to suppress transformation of cells, tumour growth and tumour progression (Bellamy, 1997). The p53 dependent pathway of apoptosis has been important in understanding not only the development of tumours but also the treatment of cancer (Bellamy, 1997). Studies indicate that effective p53 mediated apoptosis is a hallmark of successful chemotherapy (Bellamy, 1997).

p53 dependent apoptosis can be inhibited by various factors. Exposure to growth factors such as Il-3 and Il-6 can inhibit p53 dependent apoptosis (Lin & Benchimol, 1995). Also, Bcl-2 or adenovirus E1B are known to block p53 activation (Lin & Benchimol, 1995). However, it is also thought that wild type p53 can transcriptionally repress Bcl-2 while inducing expression of Bax, one of the target genes (Ko & Prives, 1996). Therefore, loss of p53 function by mutation would be expected to lead to an accumulation of Bcl-2 and a decreased level of Bax. However, Krajewski et al. (1997), in a study of breast cancer, reported a strong inverse correlation between immunoreactive p53 expression and immunoreactive Bcl-2 expression. This poses an interesting problem in the interpretation because nuclear immunopositive expression of p53 is generally thought to signify the presence of a gene mutation in which the mutant protein has a relatively long half-life . Therefore, this study would indicate that mutant p53 is also effective in reducing Bcl-2 expression. On the other hand, this inverse relationship between immunoreactive expression of Bcl-2 and p53 may indicate that wt p53 may actually be substantially upregulated in breast cancer cells. Krajewski et al. (1997) found no relationship between p53 and Bax expression. A number of other studies in breast cancer have confirmed this inverse correlation between p53 and Bcl-2 expression. Barbareschi et al. (1996) examined 178 node-negative breast cancers and found Bcl-2 positive cells expression were inversely associated with p53 overexpression. Lee et al. (1996), Berardo et al. (1996) and Haldar et al. (1996), found similar inverse asociations between p53 and Bcl-2 expression. The present study likewise examines the association between p53 and Bcl-2 and their influence in local recurrence.

4.1.2 p53 and Proliferation

As discussed earlier, p53 regulates proliferation and apoptosis by activating or suppressing other genes required for either process (Lewin, 1997). The wild type p53 gene inhibits proliferation by influencing certain cell cycle regulators (Lewin, 1997). For instance, the cdk-cyclin kinase inhibitor p21, which binds to all cdks and arrests the cell cycle at any point within G1-S, is upregulated by p53 (Ko & Prives). As described earlier, p21-/- cells were incapable of arresting cell cycle progression in response to irradiation, suggesting the importance of p21 as a regulator of cell progression and as a critical target for p53 (Ko & Prives). Interactions of p53 with other cell cycle regulators remains unknown ((Ko & Prives).

4.2 p53 and Tumourigenesis

p53 mutations occur with high frequency in various types of human cancers. More than 2500 tumours and tumour cell lines have revealed some form of p53 mutation (Ko & Prives, 1996). Bellamy (1997) proposes that the wide occurrence of defective p53 derives from three properties: (1) wt p53 is highly vulnerable to dysfunction caused by even a single base charge in the coding sequence; (2) a single abnormal p53 allele or loss of allele can alter the phenotype; (3) the participation of p53 in multiple biological pathways makes it an Achilles heel of cancer suppression since a defect in its function can radically reduce cellular defence against carcinogenesis.

4.3 p53 Expression and Breast Carcinoma

p53 overexpression is detectable in 30-45% of primary breast carcinomas (Molina et al., 1998). Furthermore, p53, as an independent prognostic marker in breast cancer, has been reported in tumours smaller than 3 cm in size (Molina et al., 1998). In a study of 200 breast specimens, p53 mutation detected immunohistochemically also showed a significant association with high grade tumours, hormone receptor negativity and poor prognosis (Porter-Jordon & Lippman, 1994). Similarly, Elledge & Allred (1994) observed that p53 accumulation is associated with a number of prognostic factors such as ER negativity and high histological grade. In another study of 304 breast cancer cases with a 7 year follow-up, p53 positive tumours were significantly associated with shortened disease free survival and overall survival. Moreover, node negative, but p53 positive cases showed a 80% 5 year survival, compared to a 93% survival for p53 negative tumours (Porter-Jordon & Lippman, 1994). Similar results were observed in a recent study which showed a significant correlation between overexpression of p53 and increased rate of recurrence in node negative and node positive patients (Molina et al., 1998).

4.4 Immunohistochemical Analysis of p53

The wt p53 protein in the cell nucleus is usually present in small quantities with a very short half-life. Mutations in p53 cause various changes in its properties. Missense mutations increase the half-life and quantity of p53 (Greenblatt et al., 1994). The prolonged half-life and accumulation of the protein, termed overexpression of p53 in the nucleus, enables immunohistochemical (IHC) detection with specific antibodies. It is generally thought, but by

no means proven, that nuclear immunopositivity for p53 is an indirect indication of a mutation. Therefore, immunohistochemical staining of tissue sections seems an appropriate means of screening for p53 mutational events which can affect patient prognosis (Greenblatt et al, 1994; Sjorgen et al., 1996).

In various tumours, expression of p53 measured immunohistochemically is associated with the expression of the proliferative markers, Ki-67 and PCNA. It is logical to expect that mutation of wt p53, which normally restricts the movement of cells through G1-S, would result in increased proliferation. Various breast cancer studies show a positive correlation between p53 immunopositivity and proliferative rate (Allred et al.,1993; Gorczyca et al., 1995; van Slooten et al.,1996).

In this study, an association between p53 expression and proliferative markers (Ki-67 and PCNA), apoptotic markers (Bax, Bcl-2), and the rate of apoptosis was investigated. The practical details of the immunohistochemical and TUNEL methods are described in the next section.

Chapter 5

Principles of Laboratory Methods

5.0 Immunohistochemistry (IHC)

Since its development in the 1940s, immunohistochemistry has become a major diagnostic tool in pathology. The underlying principle of all immunohistochemical reactions is the specific recognition by an antibody of a cell or tissue antigen, followed by a visualization of the attached antibody (Elias, 1990). The site of the antigen-antibody binding can be identified either by a direct or an indirect method. In the direct method, the primary antibody specific to the antigen is conjugated directly to an enzyme such as horseradish peroxidase or alkaline phosphatase which uses a chromagen substrate for visualization of staining (Bancroft & Stevens 1996). Enzymes, the most commonly used labels in combination with a chromagen, yield a stable coloured reaction easily visible under the light microscope.

The indirect method, involves application of an unconjugated primary antibody, followed by a labelled antibody directed against the primary antibody (Bancroft & Stevens 1996). In the present study an indirect method, called the labelled avidin-biotin technique was used to identify the expression of apoptotic and proliferative markers in formalin fixed breast tissues embedded in paraffin wax.

5.0.1 The Avidin Biotin Technique

The Avidin Biotin method utilizes three reagents: primary antibody, biotinylated secondary antibody, and Streptavidin conjugated with horseradish peroxidase (Carson, 1997). The unlabelled primary antibody (raised in a goat or a rabbit) specific for the antigen, is

applied to the tissue. To detect the primary antibody, biotinylated secondary antibody, made from a second species, specific for the primary antibody derived from the first species, binds to the primary antibody (Carson, 1997). When streptavidin conjugated with the marker enzyme horseradish peroxidase is applied, it binds to the biotinylated secondary antibody. Horseradish peroxidase interacts with the chromagen Diaminobenzidene (DAB), yielding a dark brown reaction end product visible in the microscope (Elias, 1990).

This method relies on the affinity of a 67 kD glycoprotein avidin for biotin, a low molecular weight vitamin. Avidin, found in egg whites, is composed of four subunits which form a tertiary structure possessing four biotin-binding hydrophobic pockets (Bancroft & Stevens, 1996). The oligosaccharide residues found in the egg white with its charged properties have some affinity to the tissue components which can result in non-specific binding. Large amounts of biotin found in liver, also contribute to further background staining (Bancroft & Stevens, 1996). To avoid non specific binding, use of a similar molecule streptavidin, which lacks oligosaccharide residues and contains neutral isoelectric points was applied to the tissues instead of avidin.

Biotin is easily conjugated to antibodies and enzyme markers. Approximately 150 biotin molecules can be attached to a single antibody molecule. The ability of a large number of biotin molecules to bind to a single antibody enables numerous streptavidin molecules to bind to the biotinylated secondary antibody and results in an increased sensitivity to the antigen-antibody binding (Bancroft & Stevens, 1996). Consequently, the labelled avidin-biotin method has been found to be a most efficient technique, capable of detecting minimal levels

of antigen expression (Gorino, 1984).

5.1 The TUNEL Method

The terminal deoxynucleotidyl transferase (TdT)- mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labelling (TUNEL) method has been utilized to identify apoptotic cells in formalin fixed, paraffin embedded tissue sections. The enzyme TdT catalyses the insertion of digoxigenin labelled dUTP molecules onto the 3'-OH end of fragmented DNA in cells undergoing apoptosis. The dUTP molecules require no template for nucleotide elongation . The incorporated nucleotides form a heteropolymer in a ratio specific for anti-digoxigenin antibody binding. Digoxigenin is derived from the digitalis plant. In animals, immunochemically-similar ligands for binding to the anti-digoxigenin antibody are rare, thus ensuring low background staining. The Fc portion of the polyclonal sheep antibody to digoxigenin was proteolitically cleaved, allowing a <1% chance of cross-reactivity. The anti-digoxigenin antibody fragment carries the enzyme peroxidase which reacts with DAB to produce an intense, coloured signal. The above information was supplied by the ONCOR Apop Tag Manual.

There are concerns that the TUNEL method does not sufficiently discriminate between necrotic cells and true apoptotic cells. Kraupp and Nedecky (1995) showed that hepatocytes dying by both apoptosis and necrosis gave a similar positive result in the TUNEL assay. As a result of the study, apoptotic cells were assessed with particular care by the author and the pathologists (see section 7.3).

Chapter 6

Research Hypotheses and Objectives

Relatively few studies have concentrated on the inter-relation of well known apoptotic markers (Bcl-2, Bax), proliferative markers (Ki-67, PCNA) and clinicopathological features (tumour score, tumour size, and lymph node status) and their potential role as predictors of local recurrence in breast cancer. Therefore, this study was undertaken to investigate the interrelation of these factors and their clinical prognostic potential.

6.0 Research Hypotheses

Since tumour growth is determined by the rate of cell proliferation and apoptosis, it was hypothesized that local recurrence of breast cancer would be predicted in either of the two situations: (1) low expression of Bcl-2 in association with a high rate of proliferation and a high rate of apoptosis; and (2) expression of mutant p53 was increased in association with a high rate of proliferation and a low rate of apoptosis.

6.1 Research Objectives

By immunohistochemistry on breast carcinoma, we wished to:

- determine the rate of apoptosis by the TUNEL method and to examine its relation to the expression of the apoptotic regulators, Bcl-2, Bax and p53.
- examine the interrelation of the pro-apoptotic factors, Bax and p53, and the anti-apoptotic factor Bcl-2 .
- examine the interrelation of proliferation factors, Ki-67 and PCNA, and the apoptotic regulators, Bcl-2, and p53

- **ascertain if one or more of the following factors determine the incidence of locally recurrent breast carcinoma within a 5 year period following initial diagnosis of invasive breast carcinoma:**
  - **Expression of PCNA and Ki-67**
  - **Rate of apoptosis**
  - **Expression of Bcl-2**
  - **Expression of Bax**
  - **Expression of mutant p53**
  - **Clinical parameters such as tumour size, tumour score, and lymph node status**

Chapter 7

Methods

7.0 Subjects

This retrospective study examined 145 female Newfoundland breast cancer patients. The patients were allocated into two groups: those with a local recurrence of tumour at the same surgical site within the 5 year period following initial surgery or those without local recurrence during this period. Local recurrence was defined as the growth of tumour at the original site of surgery or in the axillary lymph nodes. The two groups were further divided into those with metastatic tumour in the axillary lymph nodes at presentation and those without lymph node metastatic tumour at presentation.

	Locally Recurrent	Non-Recurrent
Lymph Node positive	46	27
Lymph Node negative	45	27

7.1 Procedure

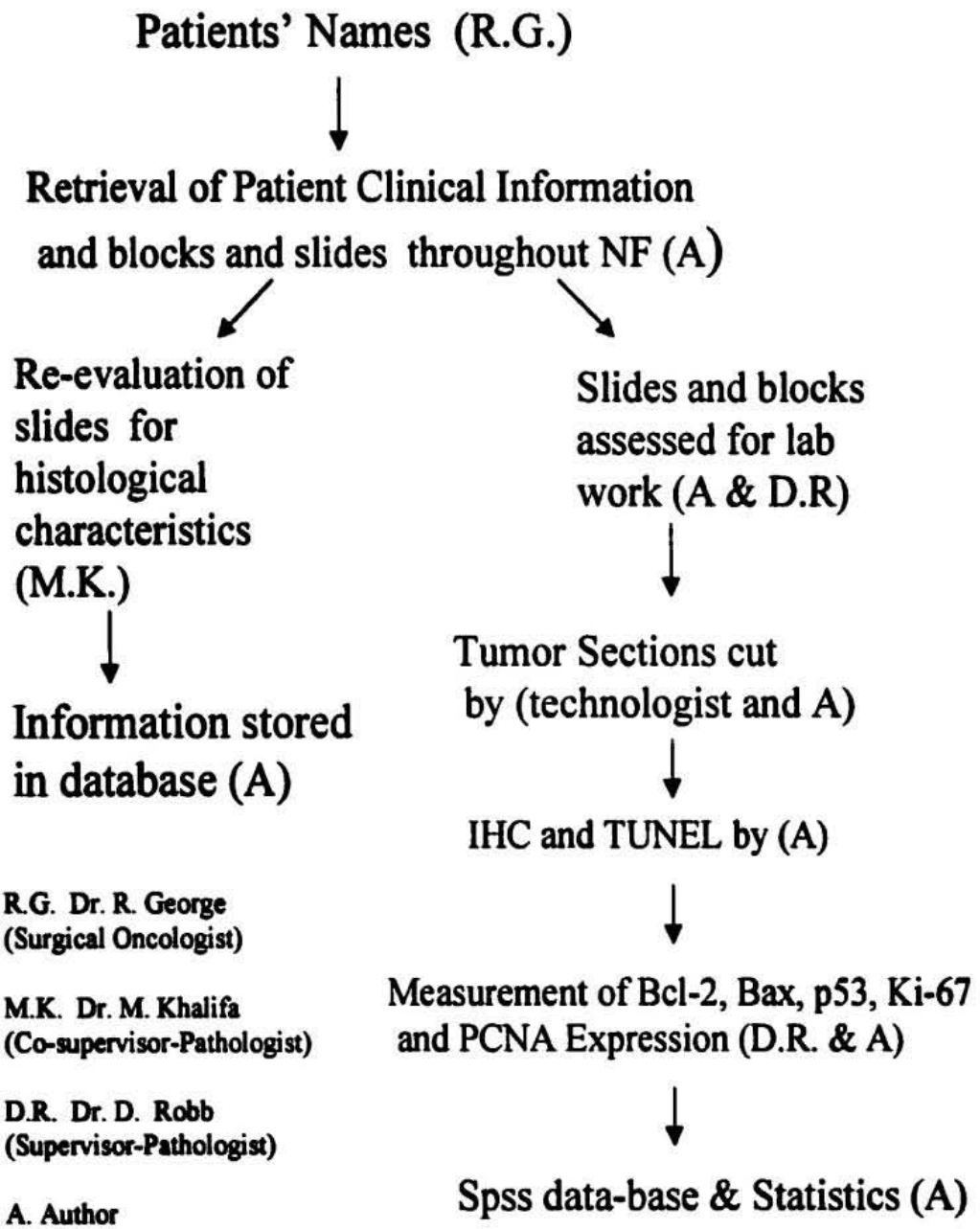
Patient names and relevant clinical data were retrieved from the Newfoundland Cancer Centre. Microscopic slides and paraffin blocks were retrieved from various centres in Newfoundland: St. John's, St. Anthony, Corner Brook, Grand Falls, and Gander. Initially, 165 breast cancer patients with local recurrence within a 5 year period were chosen for the study. Twenty of the patients were eliminated from the study due lack of relevant clinical data,

missing slides or mouldy paraffin blocks. The following clinical data retrieved from patients' charts were stored on the Microsoft Access Database:

- Tumour Size
- Lymph node status
- Presence or absence of local recurrence
- Tumour Grade
- Patient treatment (surgery, chemotherapy, radiation)

In addition all cases were re-examined microscopically by one pathologist and histologically graded according to the Nottingham Modification of the Bloom and Richardson system (Galea et al., 1992). The Hematoxylin & Eosin (H&E) slides were also examined and evaluated in terms of a "non-fatty" constitution and presence of tumour cells in at least one area of the tissue. The paraffin embedded tumour tissue corresponding to the particular H&E slide was then adopted for this study and analysed for the presence of the biological markers evaluated in this study. The selected patients and their paraffin embedded tumour specimens if selected were represented by a number from 1-145 to observe patient confidentiality and to prevent bias during slide examination of the apoptotic and proliferative factors. All procedural assessment, retrieval, and evaluations of slides and blocks were performed by the author and the two pathologist (see chart 1 for contributions by individuals during the progress of this study).

Chart 1: Contributions of individuals involved in the progress of this study



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(Supervisor-Pathologist)

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7.2 Pretreatment of Slides

The slides (75 x 25; J Melvin Freed Brand) to be used were placed for 2 min in 150 ml Histogrip (Zymed) diluted 1:50 in acetone. The slides were washed in three staining dishes of distilled water (10 dips each), and placed in a 55°C oven for two hours. Five micron thick tissue sections were cut on a microtome and mounted on the slides treated with Histogrip. The tissue sections were then used for *in situ* Terminal Deoxynucleotidyl Transferase Mediated Nick End Labelling (TUNEL) and Immunohistochemistry (IHC).

7.3 Terminal Deoxynucleotidyl Transferase Mediated Nick End Labelling (TUNEL)

The rate of apoptosis was determined by the TUNEL method using the Apop Tag Plus Kit (ONCOR). Reagents from the Apop Tag Plus Kit included Equilibriation Buffer, Reaction Buffer, TdT Enzyme, Stop/Wash Buffer, and Anti-Digoxigenin-Peroxidase.

Paraffin was removed from the tissue sections by placing the specimens in 300 ml of xylene, twice, for five minutes each. The deparaffinized tissue was then placed in 300 ml of absolute ethanol, twice, for five minutes. each. The specimen was placed in 300 ml of 95% ethanol followed by 300 ml of 70% ethanol for 3 minutes each. The tissue was then placed in running tap water, and finally rinsed with 10-15 ml of phosphate buffered saline (PBS, pH 7.4)(6.8995g/L of 50mM sodium Phosphate and 11.688g/L of 200mM NaCl).

To expose the free 3'OH group in DNA strands, the sections were digested in a solution of 50 µl of proteinase K (Sigma; 20µg/ ml in PBS) for thirty minutes in a humidifying chamber at room temperature. Sufficient proteinase K solution was added to cover the entire area of the tissue section with the use of a coverslip. Initial tests indicated

thirty minutes as the optimal period for digestion of breast tissue. Following the 30 minute incubation, the enzyme was removed with deionized water to prevent deterioration of the tissue.

The tissues were treated with 10 ml of 3% hydrogen peroxide (Health Care Stores) for five minutes at room temperature to eliminate endogenous peroxidase activity. Sections were rinsed in PBS and then placed in 10-15ml of PBS for 5 minutes to remove hydrogen peroxidase. This procedure was repeated once. Seventy-five microlitres of the Equilibration Buffer (supplied in the kit) was added to the specimen for 30 seconds at room temperature. A coverslip was placed on the tissue to spread the reagent evenly and to avoid evaporation. Excess buffer was drained off without rinsing by placing the slide on an incline.

A mixture of Reaction Buffer (supplied in the kit and containing digoxigenin labelled nucleotides), and TdT enzyme solution also supplied in the kit (30 µl of Reaction buffer and 16 µl of enzyme solution), was applied to all sections. The sections were coverslipped and incubated in a 37°C oven for 1 hour.

Coverslips were removed and the tissues were placed in a coplin jar containing 100 ml of stop/wash buffer for 10 minutes at room temperature. The stop/wash buffer (1 ml of the stock concentrated buffer diluted in 34 ml of distilled water) stopped the enzymatic reaction by removing any remaining enzyme. The specimens were rinsed and placed in 15 ml of PBS for 3 minutes. This procedure was repeated two more times. Fifty- five microlitres of the anti-digoxigenin peroxidase antibody (provided in the kit) was added to each of the specimens which were coverslipped and incubated for thirty minutes at room temperature.

The specimens were rinsed and placed in 15 ml of PBS for 5 minutes. This process was performed two more times. One microlitre of 30% hydrogen peroxide (Sigma) was added to 2 ml diamino benzidine (DAB; Sigma) solution prepared by diluting 10ml of DAB stock solution (10 mg DAB tablet for 10 ml PBS) with 15ml PBS. Three to four drops of DAB was added to each slide for 2-3 minutes at room temperature.

The specimens were washed in water three times for a minute each. The tissues were counterstained in 10 ml of methyl green solution (1.5ml of 1% methyl green (Sigma) in 0.1 M sodium acetate pH 4.0), for 10 minutes at room temperature.

The specimens were washed by dipping the slides seven times each in two staining dishes containing 200 ml of water. The slides were then placed in a staining dish containing 200 ml of water for 30 sec without agitation. This process was repeated using 200 ml of 100% butanol (Sigma). Finally, the specimens were consecutively placed for two to three minutes each in two staining dishes containing 200 ml of xylene (Health Care Stores), and then mounted in permount (Stephens Scientific) and coverslipped.

Slides were examined under the microscope by the author and two pathologists. The rate of apoptosis was measured by counting the number of stained apoptotic cells in 4-5 adjacent high power fields each consisting of at least 1000 cells. The mean rate of apoptosis (total number of apoptotic cells per number of adjacent power fields [40x] used in the tissue) was calculated.

7.4 Immunohistochemistry

This method utilizes three reagents: primary antibody specific for the antigen, secondary antibody conjugated to biotin, and peroxidase labelled streptavidin. This procedure was applied to the detection of the apoptotic factors: Bcl-2 (anti-apoptotic gene), Bax (apoptotic gene), p53 (tumour suppressor gene), Ki-67 (proliferative marker), and PCNA (proliferating nuclear antigen- a proliferative marker).

Paraffin was removed from the tissue sections by placing the specimens in 300 ml of xylene, twice, for five minutes each. The tissues were dipped in three staining dishes containing a series of ethanol concentrations: 10 dips in 300 ml absolute alcohol , 10 dips in 95% alcohol, and 10 dips in 70% alcohol. The specimens were rehydrated in running water for one to two minutes.

Tissue sections were then placed in a staining dish containing 300 ml of 3% hydrogen peroxide (Gibco) for twenty minutes to block endogenous peroxidase. Following the twenty minute treatment, the sections were placed in running tap water for one to two minutes.

For antigen retrieval tissue sections were initially placed in a beaker containing 500 ml of boiling 6M citrate buffer (Sigma) for eight to ten minutes and then allowed to cool for ten minutes by removing the beaker from the hot plate. The slides were removed and placed for 2-4 minutes in a staining dish containing 300 ml of tap water. This procedure was carried out for all antigens except PCNA.

The tissues were placed in the humidifying chamber and rinsed with PBS (8g sodium chloride; Sigma, 1.3g disodium phosphate; Sigma, 4.0g monosodium phosphate; Sigma in 1L

water). The excess PBS was drained, and the slides were dried with a clean gauze. To prevent non-specific background staining, two to three drops of 5% goat serum (Dimensions; diluted 1:20 with water) was applied to all the tissues and incubated for 20 minutes. The goat serum was gently tapped off the slide and the primary antibody was added.

7.4.1 Application of Primary Antibodies

Monoclonal mouse antibody for p53 (Cedarlane; Pab 1801) and Bcl-2 (Calbiochem) was diluted 1:40 and 1:25 respectively in 1% bovine serum albumin (BSA) (Sigma; 1g BSA in 100 ml of PBS). The monoclonal mouse antibodies for the two proliferative markers, Ki-67 (Oncogene; MIB) and PCNA (Oncogene; PC10) were diluted 1:80 in 1% BSA. Tissue sections staining with primary antibodies for p53, Bcl-2, and Ki-67 were incubated for twenty-four hours in a humidifying chamber. Tissue sections staining for PCNA were incubated for 1 hr. Polyclonal rabbit antibody for Bax (Calbiochem; Ab-1) was diluted 1:25 in 1% BSA and sections stained with this antibody were incubated for twenty four hours. Two or three drops of diluted antibody solution was applied to each section to ensure that the sections were completely covered. Following incubation, the slides were rinsed twice with 10-15 ml PBS and later dried with gauze.

7.4.2 Application of Biotin Labelled Secondary Antibody

Goat anti-mouse antibody (Intermedico) 1:2000 (.5 µl in 1 ml of diluting buffer (500ml 0.5M PBS, .25g Thimerisol, 1 ml Triton X-100, 1.25 ml Tween, 5 g Casein; Sigma) was applied to sections stained for p53, Bcl-2, Ki-67, and PCNA. Goat anti-rabbit serum (Intermedico) diluted 1:2000 was applied to sections stained for Bax. Secondary antibody

incubation was carried out for half an hour at room temperature.

Excess secondary antibody was removed with two rinses of 10-15 ml PBS. The slides were again wiped dry with a gauze. Two to three drops of the solution of Streptavidin conjugated with peroxidase (Intermedico) (0.5ul per 1 ml distilled water) was applied and incubated for half an hour.

Following treatment in streptavidin, the tissues were rinsed with 10-15 ml PBS. Excess PBS was removed by wiping the slides around the tissue. Two to three drops of DAB (10mg DAB tablet in 15 ml PBS (Sigma), plus 2µl 30%hydrogen peroxide) was applied to the sections: 4 minutes for tissues stained with anti-p53, 3 minutes for tissues stained with anti-Bax, anti-PCNA and anti- Ki-67.

The tissues were placed in a staining dish containing 300 ml of haematoxylin (24 g haematoxylin, 360g ammonium aluminum. sulphate,150 ml 95% ethanol in 2400 ml water) for three minutes. The tissues were rinsed in running tap water for one minute and then dipped once in a staining dish containing 300 ml of 1% acid alcohol solution (10 ml 12 M HCL, 1L of 70% alcohol) . The sections were rinsed in water and then placed in a staining dish containing 300 ml of Scott's Tap water (7 g sodium bicarbonate (Gibco), 40 g magnesium sulphate (Gibco), 1 litre water) for one minute. This was followed by a final wash in water. The sections were dipped 10 times each in a series of staining dishes containing 300 mls of 70%, 95% and finally absolute alcohol. Finally, the specimens were consecutively placed for two to three minutes each in two staining dishes containing 200 ml of xylene, and then mounted in permount (Stephens Scientific) and coverslipped.

7.4.3 Measurement of Antigen Expression

Antigen expression was quantified with the aid of a pathologist in terms of the frequency of positive nuclear or cytoplasmic staining in a given microscopic field. The frequency was scored as 0 = no expression, 1 = less than 10% of cells were stained, 2 = 10-50 % of cells were stained and 3 = 50% or greater of cells were stained. The scores of the tumour specimens were assigned by the author and the pathologist.

7.5 Statistical Analysis

Analysis of the inter-relation of factors were calculated using the SPSS (7.5 statistical package) Chi-square test (X^2). This test is the most appropriate to measure the associations between the variables because of the categorical nature of the data. However the X^2 test could not be performed because the raw data revealed that the sample population in each of the 4 immunohistochemical categories was too small for statistical calculation. Thus, the scores of the immunohistochemical staining were recoded (1 = <10% of cells stained and 2 = >10% of cells stained), thereby increasing the sample population to calculate the X^2 test.

The mean rate of apoptosis (TUNEL score) for each tissue sample (total number of apoptotic cells per number of adjacent power fields used in the tissue) was initially recorded as a nominal value. To place the TUNEL scores into two categories so that they could be entered into the analysis, the mean number of apoptotic cells for all 145 tissue samples (sum total of the mean apoptotic cells per tissue over the total number of tissues) was first calculated ($\bar{x} = 6$). The mean score was then represented categorically for each tissue sample by a 0 or 1; 0 = less than the mean number of apoptotic cells ($\bar{x} < 6$), while 1 = greater than

the mean number of apoptotic cells ($\bar{x} \geq 6$).

While the X^2 calculates the associations between variables, it does not indicate the direction of the relationship. Thus, the Phi Statistic, which measures the direction of association (as negative or positive) between groups, was also obtained using SPSS. The Phi Statistic was calculated only if a significant association between two factors were observed in the X^2 analysis . Due to the large number of tests performed, a critical p-value of $\alpha = 0.01$ was implemented to control for the overall probability of Type I error. Furthermore, for sample sizes less than 25, Fisher's exact test was calculated. Associations between any two factors involving apoptotic, proliferative and/or clinical parameters were tested for significance using the chi-square (χ^2) test. All the raw data relating to this study is listed in Appendix.

Chapter 8

Results

8.0 Cellular expression of Bax, Bcl-2, p53, PCNA and Ki-67

Immunohistochemical staining of the breast tissues identified the proliferative and apoptotic markers. Table 8.0 illustrates the number of cases identified for each of the markers in the 4 groups (0 = no expression, 1 = less than 10% of cells stained, 2 = 10-50% of cells stained and 3 = greater than 50% of cells stained) prior to recoding. Table 8.0 also indicates the number of samples that could not be stained for each of the markers. These samples could not be assessed because of the fatty constitution of the tissue, resulting in the tissue falling off the slide or being folded, thus making quantitation impossible even when the staining procedures were repeated for these samples.

As discussed earlier, tumour cells were recoded as 0 (< 10%) or 1 (>10%) depending on the cytoplasmic (Bcl-2 or Bax; see Figure 8.1a and 8.1b) or nuclear frequency (p53, PCNA, and Ki-67; see Figure 8.1c, 8.1d and 8.1e) of cells stained. However, in one of the analyses (section 8.4.2), the association between tumor grade and the presence (>5%) or absence (0%) of p53 was examined rather than the frequency of its nuclear distribution (< 10% or >10%).

The rate of apoptosis was scored as 0 (less than the mean number of apoptotic cells; $\bar{x} < 6$) or 1 (greater than or equal to the mean number of apoptotic cells; $\bar{x} \geq 6$; see figure 8.1f for an example of an apoptotic cell identified by the TUNEL method). The TUNEL method was successful in staining 142 of the 145 cases. The samples that could not be

stained in the TUNEL method coincided with those that could not be stained using the immunohistochemical method.

Results of associations between the apoptotic, proliferative and clinical factors are tabulated individually in Appendix. The number of samples assessed in each of the analyses varies due to the nature of the χ^2 statistic. The SPSS calculates χ^2 with equal number of subjects in each of the categories. It eliminates a subject if one of the values is absent. Results of the significant associations are summarized in Table 2. (see Section 8.6).

Table 8.0: The scores and the total number of cases used to identify the apoptotic and proliferative markers.

Markers	0 - No Expression	1 <10%	2 10 - 50%	3 >50%	Total Cases	# of Missing Cases	Total
Bax	47	30	4	63	144	1	145
Bcl-2	72	4	4	63	143	2	145
P53	106	17	7	14	144	1	145
Ki-67	22	93	20	10	145	0	145
PCNA	42	51	17	35	145	0	145

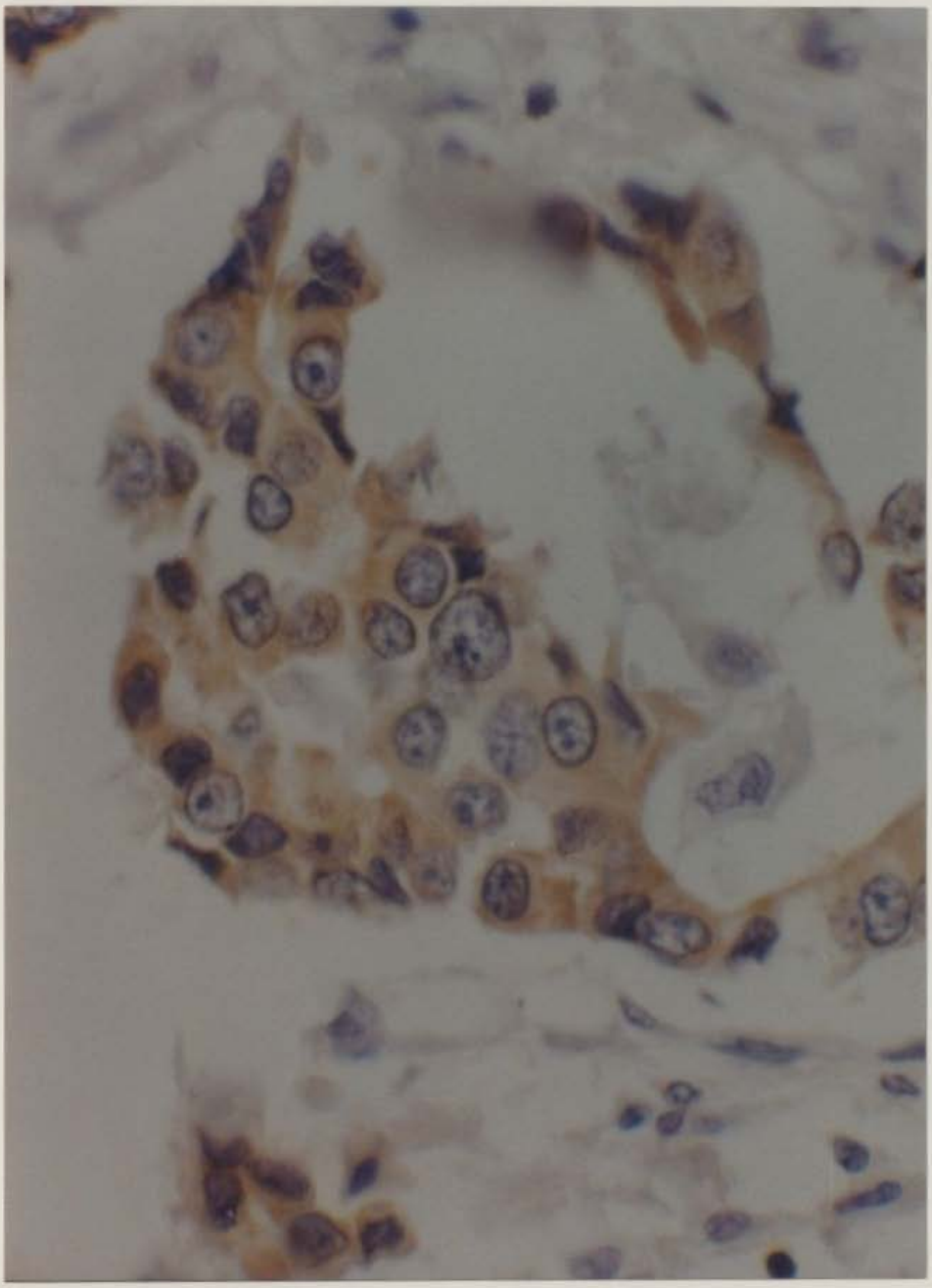

Figure 8.1a: Expression of Bcl-2 in Breast Carcinoma. Moderate to Strong Cytoplasmic Staining (≥10%) present in Cells.

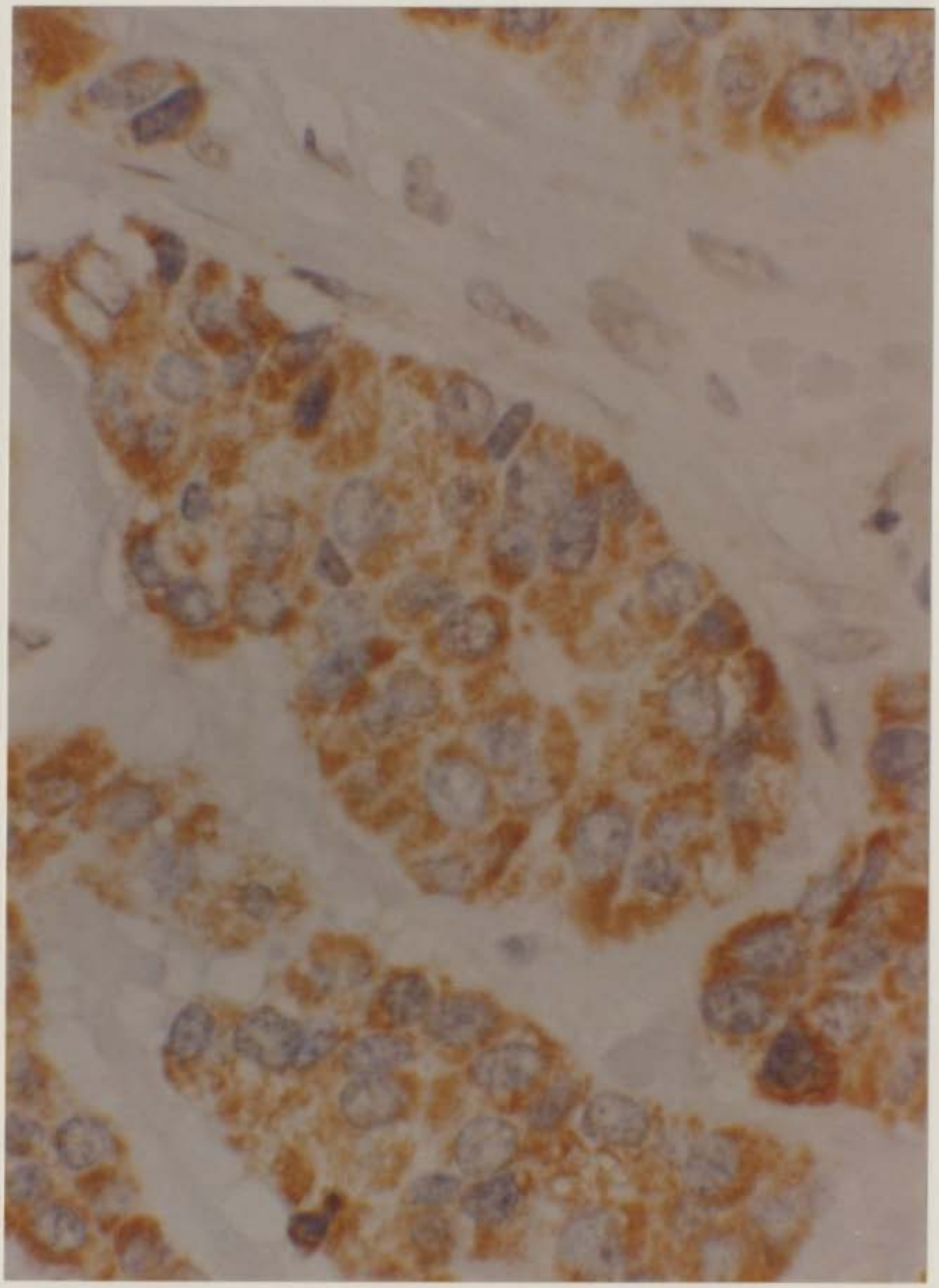

Figure 8.1b:Expression of Bax in Breast Carcinoma. Moderate to Strong (≥10%) Cytoplasmic Staining present in Cells.

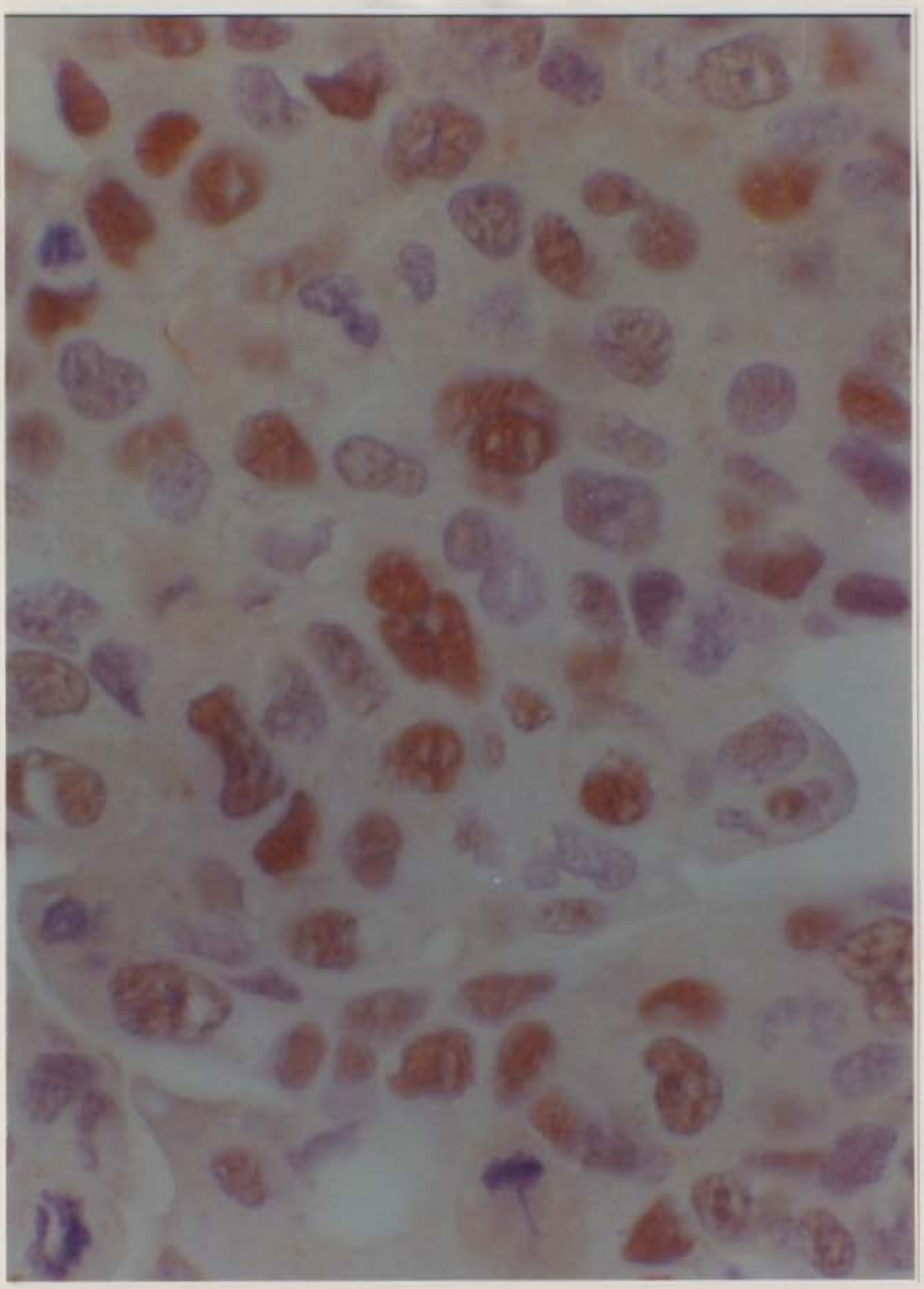

Figure 8.1c: Expression of Mutant p53 in Breast Carcinoma. Moderate to Strong (≥10%) Expression of Nuclear Staining Present in Cells.

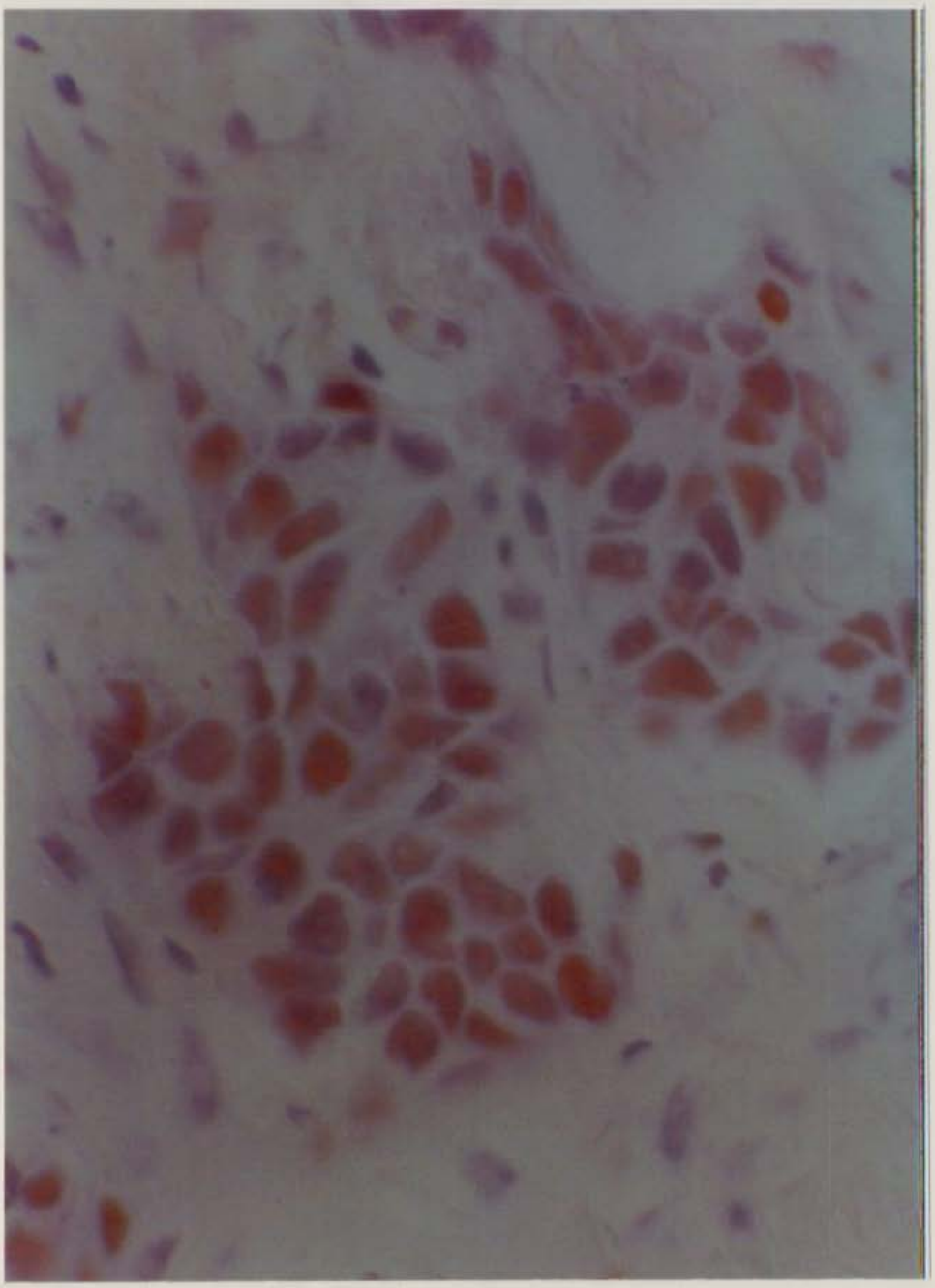

Figure 8.1d: Expression of PCNA in Breast Carcinoma. Moderate to Strong (≥10%) Expression of Nuclear Staining Present in Cells.

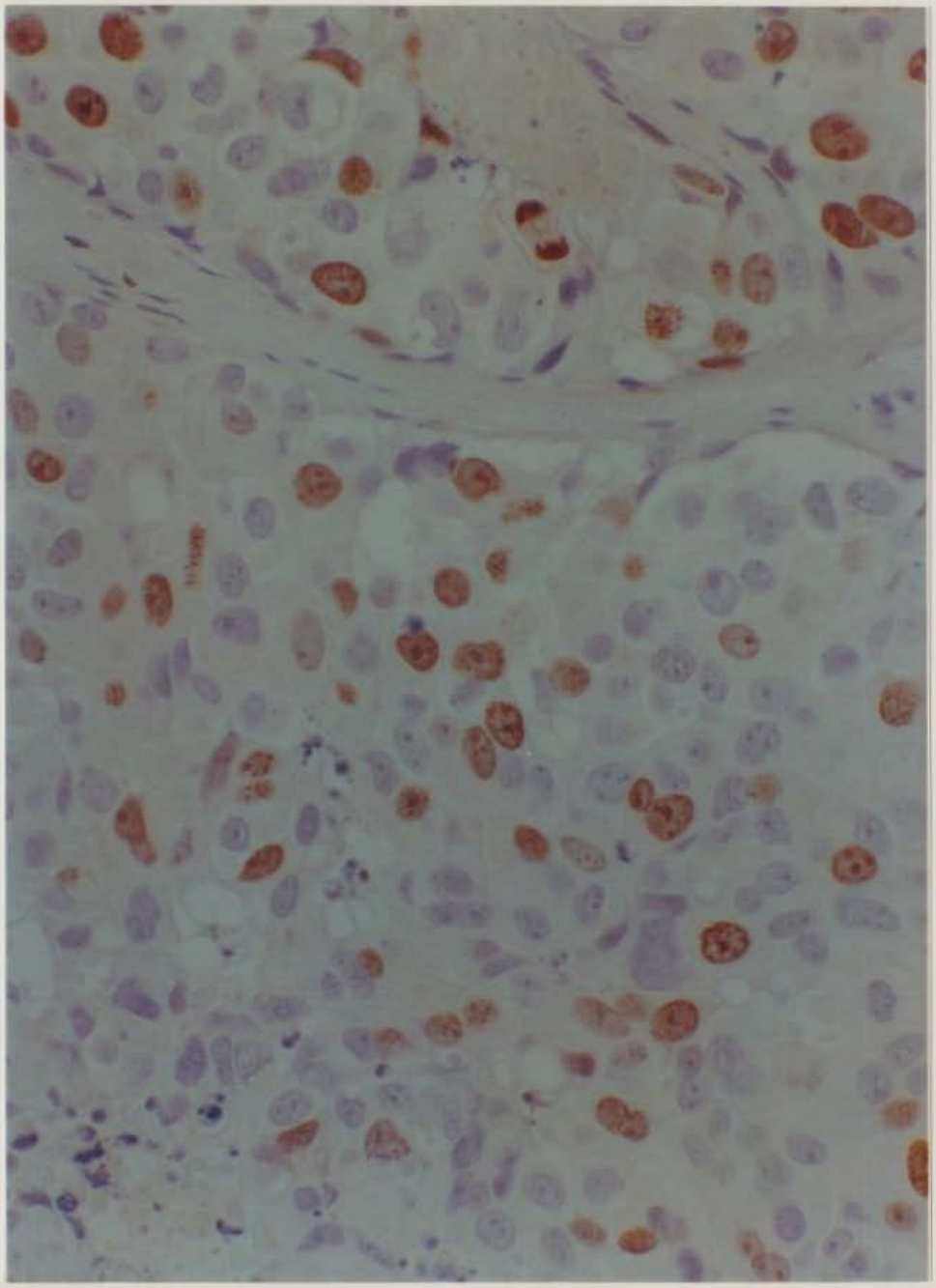

Figure 8.1e: Expression of Ki-67 in Breast Carcinoma. Moderate to Strong (≥10%) Nuclear Staining Present in Cells.

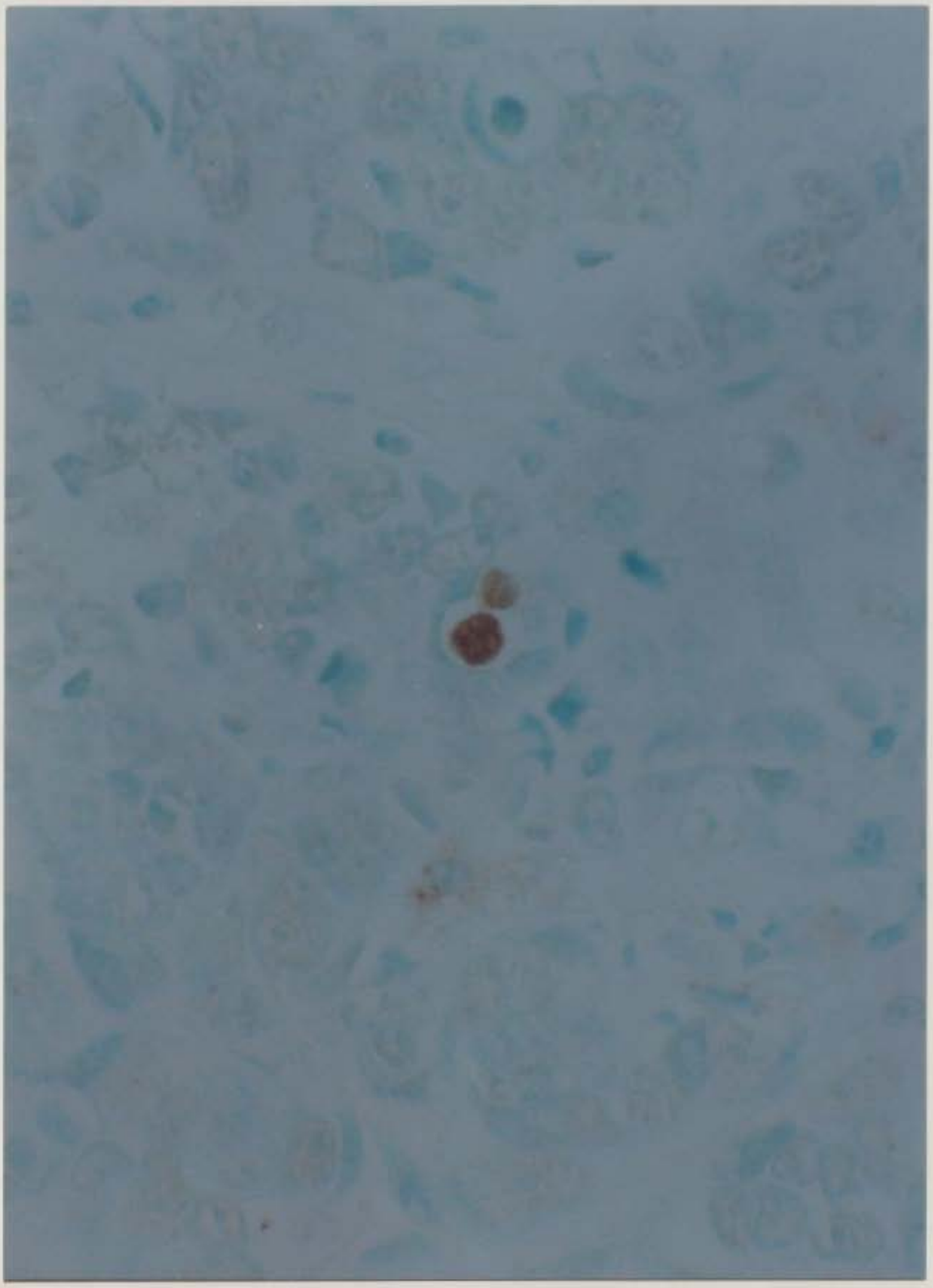

Figure 8.1f: A Cell undergoing Apoptotis as detected by the TUNEL Method.

8.1 Relation between pro-apoptotic factors, anti-apoptotic factors and apoptotic rate

8.1.1 Bcl-2

There was no significant association between Bcl-2 cytoplasmic expression and the rate of apoptosis as measured by the TUNEL method ($p > .01$; see Table 10.1 in Appendix). Bcl-2 cytoplasmic expression was positively associated with Bax cytoplasmic expression ($\chi^2 = 7.017$, $df = 1$, $p \leq .01$, phi = 0.222; see Figure 8.1.1 and Table 10.2 in Appendix). The χ^2 test did not show an inverse association between Bcl-2 and p53 expression $p > .01$; see Table 10.3 in Appendix1).

8.1.2 Bax

Analysis of data showed no significant association between Bax cytoplasmic expression and p53 expression or the rate of apoptosis ($p > .01$; see Table 10.4 and Table 10.5 respectively in Appendix).

8.1.3 p53

Analysis of data did not show a significant association between the rate of apoptosis and p53 expression in the nucleus ($p > .01$; see Table 10.6 in Appendix) .

8.2 Relation between proliferative and apoptotic factors

Data analysis indicated a positive association between the two proliferative factors, Ki-67 and PCNA ($\chi^2 = 9.582$, $df = 1$, $p \leq .01$, phi = .257; see Table 10.7 in Appendix). There was no significant association between Bcl-2 expression and Ki-67 ($p > .01$; see Table 10.8 in Appendix).

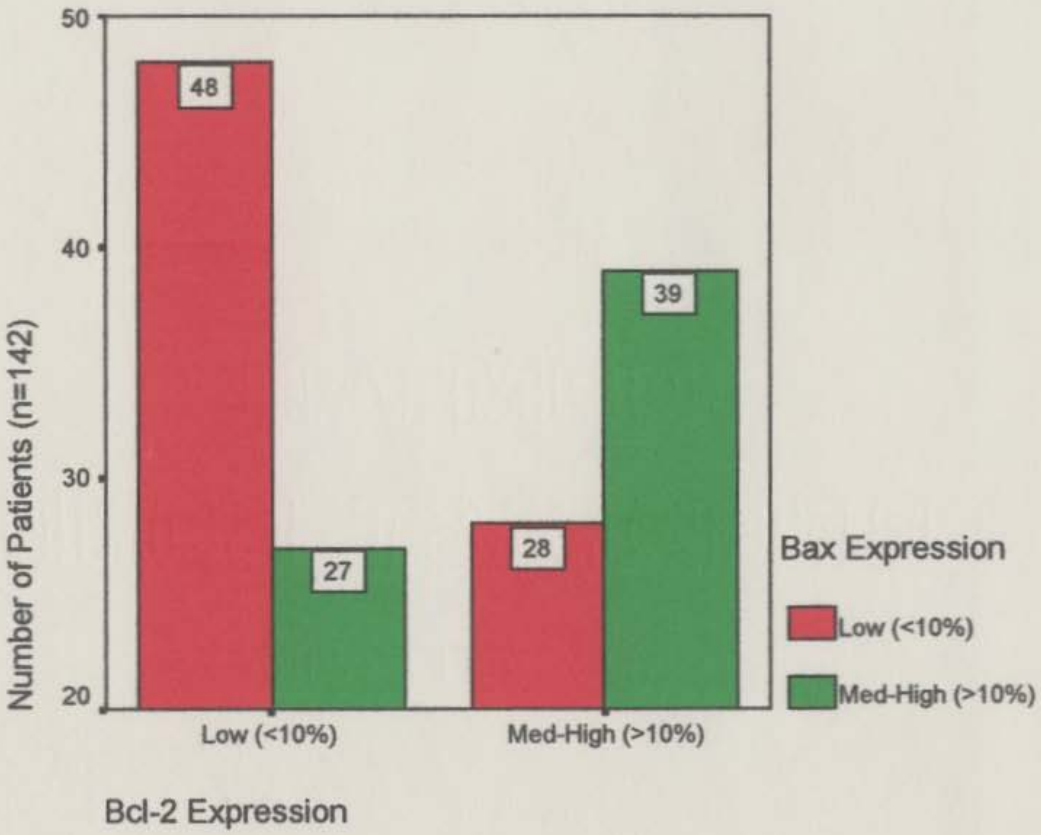

Figure 8.1.1: Association of the number of patients who exhibit low and medium-high cytoplasmic expression of Bcl-2 with number of patients who exhibit low and medium-high cytoplasmic expression of Bax ($p = .008$).

The χ^2-test did show a significant association between Bcl-2 expression and PCNA (χ^2 = 7.077, df = 1, p ≤.01, phi = .222; see Table 10.9 in Appendix). In contrast, expression of nuclear p53 showed a positive association with expression of both proliferative markers, Ki-67 (χ^2 = 25.145, df = 1, p ≤ .01, phi = .418; see Table 10.10 in Appendix) and PCNA (χ^2 = 9.949, df = 3, p ≤ .01, phi = .263; see Table 10.11 in Appendix).

8.4 Apoptotic and proliferative factors related to tumour pathological features and local recurrence

8.4.1 Bcl-2

There was no significant association between local recurrence and expression of Bcl-2 (see Table 10.12 in Appendix). However, there was a significant association between medium to high expression of Bcl-2 and low tumour grade (χ^2 = 23.284, df = 2, p ≤ .01; see Figure 8.4.1 and Table 10.13 in Appendix). The data did not show a significant association between Bcl-2 expression and tumour size (p > .01; see Table 10.14 in Appendix).

8.4.2 p53

The χ^2 test showed no significant association between local recurrence and increased expression of p53 (p > .01; Table 10.15 in Appendix). The analysis showed no association between expression of p53 and tumour size (p > .01; Table 10.16 in Appendix). There was a significant positive association between p53 expression and high tumour grade (χ^2 = 12.967, df = 2, p ≤ .01 phi = .310; Figure 8.4.2 and Table 10.17 in Appendix).

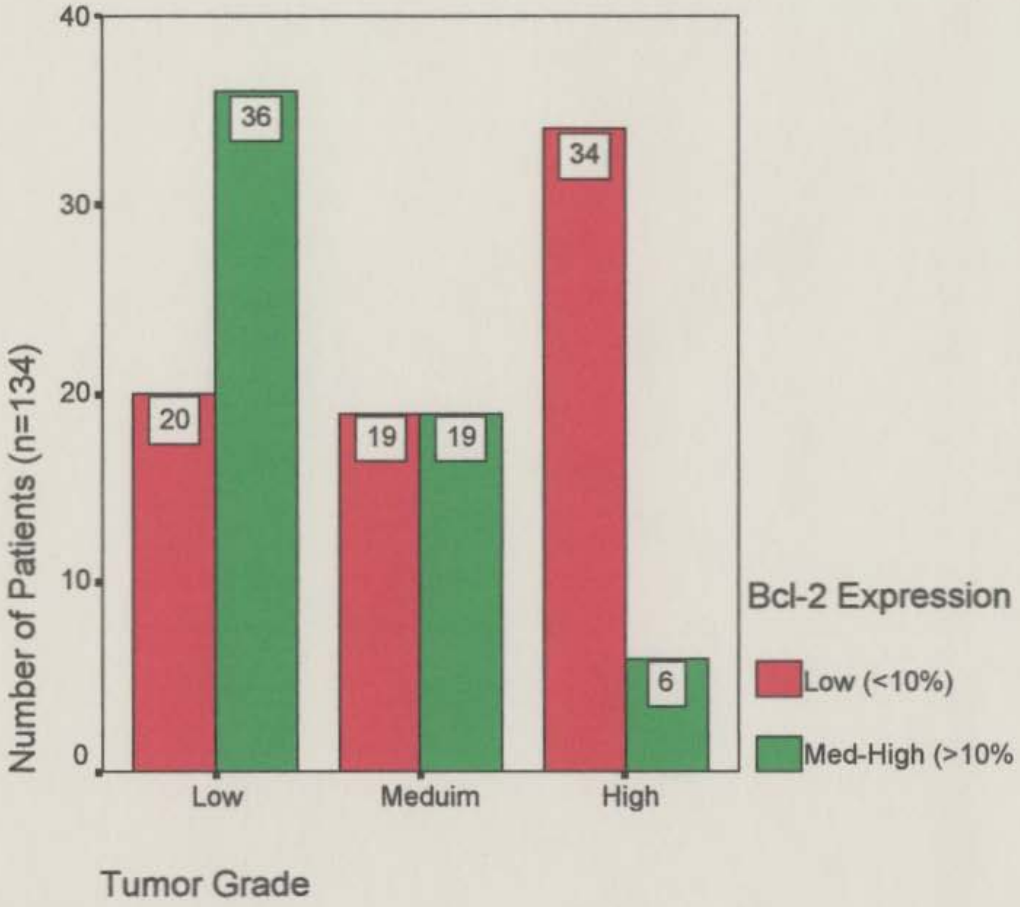

Figure 8.4.1: Association between the number of patients who exhibit low and medium-high cytoplasmic expression of Bcl-2 with number of patients who exhibit low, medium or high tumour grade (classified according to the Bloom-Richardson Method) (p = .000) .

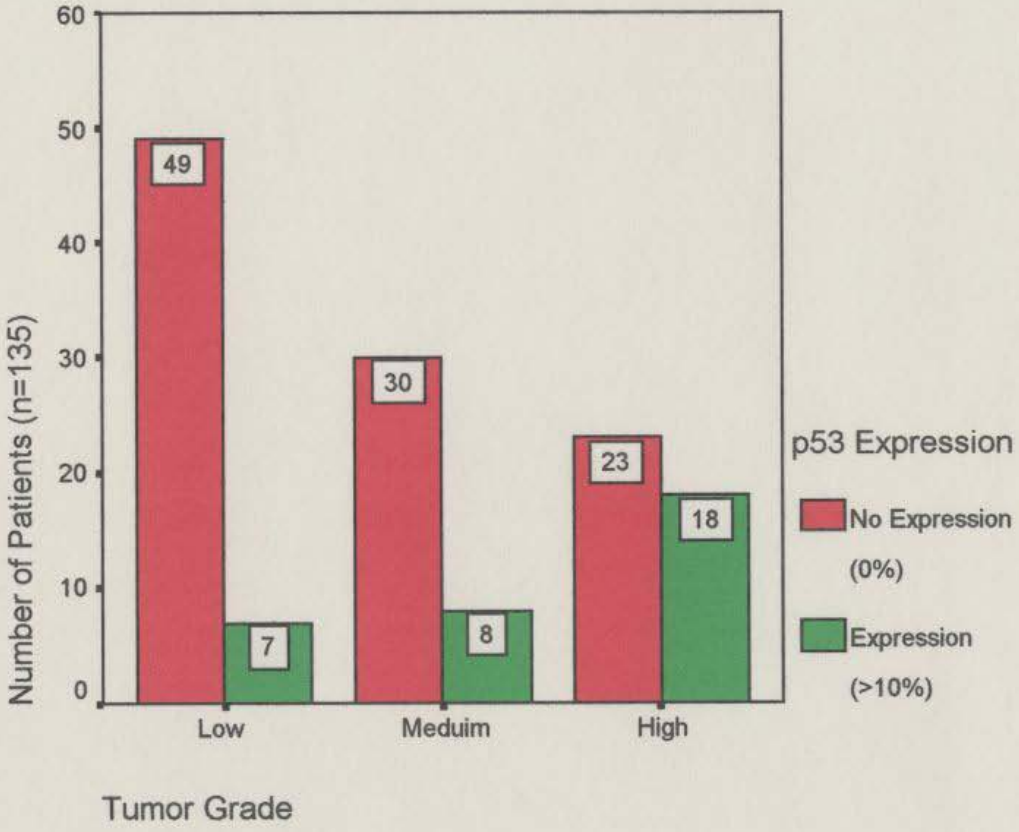

Figure 8.4.2: Association between the number of patients who exhibit low and medium-high expression of nuclear p53 with number of patients who exhibit low, medium or high tumour grade (classified according to the Bloom-Richardson Method) ($p = .002$) .

8.4.3 Ki-67 and PCNA

Analysis of data showed no association between recurrence of breast carcinoma and expression of PCNA or Ki-67 ($p > .01$; see Tables 10.18 and 10.19 respectively in Appendix). The expression of Ki-67 was associated with tumour grade ($\chi^2 = 16.102$, $df = 2$, $p \leq .01$, phi = .344) but not tumour size ($p > .01$; see Tables 10.20 and 10.21 respectively in Appendix). Data indicated a positive association between the expression of PCNA and tumour size ($\chi^2 = 5.814$, $df = 1$, $p \leq .01$, phi = .204; see Table 10.22 in Appendix). There was no association between PCNA and tumour grade ($p > .01$; see Table 10.23 in Appendix). PCNA expression was associated with absence of lymph node metastases ($\chi^2 = 5.580$, $df = 1$, $p \leq .01$, phi = .196; see Table 10.24 in Appendix).

8.5 Interrelation between tumour pathological features and local recurrence of breast carcinoma

Tumour size showed a positive association with local recurrence ($\chi^2 = 6.610$, $df=1$, $p \leq .01$; see Figure 8.5 and Table 10.25 in Appendix) There was no significant association between tumour grade and local recurrence, or lymph node status and local recurrence ($p > .01$; see Tables 10.26 and 10.27 in Appendix).

8.6 Summary of significant associations found in the study (see Table 8.6).

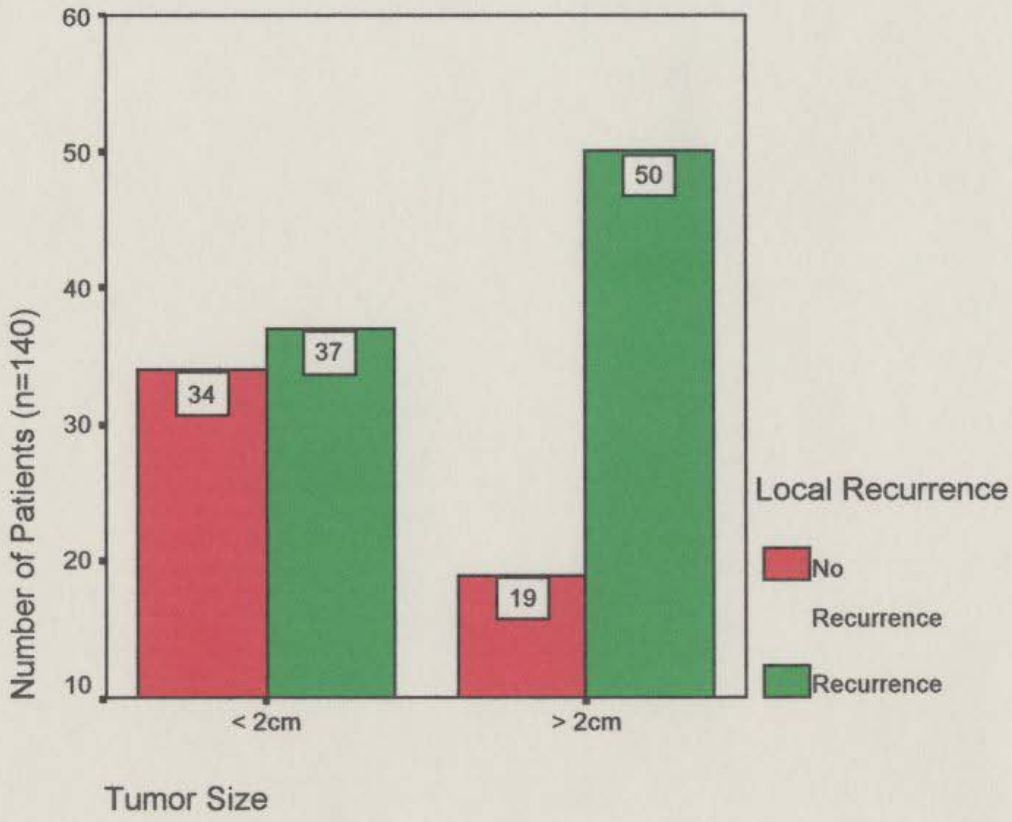

Figure 8.5: Association between local recurrence of breast carcinoma and tumour size in patients with tumors greater than 2 cm ($p = .013$).

Table 8.6: Summary of significant associations between apoptotic, proliferative and clinicopathological factors.

Factors	χ^2	Sig	Phi
Bcl-2 cytoplasmic expression * Bax cytoplasmic expression	7.017	.008	.222
PCNA expression * Ki-67 expression	9.582	.002	.257
Bcl-2 cytoplasmic expression * PCNA expression	7.077	.008	.222
p53 nuclear expression * Ki-67 expression	25.145	.000 .000 †	.418
p53 nuclear expression * PCNA expression	9.949	.002 .003 †	.263
Bcl-2 cytoplasmic expression * Tumour Grade	23.284	.000	.417
p53 nuclear staining * Tumour Grade	12.967	.002	.310
Ki-67 expression * Tumour Grade	16.102	.000	.344
PCNA expression * Tumour Size	5.814	.016	.204
PCNA expression * Lymph Node Status	5.580	.018	.196
Tumour Size * Local Recurrence	6.160	.013	.210

† P value for Fishers Exact Test (n<10)

Chapter 9

Discussion

Prior to discussing the results obtained from this retrospective study, the limitations of using the IHC and the TUNEL assays in detecting specific markers in paraffin embedded breast tissues have been addressed. It is important to note these limitations before interpreting the results of the study.

9.0 Assessment of the Laboratory Techniques

The quality of immunohistochemical staining which influences the outcome of data analysis is determined by a number of factors. The factors that play a crucial role in immunohistochemistry include the affinity of the antibodies for the antigens fixed in the tissue sample and the concentration of the cellular antigens (Bancroft & Stevens, 1996). Another very important factor is the unpredictable effects of fixatives such as formalin on the immunoreactivity of the tissue antigens (Bancroft & Stevens, 1996).

9.0.1 Fixation and Embedment

Formalin, a universal fixative, is needed to prevent disintegration of the sections during staining. It provides consistent and adequate morphological preservation (Elias, 1990). However, formalin lowers or sometimes completely abolishes the immunoreactivity of the tissue antigens by forming methylene bridge cross-links between reactive sites on the same protein molecule or between adjacent protein molecules (Bancroft & Stevens, 1996). Consequently, the intermolecular and intramolecular cross-links may alter the primary and tertiary structure of the antigens enough to affect the binding of a specific antibody (Bancroft

& Stevens, 1996). Time, temperature, fixative, concentration, and the availability of nearby proteins to be cross-linked are the variables related to the extent of marking (Bancroft & Stevens, 1996).

One approach to restoring the loss of antigenicity caused by fixation or embedding media such as paraffin wax is antigen retrieval by heat treatment (Cattoretti et al., 1993). This treatment involves boiling dewaxed formalin-fixed paraffin sections in 0.01M citrate buffer (Cattoretti et al., 1993). Digestion by citrate buffer unmasks and restores the immunoreactive sites and enhances the antigen-antibody interaction. Over treatment can produce very severe tissue damage. Thus a set boiling time may limit this damaging process (Cattoretti et al., 1993). The heat treatment method of antigen retrieval seems more reliable and reproducible than the alternative method using proteolytic enzyme digestion (Bancroft & Stevens, 1996). This heat treatment method also allows greater range of dilution of some primary antibodies (Cattoretti et al., 1993).

Other factors, such as background staining caused by binding sites that show non-specific affinity for immunoglobulin and endogenous peroxidase activity, can also affect the quality of the stains (Elias, 1990). These factors can be eliminated by applying reagents such as hydrogen peroxide, which quenches the endogenous peroxidase activity, and blocking serum (usually from the species in which the second antibody is raised), which is effective in eliminating the non-specific binding (Elias, 1990). Although the quality of stains has improved as a result of reducing the above mentioned confounding factors, there remain certain inherent factors that restrict the use of the immunohistochemical method.

9.0.2 Limitations of the IHC method

There are several limitations of the IHC method. First, the specificity of an antibody binding to the antigen is difficult to prove in IHC. The only assurance that an antigen has been recognized with IHC staining is if the staining is inhibited when a highly purified form of the antigen of interest is added to a working solution of the specific antibody (Elias, 1990). However, laboratories performing the IHC studies seldom have the purified antigen available to perform preliminary tests. A second limitation is that assessing the expression of certain markers like Bax, Bcl-2 and p53 does not necessarily mean that the specific antigen marker is biologically functional (Elias, 1990). Thirdly, the examination of one tissue slide per tumour may not be representative because of the biological heterogeneity of the tumour (Elias, 1990). The latter problem was partly circumvented in this study because the pathologist reviewed all the slides and chose the most representative (a section in which all the histological characteristics of the tumour were evident) of the tumour samples. However, some of the samples collected from the various hospitals had a limited number of tumour slides, and some were either missing or required for future use by the hospitals. Hence, the pathologist had to choose from a limited pool of samples.

The collection of tissue from various hospitals in Newfoundland was a fourth limitation since variations in quality and fixation of tumour tissue, as well as storage of the tissue samples may have affected the results of IHC. Finally, the IHC method is unable to quantify the positive stains objectively because the scoring criteria are subjective and qualitative. This naturally incurs some limitation on the accuracy and reproducibility of the

results. A review of the studies on breast cancer that used the IHC method indicate various scoring systems for assessing the degree of staining. For example, van der Kooy et al. (1996) scored dark nuclear stains of more than 10% of tumour cells as positive for p53 overexpression, while cells stained between 1-10% were considered as negative p53 staining. In the present study, an overexpression was defined as more than 10% of cells positively stained for p53 while a low or absent expression confounded to 0-10% of positive cells. In another study, Sjogren et al. (1996) classified the tumour cells in terms of frequency where 1 represented less than one third of the cells staining positive, 2 signified between one third and two thirds of positive cells, and 3 was greater than two thirds of positive cells. The variations in the scoring of the different parameters in the studies may have yielded different results and conclusions. If the criteria for scoring were similar among studies the results might be more consistent.

Similar to Sjogren's et al. (1996) study, the present study initially classified the positive tumour cells in a tissue into groups based on their frequency of expression. This grouping method limits the influence of individual values that could be given to a specific sample and restricts the type of statistical analysis.

9.0.3 The TUNEL Method

The terminal deoxynucleotidyl transferase (TdT)- mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labelling (TUNEL) method has been utilized to identify apoptotic cells. The labelled dUTP molecules are added in sequence at the 3'-OH end of fragmented DNA through the action of the enzyme TdT. Assessing the apoptotic cells by the TUNEL

method has caused a number of concerns since the advent of the study by Kraupp & Nedecky, (1995) study. They observed that the TUNEL method did not distinguish between hepatocytes dying of apoptosis and hepatocytes dying of necrosis. In the present study, the apoptotic cells were scored only if the two pathologist and the author were in agreement. However, errors must inevitably arise as the morphological detection of apoptosis is inherently subjective. As with the IHC method, quantification of the number of apoptotic cells was problematic since the rate of apoptosis may have a heterogenous distribution throughout the tumours, thereby making precise quantification difficult. A review of the studies that used the TUNEL method indicate problems of subjectivity and variation in the scoring similar to those in the IHC method. The development of a consistent method of objectively quantifying the data for both the IHC and the TUNEL method could increase the validity and reliability of the results.

9.1 Relation Between Pro-Apoptotic and Anti-Apoptotic Factors

9.1.1 Bcl-2 and Bax

It has been proposed that the relative sensitivity of cells to apoptotic stimuli is governed by the inverse ratio of Bax:Bcl-2 (Reed, 1995). The ratio of Bax to Bcl-2 expression has a profound clinical significance in determining the sensitivity of cells to radiation, or chemotherapy ((Reed, 1995). Apoptosis, necessary for a good therapeutic response to chemotherapy or radiation could be directly influenced by the reduction of pro-apoptotic Bax relative to that of anti-apoptotic Bcl-2 expression, therefore providing a selective advantage for tumour survival (Reed, 1995).

In the present study, analysis of Bax and Bcl-2 expression in invasive carcinoma showed a positive association rather than the expected inverse association (see Table 10.2 of Appendix; Figure 9.2.1a). The results from this study coincide with the results of similar studies by Krajewski et al. (1995 & 1997) on the immunohistochemical detection of Bcl-2 and Bax expression in breast carcinoma. The positive association between the two apoptotic factors suggests that the expression of Bax and Bcl-2 may be coregulated, thus providing a potential explanation for the fact that both Bcl-2 and Bax expression tend to decline during progression of breast cancer (Krajewski et al., 1997). Although undetermined, coregulation of Bax and Bcl-2 levels could be controlled by transcriptional and posttranscriptional mechanisms (Krajewski et al., 1997). Support for a possible post-transcriptional mechanism of coregulation is provided by a study that demonstrated that Bcl-2 can prolong the half-life of the Bax protein in some cell lines, probably because of the ability of Bcl-2 to heterodimerize with Bax (Krajewski et al., 1997). The role of estrogen as a common mechanism of transcriptional regulation of Bcl-2 and Bax has been considered in light of the fact that estrogen upregulates Bcl-2 expression in MCF7 breast cancer cell lines (Teixeria et al.,1995). However, estrogen does not regulate Bax expression in MCF7 cells indicating that estrogen is not the common mechanism for the co-regulation of Bax and Bcl-2 (Teixeria et al.,1995).

The concept of coregulation substantiates the fact that the most critical factor determining the sensitivity of tumour cells to apoptosis may be the actual ratio of pro-apoptotic to antiapoptotic factors of the Bcl-2 family (Reed, 1995). The observations of the

various studies, including the present, regarding the expression of Bax and Bcl-2 suggest that it may be insufficient to examine the expressions of Bcl-2 and Bax in isolation without the knowledge of other members in the Bcl-2 family such as Mcl-1 and Bcl-X_L (not measured in this study) and their influence in apoptosis (Reed, 1995).

Immunohistochemical techniques are semi-quantitative and may therefore, not be suited for the precise measurement of the expression of factors necessary to unravel the complex interrelationship of the various pro-apoptotic and anti-apoptotic factors, their relation to tumour progression, and their influence on the process of apoptosis. One approach to the problem of measurement of apoptotic factors would be the use of semi-quantitative or quantitative RT-PCR. This technique holds promise for retrospective studies now that good quality RNA can be successfully isolated from paraffin embedded tissue (Robb, 1999).

9.1.2 Bcl-2 and Apoptotic Rate

Several studies have confirmed that Bcl-2 expression inhibits the process of apoptosis (Craig, 1995; Bargou, 1995; Selvakumaran, 1994). Mustonen et al. (1997) showed an inverse relationship between the extent of apoptosis, measured by the TUNEL assay, and Bcl-2 expression in breast carcinoma. Similarly, van Slooten et al. (1998), found that high expression of Bcl-2 in invasive breast carcinoma was associated with a low rate of apoptosis. In contrast to the inverse associations found in the above mentioned studies, analysis of the data from the present study (see Table 10.1 in Appendix) showed no significant association between Bcl-2 expression and the proportion of apoptotic cells in breast carcinoma. Berardo et al., (1998) also found no significant association between Bcl-2 and the proportion of

apoptotic cells in breast cancer.

The present study, as well as the studies by Mustonen et al. (1997), van Slooten et al. (1998) and Berardo et al. (1998), measured the proportion of apoptotic cells by the TUNEL method. Mustonen et al. (1997) measured the apoptotic index (sum of apoptotic cells in a given area) by estimating the number of tumour cells in 10 high power fields and counting the total number of apoptotic events in these fields. The percentage of apoptotic cells were then obtained by dividing the number of apoptotic cells by the estimated number of tumour cells. Van Slooten et al. (1998) used a grid frame to measure the rate of apoptosis. Within each random section of the grid (which consisted of 1000 tumour cells) the positive apoptotic cells were scored and expressed as a percentage of the total tumour cells. Berardo et al's.(1998) study used a grid subdivided into 10 x10 units each containing at least 100 tumour cells. The apoptotic cells were counted in each section and expressed as a percentage of the total. In the present study, the apoptotic cells were calculated within 4-5 fields (40X) with each field containing at least 1000 tumour cells. The apoptotic rate in each case was defined as the mean number of apoptotic cells for all 145 tissue samples (sum total of the mean apoptotic cells per tissue over the total number of tissues) was first calculated ($\bar{x} = 6$). The mean scores were then divided into two categories: 0 = less than the total overall mean for all tissues analysed, and 1 = equal to or greater than the mean score. These differences in the scoring systems could account for the variability in the results obtained in different studies.

Reproducibility could be achieved if the scoring system were more stringent and uniform. The grid method seems more reliable than simply estimating a certain number of cells

and counting the number of apoptotic cells within each field. In addition, a scoring method could be made more reliable if there were a means for distinguishing whether or not a cell is undergoing apoptosis or necrosis. In one study, semi-quantitative end-labelling of DNA fragments revealed a significant inverse association with the expression of Bcl-2 (Diaz-Cano et al., 1997). In this method, DNA was extracted by the phenol/chloroform method and run on an agarose gel. Specimens displaying a marked laddering pattern were considered to have undergone apoptosis. Though less subjective, this method of using end-labelling of DNA fragments to detect apoptosis is not applicable to formalin-fixed tissue.

9.1.3 Bcl-2 and p53

Studies to date indicate that wt p53 can transcriptionally repress Bcl-2 expression while inducing expression of Bax. In p53 knock-out transgenic mice, absence of wt p53 was associated with increased expression of Bcl-2 and decreased expression of Bax (Myashita et al., 1994). Several studies have confirmed this inverse association between Bcl-2 and mutant p53 as detected immunohistochemically (Krajewski et al.,1997; Silverstini et al., 1994; Joensuu et al.,1994; Haldar et al.,1994; Gorczyca et al.,1995; Lee et al.,1996; Barbareschi et al.,1996; and Berardo et al.,1998). Analysis of the present data also indicated a strong inverse trend between p53 and Bcl-2. However, the association was not significant (see Table 10.3 in Appendix).

There have been concerns regarding the identification of p53 mutations by immunohistochemistry. Deletions and nonsense and frameshift mutations prevent synthesis of the p53 protein (Greenblatt et al.,1994; Ko & Prives,1996; Gangopadhy et al., 1997).

Immunohistochemistry cannot detect these alterations (Greenblatt et al.,1994). However, the most frequent type of p53 mutation is a missense substitution that alters a single amino acid in the p53 protein (Greenblatt et al.,1994). This stabilizes the protein, thereby increasing the half-life so that it can be detected immunohistochemically (Greenblatt et al.,1994). This phenomenon is frequently referred to in the literature as 'p53 overexpression' or 'p53 accumulation'. Sjogren et al. (1995), studying breast carcinoma, found a lack of concordance between p53 detection by immunohistochemistry and mutation detected by cDNA based sequencing. Several tumours displaying p53 immunopositivity had negative DNA sequence data and vice versa. In addition, Sjogren showed that patients with negative DNA sequence data but immunopositive results had better prognosis relative to those with positive cDNA sequence data and negative immunohistochemical results. Other studies, however, indicate correlations between p53 immunopositivity and p53 mutations observed by DNA sequencing (Harris & Hollstein, 1993; Marchetti et al., 1993).

To validate the detection of p53 mutations immunohistochemically, more conclusive methods such as immunoblotting and Single Strand Conformation Polymorphism (SSCP) could be performed concurrently. Even though the present study did not use either method to detect p53 mutations, other studies have confirmed the inverse association between p53 mutational events and Bcl-2 expression detected by immunohistochemistry (Lee et al.,1996; Barbareschi et al.,1996; and Berardo et al.,1998).

9.1.4 Bax and p53

Bax, the pro-apoptotic gene, contains several target sequences for wt p53 binding and hence is strongly transactivated by wt p53 (Krajewski et al., 1995). As previously discussed, mice deficient in wt p53 revealed a reduction in an immunohistochemical Bax expression, confirming the importance of wild type p53 as a regulator of Bax (Ko & Prives, 1996). Therefore, in the context of our study, loss of p53 function (as indicated by immunopositivity) should result in decreased Bax expression. Analysis of the present data showed no association between p53 accumulation and reduced Bax expression (see Table 10.4 in Appendix). This finding is similar to that of Krajewski et al. (1995 & 1997) who demonstrated that in breast cancer, immunohistochemical detection of mutant p53 by itself is insufficient to cause diminished Bax expression.

The negative results from this study suggests the presence of other apoptotic regulators that could influence the expression of Bax and that p53 may not be the dominant regulator of Bax expression in breast carcinoma. However, it is important to note that studies have indicated a p53 dependent increase in Bax expression in response to DNA damage by chemotherapeutic drugs or by radiation (Krajewski et al., 1997). Again, as in the case of Bcl-2, the true relationship between p53 and Bax expression may only be clarified by the use of a combination of molecular and immunohistochemical techniques.

9.1.5 Bax and Apoptotic Rate

Bax is a primary response gene involved in a p53 regulated pathway for induction of apoptosis (Ko & Prives, 1996). Thus, immunopositive staining of Bax was expected to be positively associated with the rate of apoptosis as measured by the TUNEL method. Data from the current study did not indicate a significant association (see Table10.5 in Appendix I). This lack of association suggests that Bax may not be the dominant activator of apoptosis and that other important pro-apoptotic genes within the Bcl-2 family may play a substantial role in this process. Another reason for the lack of association could be that apoptosis may have occurred independently of the p53 pathway. Other pathways, such as ceramide-mediated apoptosis or capase regulated apoptosis, may contribute to the apoptotic response in the cells.

Review of the literature shows very little research regarding the influence of Bax on the rate of apoptosis in breast carcinoma. However, there have been studies which indicate a positive association between Bax and TUNEL score in other types of cancer. One study, by Brambilla et al. (1996), revealed that Bax down-regulation was associated with low rates of apoptosis measured by the TUNEL method in lung cancer. A study that did examine this association in breast cancer found a positive relationship between Bax and the rate of apoptosis in breast cancer cell lines or tissues in the presence of chemotherapeutic drugs and any external stimulus which damages the genomic DNA (Han et al., 1998). Although this result, on the chemotherapeutic induction of apoptosis is important because it supports the idea that Bax is a pro-apoptotic gene, it does not strictly apply to this study, since the

purpose was to examine the rate of apoptosis in the context of tumour growth and proliferation without reference to treatment.

Absence of a significant association between the expression of Bax and the rate of apoptosis coincides with the lack of association between Bcl-2 and rate of apoptosis. Thus, in breast carcinoma, the process of apoptosis as detected by the TUNEL method seems to be an event independent of the mechanism whereby Bax and Bcl-2 expression appear to be coregulated. However, as discussed earlier, detection of apoptotic cells by the TUNEL method may not be completely reliable. Kraupp and Nedecky (1995) showed that hepatocytes, dying by both apoptosis and necrosis, gave a similar positive result in the TUNEL assay. Thus, the TUNEL assay did not differentiate between the two processes. Stahelin et al. (1998) have proposed that false positive staining in the TUNEL assay may be due to the release of endogenous endonucleases following proteinase treatment. The false positive staining in the TUNEL assay can be abolished by prior treatment of tissue sections with diethylcarbonate (Stahelin et al., 1998). These studies suggest that further investigations into the reliability of the TUNEL method need to be conducted.

9.1.6 p53 and Apoptotic Rate

Wild type p53 is known to promote apoptosis (Ko & Prives, 1996). Therefore, loss of p53 function which corresponds to immunohistochemical accumulation of p53 should be associated with low rates of apoptosis (Greenblatt, 1994). Analysis of study in the present data did not reveal an association between apoptotic rate and p53 function (see Table 10.6 in Appendix). Our findings suggest that the rate of apoptosis as determined by the TUNEL

assay is independent of p53 status, a conclusion supported by Bodis et al. (1996). In contrast, Shen et al. (1997) found that the differing rates of apoptosis in intraductal and infiltrating carcinoma could be regulated by altered p53 expression. Similarly, Shen et al. (1998) revealed a positive correlation between immunopositive p53 cells and rate of apoptosis, as measured by the TUNEL method, in intraductal and invasive ductal breast carcinoma. The absence of an association between p53 and rate of apoptosis may also reflect the existence of alternative pathways independent of the activation of specific target genes by p53.

Once again, it must be emphasized that the negative result could be a methodological problem with the TUNEL method or with immunohistochemical analysis of p53 expression. It is also important to recall that p53 immunopositive cells represents just one form of mutation, namely missense. Hence, the proportion of apoptotic cells in relation to this type of mutation could be smaller than in comparison to other types of mutation. Although an association between p53 and rate of apoptosis was not observed, the outcome does validate the results of the previous sections which indicated a lack of association between Bax and p53 or between Bax and the TUNEL score. Since wt p53 activates Bax for the induction of apoptosis, and no association was found between the two parameters, it is probable that there would be no association between Bax and the TUNEL score or between p53 and the TUNEL score. The results of the present data confirmed this rationale.

In conclusion, analysis of the relations between the various apoptotic factors studied suggest that in breast carcinoma apoptosis is a distinct event independent of Bax, Bcl-2 and mutant p53 expression. Therefore, our findings suggest the need for further studies to assess

if other apoptotic factors such as Bcl-X_L, Mcl-1 and Bad play a significant role in the apoptotic mechanism of breast carcinoma. We are cognizant of the fact that our results may be influenced by inherent errors and limitations: (1) the semi-quantitative nature of the immunohistochemical method; (2) false positive results arising in the TUNEL assay; and (3) errors in immunohistochemical identification arising from the wild-type p53 with prolonged half-life mimicking as mutant p53.

9.2 Relation Between Proliferative and Apoptotic Factors

9.2.1 PCNA and Ki-67

Data from the present study showed a positive correlation between the two proliferative markers PCNA and Ki-67 (see Table 10.7 in Appendix). Dawson et al. (1990) and Hall & Levinson. (1990) also found a positive association between PCNA and Ki-67 immunopositivity in breast carcinoma. However, in another study, Leonardi et al. (1992) found no association between the two parameters. Leonardi suggested that Dawson obtained a positive result because his study was performed with a different PCNA antibody, 19A2. However, the variation in the results may not be due to differences in the antibodies. Leonardi's study, as well as the present study, used the antibody PC10 to identify the PCNA, yet our study, in contrast to that of Leonardi, showed a clear positive correlation between the two proliferation factors. Dervan et al. (1992) found a positive association between PCNA and Ki-67, though Ki-67 was identified in fresh tissue while PCNA was identified in formalin fixed paraffin-embedded tissue of the same tumour. These findings not only confirm the results of the present study but also indicate the reliability of using the immunohistochemical

method in measuring the two proliferative markers.

9.2.2 Bcl-2 and PCNA, Ki-67

A strong inverse correlation between Bcl-2 and proliferative activity has been reported in a number of studies. This is probably related to the fact that Bcl-2 is known to promote the retention of cells in the G0/G1 phase of the cell cycle (Reed, 1995). Van Slooten et al. (1998; 1996), Silverstini et al. (1994;1996) and Gorczca et al. (1995) found a strong inverse correlation between Bcl-2 expression and proliferation as identified by PCNA or Ki-67. These studies suggest that highly proliferative tumours are associated with an upregulation of apoptotic mechanism resulting from a decrease in anti-apoptotic Bcl-2 expression. Data from this study, did not show an inverse association (see Table 10.8 & 10.9 respectively in Appendix I). However results from this study indicated that Bcl-2 expression was positively associated with PCNA expression (see Table 10.9 in Appendix). An possible explanation for this outcome, could be that high expression of Bcl-2 in breast cancer cells is associated with loss of normal wt p53 activity which restricts entry of cells in the S-phase of the cell cycle. The variation in the result between Bcl-2 and the proliferative marker, Ki-67, was not due to the scoring system. In comparison to our scoring method , van Slooten found a significant inverse association between Ki-67 and Bcl-2 despite using a more stringent (overexpression as greater than 20% of Ki-67 immunopositive cells while in the present study, Ki-67 immunopositivity of 10% or greater represented an overexpression) scoring system.

9.2.3 p53 and PCNA, Ki-67

The tumour suppressor gene p53 appears to inhibit cell proliferation by restricting entry of cells into the S-phase of the cell cycle (Ko & Prives, 1996; Bellamy,1997). Thus, loss of p53 function leads to an increase in cellular proliferation (Ko & Prives, 1996, Krajewski, 1996). As expected, the present findings of this study suggest that accumulation of immunopositive p53 cells (which indicates loss of p53 function) was associated with increased proliferation as detected by PCNA and Ki-67 (see Table 10.10 and 10.11 respectively in Appendix I). Other studies by Allred et al., (1993), Haerslev & Jacobsen, (1995) and van Slooten et al., (1996) have also shown that expression of mutant p53 protein was associated with high proliferation.

The association between loss of p53 function and increased proliferation seems reliable in light of the fact that p53 overexpression was not associated with the rate of apoptosis. Studies have confirmed the dual action of the tumour suppressor gene: death or growth arrest (Ko & Prives, 1996). Perhaps in this group of subjects the mechanism of growth arrest was favoured over cell death. As a result, loss of wt p53 function was associated with increased proliferation and not cell death as measured by the TUNEL method.

Van Slooten et al. (1998) found that overexpression of Bcl-2 tends to accumulate cells in the G0/G1 phase. Thus, if the mechanism of growth arrest was favoured in this study, increased proliferation should associate with mutational events in p53 and reduction in Bcl-2 expression. The data from the present study did not indicate this inverse association between Bcl-2 and proliferation, suggesting that the tumour cells are not dependent on Bcl-2 for their

survival. This supposition is confirmed by the fact that the data showed no association between Bcl-2 expression and rate of apoptosis. Another possibility could be the involvement of other Bcl-2 family members in the proliferation process. However, this possibility remains unexamined and therefore unknown. Therefore, it is important to determine the inter-relation of several apoptotic and proliferative markers in order to determine the clinical significance of the individual markers as predictors of local recurrence in breast cancer.

9.3 Apoptotic and Proliferative Factors Related To Clinicopathological Characteristics and Local Recurrence

9.3.1 Bcl-2

Studies examining the prognostic value of the Bcl-2 expression in breast carcinoma have yielded different results. Since Bcl-2 retards cell growth by inducing retention of cells in the G0\G1 phase of the cell cycle (Reed, 1995), it might be expected that low expression of Bcl-2 would be significantly associated with increased frequency of local recurrence of breast carcinoma. However, data from the present study showed no association between decreased expression of Bcl-2 and local recurrence (see Table 10.12 in Appendix I). This finding agrees with the study of Joensuu et al. (1994) which showed that Bcl-2 expression in breast carcinoma was not an independent prognostic indicator of either disease free survival or overall survival in 174 women. In contrast, Hellman et al's. (1995) study found a prognostic value of Bcl-2 expression in predicting disease-free survival or overall survival in lymph node positive patients. However, Bcl-2 did not predict disease-free survival or overall survival in lymph node negative patients. In one of the largest studies by Berardo et al.

(1998), the prognostic influence of Bcl-2 in primary breast tumour specimens from a large group of 979 lymph node positive patients was examined. Berardo's result showed that high Bcl-2 expression led to longer survival. This effect of Bcl-2 to prolong disease free survival may be associated with its ability to hold cells in the G0/G1 phase, reduce proliferation and promote tissue differentiation. Similar results were indicated by Kobayashi et al. (1997) and van Slooten et al. (1996).

Although the above studies showed Bcl-2 to be a valuable prognostic indicator, in a multivariate analyses Bcl-2 failed to maintain an independent prognostic role in relapse-free or overall survival (Silverstini et al., 1994; Joensuu et al., 1994; van Slooten et al., 1996). In the study by Berardo et al. (1998), however, a multivariate analysis did show that high Bcl-2 expression is associated with better survival. The varied nature of the results of these studies suggest that Bcl-2 does not satisfy the criteria of an independent prognostic tool, for Bcl-2 cannot consistently discriminate independently between groups of patients with better or worse prognosis.

Although Bcl-2 appears not to function as an independent prognostic indicator, its expression has been found to associate with other clinicopathological features such as tumour score, ER status and tumour size. Data from the present study showed that increased expression of Bcl-2 was associated with low tumour score, which is representative of a good prognosis, for tumours with high scores are characteristically undifferentiated and probably independent of Bcl-2 for survival (see Table 10.13 in Appendix). Other studies have reported similar correlations (van Slooten et al., 1998; Lee et al., 1996; Hellmans et al., 1995; Berardo

et al., 1998; Kobayashi et al., 1997), indicating a correlation between Bcl-2 expression and small tumour size and the presence of ER positivity.

The findings from the present study did not exhibit an association between Bcl-2 expression and small tumour size (see Table 10.14 in Appendix). Similarly, Leek et al. (1994) and Silverstini et al. (1995) found little correlation between Bcl-2 and tumour size. Associations between Bcl-2 and ER status were not analysed in this retrospective study since the charts of the patients were dispersed throughout Newfoundland. Moreover much of the information on the ER status of patients was incomplete or lost.

The findings from the present study, and others, suggests that although Bcl-2 cannot predict disease-free or overall survival, it can still function as a marker for better prognosis with regards to tumour differentiaiton.

9.3.2 p53

A number of studies have examined the relationship between nuclear accumulation of mutant p53 protein, as detected by immunohistochemistry, and various pathological factors and prognosis. In breast carcinoma some of the studies have indicated that mutant p53 expression is an independent marker of poor prognosis. In a group of 700 patients, disease free survival at 5 years was 80% for p53 negative tumours, 72% for low positive to intermediate positive tumours and 58% for p53 strongly positive tumours (Elledge & Allred, 1994). Similar findings by Thor & Yandell (1993) and Allred et al. (1993) verify the role of p53 as an independent prognostic tool. In comparison, findings from the present study did not indicate a significant association between p53 expression and local recurrence (see Table10.15

in Appendix). Similar results have been obtained by Ostrowski et al. (1991) and Haerslev & Jacobsen (1995) who found no correlation between mutant p53 expression and disease-free survival. A reason for these varying results could be related to differences in the immunohistochemical scoring methods. There are some similarities between the scoring method of Haerslev et al. (1996) and the present study. Immunopositive cells in Haerslev's study were classified into 5 groups indicating the frequency to which immunopositive cells were stained; 1=less than 1%, 2= 1-10%, 3 = 11-25%, 4= 26-50% and 5= greater than 50%. In the present study, the expression of mutant p53 was also rated to indicate the extent of staining. However, we only divided into two groups: 1= less than 10% of cells , 2 = greater than 10%. Silverstini et al. (1994) and Liponnen et al. (1994) who both found p53 mutant expression in breast cancer a significant prognostic factor, simply classified immunopositivity as either positive or negative. However we still found no significant association of p53 expression in relation to local recurrence when our data was re-analysed using the Silverstini's and Liponnen's method where p53 expression was classified as positive or negative. This suggests that the scoring system was not responsible for the different outcomes.

Another possibility for the differing findings could be related to cross-cultural differences in the type of p53 mutations found in the breast cancer patients. Sommer et al. (1992) found that the pattern of p53 mutation in breast cancer in 53 white Midwestern women differed from patterns observed in women of other countries. Missense mutations (responsible for p53 overexpression) were lower (44%) in breast cancers of the 53 U.S.

midwestern women than in Japanese, French and Scottish women. This finding suggests that various endogenous or environmental factors could contribute to varied p53 mutations in the mammary tissues among women of different countries. Thus, it is possible that the pattern of p53 mutation in Newfoundland women may be different and therefore a substantial proportion of cases may go undetected by immunohistochemistry. This further emphasizes the need to use molecular screening (e.g. SSCP and cDNA sequencing) in all studies examining the effect of p53 on clinical outcome.

Accumulation of p53 protein has also been associated with a number of other poor prognostic factors including ER negativity (Thor & Yandell, 1993), PR negativity (Allred et al., 1993) high histological grade (Elledge & Allred, 1994; Haerslev & Jacobsen, 1995), and high proliferation rate (Silverstini et al., 1993). In the present study accumulation of p53 protein was associated with proliferation rate (see Table 10.10 & 10.11 in Appendix) and high histological grade of tumour (see Table 10.17 in Appendix). This finding is consistent with high tumour proliferation resulting from loss of wild type p53 function leading to unrestricted entry of tumour cells into the S-Phase of the cell cycle. There was no significant association between p53 accumulation and tumour size (see Table 10.16 in Appendix) . This outcome seems consistent with the results of Haerslev & Jacobsen (1994), Ellegde & Allred (1994) and Molina et al., (1998).

The findings of these studies strongly suggest that p53, especially when combined with the other prognostic factors, may be a significant predictive marker of clinical outcome. The p53 protein seems to be a more reliable prognostic tool than the Bcl-2 protein since p53 can

independently predict the occurrence of disease free survival or overall survival in a multivariate analysis. However, in order to unequivocally define the role of p53 as an independent prognostic parameter, more studies are required using molecular screening, defined antibodies, and a standardized scoring system.

9.3.3 PCNA and Ki-67

As with the apoptotic markers, neither of the proliferative markers in this study predicted local recurrence of breast carcinoma (see Tables 10.18 & 10.19 in Appendix). A number of studies have, however, shown associations between certain clinicopathological parameters and expression of proliferative markers. Sheen-Chen et al. (1997) found that presence of PCNA positive cells of breast cancer was closely related to histological grade and tumour size. Using multivariate analysis, Sheen-Chen's study showed PCNA expression as an independent prognostic indicator. Similarly, results from the present study showed a significant association between PCNA expression and tumour size (see Table 10.22 in Appendix) PCNA expression and lymph node status (see Table 10.24 in Appendix). However, there was no significant association between PCNA and tumour score (see Table 10.23 in Appendix). In contrast Tahan et al. (1993) found no correlation between PCNA expression and tumour size, and lymph node involvement. However, study by Tahan et al. (1993) did indicate a significant difference in the overall survival between high and low PCNA expression groups: the high PCNA group indicated poor overall survival compared with the low PCNA group. Similar findings were obtained by Horiguchi's et al. (1997) study supported with Tahan's findings.

In the current study, Ki-67, the second proliferative marker, showed a positive association to tumour score but not to tumour size, recurrence or lymph node status (see Tables 10.19-10.21 in Appendix). There are however, certain studies that do indicate an association between Ki-67 immunopositivity and clinical prognostic parameters such as tumour size and lymph node status. Pierga et al. (1996) showed that high Ki-67 expression was significantly associated with lymph node involvement and tumour score but in a univariate analysis, Ki-67 was not an independent prognostic indicator. In contrast, Weikel et al. (1991) found no correlation between Ki-67 and tumour size or lymph node status, but there was a significant correlation between Ki-67 and tumour grade.

In studies where both markers were examined, PCNA and Ki-67 expression revealed different results. Leonardi et al. (1991) showed that PCNA did not correlate with Ki-67 expression or any other clinical prognostic parameter. However, Ki-67 was associated with high tumour grade (though not with tumour size or lymph node status). In contrast, Haerslev et al. (1996) found that high Ki-67 score was associated with PCNA over-expression. Both proliferative markers correlated with clinical parameters such as large tumour size and high histological grade. However, in a multivariate analysis, neither of the markers attained independent prognostic significance. In the present study a significant positive correlation between expression of Ki-67 and PCNA was obtained (see Table 10.7 in Appendix). Results showed a positive association between PCNA and tumour size; there was no association between Ki-67 and tumour size (see Tables 10.22 & 10.20 in Appendix). However, there was a positive association between Ki-67 expression and tumour score; there was no association

between PCNA and tumour score (see Tables 10.21 & 10.23 in Appendix).

The differing results obtained from the studies on the immunohistochemical expression of proliferation markers highlight the potential problems in attempting to interpret associations with other parameters. One possible reason for the diverse results could be the different scoring methods used by researchers. Haerslev et al.(1996) defined overexpression of Ki-67 as greater than 1% immunopositivity of the total cell population while PCNA overexpression was represented as greater than 25% immunopositivity. Pierga et al. (1996), classified immunopositivity greater than 8% as an overexpression of Ki-67 score while Sheen-Chen et al. (1997) defined high PCNA expression as 35% or greater. These differences in the scoring systems may typically result in different findings. As in the other immunohistochemical staining procedures, there is a great need to standardize scoring methods for the purpose of reliable clinicopathological correlation.

To assess whether the scoring method could be a cause for the differences observed in this study and that of Haerslev's, an additional analysis was performed in which immunopositive Ki-67 stains greater than 1% were considered as overexpression and analysed with the clinicopathological variables. The 1% cut-off value was chosen to assimilate Haerslev's et al. (1996) study. However, the results from the statistical analysis did not exhibit a significant difference from the initial analysis, thereby suggesting that, like the p53 expression, differences in the scoring systems did not, in fact, account for the differences in the results. Similar findings were obtained for PCNA.

Other reasons for the differences in findings between studies may be related to

immunostaining methods, the type of antibody, or the age of the paraffin blocks or fixation procedures.

9.4 Inter-relation Between Clinicopathological Parameters and Local Recurrence

To date, studies have confirmed that there are four clinical parameters that influence prognosis in breast cancer: tumour grade, tumour size, ER status and lymph node involvement. These parameters can reliably and independently predict disease free or overall survival (Greenfield & Mullholland, 1997). A potential prognostic parameter for breast carcinoma is assessed by its ability to: (1) independently predict disease free or overall survival (2) show correlation to other known independent prognostic factors such as tumour size, lymph node status and tumour grade. Haerslev et al., (1996) found that while PCNA and Ki-67 failed to be of independent prognostic significance, histopathological parameters such as tumour size and tumour grade were able to predict the survival of the subjects. Other studies have found that recurrence free survival was predicted by tumour size and lymph node status and not by the rate of apoptosis or p53 or Bcl-2 expression (Lipponen et al., 1994; Haerslev & Jacobsen, 1995; Hellmans et al., 1995).

The present study found that Bcl-2 was inversely associated with tumour grade while increased expression of mutant p53 was positively associated with tumour grade. Although associations between the two biological markers and tumour score were determined, further analysis of tumour score and local recurrence indicated no significant association between these two clinical parameters. Tumour size was positively associated with proliferation rate as detected by PCNA expression (see Table 10.22 in Appendix). In addition, tumours larger

than 2cm were significantly associated with a local recurrence (see Table 10.25 in Appendix).

Rubins & Farber (1994) found that women with no axillary lymph node involvement had a 5 year survival rate of 80% when compared to those with positive lymph node involvement whose survival rate fell to 21% in the presence of 4 or more affected lymph nodes. McGuire (1987) reported a strong inverse correlation between the probability of disease free survival and the number of positive nodes. In the current study, there was no association between lymph node status and local recurrence (see Table 10.27 in Appendix). Carter et al. (1989) in a study of 24,740 breast cancer cases, found that lymph node involvment along with tumour size were independent yet additive prognostic indicators of breast carcinomas. With regards to tumour size, Carter et al. (1989) also reported that survival rates varied from 45.5% for tumours larger than 5 cm with positive nodes, to 96.3% for tumours less than 2cm with no lymph nodes involved. The study indicated that disease free survival decreased as the tumour size increased, regardless of the lymph node status. Also, as lymph node involvement increased, disease free survival decreased regardless of tumour size. In this study there was no association between local recurrence and the combined score of tumour size and lymph node status. However, Carter pre-selected the 24,740 cases from a pool of 63,316 cases using tumour size of either 2cm or 5cm and absence or presence (if a minimum of eight nodes were involved) of lymph node involvment. In contrast, in the present study there was no selection of cases with regards to specific tumour size and number of positive lymph nodes. This could be one reason for the lack of association.

In summary, only tumour size, a clinicopathological parameter, seemed reliable in

predicting the clinical outcome. Though associations between some of the apoptotic markers and clinical parameters were observed, neither of the markers were capable of predicting the local recurrence of breast cancer.

One unexpected finding in the present study was that, although significant associations between tumour histological score and Bcl-2, p53 and Ki-67 were observed, there was no association between tumour score and local recurrence. Another unanticipated finding from the present study was that, with the exception of PCNA, there was a lack of association between tumour size and the other biological markers. Although studies have consistently observed the prognostic value of tumour size in independently predicting disease-free survival (Carter et al., 1989), it was surprising that there was no inter-relation between the apoptotic markers and size of the tumour. One reason could be that the markers examined had a negligible effect on the progress of tumour growth and that other markers not examined in this study play a larger role in regulating the growth of the tumours. Aside from this suggestion, it seems that tumour size is a reliable parameter for predicting local recurrence. However in should be noted that in practice, the accurate measurement of tumour size is difficult.

The diverse scoring systems, as well as the inability to objectively quantitate the expression of immunopositivity, remains a significant concern in determining the predictive value of any prognostic marker.

Chapter 10

CONCLUSION

10.1

Prognostic factors that can predict the clinical outcome of patients with breast cancer are effective in three situations. Firstly, they are helpful in identifying patients with good prognosis following surgery, thereby dispensing with the possible need for expensive adjuvant therapy. Secondly, they can be used to identify patients with poor prognosis who require more aggressive adjuvant therapy. Thirdly, they enable the identification of patients who are more likely to benefit from specific types of therapy (Clark, 1994). To date, extensive investigations have been performed on the use of parameters relating to tumour differentiation, tumour growth, and tumour aggressiveness as prognostic factors. However, a number of conflicting findings have been obtained reflecting the difficulties encountered in developing laboratory methods capable of precise quantitative measurement of parameters in complex biological tissue.

The present retrospective study examined the inter-relation between the proliferative and apoptotic markers and their role in the progression of tumour growth and clinical outcome (see Figure 10.1). This study confirms the dual function of the tumour suppressor gene, p53. Expression of p53 appears to occupy a distinct central role in the control of proliferation. Loss of p53 function, as evidenced by nuclear positivity, leads to increased proliferation (see Tables 10.10 & 10.11 in Appendix) which, in turn, is associated with increase in tumour size and tumour grade (see Tables 10.21 & 10.22 in Appendix).

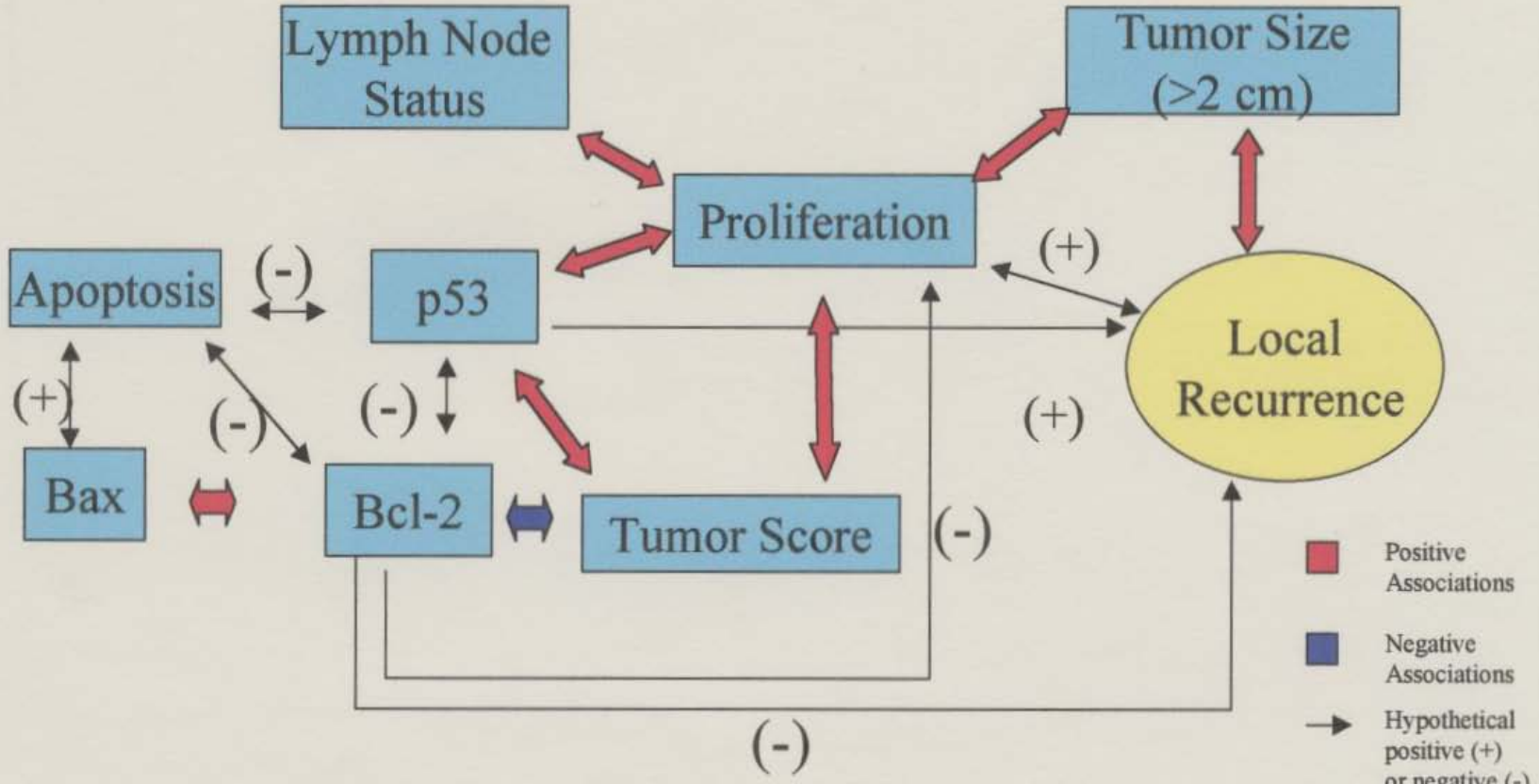

Figure 10.1: Inter-relation of Apoptotic and Proliferative Factors in Locally Recurrent Breast Carcinoma

Increased tumour size predisposes to risk of local recurrence. Thus, it appears that large tumour size is the terminal event in a series of biological changes, initiated by mutational events in p53, which predisposes to local recurrence. Although associations were found between p53 overexpression and proliferation rate and not between p53 and local recurrence, it is quite likely that the latter association could be revealed by the use of more sophisticated laboratory techniques. Overexpression of p53 is correlated with high tumour histological grade consistent with an induced state of high proliferation. Expression of Bcl-2 is reciprocally associated with tumour histological grade, supporting the view that Bcl-2 is an effective agent in promoting tumour differentiation and better prognosis. Tumour grade, however, was not associated with tumour size, or local recurrence. A surprising finding was the positive association between Bcl-2 and Bax expression, indicating some sort of co-regulation of these factors and the possibility that the ratio of Bax to Bcl-2 expression, not the absolute values, is a critical factor in regulating apoptosis. No association was found in this study between apoptotic rate and expression of Bax, Bcl-2 or p53, or to any other parameter examined. It is possible that the process of apoptosis operates by a totally different mechanism or is determined by apoptotic factors other than Bcl-2 or Bax. This speculation can only be clarified by a detailed study of apoptosis in relation to the expression of the various known apoptotic factors such as Mcl-1, Bcl-X, Bad and Bik using quantitative laboratory techniques.

Many of the biological markers used in this study still need to be examined in a large scale, prospective, confirmatory trial with node negative patients who have the highest

likelihood of disease free survival. If such a large study were to be performed, some of the methodological problems need to be addressed and resolved in order to validate the results.

Most of the studies examine specific prognostic factors by means of IHC staining with occasional use of molecular techniques. The IHC method, though easy and cost-efficient, contains certain draw-backs that hinder a reliable and reproducible result. Studies that examine a putative factor by the IHC method should set a standard cutpoint selection (low values vs. high values or positive vs. negative outcome) where the number of immunopositive cells are placed in specific groups. However, because of the inherent heterogeneity of the tumours, setting a rigid cutpoint may create a problem. A problem of miscalculation may occur when counting the number of cells in several fields. Therefore, the use of a grid which can partition a group of 1000 cells per section may aid the process of counting the cells objectively.

Another problem to overcome is the current use of a variety of antibodies and IHC methods in identifying cellular antigenic expression. The differences in these methods may produce divergent results. Also, placing immunopositive cells in groups limits the use of the non-categorical statistical analyses, though the X^2 analysis is a reliable non-parametric test and can be applied when the data analyzed is ranked or classified (Daniel, 1995). A review of the literatures that examined the prognostic value of specific factors reveal the many methods of analyzing the data. Currently, few comparative studies have been performed to determine which statistical methods might be optimal in different circumstances.

To date, the search is ongoing for a prognostic tool that fulfils the three criteria set by Carter et al. (1994). Future progress in determining such prognostic factors would require large data sets with a long term follow-up and a reliable method of quantifying the outcome so that it is reproducible. Also, to determine the optimal analysis for examining the value of the prognostic factors, various statistical methods must be applied to the same data. These standards need to be attained to help identify reliable and valid prognostic indicators.

This study provides further insight about the inter-relations between the apoptotic and proliferative factors. We have failed to confirm the role of p53 and Bcl-2 as independent prognostic factors. Future prospects entail a study of the apoptotic and proliferative markers using flow cytometry and molecular techniques to ensure reliable clinical correlation. From such a study we predict that a combination of p53 loss of function, low Bcl-2 expression and high proliferation identifies patients at risk for local recurrence of breast cancer.

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Appendix I

Table 10.1: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression as detected by the immunohistochemical staining and the rate of apoptosis.

		Bcl-2 Cytoplasmic Expression (n=142)		
Rate of Apoptosis (n=142)		Low	Med-High	Total
	Low	64	63	127
	Med-High	11	4	15
	Total	75	67	142
Statistical Significance	χ^2 =2.833 df = 1 p = .092 p = .108*			

* p-value calculated using Fisher's exact test

Table 10.2: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression and Bax cytoplasmic expression as detected by immunohistochemical staining.

		Bcl-2 Cytoplasmic Expression (n=142)		
Bax Cytoplasmic Expression (n=142)		Low	Med-High	Total
	Low	48	28	76
	Med-High	27	39	66
	Total	75	67	142
Statistical Significance	$\chi^2 = 7.017$ df = 1 p = .008 phi = .222			

Table 10.3: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression and p53 nuclear expression as detected by immunohistochemical staining.

		Bcl-2 Cytoplasmic Expression (n=142)		
p53 Expression (n=142)		Low	Med-High	Total
	Low	60	62	122
	Med-High	15	5	20
	Total	75	67	142
Statistical Significance	$\chi^2 = 4.597$ df = 1 p = .032 phi = -.180			

Table 10.4: Nonparametric analysis of the association between Bax cytoplasmic expression and p53 nuclear expression as detected by immunohistochemistry.

		Bax Cytoplasmic Expression (n=144)		
p53 Nuclear Expression (n=144)		Low	Med-High	Total
	Low	66	57	123
	Med-High	11	10	21
	Total	77	67	144
Statistical Significance	$\chi^2 = .012$ df = 1 p = .914 p = 1.000*			

* p-value calculated using Fisher's exact test

Table 10.5: Nonparametric analysis of the association between Bax cytoplasmic expression as detected by immunohistochemical staining and the rate of apoptosis.

		Bax Cytoplasmic Expression (n=141)		
Rate of Apoptosis (n=141)		Low	Med-High	Total
	Low	66	10	76
	Med-High	60	5	65
	Total	126	15	141
Statistical Significance	$\chi^2 = 1.101$ df = 1 p = .294 p = .413 *			

* p-value calculated using Fisher's exact test

Table 10.6: Nonparametric analysis of the association between p53 nuclear expression as detected by immunohistochemical staining and rate of apoptosis.

		Rate of Apoptosis (n=141)		
p53 Expression (n=141)		Low	Med-High	Total
	Low	110	11	121
	Med-High	16	4	20
	Total	126	15	141
Statistical Significance	$\chi^2 = 2.149$ df = 1 p = .143 p = .230 *			

* p-value calculated using Fisher's exact test

Table 10.7: Nonparametric analysis of the association between the two proliferative markers, Ki-67 and PCNA nuclear expression as detected by the immunohistochemical staining.

		PCNA Expression (n=145)		
Ki-67 Expression (n=145)		Low	Med-High	Total
	Low	81	34	115
	Med-High	12	18	30
	Total	93	52	145
Statistical Significance	$\chi^2 = 9.582$ df = 1 p = .002 phi = .257			

Table 10.8: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression and the nuclear expression of the proliferative marker, Ki-67 as detected by immunohistochemical staining.

		Bcl-2 Expression (n=143)		
		Low	Med-High	Total
Ki-67 Expression (n=143)	Low	58	56	114
	Med-High	18	11	29
	Total	76	67	143
Statistical Significance	$\chi^2 = 1.163$ df=1 p = .281			

Table 10.9: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression and PCNA nuclear expression as detected by immunohistochemical staining.

		Bcl-2 Expression (n=143)		
PCNA Expression (n=143)		Low	Med-High	Total
	Low	56	35	91
	Med-High	20	32	52
	Total	76	67	143
Statistical Significance	$\chi^2 = 7.077$ df = 1 p = .008 phi = .222			

Table 10.10: Nonparametric analysis of the association between p53 nuclear expression and Ki-67 nuclear expression as detected by immunohistochemical staining.

		p53 Expression (n=144)		
Ki-67 Expression (n=144)		Low	Med-High	Total
	Low	106	8	114
	Med-High	17	13	30
	Total	123	21	144
Statistical Significance	$\chi^2 = 25.145$ df = 1 p = .000 phi = .418			

Table 10.11: Nonparametric analysis of the association between p53 nuclear expression and PCNA nuclear expression as detected by immunohistochemical staining.

		p53 Expression (n=144)		
PCNA Expression (n=144)		Low	Med-High	Total
	Low	85	7	92
	Med-High	38	14	52
	Total	123	21	144
Statistical Significance	$\chi^2 = 9.949$ df=1 p = .002 p = .003* phi =.263			

* p-value calculated using Fisher's exact test

Table 10.12: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression as detected by immunohistochemical staining and local recurrence of breast cancer

		Bcl-2 Cytoplasmic Expression (n=143)		
Recurrence (n=143)		Low	Med-High	Total
	No Recurrence	26	28	54
	Recurrence	50	39	89
	Total	76	67	143
Statistical Significance	$\chi^2 = .871$ df = 1 p = .351			

Table 10.13: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression as detected by immunohistochemical staining and Tumour grade.

		Bcl-2 Expression (n=134)		
		Low	Med-High	Total
Tumour Grade (n=134)	Low	20	36	56
	Med	19	19	38
	High	34	6	40
	Total	73	61	134
Statistical Significance	χ^2 =23.284 df=2 p=.000 phi=.417			

Table 10.14: Nonparametric analysis of the association between Bcl-2 cytoplasmic expression as detected by immunohistochemical staining and tumour size.

		Bcl-2 Cytoplasmic Expression (n=138)		
Tumour Size (n=138)		Low	Med-High	Total
	< 2cm	38	31	69
	> 2cm	36	33	69
	Total	74	64	138
Statistical Significance	$\chi^2 = .117$ df = 1 p = .773			

Table 10.15: Nonparametric analysis of the association between p53 nuclear expression and local recurrence of breast carcinoma.

		p53 Expression (n=144)		
Local Recurrence (n=144)		Low	Med-High	Total
	No Recurrence	49	5	54
	Recurrence	74	16	90
	Total	123	21	144
Statistical Significance	χ^2 =1.966 df=1 p = .161 p = .223 *			

* p-value calculated using Fisher's exact test

Table 10.16: Nonparametric analysis of the association between p53 nuclear expression as detected by immunohistochemical staining and tumour size.

		p53 Expression (n=140)		
Tumour Size (n=140)		Low	Med-High	Total
	< 2cm	63	8	71
	> 2cm	57	12	69
	Total	120	20	140
Statistical Significance	χ^2 =1.072 df = 1 p = .301 p =.341 *			

* p-value calculated using Fisher's exact test

Table 10.17: Nonparametric analysis of the association between p53 nuclear expression as detected by immunohistochemical staining and Tumour grade.

		p53 Expression (n=135)		
Tumour Grade (n=135)		Absence of p53 Expression (0%)	Presence of p53 Expression (>5%)	Total
	Low	49	7	56
	Med	30	8	38
	High	23	18	41
	Total	102	33	135
Statistical Significance	$\chi^2 = 12.967$ df = 2 p = .002 phi= .310			

Table 10.18: Nonparametric analysis of the association between PCNA expression as detected by immunohistochemical staining and local recurrence of breast carcinoma.

		PCNA Expression (n=145)		
Recurrence (n=145)		Low	Med-High	Total
	No Recurrence	38	16	54
	Recurrence	55	36	91
	Total	93	52	145
Statistical Significance	$\chi^2 = 1.453$ df = 1 p = .228			

Table 10.19: Nonparametric analysis of the association between Ki-67 nuclear expression as detected by immunohistochemical staining and local recurrence of breast carcinoma.

		Ki-67 Expression (n=145)		
Recurrence (n=145)		Low	Med-High	Total
	No Recurrence	46	8	54
	Recurrence	69	22	91
	Total	115	30	145
Statistical Significance	$\chi^2 = 1.810$ df = 1 p = .179 p = .208*			

* p-value calculated using Fisher's exact test

Table 10.20: Nonparametric analysis of the association between Ki-67 nuclear expression as detected by immunohistochemical staining and tumour grade.

		Ki-67 Expression (n=136)		
Tumour Grade (n=136)		Low	Med-High	Total
	Low	54	2	56
	Med	27	11	38
	High	28	14	42
	Total	109	27	136
Statistical Significance	$\chi^2 = 16.102$ df=2 p=.000 phi = .344			

Table 10.21: Nonparametric analysis of the association between Ki-67 nuclear expression as detected by immunohistochemical staining and tumour size.

		Ki-67 Expression (n=140)		
Tumour Size (n=140)		Low	Med-High	Total
	< 2cm	59	12	71
	> 2cm	52	17	69
	Total	111	29	140
Statistical Significance	$\chi^2 = 1.275$ df = 1 p = .259			

* p-value calculated using Fisher's exact test

Table 10.22: Nonparametric analysis of the association between PCNA nuclear expression as detected by immunohistochemical staining and tumour size.

		PCNA Expression (n=140)		
Tumour Size (n=140)		Low	Med-High	Total
	< 2cm	52	19	71
	> 2cm	37	32	69
	Total	89	51	140
Statistical Significance	$\chi^2 = 5.814$ df =1 p =.016 phi=.204			

Table 10.23: Nonparametric analysis of the association between PCNA nuclear expression as detected by immunohistochemical staining and tumour grade.

		PCNA Expression (n=136)		
		Low	Med-High	Total
Tumour Grade (n=136)	Low	41	15	56
	Med	18	20	38
	High	29	13	42
	Total	88	48	136
Statistical Significance	$\chi^2 = 7.123$ df=2 p = .028 phi = .229			

Table 10.24: Nonparametric analysis of the association between PCNA nuclear expression as detected by immunohistochemical staining and lymph node status.

		PCNA Expression (n=145)		
Lymph node Status (n=145)		Low	Med-High	Total
	No Involvement	53	19	72
	Involvement	40	33	73
	Total	93	52	145
Statistical Significance	$\chi^2 = 5.580$ df=1 p=.018 phi=.196			

Table 10.25: Nonparametric analysis of the association between tumour size and local recurrence of breast carcinoma.

		Tumour Size (n=140)		
Recurrence (n=140)		<2cm	>2cm	Total
	No Recurrence	34	19	53
	Recurrence	37	50	87
	Total	71	69	140
Statistical Significance	$\chi^2 = 6.160$ df = 1 p =.013 phi=.210			

Table 10.26: Nonparametric analysis of the association between local recurrence of breast carcinoma and tumour grade.

		Recurrence (n=136)		
Tumour Grade (n=136)		Recurrence	No Recurrence	Total
	Low	31	25	56
	Med	22	16	38
	High	29	13	42
	Total	82	54	136
Statistical Significance	χ^2 =2.006 df =2 p =.367			

Table 10.27: Nonparametric analysis of the association between Recurrence and lymph node status.

		Recurrence (n=145)		
Lymph node Status (n=145)		Recurrence	No Recurrence	Total
	No Involvment	27	27	72
	Involvment	45	46	73
	Total	91	54	145
Statistical Significance	$\chi^2 = .004$ df = 1 p = .949			

	subjects	bcl2cyfr	baxcyfr	p53	tunel	tscore	ki67
1	1.00	3.00	3.00	.00	5.75	2.00	1.00
2	1.00	3.00	.00	.00	.25	1.00	1.00
3	1.00	.00	3.00	.00	.00	1.00	3.00
4	1.00	.00	3.00	.00	.00	1.00	1.00
5	1.00	3.00	.00	.00	.00	1.00	2.00
6	1.00	3.00	3.00	.00	.83	1.00	.00
7	1.00	3.00	.00	.00	3.00	2.00	1.00
8	1.00	.00	3.00	.00	.66	1.00	2.00
9	1.00	.00	1.00	.00	3.60	2.00	1.00
10	1.00	3.00	3.00	.00	9.50	3.00	1.00
11	1.00	3.00	1.00	.00	4.00	2.00	1.00
12	1.00	.00	3.00	3.00	.60	1.00	1.00
13	1.00	3.00	3.00	.00	.75	1.00	1.00
14	1.00	3.00	1.00	.00	3.50	2.00	1.00
15	1.00	3.00	1.00	.00	2.70	2.00	2.00
16	1.00	.00	.00	.00	2.10	2.00	1.00
17	1.00	3.00	3.00	1.00	3.50	2.00	1.00
18	1.00	.00	.00	.00	4.60	2.00	1.00
19	1.00	.00	1.00	.00	8.80	3.00	2.00
20	1.00	3.00	.00	.00	11.80	3.00	3.00
21	1.00	.00	.00	.00	.00	1.00	1.00
22	1.00	2.00	3.00	1.00	1.16	1.00	1.00
23	1.00	3.00	3.00	1.00	.00	1.00	1.00
24	1.00	.00	3.00	.00	.33	1.00	1.00
25	1.00	3.00	2.00	.00	.33	1.00	1.00
26	1.00	3.00	3.00	.00	.00	1.00	1.00
27	1.00	3.00	.00	.00	2.10	2.00	1.00
28	2.00	3.00	3.00	.00	.00	1.00	.00
29	2.00	3.00	1.00	.00	.25	1.00	1.00
30	2.00	3.00	.00	.00	2.30	2.00	1.00

	pcna	tscore1	baxcyf1	bcl2cyf1	tumsize	tumscore	recurren
1	1.00	1.00	1.00	1.00	2.50	5.00	.00
2	1.00	1.00	.00	1.00	1.30	4.00	.00
3	.00	1.00	1.00	.00	2.00	9.00	.00
4	.00	1.00	1.00	.00	3.50	5.00	.00
5	.00	1.00	.00	1.00	2.50	7.00	.00
6	1.00	1.00	1.00	1.00	3.50	5.00	.00
7	1.00	1.00	.00	1.00	2.00	6.00	.00
8	1.00	1.00	1.00	.00	1.00	4.00	.00
9	1.00	1.00	.00	.00	1.20	8.00	.00
10	1.00	2.00	1.00	1.00	2.50	4.00	.00
11	2.00	1.00	.00	1.00	3.00	6.00	.00
12	1.00	1.00	1.00	.00	2.00	8.00	.00
13	1.00	1.00	1.00	1.00	2.00	4.00	.00
14	1.00	1.00	.00	1.00	1.80	7.00	.00
15	3.00	1.00	.00	1.00	1.40	6.00	.00
16	3.00	1.00	.00	.00	2.00	8.00	.00
17	3.00	1.00	1.00	1.00	3.40	8.00	.00
18	3.00	1.00	.00	.00	2.80	9.00	.00
19	.00	2.00	.00	.00	1.00	9.00	.00
20	1.00	2.00	.00	1.00	.80	6.00	.00
21	1.00	1.00	.00	.00	1.70	8.00	.00
22	3.00	1.00	1.00	1.00	2.20	8.00	.00
23	3.00	1.00	1.00	1.00	1.00	5.00	.00
24	1.00	1.00	1.00	.00	5.50	7.00	.00
25	3.00	1.00	1.00	1.00	2.50	4.00	.00
26	1.00	1.00	1.00	1.00	2.00	5.00	.00
27	2.00	1.00	.00	1.00	2.60	4.00	.00
28	1.00	1.00	1.00	1.00	2.80	5.00	.00
29	2.00	1.00	.00	1.00	3.00	5.00	.00
30	.00	1.00	.00	1.00	1.80	5.00	.00

	lymhstat	tumorsc1	porabcl2	porap53	sizoftum	p532	pcna2
1	1.00	1.00	1.00	.00	2.00	.00	.00
2	1.00	1.00	1.00	.00	1.00	.00	.00
3	1.00	3.00	.00	.00	1.00	.00	.00
4	1.00	1.00	.00	.00	2.00	.00	.00
5	1.00	2.00	1.00	.00	2.00	.00	.00
6	1.00	1.00	1.00	.00	2.00	.00	.00
7	1.00	2.00	1.00	.00	1.00	.00	.00
8	1.00	1.00	.00	.00	1.00	.00	.00
9	1.00	3.00	.00	.00	1.00	.00	.00
10	1.00	1.00	1.00	.00	2.00	.00	.00
11	1.00	2.00	1.00	.00	2.00	.00	1.00
12	1.00	3.00	.00	1.00	1.00	1.00	.00
13	1.00	1.00	1.00	.00	1.00	.00	.00
14	1.00	2.00	1.00	.00	1.00	.00	.00
15	1.00	2.00	1.00	.00	1.00	.00	1.00
16	1.00	3.00	.00	.00	1.00	.00	1.00
17	1.00	3.00	1.00	1.00	2.00	.00	1.00
18	1.00	3.00	.00	.00	2.00	.00	1.00
19	1.00	3.00	.00	.00	1.00	.00	.00
20	1.00	2.00	1.00	.00	1.00	.00	.00
21	1.00	3.00	.00	.00	1.00	.00	.00
22	1.00	3.00	1.00	1.00	2.00	.00	1.00
23	1.00	1.00	1.00	1.00	1.00	.00	1.00
24	1.00	2.00	.00	.00	2.00	.00	.00
25	1.00	1.00	1.00	.00	2.00	.00	1.00
26	1.00	1.00	1.00	.00	1.00	.00	.00
27	1.00	1.00	1.00	.00	2.00	.00	1.00
28	.00	1.00	1.00	.00	2.00	.00	.00
29	.00	1.00	1.00	.00	2.00	.00	1.00
30	.00	1.00	1.00	.00	1.00	.00	.00

	ki672
1	.00
2	.00
3	1.00
4	.00
5	1.00
6	.00
7	.00
8	1.00
9	.00
10	.00
11	.00
12	.00
13	.00
14	.00
15	1.00
16	.00
17	.00
18	.00
19	1.00
20	1.00
21	.00
22	.00
23	.00
24	.00
25	.00
26	.00
27	.00
28	.00
29	.00
30	.00

	subjects	bcl2cyfr	baxcyfr	p53	tunel	tscore	ki67
31	2.00	.00	1.00	.00	.00	1.00	1.00
32	2.00	.00	3.00	.00	.00	1.00	.00
33	2.00	.00	3.00	3.00	5.50	2.00	1.00
34	2.00	3.00	3.00	.00	9.40	3.00	1.00
35	2.00	.00	1.00	3.00	6.30	3.00	1.00
36	2.00	3.00	.00	.00	.00	1.00	1.00
37	2.00	.00	3.00	1.00	.00	1.00	1.00
38	2.00	3.00	3.00	.00	.00	1.00	.00
39	2.00	.00	3.00	2.00	6.00	3.00	1.00
40	2.00	.00	.00	.00	.00	1.00	.00
41	2.00	3.00	3.00	.00	3.00	2.00	.00
42	2.00	3.00	3.00	.00	.00	1.00	1.00
43	2.00	.00	.00	3.00	4.60	3.00	2.00
44	2.00	.00	.00	.00	2.10	2.00	1.00
45	2.00	.00	.00	.00	.80	1.00	.00
46	2.00	1.00	2.00	.00	1.30	1.00	1.00
47	2.00	3.00	3.00	1.00	3.00	2.00	1.00
48	2.00	.00	.00	.00	.83	1.00	1.00
49	2.00	.00	1.00	.00	4.00	2.00	3.00
50	2.00	.00	.00	.00	.60	1.00	1.00
51	2.00	.00	.00	.00	2.80	2.00	1.00
52	2.00	3.00	.00	.00	.83	1.00	1.00
53	2.00	1.00	.00	1.00	.50	1.00	1.00
54	2.00	3.00	3.00	.00	.16	1.00	1.00
55	3.00	3.00	.00	.00	.60	1.00	.00
56	3.00	999.00	1.00	.00	999.00	999.00	1.00
57	3.00	3.00	1.00	.00	8.50	3.00	2.00
58	3.00	.00	3.00	.00	1.30	1.00	1.00
59	3.00	3.00	3.00	.00	.00	1.00	.00
60	3.00	.00	.00	.00	3.50	2.00	1.00

	pcna	tscore1	baxcyf1	bcl2cyf1	tumsize	tumscore	recurren
31	.00	1.00	.00	.00	10.00	8.00	.00
32	.00	1.00	1.00	.00	1.20	6.00	.00
33	3.00	1.00	1.00	.00	1.20	6.00	.00
34	1.00	2.00	1.00	1.00	1.70	5.00	.00
35	.00	2.00	.00	.00	3.00	4.00	.00
36	.00	1.00	.00	1.00	2.00	4.00	.00
37	.00	1.00	1.00	.00	1.80	5.00	.00
38	.00	1.00	1.00	1.00	2.00	5.00	.00
39	.00	2.00	1.00	.00	2.00	9.00	.00
40	1.00	1.00	.00	.00	3.00	5.00	.00
41	.00	1.00	1.00	1.00	2.50	5.00	.00
42	.00	1.00	1.00	1.00	1.70	5.00	.00
43	1.00	2.00	.00	.00	1.20	9.00	.00
44	1.00	1.00	.00	.00	2.00	6.00	.00
45	.00	1.00	.00	.00	1.50	4.00	.00
46	1.00	1.00	1.00	.00	2.00	5.00	.00
47	2.00	1.00	1.00	1.00	1.30	6.00	.00
48	3.00	1.00	.00	.00	2.80	8.00	.00
49	2.00	1.00	.00	.00	1.50	6.00	.00
50	.00	1.00	.00	.00	1.50	6.00	.00
51	1.00	1.00	.00	.00	2.00	7.00	.00
52	2.00	1.00	.00	1.00	1.00	6.00	.00
53	.00	1.00	.00	.00	1.00	7.00	.00
54	3.00	1.00	1.00	1.00	3.00	4.00	.00
55	2.00	1.00	.00	1.00	1.80	5.00	1.00
56	1.00	.	.00	999.00	.10	9.00	1.00
57	3.00	2.00	.00	1.00	2.20	7.00	1.00
58	.00	1.00	1.00	.00	2.20	5.00	1.00
59	.00	1.00	1.00	1.00	1.30	6.00	1.00
60	1.00	1.00	.00	.00	2.50	8.00	1.00

	lymhstat	tumorsc1	porabcl2	porap53	sizoftum	p532	pcna2
31	.00	3.00	.00	.00	.	.00	.00
32	.00	2.00	.00	.00	1.00	.00	.00
33	.00	2.00	.00	1.00	1.00	1.00	1.00
34	.00	1.00	1.00	.00	1.00	.00	.00
35	.00	1.00	.00	1.00	2.00	1.00	.00
36	.00	1.00	1.00	.00	1.00	.00	.00
37	.00	1.00	.00	1.00	1.00	.00	.00
38	.00	1.00	1.00	.00	1.00	.00	.00
39	.00	3.00	.00	1.00	1.00	1.00	.00
40	.00	1.00	.00	.00	2.00	.00	.00
41	.00	1.00	1.00	.00	2.00	.00	.00
42	.00	1.00	1.00	.00	1.00	.00	.00
43	.00	3.00	.00	1.00	1.00	1.00	.00
44	.00	2.00	.00	.00	1.00	.00	.00
45	.00	1.00	.00	.00	1.00	.00	.00
46	.00	1.00	.00	.00	1.00	.00	.00
47	.00	2.00	1.00	1.00	1.00	.00	1.00
48	.00	3.00	.00	.00	2.00	.00	1.00
49	.00	2.00	.00	.00	1.00	.00	1.00
50	.00	2.00	.00	.00	1.00	.00	.00
51	.00	2.00	.00	.00	1.00	.00	.00
52	.00	2.00	1.00	.00	1.00	.00	1.00
53	.00	2.00	.00	1.00	1.00	.00	.00
54	.00	1.00	1.00	.00	2.00	.00	1.00
55	1.00	1.00	1.00	.00	1.00	.00	1.00
56	1.00	3.00	.	.00	1.00	.00	.00
57	1.00	2.00	1.00	.00	2.00	.00	1.00
58	1.00	1.00	.00	.00	2.00	.00	.00
59	1.00	2.00	1.00	.00	1.00	.00	00
60	1.00	3.00	.00	.00	2.00	.00	.00

	ki672
31	.00
32	.00
33	.00
34	.00
35	.00
36	.00
37	.00
38	.00
39	.00
40	.00
41	.00
42	.00
43	1.00
44	.00
45	.00
46	.00
47	.00
48	.00
49	1.00
50	.00
51	.00
52	.00
53	.00
54	.00
55	.00
56	.00
57	1.00
58	.00
59	.00
60	.00

	subjects	bcl2cyfr	baxcyfr	p53	tunel	tscore	ki67
61	3.00	3.00	3.00	.00	1.10	1.00	.00
62	3.00	.00	.00	1.00	11.50	3.00	1.00
63	3.00	.00	1.00	3.00	2.00	1.00	2.00
64	3.00	3.00	1.00	.00	1.50	1.00	1.00
65	3.00	.00	.00	.00	.00	1.00	1.00
66	3.00	.00	3.00	3.00	.50	1.00	1.00
67	3.00	2.00	.00	1.00	3.00	2.00	2.00
68	3.00	3.00	1.00	.00	3.30	2.00	1.00
69	3.00	.00	.00	3.00	3.10	2.00	3.00
70	3.00	2.00	3.00	.00	.16	1.00	.00
71	3.00	.00	1.00	.00	.50	1.00	.00
72	3.00	.00	.00	3.00	5.60	2.00	3.00
73	3.00	.00	1.00	.00	1.50	1.00	1.00
74	3.00	.00	1.00	.00	12.50	3.00	1.00
75	3.00	.00	.00	2.00	1.40	1.00	2.00
76	3.00	.00	3.00	.00	11.40	3.00	1.00
77	3.00	.00	.00	.00	8.60	3.00	.00
78	3.00	3.00	.00	.00	.16	1.00	1.00
79	3.00	3.00	3.00	.00	.16	1.00	1.00
80	3.00	.00	3.00	.00	.16	1.00	1.00
81	3.00	3.00	3.00	1.00	1.66	1.00	1.00
82	3.00	.00	3.00	.00	.00	1.00	1.00
83	3.00	3.00	3.00	1.00	.50	1.00	1.00
84	3.00	.00	1.00	1.00	1.10	1.00	1.00
85	3.00	3.00	1.00	.00	.25	1.00	1.00
86	3.00	.00	1.00	.00	.66	1.00	.00
87	3.00	.00	1.00	.00	4.30	3.00	1.00
88	3.00	3.00	.00	.00	3.10	2.00	1.00
89	3.00	.00	.00	.00	2.10	2.00	1.00
90	3.00	.00	3.00	.00	.80	1.00	2.00

	pcna	tscore1	baxcyf1	bcl2cyf1	tumsize	tumscore	recurren
61	2.00	1.00	1.00	1.00	1.20	4.00	1.00
62	1.00	2.00	.00	.00	1.80	8.00	1.00
63	2.00	1.00	.00	.00	1.50	7.00	1.00
64	1.00	1.00	.00	1.00	4.00	4.00	1.00
65	.00	1.00	.00	.00	2.00	8.00	1.00
66	2.00	1.00	1.00	.00	5.30	.00	1.00
67	2.00	1.00	.00	1.00	3.00	8.00	1.00
68	.00	1.00	.00	1.00	4.00	5.00	1.00
69	3.00	1.00	.00	.00	1.80	8.00	1.00
70	3.00	1.00	1.00	1.00	1.60	5.00	1.00
71	.00	1.00	.00	.00	3.00	9.00	1.00
72	3.00	1.00	.00	.00	2.20	7.00	1.00
73	1.00	1.00	.00	.00	2.50	8.00	1.00
74	1.00	2.00	.00	.00	1.80	8.00	1.00
75	3.00	1.00	.00	.00	3.00	5.00	1.00
76	2.00	2.00	1.00	.00	7.00	9.00	1.00
77	3.00	2.00	.00	.00	3.50	7.00	1.00
78	1.00	1.00	.00	1.00	2.00	7.00	1.00
79	3.00	1.00	1.00	1.00	3.00	4.00	1.00
80	1.00	1.00	1.00	.00	1.60	6.00	1.00
81	3.00	1.00	1.00	1.00	2.60	7.00	1.00
82	3.00	1.00	1.00	.00	.50	4.00	1.00
83	.00	1.00	1.00	1.00	3.00	8.00	1.00
84	.00	1.00	.00	.00	2.80	4.00	1.00
85	3.00	1.00	.00	1.00	8.00	5.00	1.00
86	1.00	1.00	.00	.00	5.00	8.00	1.00
87	.00	2.00	.00	.00	1.00	.00	1.00
88	3.00	1.00	.00	1.00	2.80	7.00	1.00
89	1.00	1.00	.00	00	5.00	8.00	1.00
90	3.00	1.00	1.00	.00	3.00	9.00	1.00

	lymhstat	tumorsc1	porabcl2	porap53	sizoftum	p532	pcna2
61	1.00	1.00	1.00	.00	1.00	.00	1.00
62	1.00	3.00	.00	1.00	1.00	.00	.00
63	1.00	2.00	.00	1.00	1.00	1.00	1.00
64	1.00	1.00	1.00	.00	2.00	.00	.00
65	1.00	3.00	.00	.00	1.00	.00	.00
66	1.00	.	.00	1.00	2.00	1.00	1.00
67	1.00	3.00	1.00	1.00	2.00	.00	1.00
68	1.00	1.00	1.00	.00	2.00	.00	.00
69	1.00	3.00	.00	1.00	1.00	1.00	1.00
70	1.00	1.00	1.00	.00	1.00	.00	1.00
71	1.00	3.00	.00	.00	2.00	.00	.00
72	1.00	2.00	.00	1.00	2.00	1.00	1.00
73	1.00	3.00	.00	.00	2.00	.00	.00
74	1.00	3.00	.00	.00	1.00	.00	.00
75	1.00	1.00	.00	1.00	2.00	1.00	1.00
76	1.00	3.00	.00	.00	2.00	.00	1.00
77	1.00	2.00	.00	.00	2.00	.00	1.00
78	1.00	2.00	1.00	.00	1.00	.00	.00
79	1.00	1.00	1.00	.00	2.00	.00	1.00
80	1.00	2.00	.00	.00	1.00	.00	.00
81	1.00	2.00	1.00	1.00	2.00	.00	1.00
82	1.00	1.00	.00	.00	1.00	.00	1.00
83	1.00	3.00	1.00	1.00	2.00	.00	.00
84	1.00	1.00	.00	1.00	2.00	.00	.00
85	1.00	1.00	1.00	.00	2.00	.00	1.00
86	1.00	3.00	.00	.00	2.00	.00	.00
87	1.00	.	.00	.00	1.00	.00	.00
88	1.00	2.00	1.00	.00	2.00	.00	1.00
89	1.00	3.00	.00	.00	2.00	.00	.00
90	1.00	3.00	.00	.00	2.00	.00	1.00

	ki672
61	.00
62	.00
63	1.00
64	.00
65	.00
66	.00
67	1.00
68	.00
69	1.00
70	.00
71	.00
72	1.00
73	.00
74	.00
75	1.00
76	.00
77	.00
78	.00
79	.00
80	.00
81	.00
82	.00
83	.00
84	.00
85	.00
86	.00
87	.00
88	.00
89	.00
90	1.00

	subjects	bcl2cyfr	baxcyfr	p53	tunel	tscore	ki67
91	3.00	.00	3.00	.00	.80	1.00	1.00
92	3.00	3.00	1.00	1.00	4.30	2.00	3.00
93	3.00	.00	.00	.00	.00	1.00	1.00
94	3.00	.00	3.00	.00	1.30	1.00	.00
95	3.00	.00	3.00	3.00	1.50	1.00	2.00
96	3.00	.00	.00	3.00	.60	1.00	3.00
97	3.00	.00	1.00	3.00	5.00	2.00	1.00
98	3.00	3.00	3.00	3.00	5.20	2.00	3.00
99	3.00	.00	.00	3.00	6.00	3.00	2.00
100	3.00	3.00	.00	.00	.50	1.00	1.00
101	4.00	3.00	3.00	2.00	2.80	2.00	2.00
102	4.00	3.00	3.00	.00	1.00	1.00	1.00
103	4.00	3.00	1.00	.00	2.80	2.00	.00
104	4.00	2.00	3.00	1.00	2.50	2.00	.00
105	4.00	999.00	3.00	3.00	999.00	999.00	2.00
106	4.00	3.00	1.00	2.00	2.00	1.00	3.00
107	4.00	.00	2.00	1.00	.16	1.00	1.00
108	4.00	3.00	3.00	.00	.16	1.00	1.00
109	4.00	.00	999.00	999.00	.66	1.00	.00
110	4.00	.00	.00	.00	2.00	1.00	2.00
111	4.00	3.00	3.00	.00	.25	1.00	1.00
112	4.00	3.00	.00	.00	.00	1.00	.00
113	4.00	3.00	3.00	.00	.16	1.00	1.00
114	4.00	1.00	3.00	.00	999.00	999.00	.00
115	4.00	3.00	3.00	.00	.16	1.00	1.00
116	4.00	.00	.00	2.00	.33	1.00	1.00
117	4.00	3.00	.00	.00	3.50	2.00	3.00
118	4.00	3.00	3.00	.00	2.50	2.00	.00
119	4.00	.00	2.00	.00	2.30	2.00	1.00
120	4.00	.00	.00	.00	.33	1.00	1.00

	pcna	tscore1	baxcyf1	bcl2cyf1	tumsize	tumscore	recurren
91	1.00	1.00	1.00	.00	2.80	4.00	1.00
92	3.00	1.00	.00	1.00	3.00	8.00	1.00
93	1.00	1.00	.00	.00	3.00	5.00	1.00
94	.00	1.00	1.00	.00	2.80	5.00	1.00
95	2.00	1.00	1.00	.00	2.20	7.00	1.00
96	3.00	1.00	.00	.00	2.50	.00	1.00
97	.00	1.00	.00	.00	3.00	8.00	1.00
98	3.00	1.00	1.00	1.00	999.00	999.00	1.00
99	3.00	2.00	.00	.00	8.00	8.00	1.00
100	3.00	1.00	.00	1.00	2.00	6.00	1.00
101	3.00	1.00	1.00	1.00	2.50	6.00	1.00
102	.00	1.00	1.00	1.00	1.00	4.00	1.00
103	2.00	1.00	.00	1.00	3.50	5.00	1.00
104	1.00	1.00	1.00	1.00	5.00	.00	1.00
105	.00	999.00	1.00	999.00	1.00	9.00	1.00
106	3.00	1.00	.00	1.00	3.00	9.00	1.00
107	1.00	1.00	1.00	.00	2.20	8.00	1.00
108	.00	1.00	1.00	1.00	1.40	5.00	1.00
109	.00	1.00	999.00	.00	999.00	9.00	1.00
110	.00	1.00	.00	.00	2.40	8.00	1.00
111	1.00	1.00	1.00	1.00	1.50	5.00	1.00
112	1.00	1.00	.00	1.00	2.50	4.00	1.00
113	.00	1.00	1.00	1.00	2.00	.00	1.00
114	.00		1.00	.00	.40	5.00	1.00
115	1.00	1.00	1.00	1.00	2.70	5.00	1.00
116	1.00	1.00	.00	.00	1.80	8.00	1.00
117	3.00	1.00	.00	1.00	1.80	6.00	1.00
118	.00	1.00	1.00	1.00	.00	.00	1.00
119	.00	1.00	1.00	.00	.60	7.00	1.00
120	.00	1.00	.00	.00	.50	6.00	1.00

	lymhstat	tumorsc1	porabcl2	porap53	sizoftum	p532	pcna2
91	1.00	1.00	.00	.00	2.00	.00	.00
92	1.00	3.00	1.00	1.00	2.00	.00	1.00
93	1.00	1.00	.00	.00	2.00	.00	.00
94	1.00	1.00	.00	.00	2.00	.00	.00
95	1.00	2.00	.00	1.00	2.00	1.00	1.00
96	1.00	.	.00	1.00	2.00	1.00	1.00
97	1.00	3.00	.00	1.00	2.00	1.00	.00
98	1.00	.	1.00	1.00	.	1.00	1.00
99	1.00	3.00	.00	1.00	2.00	1.00	1.00
100	1.00	2.00	1.00	.00	1.00	.00	1.00
101	.00	2.00	1.00	1.00	2.00	1.00	1.00
102	.00	1.00	1.00	.00	1.00	.00	.00
103	.00	1.00	1.00	.00	2.00	.00	1.00
104	.00	.	1.00	1.00	2.00	.00	.00
105	.00	3.00	.	1.00	1.00	1.00	.00
106	.00	3.00	1.00	1.00	2.00	1.00	1.00
107	.00	3.00	.00	1.00	2.00	.00	.00
108	.00	1.00	1.00	.00	1.00	.00	.00
109	.00	3.00	.00	.	.	.	.00
110	.00	3.00	.00	.00	2.00	.00	.00
111	.00	1.00	1.00	.00	1.00	.00	.00
112	.00	1.00	1.00	.00	2.00	.00	.00
113	.00	.	1.00	.00	1.00	.00	.00
114	.00	1.00	.00	.00	1.00	.00	.00
115	.00	1.00	1.00	.00	2.00	.00	.00
116	.00	3.00	.00	1.00	1.00	1.00	.00
117	.00	2.00	1.00	.00	1.00	.00	1.00
118	.00	.	1.00	.00	.	.00	.00
119	.00	2.00	.00	.00	1.00	.00	.00
120	.00	2.00	.00	.00	1.00	.00	.00

	ki672
91	.00
92	1.00
93	.00
94	.00
95	1.00
96	1.00
97	.00
98	1.00
99	1.00
100	.00
101	1.00
102	.00
103	.00
104	.00
105	1.00
106	1.00
107	.00
108	.00
109	.00
110	1.00
111	.00
112	.00
113	.00
114	.00
115	.00
116	.00
117	1.00
118	.00
119	.00
120	.00

	subjects	bcl2cyfr	baxcyfr	p53	tunel	tscore	ki67
121	4.00	.00	.00	.00	.00	1.00	1.00
122	4.00	.00	3.00	.00	12.80	3.00	.00
123	4.00	1.00	3.00	.00	.00	1.00	1.00
124	4.00	.00	1.00	.00	.83	1.00	1.00
125	4.00	.00	3.00	.00	.16	1.00	1.00
126	4.00	3.00	3.00	.00	.00	1.00	1.00
127	4.00	3.00	3.00	.00	.00	1.00	1.00
128	4.00	3.00	1.00	.00	.33	1.00	1.00
129	4.00	3.00	3.00	.00	.16	1.00	1.00
130	4.00	.00	3.00	.00	.00	1.00	1.00
131	4.00	3.00	3.00	.00	1.30	1.00	1.00
132	4.00	.00	1.00	1.00	1.00	1.00	1.00
133	4.00	3.00	3.00	.00	.33	1.00	1.00
134	4.00	.00	3.00	.00	.00	1.00	1.00
135	4.00	3.00	3.00	.00	.33	1.00	1.00
136	4.00	3.00	1.00	.00	2.30	2.00	1.00
137	4.00	.00	.00	1.00	.16	1.00	2.00
138	4.00	.00	.00	.00	1.80	1.00	1.00
139	4.00	.00	.00	.00	1.00	1.00	2.00
140	4.00	.00	.00	.00	2.30	2.00	1.00
141	4.00	.00	.00	1.00	5.00	2.00	2.00
142	4.00	3.00	3.00	2.00	1.00	1.00	1.00
143	4.00	.00	.00	.00	1.30	1.00	2.00
144	4.00	3.00	3.00	2.00	.00	1.00	2.00
145	4.00	.00	1.00	.00	1.50	1.00	1.00

	pcna	tscore1	baxcyf1	bcl2cyf1	tumsize	tumscore	recurren
121	1.00	1.00	.00	.00	5.00	5.00	1.00
122	1.00	2.00	1.00	.00	2.10	7.00	1.00
123	.00	1.00	1.00	.00	1.40	5.00	1.00
124	2.00	1.00	.00	.00	2.00	8.00	1.00
125	.00	1.00	1.00	.00	3.00	8.00	1.00
126	3.00	1.00	1.00	1.00	1.00	7.00	1.00
127	3.00	1.00	1.00	1.00	2.00	5.00	1.00
128	1.00	1.00	.00	1.00	1.40	4.00	1.00
129	.00	1.00	1.00	1.00	2.20	6.00	1.00
130	1.00	1.00	1.00	.00	1.10	8.00	1.00
131	1.00	1.00	1.00	1.00	2.00	4.00	1.00
132	1.00	1.00	.00	.00	1.50	5.00	1.00
133	1.00	1.00	1.00	1.00	2.00	5.00	1.00
134	2.00	1.00	1.00	.00	1.30	7.00	1.00
135	1.00	1.00	1.00	1.00	.00	.00	1.00
136	3.00	1.00	.00	1.00	4.00	6.00	1.00
137	1.00	1.00	.00	.00	2.50	8.00	1.00
138	1.00	1.00	.00	.00	3.00	5.00	1.00
139	.00	1.00	.00	.00	1.80	6.00	1.00
140	1.00	1.00	.00	.00	1.50	5.00	1.00
141	.00	1.00	.00	.00	2.80	8.00	1.00
142	3.00	1.00	1.00	1.00	2.50	5.00	1.00
143	1.00	1.00	.00	.00	3.00	8.00	1.00
144	3.00	1.00	1.00	1.00	2.20	.00	1.00
145	2.00	1.00	.00	.00	2.80	7.00	1.00

	lymhstat	tumorsc1	porabcl2	porap53	sizoftum	p532	pcna2
121	.00	1.00	.00	.00	2.00	.00	.00
122	.00	2.00	.00	.00	2.00	.00	.00
123	.00	1.00	.00	.00	1.00	.00	.00
124	.00	3.00	.00	.00	1.00	.00	1.00
125	.00	3.00	.00	.00	2.00	.00	.00
126	.00	2.00	1.00	.00	1.00	.00	1.00
127	.00	1.00	1.00	.00	1.00	.00	1.00
128	.00	1.00	1.00	.00	1.00	.00	.00
129	.00	2.00	1.00	.00	2.00	.00	.00
130	.00	3.00	.00	.00	1.00	.00	.00
131	.00	1.00	1.00	.00	1.00	.00	.00
132	.00	1.00	.00	1.00	1.00	.00	.00
133	.00	1.00	1.00	.00	1.00	.00	.00
134	.00	2.00	.00	.00	1.00	.00	1.00
135	.00	.	1.00	.00	.	.00	.00
136	.00	2.00	1.00	.00	2.00	.00	1.00
137	.00	3.00	.00	1.00	2.00	.00	.00
138	.00	1.00	.00	.00	2.00	.00	.00
139	.00	2.00	.00	.00	1.00	.00	.00
140	.00	1.00	.00	.00	1.00	.00	.00
141	.00	3.00	.00	1.00	2.00	.00	.00
142	.00	1.00	1.00	1.00	2.00	1.00	1.00
143	.00	3.00	.00	.00	2.00	.00	.00
144	.00	.	1.00	1.00	2.00	1.00	1.00
145	.00	2.00	.00	.00	2.00	.00	1.00

	ki672
121	.00
122	.00
123	.00
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127	.00
128	.00
129	.00
130	.00
131	.00
132	.00
133	.00
134	.00
135	.00
136	.00
137	1.00
138	.00
139	1.00
140	.00
141	1.00
142	.00
143	1.00
144	1.00
145	.00