MUTATION SPECTRUM OF THE APC GENE IN THE NEWFOUNDLAND PATIENTS WITH APC-ASSOCIATED POLYPOSIS CONDITIONS

AIHUA MA

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Mutation Spectrum of the *APC* Gene in the Newfoundland Patients with *APC*-associated Polyposis Conditions

by

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

Discipline of Genetics, Faculty of Medicine
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Abstract

Familial adenomatous polyposis (FAP) is an autosomal dominant colon cancer predisposition that results from germline mutations in the adenomatous polyposis coli (*APC*) gene. FAP shows substantial phenotypic variability: classical FAP patients develop more than 100 colorectal adenomas, whereas those with attenuated FAP (AFAP) have fewer than 100 adenomas and those with multiple adenomas present fewer than 50 polyps. The incidence of colorectal cancer (CRC) in Newfoundland is 27% higher than the national average. However, the mutation spectrum in this population has not been well characterized. Using direct DNA sequencing and multiple ligation-dependent probe amplification (MLPA), we performed mutation scanning of the *APC* gene in 48 unrelated Newfoundland patients with FAP/AFAP/multiple adenomas. Three previously described and one novel truncating mutation were identified in four FAP patients (44 %). Exon14 deletion was detected in one patient with AFAP (5%). Two previously known missense variants were found in 15 individuals. In addition, eight silent variants were also identified in studied patients and four of them are novel. Our results suggest: 1) the genetic predisposition to FAP in Newfoundland population is similar to that in other populations; 2) germline *APC* mutation may not be the major cause for AFAP; 3) the search for exonic deletion of the *APC* gene is necessary for mutation study on patients with AFAP.

Keywords: familial adenomatous polyposis (FAP); attenuated familial adenomatous polyposis (AFAP); multiple colorectal adenomas; adenomatous polyposis coli (*APC)*; DNA sequencing; multiple ligation-dependent probe amplification: MLPA; germline mutations; Newfoundland.

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Abbreviations

AFAP	attenuated familial adenomatous polyposis
APC	adenomatous polyposis coli
bp	base pairs
CHRPE	congenital hypertrophy of retinal pigment epithelium
CIN	chromosomal instability
CRC	colorectal cancer
EB1	end-binding protein 1
ECM	extracellular matrix
ECMs	extracolonic manifestations
ESE	exonic splicing enhancer
FAP	familial adenomatous polyposis
FISH	fluorescent in situ hybridization
hDLG	human discs large tumor suppressor protein
HNPCC	hereditary non-polyposis colorectal cancer
IBD	inflammatory bowel disease
LOH	loss of heterozygosity
MAP	MYH associated polyposis
MAPH	multiplex amplification and probe hybridization
MCR	mutation cluster region
MLPA	multiple ligation-dependent probe amplification

MYH	Mut Y Homolog
PCR	polymerase chain reaction
PTT	protein truncation test
SAP	shrimp alkaline phosphatase
SLS	sample loading solution
UC	ulcerative colitis

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Chapter 1

Introduction and Overview

Colorectal cancer (CRC) is a common cancer that affects the digestive system, and is responsible for 10% of all cancer deaths. Approximately two-thirds of CRCs are found in the large intestine and one-third in the rectum (http//www.medicinenet.com/colon cancer/). CRC usually develops from small noncancerous adenomatous polyps found in the bowel. Over time, some of these polyps become cancerous due to the sequential accumulation of mutations. Symptoms of CRC may include change in bowel habits, stool streaked or mixed with blood, discomfort or pain in the lower abdomen and tiredness (Canadian Cancer Society. 1993). CRC represents a major health burden in Western countries. Globally, CRC is the third leading cause of cancer in males and the fourth leading cause of cancer in females. The incidence of CRC differs around the world. It is common in the Western world, but rare in Asia and Africa. CRC is the second leading cause of cancer death in Canada (http//www.medicinenet.com/colon cancer/). The population of Newfoundland and Labrador has one of the highest rates of colorectal cancer in North America (Woods et al. 2005).

Although the etiology of CRC remains uncertain, a number of pre-existing medical conditions, environmental and genetic factors can be involved. Environmental factors, for example, diets high in fat and low in fiber, are implicated in the pathogenesis of CRC (Giovannucci and Willett 1994; Winawer et al. 1997). Other risk factors for CRC include inflammatory bowel disease (IBD), Crohn's disease and Ulcerative colitis (UC) (Potter.1999). Investigations have shown that the majority of CRC cases are sporadic with no significant family history of CRC. However, some individuals with CRC are

shown to have an inherited susceptibility, therefore, a whole family is identified as at high risk. Hence, understanding an inherited susceptibility to CRC cancer is a key to the identification of individuals at high risk and recommendation of effective surveillance procedures to them.

A scheme for types of CRC susceptibility is illustrated in Figure 1. Chance and the environment probably account for at least 70% of all sporadic cases, while the inherited susceptibilities to CRC including familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC)) and other polyposis syndromes, in total, account for no more than 5%. However, the remainder, perhaps about 25% represents a "multifactorial" contribution that has no identifiable hereditary cause (Walter and Bodmer. 2006). There may be other genes not yet identified, particularly those with reduced penetrance, or a multifactorial predisposition to CRC (Park et al.1999; Wei et al. 2003; Woods et al. 2005).

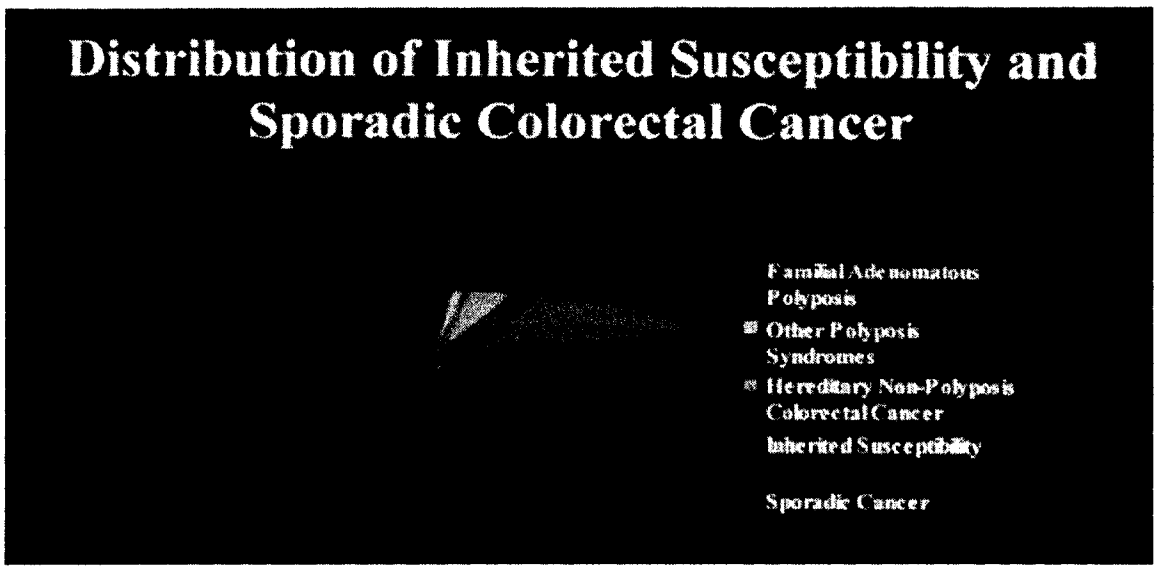

Figure 1 Scheme for inherited susceptibility to colorectal cancer (Walter et al. 2006)

1.1 *APC*-associated disorders involving an inherited susceptibility to CRC

Adenomatous polyposis coli (*APC*) - associated disorders involving an inherited susceptibility to CRC include familial adenomatous polyposis (FAP), attenuated FAP (AFAP) and multiple colorectal adenomas.

1.1.1 Familial adenomatous polyposis (FAP)

FAP (MIM 175100) is a hereditary colon cancer syndrome, first clearly described as a dominantly inherited Mendelian trait by Lockhart-Mummery in 1925 (Lockhart-Mummery. 1925). The incidence of FAP is estimated at about 1 in 8000 live newborns,

and FAP affects both sexes equally (Fearnhead et al. 2001). The most common symptoms of FAP are rectal bleeding, diarrhea, abdominal pain, mucous discharge and some symptoms related to cancer, such as weight loss, anemia, intestinal obstruction (Lynch and de la Chapelle. 2003). Some affected individuals may have extra-colonic features, such as bony growths (benign bone tumors called osteomas), which give early evidence that they are affected. Patients with FAP typically develop hundreds to thousands of adenomatous polyps throughout the entire colon and rectum. The diagnosis can be made earlier when fewer than 100 polyps are present if there is a first-degree relative with FAP. Investigations have demonstrated that almost all individuals over 40 years of age with FAP would inevitably develop colon cancer from these polyps unless they undertake prophylactic colectomy (Nagase et al. 1992). Other investigators revealed that the penetrance of FAP for inherited cases was close to 100% in the 40's (Bisgaard et al. 1994). Therefore, the risk of cancer is virtually 100% if the polyps are not detected and removed in time. Among all FAP patients, approximately 75-80% of FAP cases are familiar, and the 20-25% of sporadic cases are the result of de novo mutations (Bisgaard et al. 1994; Ripa et al. 2002). This is why some individuals with FAP have no previous family history of FAP.

In addition to colorectal polyps and cancer, the variable phenotype of FAP can include various extracolonic manifestations (ECMs) (Giardiello et al. 1994). Congenital hypertrophy of the retinal pigment epithelium (CHRPE) occurs in about 60% of FAP kindreds (Blair and Trempe. 1980). CHRPE has no impact on sight, nor any malignant

potential. It can, however, be detected by ophthalmoscopy from birth, thus helping to identify individuals at risk of FAP from an early age in those kindreds where CHRPE is present (Diaz-Llopis and Menezo.1988). Currently, it is regarded as a reliable early marker for FAP in relevant families (Berk et al. 1988; Valanzano et al. 1996). Other extra-colonic manifestations of FAP include upper gastrointestinal tumors (adenomas or carcinomas of the small intestine, fundic gland polyps or carcinomas of the stomach), intra-abdominal desmoid tumors, osteomas (bony tumors), and benign abnormalities, such as dental abnormalities, or epidermal cysts (Goss and Groden. 2000; Fearnhead et al. 2001). Other malignancies seen in a relatively small proportion of FAP patients include non-medullary thyroid cancer (usually papillary thyroid cancer), hepatoblastoma, medulloblastoma and duodenal carcinoma (Fearnhead et al. 2001; Nandakumar et al. 2004). Peri-ampullary carcinoma is the commonest cause of death in FAP patients who have undergone prophylactic colectomy (Jagelman et al. 1988; Offerhaus et al. 1992), making endoscopic screening of the upper gastrointestinal tract mandatory in FAP patients.

Importantly, studies show that there is significant variation in the associated phenotypic features of FAP, both between carriers with different mutations and, to a lesser extent, within family members with the same mutation (Giardiello et al. 1994, Nugent et al. 1994). Although the extra-colonic manifestations of this syndrome and its variants have been know for many years (Bussey.1990), and they are to some extent helpful as

diagnostic features, the diagnosis still relies largely on the detection of numerous colorectal polyps during the second or third decade of life.

FAP follows an autosomal dominant inheritance pattern, caused by germline mutation in the adenomatous polyposis coli (*APC*) gene on chromosome 5q21-22.

Table1. Benign and malignant lesions associated with familial adenomatous polyposis (Nandakumar et al. 2004).

Malignant tumours (lifetime risk)	**Other lesions**
Duodenal (5-11%)	Osteomas
Pancreatic (2%)	Radiopaque jaw lesions
Thyroid (2%)	Supernumerary teeth
Brain (medulloblastoma) (<1%)	Lipomas, fibromas, epidermoid cysts
Hepatoblastoma (0.7% of children <5 yrs)	Desmoid tumours
	Gastric adenomas / fundic gland polyp
	Duodenal, jejunal and ileal adenomas
	Nasopharyngeal angiofibromas

1.1.2 Attenuated FAP (AFAP)

AFAP or attenuated adenomatous polyposis coli (AAPC) is a more variable version of classical FAP. Affected individuals often present with fewer polyps (<100 colonic adenomas), and tend to be older at the diagnosis of their polyps (average age of 44 years). However, within a family there may be variable age at onset and variable number of

polyps (from <100 to hundreds or thousands) (Spirio et al. 1999; Plawski et al. 2007). Individuals with AFAP are still at very high risk of developing colon cancer, but the average age of colon cancer diagnosis is 50-55, 10 to 15 years later than in classical FAP (Spirio et al. 1993; Friedl et al. 1996; Giardiello et al. 1997). In some AFAP patients, extra-colonic features are infrequent (Rozen et al. 1999), although other AFAP patients, such as those with hereditary desmoid disease, have severe extra-colonic disease (Eccles et al. 1996; Scott et al. 1996). The incidence and frequency of AFAP is thought to be up to 10% of adenomatous polyposis families (Vasen. 2000). However, the true incidence and frequency of AFAP remains unknown. Similar to FAP, AFAP follows an autosomal dominant inheritance pattern, and is caused by germline mutations in the *APC* gene.

1.1.3 Multiple colorectal adenomas

Patients with multiple colorectal adenomas have a phenotype like AFAP, with 3 to 99 polyps throughout the colorectum. Affected individuals are also at high risk of developing colorectal cancer. The condition can be inherited as a Mendelian trait, either autosomal dominant or recessive, but can also occur in the form of isolated cases. Some patients with the multiple adenoma phenotype are classified as having attenuated polyposis (AFAP) owing to a germline *APC* mutation, usually in exons 1-4, exon 9, or the 3'-end (distal to codon 1580) (Knudsen et al. 2003; Young et al. 1998; Soravia et al. 1998; Sieber et al. 2006). However, most multiple adenoma patients have no identifiable

germline *APC* mutations and do not have the extracolonic manifestations sometimes associated with AFAP (Vasen. 2000; Sieber et al. 2002).

Studies have shown that a substantial proportion of multiple adenoma patients are associated with a novel type of DNA repair defect. Recent studies indicate that mutations in the base excision repair gene - human *MutY* homologue (*MYH*) cause a new autosomal recessive form of polyposis characterized by the presence of a variable number of colorectal adenomas, referred to as *MYH* associated polyposis (MAP). Oliver et al (2003) reported that of 152 patients with 3 to 100 adenomas, about 5% had disease attributable to *MYH* mutations. However, they frequently do not have an autosomal dominant family history of polyposis, (Jones et al. 2002; Sieber et al. 2003; Galiatsatos et al. 2006).

The cause of the phenotype of multiple colorectal adenomas is probably genetically heterogeneous. It is difficult to distinguish between patients with *APC* mutations and those with biallelic *MYH* mutations on the basis of clinicopathological features, although family history can be useful. Therefore, the cause of the phenotype of multiple colorectal adenomas should be elucidated by molecular classification. Patients with multiple adenomas should be classified as having *APC*-associated or *MYH*-associated polyposis.

1.2 Model for genetic alterations in the developments of *APC*-associated colorectal cancer

It is well established that development of CRC is a complex and multi-step process, in which several defective genes coordinate with each other in tumorigenesis. CRC usually develops from an adenoma (commonly known as a polyp) on the inner lining of the gastrointestinal tract. Abnormal cells arising within these adenomas will eventually progress to adenocarcinomas through histologically distinct stages, the "adenoma to carcinoma sequence" (O'Brien et al. 1990, Muto et al. 1995). Fearon and Vogelstein (1990) proposed a model that has improved the understanding of the molecular genetics of sporadic CRC (Figure 2). This model states that a series of genetic changes take place in order for the development of CRC. The process includes the interdependence of different pathways and involvement of many more gene mutations than previously recognized (Bodmer. 1996; Ilyas et al.1999). These changes include major chromosomal alterations, germline and somatic mutations. Loss of heterozygosity (LOH) commonly but variably occurs on chromosomes 5q, 8p, 17p, 18q and 22q during the adenoma-carcinoma sequence (Nowak. 2002). Mutations occur in tumor suppressor genes (e.g. *APC, DCC, SMAD4*, and *P53*) and proto-oncogenes (e.g. *K-ras*), which are also involved in the development of hereditary CRCs (Fearon and Vogelstein. 1990; Fodde. 2002). The *APC* gene, the primary cause of FAP, is described as a gatekeeper, because evidence reveals that mutations in this gene perhaps set a stage for mutations in other genes such as *K-ras, DCC*, and *p53* (Groden et al. 1991; Nishisho et al. 1991). An *APC* gene mutation appears to be an early and critical event in the progression of normal colonic epithelium to adenoma, and then to carcinoma by disturbance of proliferation regulation of colonic cells (Kinzler and Vogelstein. 1996; Fearnhead et al. 2001). However, this genetic model

is also controversial. Smith et al (2002) analyzed the mutation spectra in *APC, p53* and *K-ras* in more than 100 patients with colorectal cancer. In contrast to the prediction of the sequential model of mutation accumulation, only 6.6% of tumors were found to carry mutations in all three genes, with 38.7% of tumors containing mutations in only one of the three genes. The most common combination of mutations was *p53* and *APC* (27.1%), whereas, mutations in *K-ras* and *p53* co-occurred less frequently than expected by chance.

Lamlum et al (1999) found that in patients carrying an *APC* germline mutation, tumor development starts in the polyps when the remaining wild-type *APC* allele is mutated by somatic mutation which is consistent with Knudson's "two hit" hypothesis. However, some contradictary observations have also been reported. Polakis (1997) noted that in some circumstances one defective gene copy alone may be enough to trigger the cancer development, because the mutant *APC* gene product can influence the function of the wild-type gene product, resulting in the total loss of *APC* function (dominant negative).

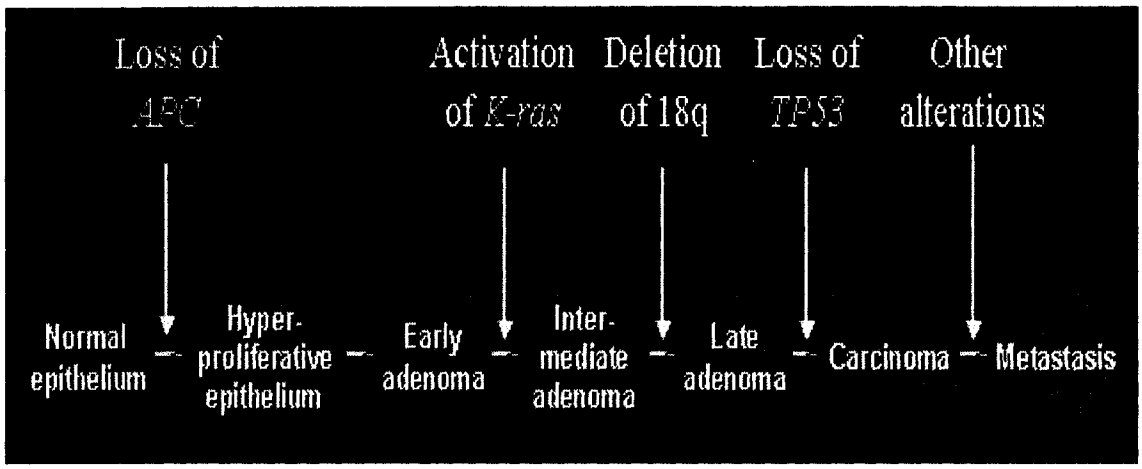

Figure 2. Model for genetic alterations in the development of colorectal cancer (Fearon and Vogelstein. 1990)

1.3 The *APC* gene and the gene product

1.3.1 *APC* gene

Herrera and Sandberg in 1986 demonstrated an interstitial deletion of the chromosomal band 5q21 in a patient with colorectal polyposis and mental retardation, but no family history of FAP (Herrera et al. 1986). This observation greatly helped to localize the *APC* gene in 1987 (Bodmer et al. 1987). Subsequent DNA linkage analysis of families with FAP led to the refined mapping of the *APC* gene to 5q21-22 (Bodmer et al. 1987; Leppert et al. 1987) (Figure 3). Loss of heterozygosity (LOH) studies had already strongly indicated the involvement of this locus in a high proportion of sporadic colorectal carcinomas (Solomon et al. 1987). Studying a smaller deletion, the location of *APC* gene

was refined by Groden et al (1991) and Kinzler et al (1991). Ultimately, the *APC* gene was cloned, characterized, and identified as the specific gene responsible for FAP by means of positional cloning (Joslyn et al. 1991; Nishisho et al. 1991).

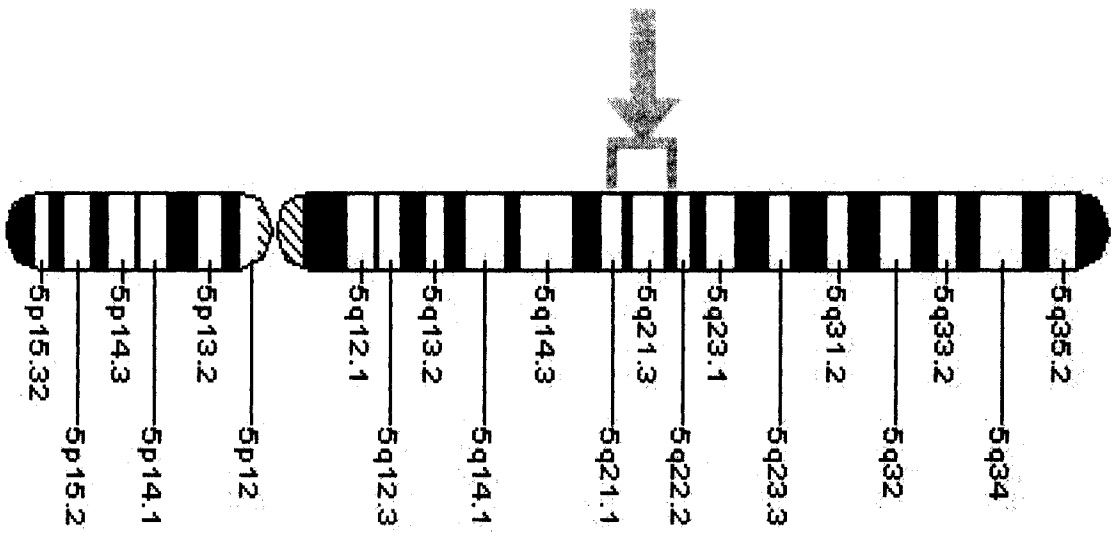

Figure 3. Chromosomal location of the *APC* gene

The *APC* gene has 15 exons with an 8535 base pair open mRNA reading frame (Van ES et al. 2001; Fearnhead et al. 2001; Foulkes et al.1995). Exon 15 is the largest exon (6500 bp length), comprising more than 75% of the coding sequence of *APC* (Powell et al. 1992; Horii et al. 1993). The *APC* gene encodes for a 312 KDa protein that consists of 2843 amino acids (Van Es et al 2001; Fearnhead et al. 2001; Foulkes. 1995). The transcriptional initiation of *APC* occurs at three sites in two distinct non-translated exons at the 5-prime end of the gene (Horii et al. 1993). At least five different forms of 5-prime noncoding sequences have been identified, which are generated by alternative splicing

(Horii et al. 1993). The splicing mechanism appears to be regulated in a tissue-specific fashion, and one transcript, expressed exclusively in brain, contains an extra exon. A diagram of the structure of the *APC* gene is presented in Figure 4.

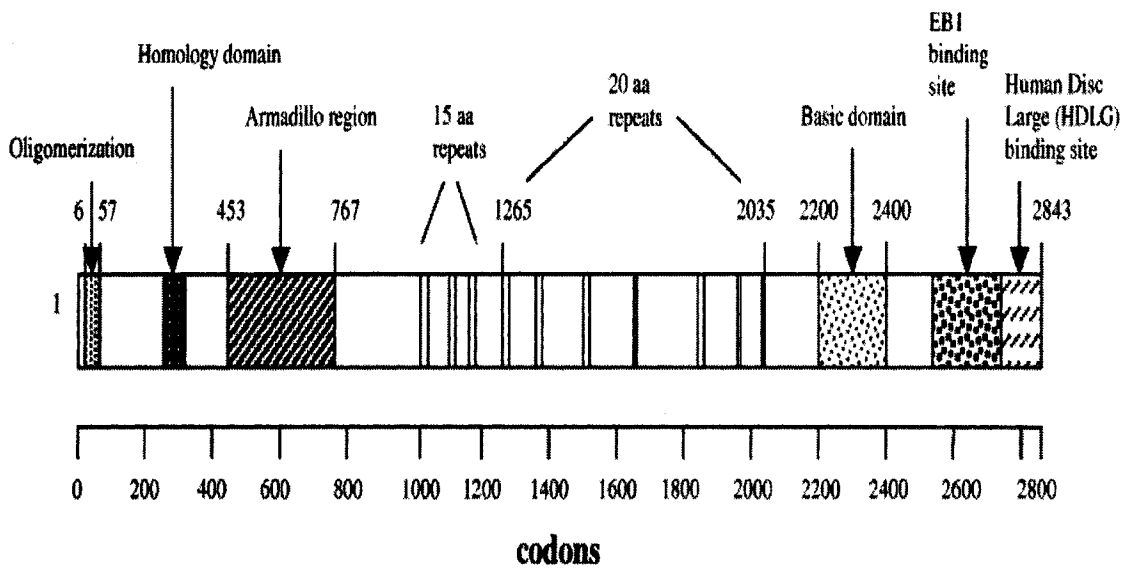

Figure 4. *APC* gene structure, cDNA (below) and important protein motifs (above).

(Van Es et al 2001)

1.3.2 The *APC* protein

The *APC* protein is a multidomain protein containing 2,843 amino acids, and expressed constitutively in a variety of fetal and adult tissues, including mammary and colorectal epithelium (Van ES et al. 2001). Within a cell, the *APC* protein exists predominantly in the cytoplasm, although nuclear localization has also been reported. It was reported to occur in several isoforms within cells, probably as a result of alternative splicing at the mRNA level (Sulekova et al. 1995). The *APC* protein is multifunctional and contains

several amino acid motifs and domains, which interact with numerous other molecules having diverse functions within the cells. The structure of the *APC* protein with different protein interaction domains is illustrated in Figure 5. At the N-terminal site, the *APC* protein contains oligomerization and Armadillo-repeat binding domains. At the C-terminal site, there are end-binding protein 1 (EB1) and human discs large tumor suppressor protein (hDLG) binding domains. Furthermore, the *APC* protein binds β–catenin through two motifs: the first contains three imperfect 15 amino acid repeats and the second comprises seven repeats of 20 amino acids, involved in the negative regulation of β–catenin protein levels in cells (Su et al. 1993; Rubinfeld et al. 1993; Rubinfeld et al. 1996; Rubinfeld et al. 1997).

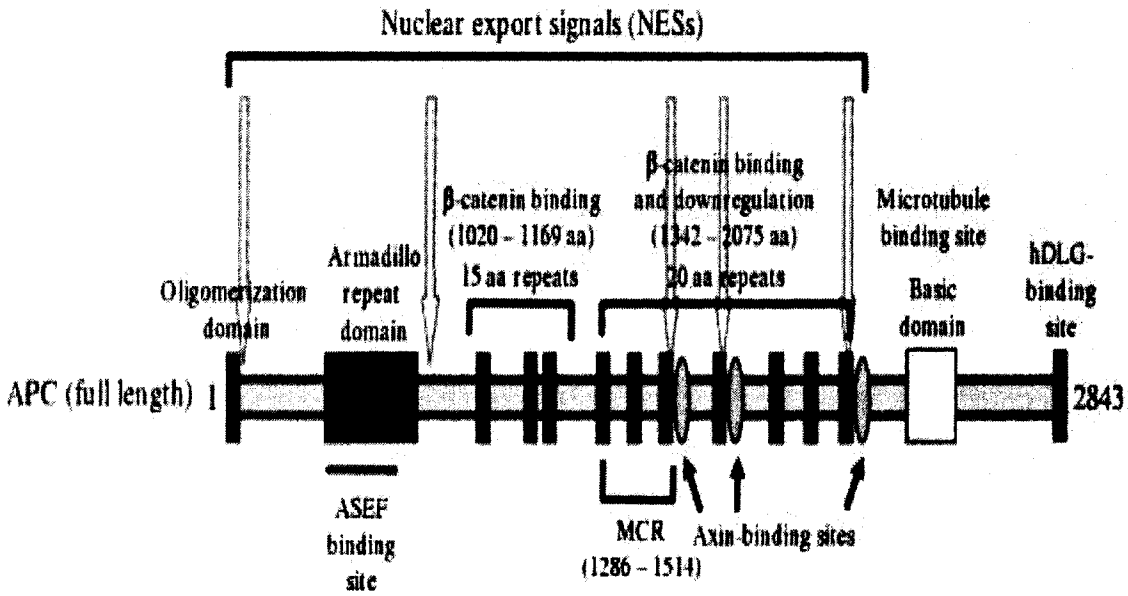

Figure 5. Structural features of the *APC* protein (Narayan et al. 2003)

1.3.3 Functions of the *APC* protein

The *APC* protein is multifunctional and plays a major role in tumor suppression by antagonizing the Wnt-signaling pathway. Inappropriate activation of this pathway through loss of *APC* function contributes to cancer progression. *APC* also has roles in cell migration, adhesion, chromosome segregation, spindle assembly, apoptosis, and neuronal differentiation.

i) *APC* protein plays a role in the control of cell proliferation

The *APC* protein plays an integral role in the Wnt-signalling pathway, especially in regard to the degradation of β-catenin within the cell cytoplasm (Bienz. 1999; Willert et al. 1998). The *APC* protein binds β-catenin and regulates intracellular levels of β-catenin through Wnt-signaling pathway, which regulates the proliferation, migration, and differentiation of cells in the intestinal epithelium. The details of Wnt-signaling pathway are illustrated in Figure 6.

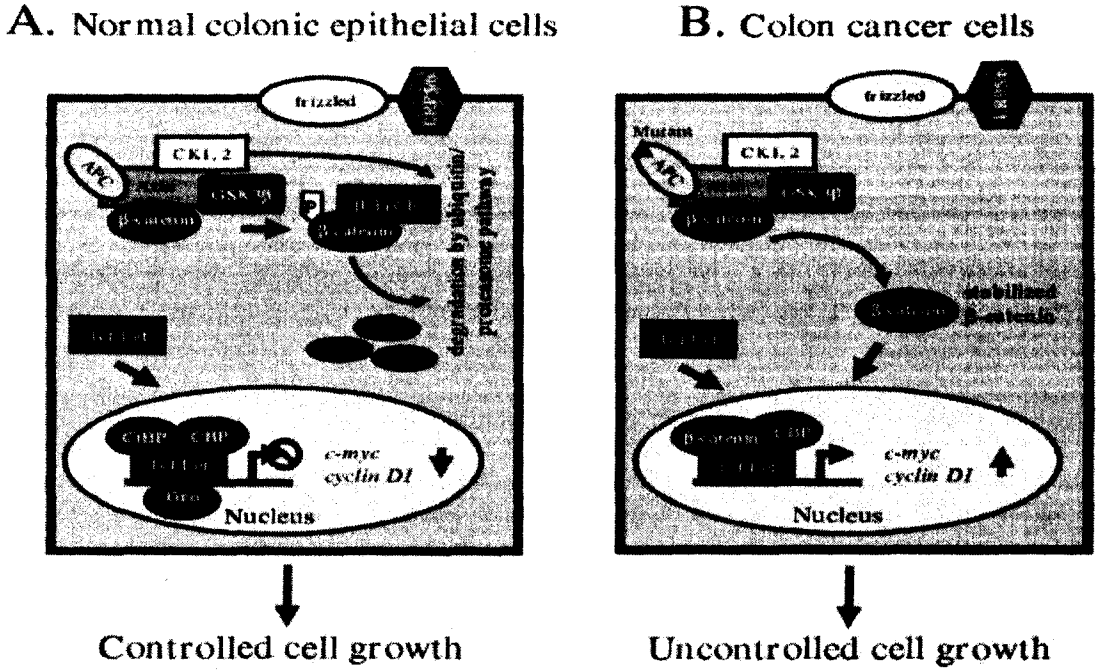

Figure 6. A model for the Wnt-signaling pathway (Narayan et al. 2003)

ii) The *APC* protein is involved in actin cytoskeletal integrity, cell-cell adhesion and cell migration

Actin cytoskeletal integrity has a very important role in maintaining the shape and adherence junctions of cells. If the balance in actin cytoskeletal integrity is destroyed, it will affect intercellular adhesion and cell migration. The *APC* protein is indirectly linked to actin cytoskeleton with β-catenin and α-catenin establishing a bridge between them (Serrano et al. 1997). It also interacts with E-cadherin through β-catenin. This forms a cell-cell adhesion complex with actin cytoskeleton, which maintains stable cell-cell adhesion (Polakis. 2000; Gumbiner. 2000) (Figure 7).

The *APC* protein also contributes to orderly migration of intestinal cells within the intestinal crypt (Moss et al. 1996; Mahmoud et al. 1997).

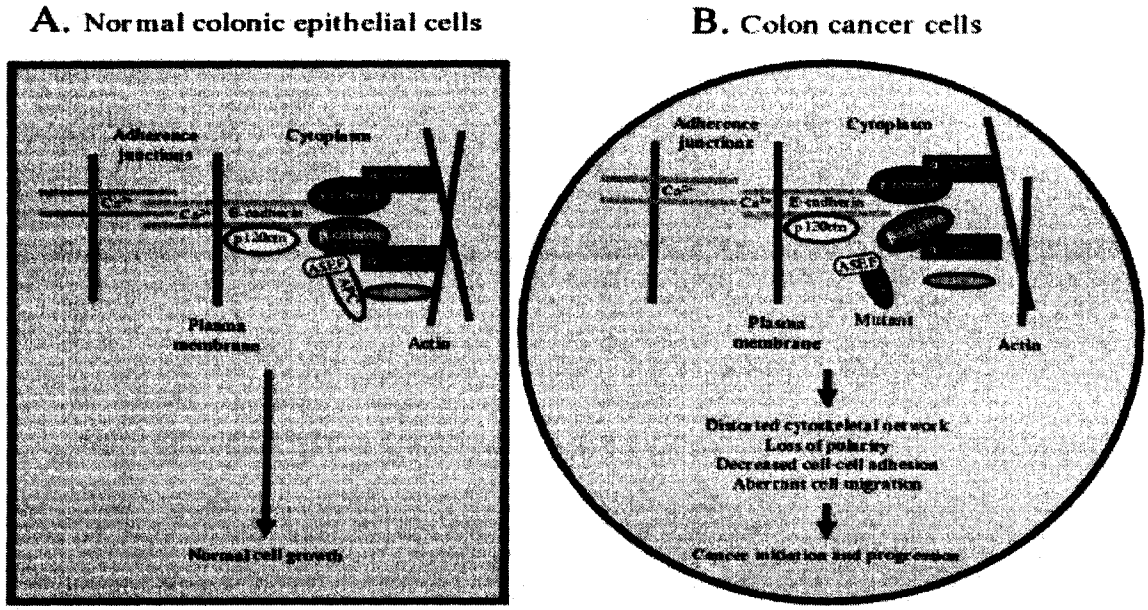

Figure 7. Mutations in *APC* gene impair actin cytoskeletal integrity, cell-cell adhesion and cell migration properties of colon cancer cells (Narayan et al. 2003)

iii) The *APC* protein is associated with chromosomal instability (CIN)

Studies demonstrate that *APC* acts as a linkage between microtubules and chromosomes, which may facilitate the spindle formation. Therefore, it is crucial in maintaining normal segregation of chromosomes at mitosis (Kaplan et al. 2001) (Figure 8).

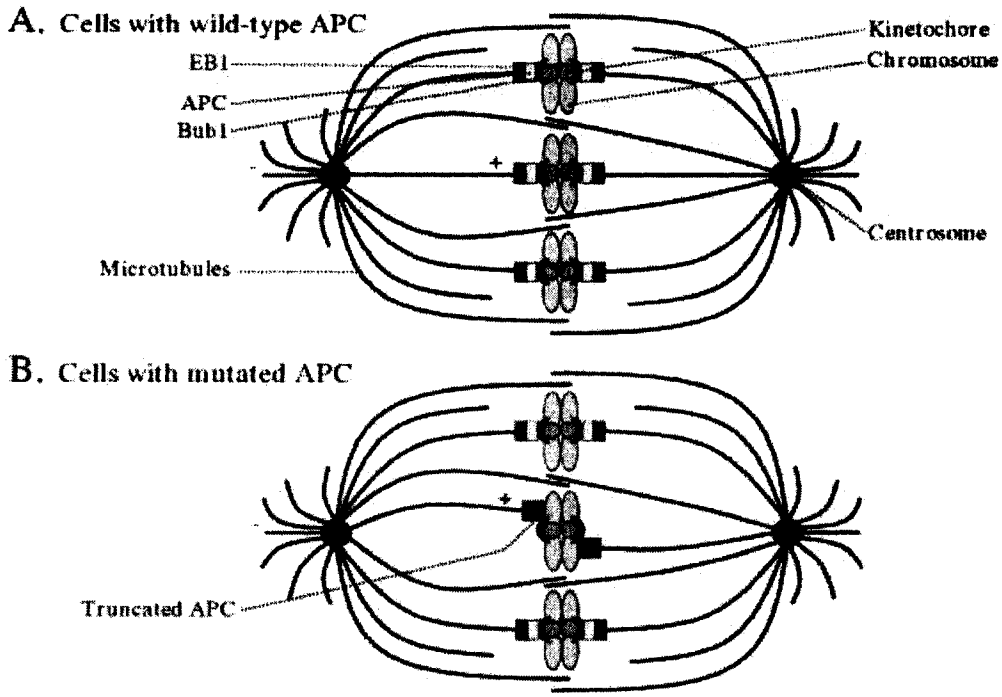

Figure 8. Chromosomal instability (CIN) in cells carrying mutations in *APC* gene (Narayan et al.2003)

iv) The *APC* protein has a role in the regulation of apoptosis

It is possible that *APC* may play an indirect role in regulation of apoptosis, as the restoration of expression of wild-type *APC* in colorectal cancer cells lacking endogenous *APC* expression has been reported to promote cell death through apoptosis (Morin et al. 1996). It is likely that *APC* may stimulate the apoptosis of cells by an indirect influence on intercellular adhesion and the extracellular matrix (ECM).

1.4 Molecular pathogenesis of FAP/AFAP/multiple colorectal adenomas

1.4.1 Germline mutations of *APC*

In approximately 80% of individuals with FAP, germline mutations can be identified within the *APC* gene (Kinzler and Vogelstein.1996; Miyoshi et al. 1992; Nagase et al.1992; Powell et al. 1993). The spectrum of identified mutations is extremely heterogeneous. To date, more than 1000 different germline mutations in the human *APC* gene have been compiled from the literature in online databases (http://perso.curie.fr/tsoussi, and http://www.cancer-genetics.org). In the majority of cases, the reported mutations cause a premature truncation of the *APC* protein (94%), usually through either a nonsense mutation (33%), or a frameshift mutation (6% small insertions, 55% small deletions) (Beroud and Soussi. 1996). Therefore, loss of function in one *APC* allele is suggested to underly the genetic susceptibility for FAP/AFAP/multiple adenomas. The reported germline mutations in *APC* are unevenly distributed over the entire gene sequence (Figure 9), and are predominantly located in the 5' end of the gene, particularly at the 5' end of exon 15 between codon 713 and 1597, referred to as the mutation cluster region (MCR) (Nagase and Nakamura. 1993). The most common germline *APC* mutations are the two 5-bp deletions, which result in frameshift mutations at codon 1061 (c.3183_3187delACAAA) and 1309 (c.3927_3931delAAAGA) (Beroud and Soussi. 1996). These two deletions account for about 11% and 17% of all germline mutations, respectively (Groden et al. 1993; Fearnhead et al. 2001). The cause of

mutational hotspots at these codons may be the repeat sequences located near these two deletions, which can cause misalignment errors in DNA replication (Mandl et al. 1994; De Rosa et al. 2003).

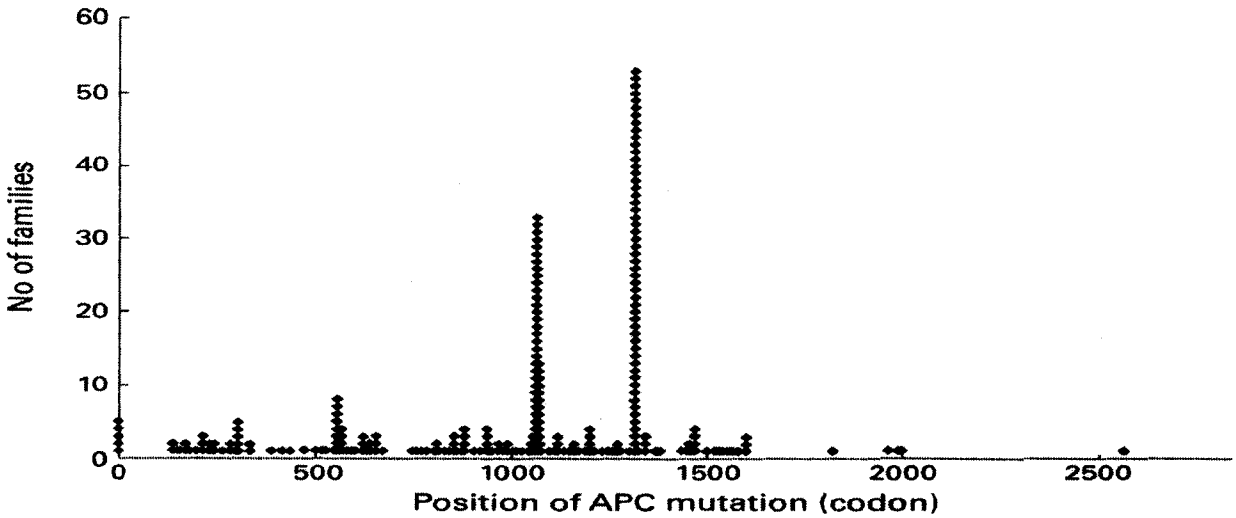

Figure 9. Distribution of identified *APC* germline mutations in 327 of 680 FAP families (Friedl et al. 2001)

Large genomic rearrangements are also found in patients with FAP, accounting for approximately 8-12% FAP cases (Sieber et al. 2002; Bunyan et al. 2004; Aretz et al. 2005; Michils et al. 2005). These genomic rearrangements may be large enough to be cytogenetically visible or may only involve one or a few exons. Cytogenetically visible interstitial deletions of chromosome 5q22, which include the *APC* gene, have been reported in individuals with adenomatous polyposis and mental retardation (Pilarski et al 1999). However, the majority of reported genomic arrangements associated with FAP/AFAP/multiple adenomas are not cytogenetically visible but are sometimes

detectable using fluorescence in situ hybridization (FISH). Deletions of exon 4, 11, 12, 14, and 15 (Su et al. 2002; Cao et al. 2001; Sieber et al. 2002), and duplication of exon 4 (McCart et al. 2006) have been described. Of these rearrangements, partial or total deletions of exon 14 are the most frequent (Su et al. 2000; Cao et al. 2001; Sieber et al. 2002). These large deletions are found more frequently in patients with classical FAP than in patients with AFAP (Sieber et al. 2002, Su et al. 2002). In Michils study (2005), large deletions were identified in 15% of classical FAP patients (4 of 27), but were absent in AFAP patients (0 of 28). However, Su et al (2002) identified deletion of the entire exon 15 of *APC* (caused by 56 kb and 73 kb deletions) in two patients. One patient presented with the typical FAP phenotype, whereas the other exhibited an AFAP phenotype.

Missense or silent variations are rarely associated with FAP. Two missense variants in *APC*, I1307K and E1317Q, are reported to be linked to an increased risk of colorectal adenoma and carcinoma. The mutation I1307K is reported to create a hypermutable region that does not lead to classic FAP, but causes an increased risk of colon cancer (Laken et al 1997). Approximately 6% of all individuals of Ashkenazi Jewish ancestry have the I1307K mutation. These individuals are predisposed to developing only a few colon polyps, but have an approximate 10-20% lifetime risk of developing colon cancer. The E1317Q mutation may be associated with a predisposition to colon adenomas and/or colon cancer (Frayling et al 1998; Lamlum et al 2000; Popat et al 2000 and Hahnloser et al 2003).

1.4.2 Genotype-phenotype correlations in FAP

Correlations between the location of a particular germline *APC* mutation and clinical features have been suggested by a large number of studies. Mutations in the central region of the *APC* gene have been correlated with a severe phenotype with thousands of polyps at a young age and with additional extracolonic manifestations (Caspari et al. 1995; Miyoshi et al. 1992). The most frequent mutation in the *APC* gene is located in this region at codon 1309. Mutations at this codon lead to FAP-related symptoms because of multiple colorectal adenomas at an average age of 20 years (Friedl et al 2001; Bertario et al 2003). Individuals with mutations between codon 168 and 1580 (excluding 1309) presented with symptoms at an average age of 30 years, and individuals with mutations 5' of codon 168 and 3' of codon 1580 presented with symptoms at an average age of 52 years (Friedl et al 2001). Profuse polyposis (an average of 5000 polyps) has been associated with mutations between codons 1250-1464 (Nagase et al 1992).

Mutations in the first or last third of the gene are associated with an attenuated polyposis with a later onset and a small number of polyps (Spirio et al. 1993; van der Luijt et al. 1996; Friedl et al. 1996). The AFAP associated regions in the *APC* gene include the 5'-end (codons 1-177 in exons 1-4), the alternatively spliced part of exon 9 (codons 311-408), and the 3'-end (distal to codon 1580) (Knudsen et al. 2003; Young et al. 1998; Soravia et al. 1998; Sieber et al. 2006) (Figure 10). Mutations at the 5' end of *APC* have been reported as the most frequent mutations associated with the AFAP. It was

speculated that the wild-type *APC* protein might be unable to form dimers with the very small truncated proteins generated from 5' end mutations, thus, causing an attenuated phenotype (Dobbie et al. 1994). This speculation was supported by another study, in which an identical 5' splice-site acceptor mutation (in *APC* intron 3) was detected in five attenuated FAP families from Newfoundland resulting from a founder effect (Spirio et al. 1999).

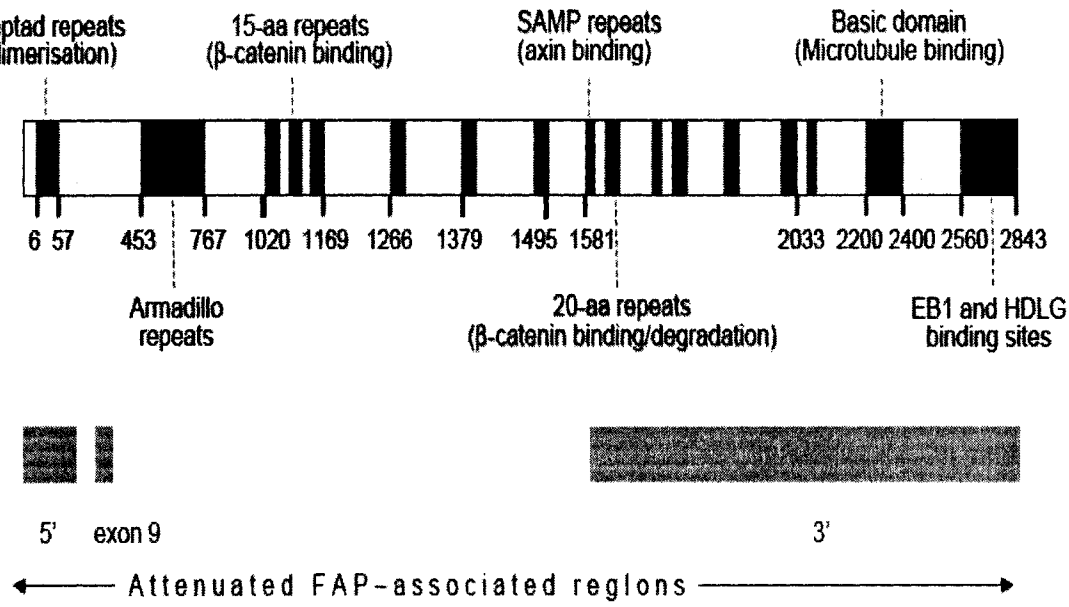

Figure 10. Attenuated FAP –associated regions in *APC* gene (Sieber et al. 2006)

Prominent extracolonic manifestations often correlate, but not completely, with more distal *APC* gene mutations. Mutations between codons 1444 and 1580 are associated with a higher incidence of desmoid tumors (Caspari et al 1995; Davies et al 1995). Mutations between codons 463 and 1387 are associated with congenital hypertrophy of the retinal

pigment epithelium (CHRPE) (Olschwang et al. 1993; Caspari et al. 1995), whereas, absence of CHRPE was correlated with mutations between codons 1444 and 1578 (Caspari et al 1995; Davies et al 1995). Individuals with mutation between codons 976 and 1067 were reported to have a fourfold increased risk in duodenal adenomas (Bertario et al. 2003). Hepatoblastoma and brain cancer were seen only in individuals with mutations in codons 457-1309. Mutations in codons 177-452 seem to be associated with absence of CHRPE, osteomas, hepatoblastomas, periampullary region tumors, or brain cancers.

1.4.3 Molecular genetic testing for mutations in *APC* gene

Because germline mutations of *APC* gene are well known to cause genetic susceptibility to FAP and AFAP, detection of *APC* germline mutations could be a powerful tool for clinical diagnosis or predictive testing for FAP/AFAP/multiple adenomas. Testing for known mutations in *APC* genes has been routinely offered in the majority of molecular diagnostic laboratories in North America, which is mainly used in confirmative, predictive and prenatal diagnosis of *APC*-associated polyposis conditions.

Mutation scanning of the entire *APC* gene by using direct DNA sequencing or other mutation scanning methods can detect up to 90% of mutations. The majority of germline mutations identified from individuals with FAP/AFAP/multiple adenomas will lead to truncation of the *APC* protein, which forms the basis for their detection by the protein

truncation test (PTT) (Powell et al. 1993). Therefore, PTT is commonly used for searching for *APC* germline mutations in most clinical diagnostic laboratories. Approximately, 80% of individuals with FAP are found to be positive for PTT. Duplications and deletions in the *APC* gene can be detected by using methods including Southern blot analysis, multiplex ligation-dependent probe amplification (MLPA), and quantitative PCR. However, these *APC* duplication/deletion analyses are still more commonly performed as a research protocol.

Germline mutations identified from the *APC* gene are highly heterogeneous and widely spread throughout the very large *APC* gene. Because of this, targeted mutation analysis, a common and efficient method for mutation analysis in molecular genetic diagnosis is not feasible. Although mutation scanning of the entire *APC* gene by using direct sequencing can detect up to 90% of mutations, this is time-consuming, costly and labor-intensive. Therefore, it is difficult for direct sequencing to be applied as a routine test in clinical service. Moreover, direct sequencing analysis cannot detect large genomic rearrangements or possible transcription defects. Southern blot is the traditional but still commonly used molecular method for analyzing large genomic rearrangements. The detection of these genomic rearrangements is based on the determination of the gene dosage changes. However, hybridization-based techniques are relatively expensive, time-consuming and large amounts of high-quality DNA are needed. Multiplex amplification and probe hybridization (MAPH) and multiplex ligation-dependent probe amplification (MLPA) are recently developed techniques used for facilitating measurement of

alterations of gene dosage, especially for detecting gene dosage changes in much smaller fragments (Armour et al. 2000; Schouten et al. 2002). These techniques have been successfully used to screen for deletions/duplications of the *APC* gene in families with FAP/AFAP, in which *APC* point mutations are not found.

1.5 FAP/AFAP/multiple colorectal adenomas in the Newfoundland population

The population of the island of Newfoundland consists mainly of descendants of English and Irish settlers who arrived in the 17th and 18th centuries (Martin et al. 2000). The geographical and social isolation of this island has ensured very little inward migration for several hundred years (Bear et al. 1987) and thus has lead to a small population (530 000 individuals; Statistics Canada 2001) with a relatively homogenous genetic background. Founder mutations have been demonstrated in a number of genetic diseases including FAP (Spirio et al. 1999).

Newfoundland has the highest incidence of CRC among all Canadian provinces, which is 27% higher than the national average (Canadian Cancer Statistics. 2003). Currently, at least 25 Newfoundland families with FAP/AFAP/multiple adenomas have been clinically identified, and there are approximately 50 families, which are suspected to have FAP, AFAP or multiple colorectal adenomas.

1.6 Rationale for the proposed study

The spectrum of *APC* mutations in a large number of families with FAP/AFAP/multiple adenomas in the Newfoundland population remain unknown: Searching for germline mutations of the *APC* gene in the Newfoundland population has only been performed in a limited number of families with clinically diagnosed FAP or AFAP or multiple adenomas and three mutations have been identified. However, molecular defects in the majority of Newfoundland families with FAP / AFAP/ multiple adenomas have not been identified. Genetic testing is therefore not possible for these families with unidentified mutations.

Members of FAP or AFAP or multiple adenomas families are at risk of developing colon cancer, and identification of genetic predisposition will be of clinical benefit: Individuals with an *APC* germline mutation will develop multiple polyps in the colon and have an 80 -100% lifetime risk that one or more polyps will progress to colon cancer. Morbidity and mortality can be significantly reduced if at-risk individuals are followed with a colonoscopy-screening program, and the colon is removed surgically when multiple polyps are identified. Since FAP/AFAP is an autosomal dominant condition, each first-degree relative of an affected person has a 50% chance of having inherited the same mutation. The screening program is most efficient when the family-specific mutation in the *APC* gene is known and genetic testing is offered to those at-risk. Then colonoscopy screening is only required by those who have the identified mutation. Therefore, genetic

testing and management that will detect or avoid CRC cancer in these families are very cost effective.

Identification of non-carriers for the family germline mutation will also have clinical and social benefits: Many individuals in high-risk families are not at increased risk of FAP or AFAP or multiple adenomas because they did not inherit the "family" mutation (non-carriers). They usually suffer an unnecessary physical challenge from routine colonoscopy screening if their non-carrier status is unknown. For those who did not inherit the family mutation, genetic testing will allow the tested individual to have a permanent release from psychosocial and physical suffering resulting from unnecessary routine colonoscopy and worry about cancer risk. There will also be savings in health care costs when unnecessary colonoscopy screening and clinical follow-up can stop.

The purpose of the study: The proposed project is a) to perform a mutation study in all Newfoundland families with patients who have clinical indication of FAP/AFAP/multiple adenomas; b) to understand the population genetics of FAP/AFAP/multiple adenomas in Newfoundland, its origin, and potentially a better understanding of the phenotypic variation associated with particular mutations in this isolated population. Mutation screening by using a combination of direct sequencing analysis and MLPA analysis will allow us to identify all possible point mutations and also to detect large genomic rearrangements in the *APC* gene. Successful identification of these mutations will be of long-term benefit to the Newfoundland population because a) genetic predisposition

screening can be offered to family members who are at risk for FAP; b) definitive genetic counseling and clinical guidance can then be provided to at-risk individuals who have the genetic testing.

Chapter 2

Patients and Methods

2.1 Clinical criteria used in phenotype classification

i) Classical FAP

- Individual with or without an autosomal dominant family history of CRC and/ or polyps who has more than 100 colorectal polyps.
- Or, individual with an autosomal dominant family history of CRC and/ or polyps and / or extra colonic features associated with FAP who has fewer than 100 colorectal polyps before age of 35 years.

ii) Attenuated FAP

- Individual who has an autosomal dominant family history of CRC and/ or polyps with a variable number of polyps (fewer than 20 polyps to 100 polyps), variable age at onset, and variable location of polyps.
- Or, individual with no family history who has 10-100 polyps

iii) Multiple colorectal adenomas

Patients of this category include those who have family history of CRC and/ or polyps (<50 polyps), but do not meet the criteria either for classical FAP or for AFAP. The majority of these patients have fewer than 10 polyps.

2.2 Patients

In total, 48 unrelated patients (29 males, 19 females) were involved in present study. These patients represent 48 different families from Newfoundland. All patients had been examined by colonoscopy or had a colectomy, and histological studies, and the diagnoses of classical FAP, AFAP or multiple colorectal adenomas for these patients were determined using the clinical criteria mentioned above.

Of the 48 selected patients, nine were classified as classical FAP; 20 as AFAP; 19 as multiple adenomas. The details of clinical features from all of the patients are presented in Table 2. Two healthy individuals (unaffected with any cancer) were included in the study panel as healthy controls.

The present study was approved by the Human Investigations Committee of the Faculty of Medicine, Memorial University of Newfoundland, and the Health Care Corporation of St. John's. All informed consents for genetic testing were obtained.

Table 2. Clinical characteristics of the 48 patients tested for *APC* germline mutations

Patient No	# of polyps/ age at diagnosis of polyps	CRC/ age at diagnosis of CRC	Extracolonic Manifestation	Family History	Phenotype
75	100-1000/ 63	No		No	FAP
195*	>1000/ 57	No		No	FAP
222	>1000/ 36	Yes/ 49	desmoids,osteomas,epidermoid	Yes	FAP
884	>1000/ 51	Yes/ 51		No	FAP
1168	100-1000/ 27	No	CHRPE, others	Yes	FAP
1213	100-1000/ 40	Yes/ 40		Yes	FAP
10912	100-1000/ 11	No	CHRPE,	Yes	FAP
11572	>1000/ 31	No	duodenal polyposis	No	FAP
12426	10-100/ 25	No	desmoids, other	Yes	FAP
18	10-100/ 54	Yes/ 54	gastric adenomas	Yes	AFAP
648*	10-100/ 45	Yes/ 45		No	AFAP
954	150/ 56	Yes/ 56		Yes	AFAP
1101	10-100/ 47	Yes/ 47	other	Yes	AFAP
1167	200/ 59	Yes/ 59		Yes	AFAP
1212	10-100/ 77	No		Yes	AFAP
1215	10-100/ 46	No		Yes	AFAP
1320	150/ 44	Yes/ 44		Yes	AFAP
1606	10-100/ 52	No		Yes	AFAP
2052	10-100/ 62	Yes/ 62		Yes	AFAP
10620	100/ 45	Yes/ 45	other	Yes	AFAP
11160	10-100/ 51	No	duodenal polyposis	Yes	AFAP
11417	10-100/ 57	No		Yes	AFAP
11468	150/ 25	No		Yes	AFAP
11988	<10/ 60	Yes/ 69		Yes	AFAP
12071*	10-100/ 38	No		Yes	AFAP
12082	10-100/ 52	No	other	Yes	AFAP
12127	10-100/ 29	No		Yes	AFAP
12204	10-100/ 40	No	other	Yes	AFAP
12597	10-100/ 55	Yes/ 65		Yes	AFAP
63	10-100/ 71	Yes/ 71		Yes	MA
213	<10/ 51	No		Yes	MA
415	<10/ 58	Yes/ 58		No	MA
464	10-100/ 54	Yes/ 56		Yes	MA
931	0/ 44	Yes/ 44		Yes	MA
1096	10-100/ 54	Yes/ 54		Yes	MA
1307	<10/ 54	Yes/ 56		Yes	MA
1349	10-100/ 47	No		Yes	MA
1685	0/ 45	Yes/ 45		Yes	MA
1744	Unknown	Yes/ 43		Yes	MA
11069	10-100/ 52	No		Yes	MA
11102	<10/ 43	No	other	Yes	MA
11308*	<10/ 61	Yes/ 61		Yes	MA
11431*	10-100/ 58	No	other	Yes	MA
11867	<10/ 79	Yes/ 79		Yes	MA
12174	<10/ 63	No		Yes	MA
12566	0/ 70	Yes/ 70		Yes	MA
12603	<10/ 54	Yes/ 57		Yes	MA
12905	<10/ 57	Yes/ 57		No	MA

FAP indicates familial adenomatous polyposis

AFAP indicates attenuated familial adenomatous polyposis

MA indicates multiple adenomas

Asterisk(*) indicates that DNA samples were not available for MLPA test

2.3 DNA extraction from whole blood

Genomic DNA was extracted from the white cells of venous blood, which was collected from subjects in Na_2-EDTA vacutainer tubes. DNA extraction was performed using a salting-out method described by Miller et al (1988) with the following modifications:

5 ml of TKM_1 buffer (10 mM Tris, 10 mM KCL, 10 mM $MgCl_2$, 2 mM EDTA, pH 7.6) was added to the blood, followed by 1.25 ml of a membrane-lysing agent, 10% Igepal (Sigma - a non-ionic detergent; Octylphenyl-polyethylene glycol). The tube was mixed by inversions, and then centrifuged at 2200 rpm for 10 minutes at room temperature. The supernatant was poured off and the nuclear pellet was saved. The pellet was then washed twice with 10ml of TKM1 buffer, re-suspended in 0.8 ml of TKM_2 buffer (10mM Tris HCl, 10mM KCl, 10mM $MgCl_2$, 4M NaCl, 2mM EDTA pH7.6), and transferred to a 1.5 ml microcentrifuge tube containing 50 µl of 10% SDS, and then incubated for 30 minutes at 50 ^{0}C for complete nuclear membrane lysis. Following this incubation, 0.4 ml of 5M NaCl was added to the tube, mixed and centrifuged at 14000 rpm for 20 minutes. The supernatant was saved and transferred to a 15 ml centrifuge tube, and 2.4 ml of 90% ethanol was added to precipitate DNA. The precipitated DNA was transferred to an

eppendorf tube and dissolved in 100-200 µl TE buffer (10mM Tris-HCl, 0.2 mM Na_2EDTA, pH 7.5). Then the concentration was determined by UV spectrophotometry measured absorbance at 260 and 280.

2.4 Mutation scanning - direct DNA sequencing

2.4.1 Primers for PCR amplification

Direct DNA sequencing was used to perform germline mutation screening for the *APC* gene in the present study. The genomic DNA sequence of the *APC* gene was amplified in a total of 38 PCR amplicons, which cover the entire coding region, the splice sites and partial 5' and 3' non–coding regions of the *APC* gene. The sequences of all 38-paired primers used for *APC* gene analysis are presented in Table 3. All the primers are described from 5' → 3'direction. The sequences of the majority of the primers were reported in a previous study (Groden et al. 1991). The remainder (denoted with *) were designed using the computer software of IDT's Primer Quest (http://scitools.idtdna.com/primerquest/) (All primers were purchased from Applied Biosystems Foster City, CA).

Table 3. PCR primers for sequence analysis of 15 exons of the *APC* gene

Exon	Forward primer 5'-3'	Reverse primer 5'-3'
1*	ccactgtttcatcctcttagatgc	atcactgtactgaggcaaggt
2*	gtgcgtgctttgagagtgatctga	gcttgttgctattctgccagtcac
3*	tttaccctgacccaagtggac	cgtttcctgggattctgaagacct
4*	ttagcactttaggtagagaagtttgc	caagcactaaagctggtttgttt
5*	ggatccagattgagtctgacacct	tagatggtggtcttccggtagcta
6	ggtagccatagtatgattatttct	ctacctatttttatacccacaaac
7	aagaaagcctacaccatttttgc	gatcattcttagaaccatcttgc
8*	cagtctttggttaagtccattctgc	cttgaactcctggcctcaagtgat
9-1	agtcgtaattttgtttctaaactc	tgaaggactcggatttcacgc
9-2	tcattcactcacagcctgatgac	gctttgaaacatgcactacgat
10	aaacatcattgctcttcaaataac	taccatgatttaaaaatccaccag
11	gatgattgtctttttcctcttgc	ctgagctatcttaagaaatacatg
12*	gaccaaggcaagtgttacacacaca	tgcagtgagctgagattgcacaac
13*	agtcaccacggctagccagaattt	aggttgcagtgagccaagatcaga
14	tagatgacccatattctgtttc	tagatgacccatattctgtttc
15-1	gttactgcatacacattgtgac	gctttttgtttcctaacatgaag
15-2	agtacaaggatgccaatattatg	acttctatctttttcagaacgag
15-3	atttgaatactacagtgttaccc	cttgtattctaatttggcataagg
15-4	ctgcccatacacattcaaacac	tgtttgggtcttgcccatctt
15-5	agtcttaaatattcagatgagcag	gtttctcttcattatattttatgcta
15-6	aagcctaccaattatagtgaacg	agctgatgacaaagatgataatg
15-7*	agaggcagaatcagctccatccaa	actgcatggttcactctgaacgga
15-8	atctccctccaaaagtggtgc	tccatctggagtactttccgtg
15-9*	agctcaaaccaagcgagaagtacc	acaatacacccgtggcaatcatcc
15-10	cccagactgcttcaaaattacc	gagcctcatctgtacttctgc
15-11	ccctccaaatgagttagctgc	ttgtggtataggttttactggtg
15-12	acccaacaaaaatcagttagatg	gtggctggtaactttagcctc
15-13	atgatgttgacctttccaggg	attgtgtaacttttcatcagttgc
15-14	aaagacataccagacagaggg	ctttttggcattgcggagct
15-15	aagatgacctgttgcaggaatg	gaatcagacgaagcttgtctagat
15-16	ccatagtaagtagtttacatcaag	aaacaggacttgtacttgagga
15-17	cagcccttcaagcaaacatg	gaggacttattccatttctacc
15-18	cagtctcctggccgaaactc	gttgactggcgtactaatacag
15-19	tggtaatggagccaataaaaagg	tgggagttttcgccatccac
15-20	tgtctctatccacacattcgt	atgtttttcatcctcacttttttgc
15-21	ggagaagaactggaagttcatc	ttgaatctttaatgtttggatttgc
15-22	tctcccacaggtaatactccc	gctagaactgaatggggtacg
15-23	caggacaaaataatcctgtccc	attttcttagtttcattcttcttcctc

2.4.2 PCR amplification and product purification

DNA PCR amplification was carried out in a total of 25 µl of PCR reaction consisting of approximately 100-200 ng genomic DNA, 2.5 µl dNTPs (10 mM), 0.5 µl (2.5 µM/µl) each primer and 0.3 µl (5 units /µl)Taq DNA polymerase, 1.5 µl (50mM) $MgCl_2$, 2.5 µl (10 mM KCl, 10 mM $(NH_4)_2SO_4$, Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100 PH 8.8) 10x buffer (All dNTPs, PCR buffer and DNA polymerase were purchased from Applied Biosystems Foster City, CA). The PCR amplifications were performed by using the Eppendorf MasterCycler (Eppendorf AG, Hambeug, Germany). All samples were amplified by using the conditions as follows: 95 ^{0}C for 5 min followed by 35 cycles of 94 ^{0}C for 1 min, 50 ^{0}C -58 ^{0}C for 1 min, 72 ^{0}C for 1 min, plus a final extension of 72 ^{0}C for 10 min.

The quality of the DNA amplification was analyzed by electrophoresis of the PCR products on 2 % ethidium bromide stained agarose gels, which were visualized under UV transilluminator. The presence of bands of the appropriate length confirmed the successful PCR amplification.

Purification of PCR products was carried out using the QIAquick Multiwell PCR purification kit (Qiagn) on a Mastercycler. Briefly, each 5 µl of PCR amplification mixture was mixed with 1µl (10 units / µl) of Exonuclease I and 2 µl (1 unit / ul) of Shrimp Alkaline Phosphatase (SAP), to remove excess primers and free dNTPs. The

reaction was carried out at 37 ^{0}C for 15 min, and terminated by heating at 80 ^{0}C for 15 min (inactivation of the enzyme within the reaction).

2.4.3 Taq-polymerase catalyzed cycle sequencing using fluorescent-labeled dye terminator reactions and post-reaction cleanup

Cycle sequencing was performed in a 20 µl PCR reaction by using CEQ DTCS-Quick Start Kit (Dye Terminator cycle sequencing) on a Mastercycler according to the manufacturer's instructions, with certain modifications. Briefly, each 4 µl of purified PCR product was added to 16 µl of mixed solution containing DNA polymerase, CEQ Dye terminators (ddUTP, ddUTP, ddGTP, ddCTP, and ddATP), dNTPs, sequencing reaction buffer (Applies Biosystems), and primers. The reaction was then subjected to 95 ^{0}C for 4 min, as an initial denaturing step, followed by 40 cycles of 95 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, and 72 ^{0}C for 1 min.

Subsequently, ethanol precipitation was used to clean up the free ddNTPs and the residual salts from each reaction. Briefly, the sequencing products were transferred to a 0.5 ml microcentrifuge tube containing 5 µl of stop solution (2µl of 3 M NaOA pH 5.2, 2 µl of 100 mM EDTA pH 8.0, and 1 µl of 20 µg /µl Glycogen), 60 µl of cold (-20 ^{0}C) 95 % ethanol was then added and the tube was centrifuged at 14, 000 rpm for 15 min. The DNA pellet was saved by discarding the supernatant, and rinsed two times with cold (-20 ^{0}C) 200 µl of 70% ethanol to remove the unincorporated dye-terminators and the residual

salts. For each rinse, cold (-20 ^{0}C) ethanol was added and followed by an immediate centrifugation at 14, 000 rpm for 2 min. Finally, the DNA pellet was dried in a sealed vacuum centrifuge for 15 min, and then re-suspended in 20 µl of sample loading solution (SLS).

2.4.4 Gel electrophoresis and data analysis on the DNA sequencer

Each 20 µl of the purified reaction mixture was loaded and analyzed on an automated CEQ Beckman 8000 Genetic Analysis System (Beckman Coulter, Inc, Fullerton, CA) according to the manufacturer's instructions. Sequence analysis was conducted using software according to the manufacture's guidelines. All analyzed DNA fragments were sequenced in both directions. Sequence results were aligned with wild type sequences achieved from Genbank (M73547), and the identified sequence variances were evaluated by the sequencher sequence alignment program (ACGT Codes). We are using HGVS nomenclature to describe mutations, i.e. numbering from the first A of the start codon, as opposed to the first residue given in the Genbank entry used.

2.5 Genomic rearrangement analysis - multiplex ligation-dependent probe amplification (MLPA)

2.5.1 MLPA test kit

The MLPA analysis was carried out using the kit SALSA P043 *APC* from MRC Holland (Amsterdam, The Netherlands, www.mrc-holland.com). The kit contains 23-paired probes, which were designed for analyzing each exon of the gene in question (exons 1–15, including exon 10A) including promoter regions. The promoter region was covered by two paired probes, and each individual exon (exon 1-14, plus exon 10A) was analyzed by a single probe. The largest exon, exon 15, was assayed by five paired probes, which are located in different regions of exon 15. Among the five paired exon 15 probes, two were specifically designed for the two mutation hotspots in exon 15 (codon 1061, codon 1309). In addition, 14-paired probes from other chromosomal regions plus further controls to check for adequate quality of test DNA and efficient ligation were used as controls.

2.5.2 DNA denaturation and hybridization of the SALSA-probes

For each sample, approximately 250 ng of genomic DNA in 5 µl of TE buffer (10 mM Tris–HCl, pH 8.5 and 1 mM EDTA) was denatured at 98°C for 10 min. Hybridization was performed by adding 1.5 µl SALSA-probes mixture (1fmol of each synthetic probe oligonucleotide in TE) and 1.5 µl MLPA buffer (1.5 M KCl, 300 mM Tris–HCl pH 8.5, 1 mM EDTA) to denaturized genomic DNA. The reaction was first incubated at 95°C for 1 min and then at 60°C for 16 hours.

2.5.3 Ligation reaction

After hybridization, ligation of annealed paired probes was performed by adding ligase-65 enzyme buffer (1 unit ligase-65 enzyme, 2.6 mM $MgCl_2$, 5 mM Tris–HCl pH 8.5, 0.013% non-ionic detergents, 0.2 mM NAD) to each of the reactions. The ligation reaction was incubated at 54°C for 15 min and then terminated by heating at 98°C for 5 min.

2.5.4 PCR amplification of the ligated probes

Ligated products were amplified by PCR. 30 µl SALSA PCR buffer was added to 10 µl of the ligation reaction. 10 µl of polymerase mixture were added when the temperature reached 60°C. The polymerase mixture contained the PCR primers (10 pmol each of unlabelled and fluorescent dye D4-labelled primer), 2.5 nmol dNTPs and 2.5 units SALSA polymerase. The PCR reaction was carried out for 35 cycles (95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min).

2.5.5 PCR products electrophoresis

The PCR products were separated by using the CEQ8000 capillary electrophoresis system (Beckman Coulter, Inc, Fullerton, CA). The amplified PCR fragments from wild

type sequence of the *APC* gene presented a peak pattern of 37 and the sizes of these peaks ranged from 95 to 445 nt.

2.5.6 Data analysis

Peak areas from each tested individual were then exported to a Microsoft Excel spreadsheet, which was designed to assess the ratios of each test peak relative to all other peaks for the same individual. Ratios of test peaks to control peaks and control peaks to other control peaks in each patient sample were compared to the same ratios obtained for two normal individuals, which were included in each run.

For normal sequences, a dosage quotient of 1.0 was expected. If a deletion or duplication was present, the dosage quotient should be less than 0.6, or greater than 1.4, respectively. All samples with suspected deletions/duplications were confirmed in a duplicate analysis, and the determination of deletion/duplication was made based on consistent results from the duplicated analyses.

Chapter 3

Results

3.1 Point mutation scanning – direct sequencing of the entire *APC* gene

The genomic DNA from 48 patients who represent 48 different families was sequenced to screen for germline point mutations in the *APC* gene. The analyzed DNA sequence of the *APC* gene included the entire coding region, all splice sites and part of the 5' and 3' non–coding regions of the gene. Thirty-eight PCR amplicons were designed to cover the entire analyzed sequence, and the sizes of these PCR segments vary from 200 to 500 bp. Among the total 15 exons of the *APC* gene, 13 were covered by an individual PCR amplicon; exon 9 was divided by two overlapping PCR segments; and exon 15, the largest exon (6571bp), was covered by 23 overlapping PCR segments. In total, four truncating mutations (c.3067dupA, c.3183_3187delACAAA, c.4348C>T and c.867delC) and two missense variants, p.V1882D (c.5465T>A) and p.G2502S (c.7504G>A) were identified. In addition, a large number of silent single base pair substitutions were also found. The four truncating mutations were all identified from the FAP patients group (4 of 9, 44 %). Three of these truncating mutations have been previously reported in other studies (Groden et al. 1993; Wallis et al.1999; Pang et al. 2001), and the c.867delC mutation is a novel finding which is specific to the Newfoundland population . The two missense variants were also reported in previous studies (Gregory et al. 2005; Okkels et al. 2006). The details of these mutations and polymorphisms from the 48 families are shown in Table 4.

Table 4. Identified sequence variants of *APC* gene in the Newfoundland families with *APC* - associated polyposis conditions

Patient	Clinical	Mutations	Polymorphisms
75	FAP		c.3165A>T (p.I1055I)
195*	FAP		c.5465T>A (p.V1822D)
222	FAP	c. 4348C>T (p. R1450X)	c.5034A>G (p.G1678G); c.5880G>A (p.P1960P)
884	FAP		c.1635G>A (p.A545A); c.5880G>A (p.P1960P)
1168	FAP		
1213	FAP		c.1458T>C (p.Y486Y) ; c.1635G>A (p.A545A) c.5034A>G (p.G1678G); c.4479G>A (p.T1493T) c.5880G>A (p.P1960P)
10912	FAP	c. 3067dupA	
11572	FAP	c.3183_3187delACAAA at codon 1061	
12426	FAP	c. 867delC	c.5465T>A (p.V1822D); c.5880G>A (p.P1960P)
18	AFAP	Exon14 del	c.5034A>G (p.G1678G); c.5880G>A (p.P1960P)
648*	AFAP		
954	AFAP		
1101	AFAP		
1167	AFAP		c.4479G>A (p.T1493T); c.5034A>G (p.G1678G) c.5880G>A (p.P1960P)
1212	AFAP		c.1458T>C (p.Y486Y); c.1635G>A (p.A545A) c.5034A>G (p.G1678G); c.5465T>A (p.V1822D) c.5880G>A (p.P1960P)
1215	AFAP		c.1458T>C (p.Y486Y); c.1635G>A (p.A545A) c.5880G>A (p.P1960P)
1320	AFAP		c.1458T>C (p.Y486Y) ; c.1635G>A (p.A545A) c.5465T>A (p.V1822D); c.5880G>A (p.P1960P)
1606	AFAP		c.5465T>A (p.V1822D)
2052	AFAP		
10620	AFAP		c.1458T>C (p.Y486Y)
11160	AFAP		c.1458T>C (p.Y486Y) ; c.1635G>A (p.A545A) c.5034A>G (p.G1678G); c.5880G>A (p.P1960P)
11417	AFAP		c.5034A>G (p.G1678G); c.5880G>A (p.P1960P)
11468	AFAP		c.1458T>C (p.Y486Y) ; c.5465T>A (p.V1822D) c.7201C>T (p.L2401L)
11988	AFAP		
12071*	AFAP		c.1635G>A (p.A545A); c.5034A>G (p.G1678G) c.5465T>A (pV1822D); c.5880G>A (p.P1960P)
12082	AFAP		
12127	AFAP		
12204	AFAP		
12597	AFAP		
63	MA		

213	MA	c.1458T>C (p.Y486Y) ;	c.5880G>A (p.P1960P)
415	MA		
464	MA	c.1458T>C (p.Y486Y) ; c.5465T>A (p.V1822D);	c.5034A>G (p.G1678G) c.5880G>A (p.P1960P)
931	MA	c.1458T>C (p.Y486Y);	c.2946G>A (p.S982S)
1096	MA	c.5034A>G (p.G1678G);	c.5880G>A (p.P1960P)
1307	MA	c.1458T>C (p.Y486Y) ;	c.1635G>A (p.A545A)
1349	MA	c.5034A>G (p.G1678G)	
1685	MA	c.1635G>A (p.A545A); c.5880G>A (p.P1960P);	c.5034A>G (p.G1678G) c.7201C>T (p.L2401L)
1744	MA	c.1458T>C (p.Y486Y);	c.4479G>A (p.T1493T)
11069	MA	c.5034A>G (p.G1678G)	
11102	MA	c.1458T>C (p.Y486Y) ;	c. 5880G>A (p.P1960P)
11308*	MA	c.1458T>C (p.Y486Y) ;	c.5465T>A (p.V1822D)
11431*	MA		
11867	MA	c.5880G>A (p.P1960P)	
12174	MA	c.5465T>A (p.V1822D)	
12566	MA		
12603	MA	c.5465T>A (p.V1822D);	c.5880G>A (p.P1960P)
12905	MA	c.7504G>A (p.G2502S);	c.7201C>T (p.L2401L)

FAP indicates familial adenomatous polyposis

AFAP indicates attenuated familial adenomatous polyposis

MA indicates multiple adenomas

Asterisk (*) indicates that DNA samples were not available for MLPA test

In the patient 10912, a single bp (A) duplication was identified from exon 15 at nucleotide position 3067 (c. 3067dupA) (Figure 11a), which causes an mRNA reading frameshift after the duplicated nucleotide (A), and generates a novel stop codon at codon 1028. Patient 10912 was diagnosed as classical FAP at age 11 based on the clinical

finding of 100-1000 adenomas and congenital hypertrophy of retinal pigment epithelium (CHRPE), as well as family history of polyposis and / or CRC.

Patient 11572 was shown to carry a five bp deletion in the exon 15 (c.3183_3187delACAAA) which leads to a mRNA reading frameshift starting from codon 1061 and generating a stop codon at codon 1062 (Figure 11b). Patient 11572 was an FAP patient. He had over 1000 polyps in his colon and duodenum at age 31. He had no family history of polyposis and /or CRC, and therefore, the identified c.3183_3187delACAAA mutation may be due to a "de novo" event.

A single base pair (C) deletion in exon 8 (c.867delC) was detected in FAP patient 12426. This single base pair deletion caused a mRNA reading frameshift starting from codon 289, and a stop codon was generated at codon 292 (Figure 11C). This patient had a medulloblastoma and colorectal adenomas at age 25. She also suffered from desmoid disease and had a family history of polyps and CRC.

Patient 222 was found to carry a C>T transition at nucleotide position 4348 (c. 4348C>T), which changes codon 1450 from CGA for arginine to a stop codon, TGA, in exon 15 (p.R1450X). This patient was diagnosed as classical FAP at age of 36 because of the clinical findings of over 1000 colorectal polyps, and a number of extracolonic manifestations including desmoids, osteomas and epidermoid cysts. He developed colon cancer at age 49.

Two known missense variants, c. 5465 A>T (p. V1882D), and c. 7504G>A (p. G2502S) were detected in our studied patients. The missense variant, c. 7504G>A (p. G2502S) was identified only in patient 12905 with multiple adenomas. The missense variant, c. 5465 A>T (p. V1882D) is a common *APC* variant, which was found in 13 of our 48 patients (27%). The frequency of this missense change in subgroups of patients with FAP, AFAP and multiple adenomas was 22% (2/9), 25% (5/20) and 36% (7/19) respectively. These two missense changes have been previously reported but the effect of these variants remains unclear.

In addition to the truncating and missense changes, we also detected eight silent variants in our study patients. These silent variants were all caused by single base substitutions in the coding region. These silent variants include c.1458 T>C (p.Y486Y), c.1635 G>A (p.A545A), c.2946G>A (p.S982S), c.3165A>T (p.I1055I), c.4479G>A (p.T1493T), c.5034 A>G (p.G1678G), c.5880 G>A (p.P1960P) and c.7201C>T (p.L2401L). Among these variants, c.2946G>A (p.S982S), c.3165A>T (p.I1055I), c.4479G>A (p.T1493T) and c.7201C>T (p.L2401L) are novel, and the others are previously known.

Table 5. Frequencies of the identified sequence variants of *APC* gene in the Newfoundland patients with *APC* - associated polyposis conditions

Silent variants	Missense variants	Frequencies (%)
c.1458 T>C (p.Y486Y)		31.3
c.1635 G>A (p.A545A)		18.8
c.2946G>A (p.S982S)*		2.1
c.3165A>T (p.I1055I)*		2.1
c.4479G>A (p.T1493T)*		6.2
c.5034 A>G (p.G1678G)		33.3
c.5880 G>A (p.P1960P)		43.8
c.7201C>T (p.L2401L)*		8.3
	c.5465T>A (p.V1822D)	29.2
	c.7504G>A (p.G2502S)	2.1

Asterisks (*) indicate that the silent variants are novel

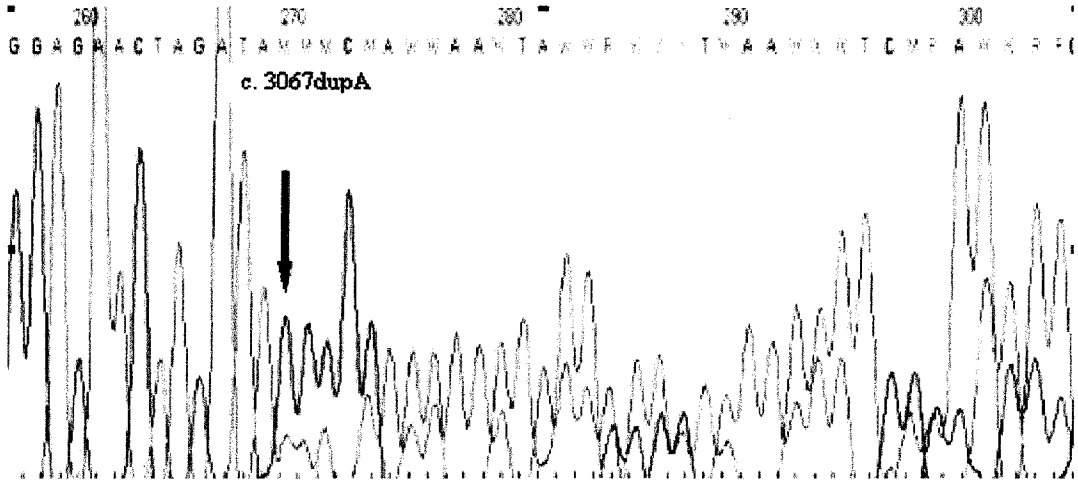

Figure 11a. Sequence result of mutation c. 3067dupA (patient 10912)

c. 3183-3187delACAAA

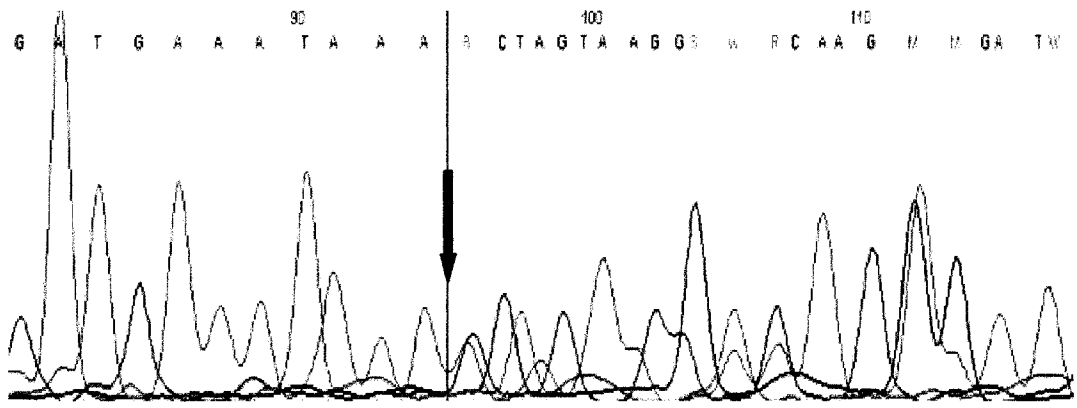

Figure 11b. Sequence result of mutation c. 3183-3187delACAAA (patient 11572)

c. 867delC

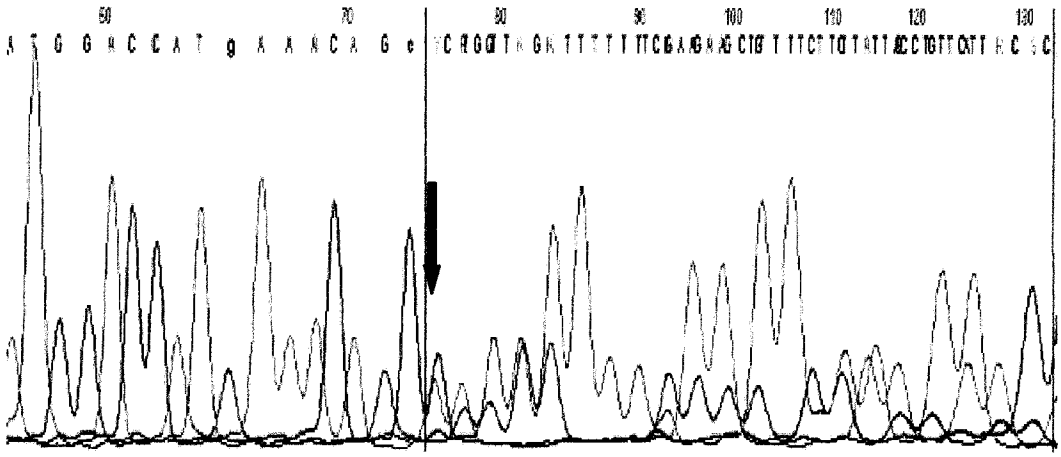

Figure 11c. Sequence result of mutation c. 867delC (patient 12426)

c. 4348C>T

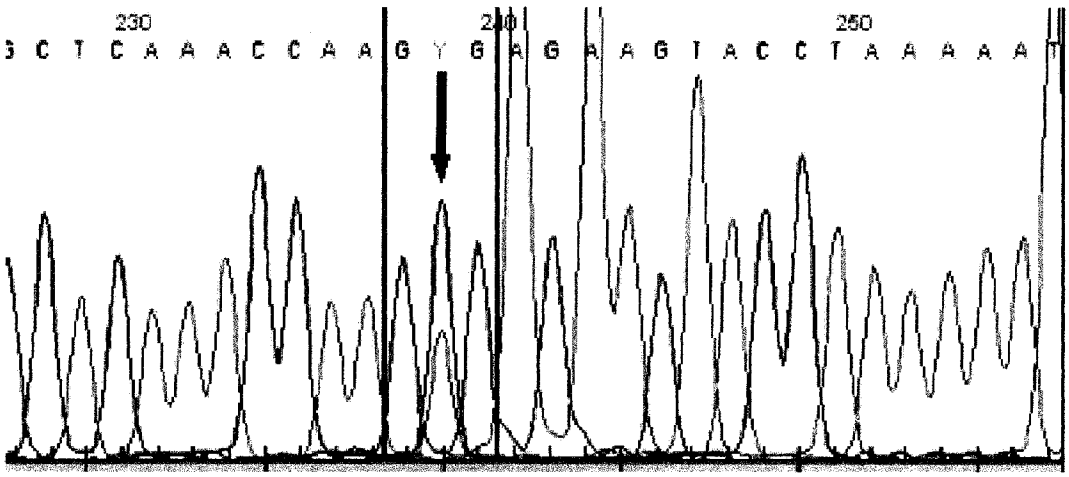

Figure 11d. Sequence result of mutation c.4348C>T (R1450X) (patient 222)

c. 5465T>A

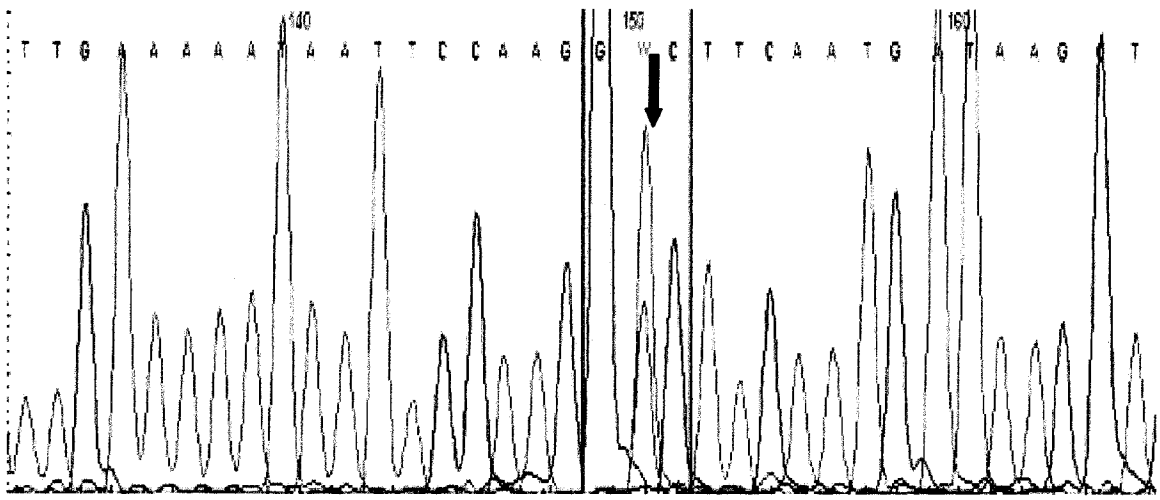

Figure 11e. Sequence result of missense mutation c.5465T>A (p.V1822D)

c. 7504G>A

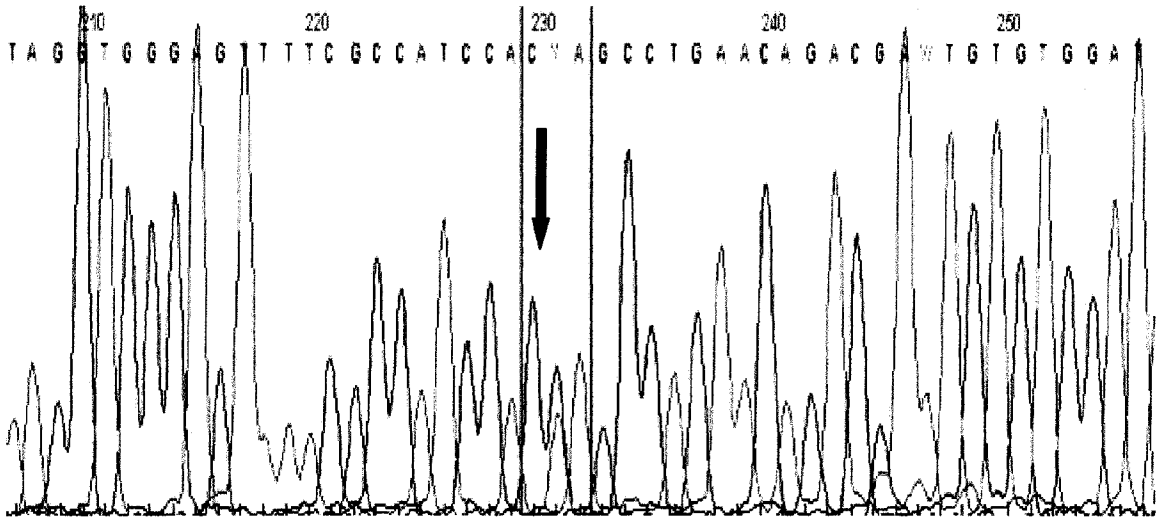

Figure 11f. Sequence result of missense mutation c.7504G>A (p.G2502S)

3.2 Searching for genomic rearrangements *in APC* - MLPA analysis

To search for *APC* germline mutations caused by exon deletion and duplication, MLPA analysis was carried out for 35 patients in whom no point mutation had been found by sequence analysis. Patient 11572 who was known to have a 5 bp deletion in exon 15 (c.3183_3187delACAAA) was also included as a positive control for MLPA analysis. Using the commercial kit for analysis of *APC* gene, MLPA analysis was performed to examine the entire *APC* gene including the promoter region. For exons 1–14, there was one PCR segment for each individual exon. Promoter region was divided into two PCR segments, and exon 15 was divided into three PCR segments, 15-1, 15-2, 15-3. Two additional PCR fragments were designed specifically to identify the two mutational hotspots [the 5 bp deletion at codon 1061(c.3183_3187delACAAA) and the 5 bp deletion at codon 1309 (c. 3927_3931delAAAGA)] in exon 15 of the *APC* gene. Based on the data from previous studies (Bunyan et al. 2004), an exonic deletion is suggested if the tested peak area is reduced greater than 40% compared with the controls. Our results showed that the peak area representing exon 14 in patient 18 was reduced more than 60% compared with the normal controls (Figure 12a), which was evident by both visual examination and calculation of peak areas. Therefore, a heterozygous exon 14 deletion was determined in patient 18. This patient was diagnosed as AFAP at age of 54 based on the findings of colorectal adenomas (at range of 10-100), CRC, gastric adenomas and family history of polyps.

Our MLPA analysis also detected a heterozygous 5 bp deletion at codon 1061 in patient 11572 (Figure 12b), which was consistent with the result obtained from our sequencing analysis. The peak area representing the 5 bp deletion (c.3183_3187delACAAA) at codon 1061 was reduced 52% compared with the corresponding control. This result for a known mutation provided good quality control for our MLPA analysis. The MLPA analysis was repeated to confirm these results.

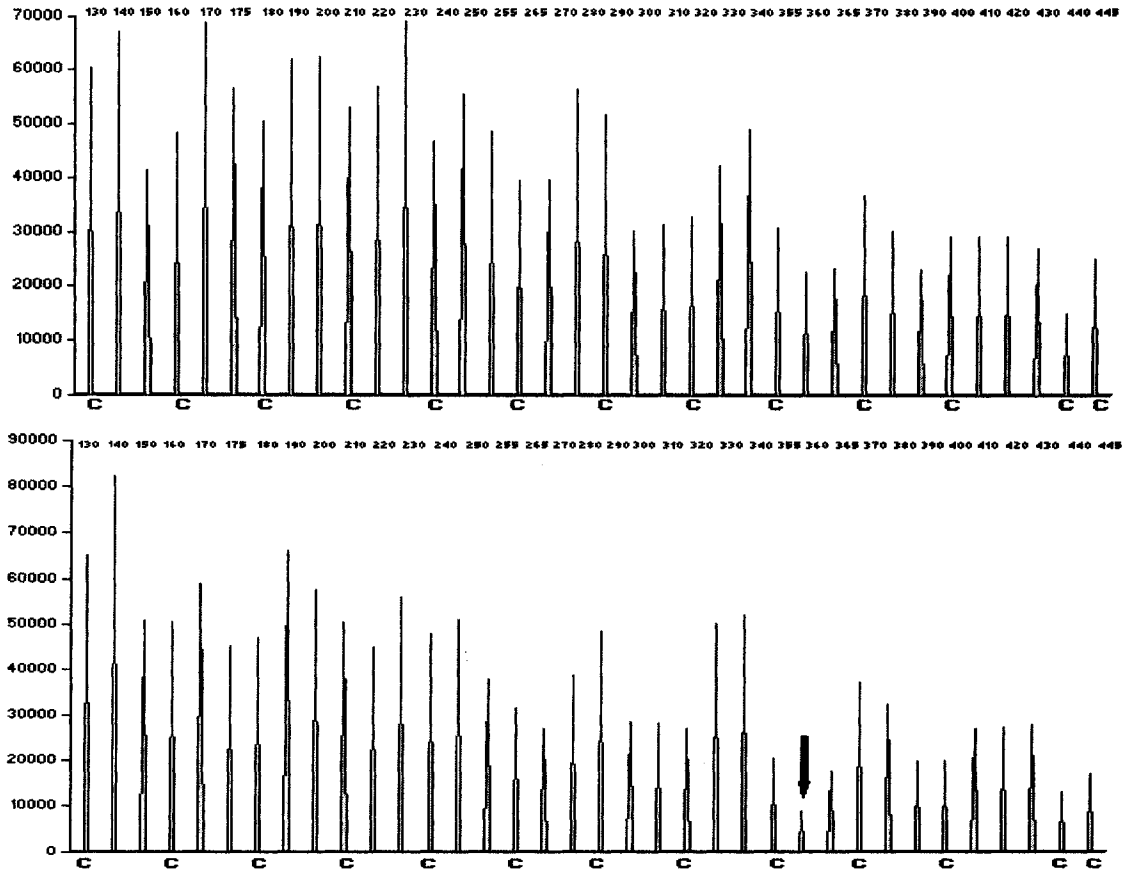

Figure12 a. MLPA result from wild type control (above) and patient 18 (below) with heterozygous *APC* exon 14 deletion. An arrow denotes the *APC* exon 14-specific peak.

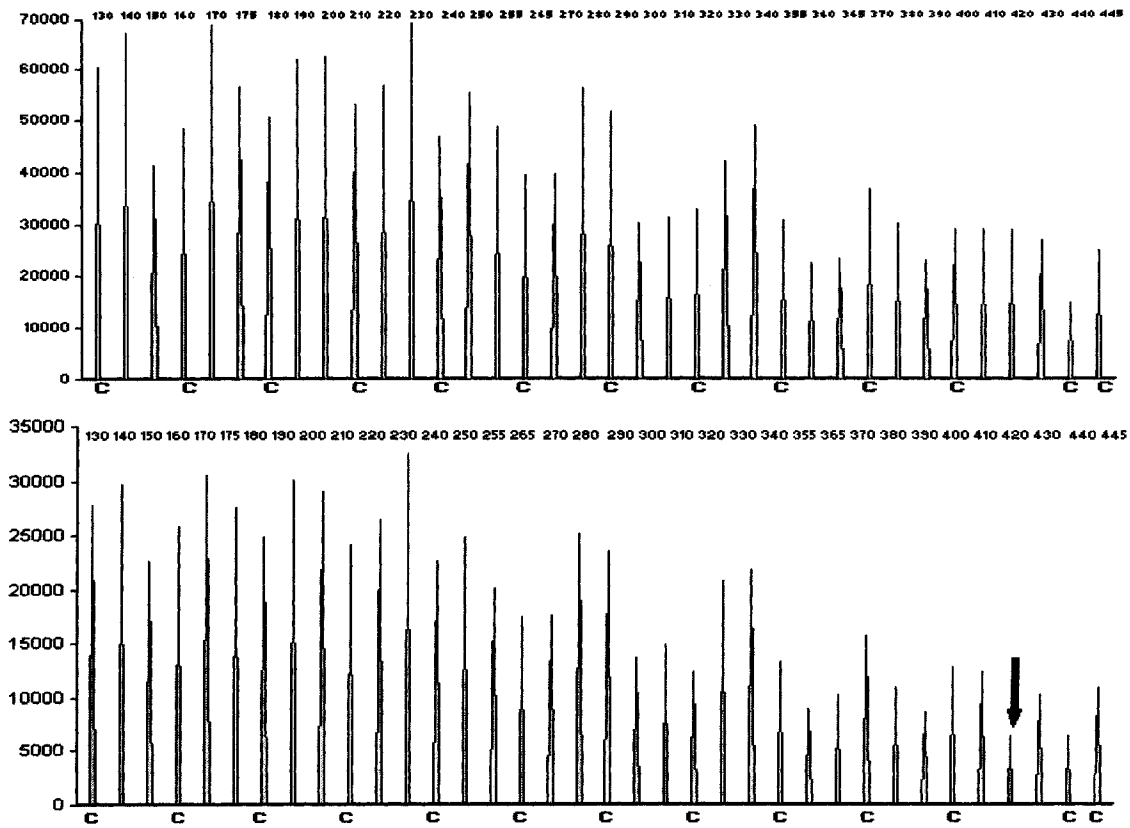

Figure12b. MLPA result from wild type control (above) and patient 11572 with heterozygous 5 bp deletion at codon 1061 of the *APC* gene (c.3183_3187delACAAA). An arrow denotes the codon 1061-specific peak.

Chapter 4

Discussion and Conclusion

4.1 Sequence variants found in the present study

To date, over 1,000 different germline mutations have been reported in patients with *APC*-associated polyposis conditions (http://perso.curie.fr/tsoussi, and http://www.cancer-genetics.org). The majority of the mutations cause a premature truncation of the *APC* protein, usually through single amino-acid substitutions (nonsense mutations) or frameshifts (small deletions or insertions). The reported mutations are spread throughout the gene, but are predominantly located in the 5' half of the gene. The most common germline *APC* mutations are the two 5-bp deletions, which result in frameshift mutations at codon 1061 (c.3183_3187delACAAA) and 1309 (c.3927_3931delAAAGA) (Beroud et al. 1996). In the present study, we identified 15 different sequence variants of the *APC* gene by screening 48 Newfoundland patients with FAP/AFAP/multiple adenoma representing 48 unrelated families. The sequence variants we identified include three small deletions/duplication (c.3067dupA, c867delC, c3183_3187delACAAA), one nonsense change (p. R1450X), one exonic deletion (del exon 14), two missense changes (p. V1822D and p. G2502S) and eight silent variants.

Protein truncating mutations

Sequence variants caused by small deletion/duplication (c.3183_3187delACAAA, c.867delC, del exon 14 and c.3067dupA) and nonsense change (p. R1450X) are predicted to cause protein truncation of *APC*. To date, the c.3067dupA, c.3183_3187delACAAA, del exon 14 and p.R1450X have been previously reported as causative mutations for FAP. However, the c.867delC is a novel finding. Because the single base pair deletion

would result in a frameshift, we think that the c.867delC should be a causative mutation for FAP in this patient. The majority of reported germline mutations found in patients with FAP cause protein truncation (Mandl et al. 1994; Paffenholz et al. 1994; van der luijt et al.1997; Wallis et al. 1999; Gebert et al. 1999; Friedl et al. 2001). Miyoshi et al (1992) examined 79 unrelated patients with FAP and detected germline mutations of the *APC* gene in 53 patients (67%), and 92% of these mutations were predicted to cause truncation of the *APC* protein. Through investigating 123 unrelated families with FAP, Gebert et al (1999) identified 85 different germline mutations from these families. All of these mutations were distributed in the 5' half of the *APC* gene between codons 213 and 1581, and were predicated to cause protein truncation of *APC*. Our results suggest that the genetic predisposition for FAP in Newfoundland population is similar to other populations, which are mainly due to loss of function mutations of *APC* gene. The exon 14 deletion identified in one AFAP patient is also predicted to cause protein truncation, but we do not know, at this moment, if this deletion causes a reading frameshift or not. We assume this deletion may at least cause partial loss of the *APC* function.

These five truncating mutations are all located in the 5' half of *APC* gene between codon 289 and 1450. Three of them are clustered at the 5' end of exon 15 between codon 1023 and 1450.

Missense variants

Disease-causing missense changes of *APC* gene are rarely reported because the functional implications of missense changes are often unclear. Generally speaking, missense alteration may be functionally important if it alters the polarity of an amino acid in highly conserved regions or in the functional domains of the encoded protein. In the *APC* protein, there are two amino acid repeat regions responsible for ß-catenin binding and down-regulation (Bright-Thomas and Hargest. 2003). The ß-catenin down-regulation domain is located in the region between codon 1324 and codon 2075 that contains seven 20 amino acid binding repeats. At least the first three binding repeats located between codons 1286 and 1513 are required for efficient ß-catenin down-regulation.

So far, only a few *APC* missense variants have been associated with FAP (Nishisho et al. 1991; Dobbie et al.1994; Laken et al. 1997; van der Luijt et al. 1997; Frayling et al.1998; Ficari et al. 2000). The missense variant, p.I1307K, for example, has been associated with increased risk of CRC but not necessarily polyposis (Laken et al. 1997). This missense variant is caused by a T>A transversion at nucleotide 3920 that converts the sequence AAATAAAA to an (A)8 tract in the *APC* coding region. This change is presumed to cause failure of the cellular transcriptional or translational machinery, thus resulting in somatic mutations by slippage when DNA replicates. In another example, the missense variant, E1317Q was reported to be associated with colon adenomas and /or colon cancer in a number of studies (Frayling et al. 1998; Lamlum et al. 2000; Popat et al. 2000; Hahnloser et al. 2003), but an opposite result was also reported. Fidder's (2005) study on Jewish patients showed that the carrier frequencies of E1317Q among CRC

patients and controls were approximately identical, which indicated that the E1317Q variant played little if any role in colorectal cancer susceptibility.

Two missense variants, V1822D and G2502S, which were identified in the present study have been previously reported and both of them are located at the 3' half of the *APC* gene. The V1822D variant is situated between the third and fourth binding repeats within the ß-catenin down-regulation domain, and therefore, is unlikely to crucially effect ß-catenin degradation (Bright-Thomas and Hargest. 2003; Rubinfeld et al. 1997). The V1822D variant was suggested to be a low-penetrance allele that increased risk of developing colorectal cancer based on an early study (Wallis et al 1999). However, such an association was not confirmed in several later association studies (Slattery et al. 2001; Tranah et al 2005). The G2502S variant is located outside the region for coordination of β-catenin down-regulation, and therefore it may not have an appreciable affect on β-catenin degradation (Bright-Thomas and Hargest. 2003; Rubinfeld et al. 1997). The G2502S might be associated with more subtle abnormalities in processing of RNA transcripts which in turn could result in the expression of differentially spliced forms of the *APC* gene, which might interfere with the functional activity of the *APC* protein (Rodney et al. 2004). However, a recent large cohort study failed to associate the G2502S variant with either colorectal cancer or adenoma (Tranah et al. 2005).

The V1822D variant was reported to have a 22% to 25% allele frequency in the general population (Slattery et al. 2001; Tranah et al. 2005), which suggested that it was a

common *APC* variant. The G2502S variant showed an allele frequency of 10% in Caucasians (SNP 500; http://snp500cancer.nci.nih.gov). In the present study, the V1822D variant was detected in 14 out of 48 patients, and the G2502S variant was only found in one patient, which gave allele frequencies of 29.2 % for V1822D and 2.1 % for G2502S respectively. The allele frequency of G2502S in Newfoundland patients is lower than in other publications (SNP 500; http://snp500cancer.nci.nih.gov). This could either be a result of a statistical bias due to the small sample size used in our study or it is possible that there is a true lower allele frequency of this variant in Newfoundland population. Our continuing study for genotyping these two variants in more patients and a large number of controls from the local population is underway, which will help to determine the allele frequencies of V1822D and G2502S in both Newfoundland patients and the normal population.

Silent variants

Single nucleotide substitution in the coding region of a gene may or may not lead to the change of an amino acid. Those that do not change the amino acid are known as silent variants. In general, the majority of the silent variants are polymorphisms, and do not play a role in disease. However, recent evidence suggests that some silent variants could be pathogenic because of skipping of an exon. In this situation, a silent variant usually occurs in the region containing a transcriptional regulatory element necessary for proper splicing known as an exonic splicing enhancer (ESE) (Aretz et al. 2004). Alteration of an

ESE even without changing an amino acid can cause exon skipping. Therefore, to determine the consequence of a missense or a silent variant, it is necessary to consider the possible amino acid change, and also whether there is a change in size of the transcript. The ESEs have been reported in a number of genes, such as the *fibrillin-1* gene, the *MLH1* gene, and the human phenylalanine hydroxylase gene (Liu et al. 1997; Nystrom-Lahti et al. 1999; Chao et al. 2001), and mutations in these ESEs are linked to the corresponding diseases. Montera et al (2001) described a distinct severe FAP phenotype within a family that was attributed to a silent mutation, c.1869G>T (p.R623R), in the middle of *APC* exon 14. This silent nucleotide substitution was related to the disruption of putative ESE motifs, inducing complete skipping of exon 14 and leading to a stable truncated *APC* protein. Aretz et al (2004) examined five rare novel missense or silent variants in the *APC* gene located close to splice sites by transcript analysis, and found that four of these variants resulted in exon skipping. These findings show a possible new model of *APC* causative mutations and demonstrate the existence of exonic sequence elements modulating the splicing of the *APC* gene. This emphasizes the importance of investigating missense and silent mutations. In the present study, we identified eight silent variants in the *APC* gene. Four of them were previously known and four were novel. The variants, 5880G>A (p.P1960P) and 5034A>G (p.G1678G) have prevalences of 43.8 % and 33.3%, respectively in our studied patients. In the near future, the prevalences of these two silent variants in the general Newfoundland population will be investigated and compared.

To determine if these silent variants predispose to polyposis conditions, we will further characterize these variants in the second phase of this study, which will include family study and transcript analysis of these variants. A family study will investigate if these silent variants cosegregate with the clinical conditions in the families. The transcript study will help us to investigate a possible ESE.

Genomic deletions

In our study, an exonic deletion was detected in one patient with AFAP. This sequence alteration was the only convincing pathogenic mutation found in AFAP patients. The exon 14 deletion has been reported in patients with FAP but not in patients with AFAP (Su et al.2000; Sieber et al, 2002; Michils et al. 2005). It may indicate that exon 14 deletion in this case is an inframe deletion rather than a frameshift change. Therefore, mutation study of the *APC* gene in AFAP patients should pay more attention to genetic defects that may mildly affect the gene function, such as inframe deletions, missense changes and some of the silent variants. Direct sequencing should not be the only method for mutation study of the *APC* gene in patients with AFAP and multiple adenomas.

4.2 Mutation detection rate

Typical FAP patients have been reported to have a 30-85 % detectable mutation rate in the *APC* gene depending on the patients examined and the methods used for mutation analysis (van der luijt et al. 1997; Wallis et al. 1999; Friedl et al. 2001; Heinimann et al. 1998; Giarola et al. 1999). The mutation detection rate in patients with FAP is usually much higher than that in patients with AFAP (Sieber et al. 2002; Aretz et al. 2005; Michils et al. 2005). In the present study, the five truncating sequence alterations (nonsense mutation and frameshift mutation) can easily be recognized as causative mutations, which give a mutation detection rate of 10.4% in the total of 48 studied patients. Among the five truncating mutations, four were detected in the patients with FAP, and one in a patient with AFAP, which gives a mutation detection rate of 44% (4/9) in patients with FAP, and 5%(1/20) in patients with AFAP. Obviously, the detection rate of *APC* mutation in our study is lower than those in previous studies. We consider several following possibilities that may have contributed to the low mutation detection rate in our study.

a) 81% (39/48) of our patients were diagnosed as either AFAP or multiple adenomas. Based on the results from previous studies, approximately 10-30% of patients with FAP and up to 90% of those with AFAP/multiple adenomas remain without a detectable *APC* germline mutation. These families are *APC* mutation-negative despite applying different screening methods (Nagase and Nakamura. 1993; Miyoshi et al. 1992; Groden et al. 1993; Armstrong et al. 1997; Giardiello et al. 1997; van der Luijt

et al. 1997; Scott et al. 2001; Spirio et al. 1993; van der Luijt et al. 1996; Lamlum et al. 2000; Heinimann et al. 2001; Friedl et al. 2001; Moisio et al. 2002; Sieber et al. 2002). Similar to other studies, detection rates of the *APC* mutation in patients with AFAP and multiple adenomas in our present study are 5% (1/20) and 0% (0/19), respectively. This suggests that the major genetic predisposition to AFAP and multiple adenomas in our group of families may be different from that for FAP. Such a difference may result from either a different way to cause *APC* function change that cannot be detected by the currently used method, or different unknown mutant gene(s) in the same or a different pathway.

b) Our sequencing analysis was restricted to the coding sequences and intron/exon junction areas of the *APC* gene. Therefore, we cannot exclude the possibility that some undetectable sequence alterations in the intronic regions and promoter region may play a role in the clinical conditions of the patients. Powell et al (1993) reported that the expression of the *APC* alleles was significantly reduced in three out of eleven *APC* mutation-negative FAP patients. This suggests that some sequence alterations in the nonexamined sequences may have a significant influence on gene expression.

c) Germline defects in gene(s) other than *APC* gene may be involved in the etiology of the clinical conditions in some of our patients. Mutations in the gene(s) that interact with the *APC* protein, or regulate the expression of *APC* may also predispose to FAP.

Some evidence supports the presence of at least one additional gene apart from *APC* causing FAP. This hypothesis is suggested based on the observations that approximately 20% of clinically typical FAP kindreds fail to show any *APC* mutations even with the best available techniques (Powell et al. 1993; Laken et al. 1999). Genes involved in the Wnt-signaling transduction pathway, such as α-catenin gene (Mcpherson et al.1994), β-catenin gene (Kraus et al. 1994), as well as axin 1 and axin 2 gene, have been suggested as the potential candidates (Peifer. 1996). There is also evidence that the base-excision-repair gene, Mut Y homolog (*MYH),* encoding a DNA glycosidase can be associated with the susceptibility to FAP (AI-Tassan et al. 2002).

d) Patients with fewer (10-100) colorectal adenomas obviously represent a heterogeneous and yet poorly characterized group between FAP and HNPCC. The phenotype in some AFAP patients sometimes can mimic HNPCC (McGarrity et al. 2000; Scott et al. 1996), and at some point AFAP was thought to be a variant of HNPCC (Lynch et al. 1996). Some HNPCC cases could be easily misdiagnosed as AFAP patients if family and medical history is incomplete (Cao et al. 2002; Lynch et al. 1996; Lynch et al. 1992).

4.3 Summary

Using direct DNA sequencing and MLPA, we performed mutation scanning of the *APC* gene in 48 selected Newfoundland patients with FAP/AFAP/multiple adenomas. These selected patients represent 48 unrelated families. In total, five truncating mutations (four frameshift mutations and one exonic mutation) were identified in five families and two missense variants were detected in 15 families. In addition, eight silent variants were also found and four of them are novel. All the frameshift changes were identified in the FAP patients (44 %). A truncating mutation caused by deletion of exon 14 was found in one patient with AFAP, this being the only convincing mutation found in our patients with AFAP (5%). Four of these mutations have been previously reported (Groden et al. 1993; Wallis et al.1999; Pang et al. 2001), and the mutation, c.867delC, is a novel finding. The two identified missense variants were also previously reported (Gregory et al. 2005; Okkels et al. 2006). Among the eight silent variants found in our patients, four are novel.

Our results suggest: 1) the genetic predisposition to FAP in Newfoundland population is similar to that identified in other populations, which is mainly due to loss of function changes of the *APC* gene; 2) germline *APC* mutation may not be the major cause for the conditions AFAP/multiple adenomas; and 3) the search for exonic deletion of the *APC* gene is necessary for mutation study of patients with AFAP. Identification of the causative germline mutations of the *APC* gene in Newfoundland patients with FAP or AFAP or multiple adenomas will lead to establishment of a direct testing method for each

of the identified mutations, which will be transferred into clinical service. The established predictive genetic testing will then be used to determine the risk status for consenting at-risk family members to provide proper prevention and/or treatment options.

References

Aaltonen LA, Salovaara R, Kristo P, et al. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med. 1998; 338:1481-7.

Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, Williams GT, Hodges AK, Davies DR, David SS, Sampson JR, Cheadle JP. Inherited variants of MYH associated with somatic G:C->T:A mutations in colorectal tumors. Nat Genet. 2002; 30:227–232.

Armour JA, Sismani C, Patsalis PC, Cross G. Measurement of locus copy number by hybridisation with amplifiable probes. Nucleic Acids Res. 2000; 28(2):605-9.

Aretz, S.; Uhlhaas, S.; Sun, Y.; Pagenstecher, C.; Mangold, E.; Caspari, R.; Moslein, G.; Schulmann, K.; Propping, P.; Friedl, W. : Familial adenomatous polyposis: aberrant splicing due to missense or silent mutations in the APC gene. Hum. Mutat. 2004; 24: 370-380.

Aretz S, Stienen D, Uhlhaas S, Pagenstecher C, Mangold E, Caspari R, Propping P, Friedl W. Large submicroscopic genomic APC deletions are a common cause of typical familial adenomatous polyposis. J Med Genet. 2005; 42:185-92.

Bear JC, Nemec TF, Kennedy JC, et al. Persistent genetic isolation in outport Newfoundland. Am J Med Genet 1987;27:807–30.

Berk T, Cohen Z, Mcleod RS, Parker JA. Congenital hypertrophy of the retinal pigment epithelium as a marker for familial adenomatous polyposis. Dis Colon Rectum.1998; 31:253-257.

Beroud, C, Soussi, T. APC gene: database of germline and somatic mutations in human tumors and cell lines. Nucleic Acids Res.1996; 24: 121-124.

Bertario L, Russo A, Sala P, Varesco L, Giarola M, Mondini P, Pierotti M, Spinelli P, Radice P. Multiple approach to the exploration of genotype-phenotype correlations in familial addenomatous polyposis. J Clin Oncol. 2003; 21:1698-707.

Bienz M. APC: the plot thickens Curr Opin Genet Dev. 1999; 9: 595-603.

Bisgaard, M. L.; Fenger, K.; Bulow, S.; Niebuhr, E.; Mohr, J. : Familial adenomatous polyposis (FAP): frequency, penetrance, and mutation rate. Hum Mutat.1994; 3: 121-125.

Blair, N. P, Trempe, C. L. Hypertrophy of the retinal pigment epithelium associated with Gardner's syndrome. Am. J. Ophthal. 1980; 90: 661-667.

Bodmer, W. F.; Bailey, C. J.; Bodmer, J.; Bussey, H. J. R.; Ellis, A.; Gorman, P.; Lucibello, F. C.; Murday, V. A.; Rider, S. H.; Scambler, P.; Sheer, D.; Solomon, E.; Spurr, N. K. Localization of the gene for familial adenomatous polyposis on chromosome 5. Nature. 1987; 328: 614-616, 1987.

Bodmer BF: The somatic evolution of cancer. The Harveian Oration of 1996. J Royal Coll Physicians Lond. 1996; 31:82-89.

Bright-Thomas RM, Hargest R. APC, ß-catenin and hTCF-4; an unholy trinity in the genesis of colorectal cancer. Eur J Surg Oncol. 2003; 29:107–17.

Bunyan DJ, Eccles DM, Sillibourne J, Wilkins E, Thomas NS, Shea-Simonds J, Duncan PJ, Curtis CE, Robinson DO, Harvey JF, Cross NC, Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification. Br J Cancer. 2004; 91: 1155-9.

Bussey,H.J.R. Historical developments in familial adenomatous polyposis. In Herrera,L. (ed.) Familial Adenomatous Polyposis. Alan R. Liss, New York, NY, 1990; pp. 1–22.

Canadian cancer statistics 2003. Toronto, Canada.

Cao Y, Pieretti M, Marshall J, Khattar NH, Chen B, Kam-Morgan L, Lynch H. Challenge in the differentiation between attenuated familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer: case report with review of the literature. Am J Gastroenterol. 2002; 97: 1822-7.

Cao, X.; Eu, K. W.; Seow-Choen, F.; Zhao, Y.; Cheah, P. Y. : Topoisomerase-I- and Alu-mediated genomic deletions of the APC gene in familial adenomatous polyposis. Hum. Genet. 2001; 108: 436-442.

Caspari R, Olschwang S, Friedl W, Mandl M, Boisson C, Boker T, Augustin A, Kadmon M, Moslein G, Thomas G, et al. Familial adenomatous polyposis: desmoid tumours and lack of ophthalmic lesions (CHRPE) associated with APC mutations beyond codon 1444. Hum Mol Genet. 1995; 4:337-40.

Davies DR, Armstrong FG,, Thakker N, Horner K, Guy SP, Clancy T, Sloan P, Blair V, Dodd C, Warnes TW, et al. Severe Gardner syndrome in families with mutations restricted to a specific region of the APC gene. Am J Hum Genet. 1995; 59:1193-201.

De Rosa M, Scarano MI, Panariello L, Morelli G, Riegler G, Rossi GB, Tempesta A, Romano G, Renda A, Pettinato G, Izzo P. The mutation spectrum of the APC gene in FAP patients from southern Italy: detection of known and four novel mutations. Hum Mutat. 2003; 21(6):655-6.

Diaz-Llopis, M.; Menezo, J. L. Congenital hypertrophy of the retinal pigment epithelium in familial adenomatous polyposis. Arch. Ophthal.1998; 106: 412-413.

Dobbie Z, Spycher M, Hurliman R, Ammann R, Ammann T, Roth J, Muller A, Muller H, Scott RJ. Mutational analysis of the first 14 exons of the adenomatous polyposis coli (*APC*) gene. Eur J Cancer. 1994; 30A:1709–1713.

Eccles DM, van der Luijt R, Breukel C, Bullman H, Bunyan D, Fisher A, Barber J, du Boulay C, Primrose J, Burn J, Fodde R. Hereditary desmoid disease due to a frameshift mutation at codon 1924 of the APC gene. Am J Hum Genet. 1996; 59(6):1193-201.

Fearnhead, NS.; Britton, M. P.; Bodmer, W. F. : The ABC of APC. Hum. Mol. Genet. 2001; 10: 721-733.

Fearon, E. R.; Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell. 1990 61: 759-767.

Ficari F, Cama A, Valanzano R, Curia MC, Palmirotta R, Aceto G, Esposito DL, Crognale S, Lombardi A, Messerini L, Mariani-Costantini R, Tonelli F, Battista P. APC gene mutations and colorectal adenomatosis in familial adenomatous polyposis. Br J Cancer. 2000; 82:348–353.

Fidder HH, Figer A, Geva R, Flex D, Schayek H, Avidan B, Meir SB, Friedman E.

Genetic analyses in consecutive Israeli Jewish colorectal cancer patients. Am J Gastroenterol. 2005;100(6):1376-80.

Fodde R. The APC gene in colorectal cancer. Eur J Cancer. 2002;38(7):867-71.

Foulkes WD. A tale of four syndromes: familial adenomatous polyposis, Gardner syndrome, attenuated APC and Turcot syndrome. QJM. 1995; 88(12):853-63.

Frayling IM, Beck NE, Ilyas M, Dove-Edwin I, Goodman P, Pack K, Bell JA, Williams CB, Hodgson SV, Thomas HJ, Talbot IC, Bodmer WF, Tomlinson IP. The APC variants I1307K and E1317Q are associated with colorectal tumors, but not always with a family history. Proc Natl Acad Sci USA. 1998; 95:10722–10727.

Friedl W, Meuschel S, Caspari R, Lamberti C, Krieger S, Sengteller M, Propping P. Attenuated familial adenomatous polyposis due to a mutation in the 3' part of the APC gene. A clue for understanding the function of the APC protein. Hum Genet. 1996; 97(5): 579-84.

Friedl W, Caspari R, Sengteller M, et al: Can APC mutation analysis contribute to therapeutic decisions in familial adenomatous polyposis? Experience from 680 FAP families. Gut. 2001; 48:515-521.

Galiatsatos P, Foulkes WD. Familial adenomatous polyposis.Am J Gastroenterol. 2006 Feb;101(2):385-98.

Gebert JF, Dupon C, Kadmon M, Hahn M, Herfarth , von Knebel Doeberitz M, Schackert HK. Combined molecular and clinical approaches for the identification of families with familial adenomatous polyposis coli. Ann Surg. 1999;229(3):350-61.

Giardiello, F. M , Krush AJ and Petersen GM.: Phenotypic variability of familial adenomatous polyposis in 11 unrelated families with identical APC gene mutation. Gastroenterology. 1994;106:1542-1547.

Giardiello, F. M.; Petersen, G. M.; Piantadosi, S.; Gruber, S. B.; Traboulsi, E. I.; Offerhaus, G. J. A.; Muro, K.; Krush, A. J.; Booker, S. V.; Luce, M. C.; Laken, S. J.; Kinzler, K. W.; Vogelstein, B.; Hamilton, S. R. APC gene mutations and extraintestinal phenotype of familial adenomatous polyposis. Gut. 1997; 40: 521-525.

Giardiello FM, Brensinger JD, Petersen GM. AGA technical review on hereditary colorectal cancer and genetic testing. Gastroenterology. 2001;121:198-213.

Giarola M, Stagi L, Presciuttini S et al: Screening for mutations of the APC gene in 66 Italian familial adenomatous polyposis patients: evidence for phenotypic differences in cases with and without identified mutation. Hum Mutat. 1999;13: 116– 123.

Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. Ann Intern Med. 1994;121(4):241-6.

Gross KH and Groden J: Biology of the adenomatous polyposis coli tumor suppressor. J Clin Oncol. 2000; 18:1967-79.

Gregory J. Tranah, Giovannucci E, Ma J, Fuchs C, and David J, Hunter: APC Asp1822Val and Gly2502Ser Polymorphisms and Risk of Colorectal Cancer and Adenoma. Cancer Epidemiol Biomarkers Prev. 2005; 14, 863-870.

Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, Joslyn G, Stevens J, Spirio L, Robertson M, Sargeant L. , Krapcho K, Wollf E, Burt R, Hughes JP,

Warrington J, McPherson J, Wasmuth J, Le Paslier D, Abderrahim H, Cohen D, Leppert M, White R. Identification and characterization of the familial adenomatous polyposis coli gene. Cell. 1991; 66:589.

Groden J., Gelbert L., Thliveris A., Nelson L., Robertson M., Joslyn G., Samowitz W., Spirio L., Carlson M., Burt R., et al Mutational analysis of patients with adenomatous polyposis: identical inactivating mutations in unrelated individuals. Am J Hum Genet. 1993; 52: 263-272.

Gumbiner BM: Regulation of cadherin adhesive activity. J Cell Biol. 2000; 148:399-404.

Hahnloser D, Petersen GM, Rabe K, Snow K, Lindor NM, Boardman L, Koch B, Doescher D, Wang L, Steenblock K, Thibodeau SN. The APC E1317Q variant in adenomatous polyps and colorectal cancers. Cancer Epidemiol Biomarkers Prev. 2003 ;12(10):1023-8.

Heinimann K, Mullhaupt B, Weber W et al: Phenotypic differences in familial adenomatous polyposis based on APC gene mutation status. Gut. 1998; 39: 675– 679.

Heinimann K, Thompson A, Locher A, et al: Nontruncating APC germ-line mutations and mismatch repair deficiency play a minor role in APC mutation-negative polyposis. Cancer Res. 2001; 61:7616-7622.

Heppner-Gross K, Groden J: Biology of adenomatous polyposis coli tumor suppressor. J Clin Oncol. 2000;18:1967-1979.

Herrera L., Kakati S., and Gibas L., et al. Brief clinical report: Gardner syndrome in a man with an interstitial deletion of 5q. Am J Med Genet. 1986; 25: 473-476.

Horii, A.;Nakatsuru, S.; Ichii, S.; Nagase, H; Nakamura, Y. Multiple forms of the APC gene transcripts and their tissue-specific expression. Hum. Molec.Genet. 1993; 2: 283-287.

Ilyas M, Straub J, Tomlinson IP, Bodmer WF. Genetic pathways in colorectal and other cancers. Eur J Cancer. 1999;35(14):1986-2002.

Jagelman DG, DeCosse JJ, Bussey HJ; the Leeds Castle Polyposis Group. Upper gastrointestinal cancer in familial adenomatous polyposis. Lancet. 1988; 1: 1149-1151.

Jones S, Emmerson P, Maynard J, Best JM, Jordan S, Williams GT, Sampson JR, Cheadle JP. Biallelic germline mutations in MYH predispose to multiple colorectal adenoma and somatic G:C→T:A mutations. Hum Mol Genet. 2002; 11:2961-67.

Joslyn G., Carlson M., and Thliveris A. Albertsen H, Gelbert H, Samowitz W, Groden J, Stevens J, Spirio L, Robertson M, Sargeant L. , Kraho K, Wollf E, Burt R, Hughes JP, Warrington J, McPherson J, Wasmuth J, Le Paslier D, Abderrahim H, Cohen D, Leppert M, White R. Identification of deletion mutations and three new genes at the familial polyposis locus. Cell. 1991;66:601-613.

Kaplan, K. B.; Burds, A. A.; Swedlow, J. R.; Bekir, S. S.; Sorger, P. K.; Nathke, I. S. A role for the adenomatous polyposis coli protein in chromosome segregation. Nature Cell Biol. 2001; 3: 492-432.

Kinzler K.W, Nilbert M., and. Su L, et al. Identification of FAP locus genes from chromosome 5q21. Science. 1991; 253: 661-665.

Kinzler K.W and. Vogelstein B. Lessons from hereditary colorectal cancer. Cell. 1996; 87: 159-170.

Knudsen AL, Bisgaard ML, Bulow S: Attenuated familial adenomatous polyposis (AFAP): A review of the literature. Fam Cancer. 2003; 2:43-55.

Kraus C, Liehr T, Hulsken J, Behrens J, Birchmeier W, Grzeschik KH, Ballhausen WG. Localization of the human β-catenin gene (CTNNB1) to 3p21: A region implicated in tumor development. Genomics. 1994; 23: 272-274.

Laken SJ, Petersen GM, Gruber SB, Oddoux C, Ostrer H, Giardiello FM, Hamilton SR, Hampel H, Markowitz A, Klimstra D, Jhanwar S, Winawer S, Offit K, Luce MC, Kinzler KW, Vogelstein B. Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC. Nat Genet. 1997;17:79–83.

Laken SJ, Papadopoulos N, Petersen GM, et al. Analysis of masked mutations in familial adenomatous polyposis. Proc Natl Acad Sci U S A. 1999; 96:2322- 6.

Lamlum, H.; Ilyas, M.; Rowan, A.; Clark, S.; Johnson, V.; Bell, J.; Frayling, I.; Efstathiou, J.; Pack, K.; Payne, S.; Roylance, R.; Gorman, P.; Sheer, D.; Neale, K.; Phillips, R.; Talbot, I.; Bodmer, W.; Tomlinson. The type of somatic mutation at APC in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet of Knudson's 'two-hit' hypothesis. Nature Med. 1999; 5: 1071-1075.

Lamlum, H.; Al Tassan, N.; Jaeger, E.; Frayling, I.; Sieber, O.; Bin Reza, F.; Eckert, M.; Rowan, A.; Barclay, E.; Atkin, W.; Williams, C.; Gilbert, J.; Cheadle, J.; Bell, J.; Houlston, R.; Bodmer, W.; Sampson, J.; Tomlinson, I. Germline APC variants in patients with multiple colorectal adenomas, with evidence for the particular importance of E1317Q. Hum Molec Genet. 2000 ; 9: 2215-2221.

Leppert M.,. Dobbs M, and. Scambler P, et al. The gene for familial polyposis coli maps to the long arm of chromosome 5. Science. 1987; 238: 1411-1413.

Liu W, Qian C, Francke U. Silent mutation induces exon skipping of fibrillin-1 gene in Marfan syndrome. Nat Genet. 1997; 16:328-329.

Lockhart-Mummery, A. Cancer and Heredity. Lancet. 1925; 1:427-429.

Lynch HT, Smyrk TC, Watson P, Lanspa SJ, Lynch PM, Jenkins JX, Rouse J, Cavalieri J, Howard L, Lynch J. Hereditary flat adenoma syndrome: a variant of familial adenomatous polyposis? Dis Colon Rectum. 1992;35(5):411-21.

Lynch HT, Smyrk T, Lynch J, Lanspa S, McGinn T,Cavalieri RJ. Genetic counseling in an extended attenauted familial adenomatous polyposis kindred. Am J Gastroenterol. 1996; 91(3):455-9.

Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med. 2003; 348(10):919-932.

Mahmoud, NN et al. Apc gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Res. 1997; 57, 5045-5050.

Mandl M, Paffenholz R, Friedl W, Caspari R, Sengteller M, Propping P. Frequency of common and novel inactivating APC mutations in 202 families with familial adenomatous polyposis. Hum Mol Genet. 1994; 3(1):181-4.

Martin LJ, Crawford MH, Koertvelyessy T, Keeping D, Collins M, Huntsman R. The population structure of ten Newfoundland outports. Hum Biol 2000;72:997–1016.

McCart A, Latchford A, Volikos E, Rowan A, Tomlinson I, Silver A. A novel exon duplication event leading to a truncating germ-line mutation of the APC gene in a familial adenomatous polyposis family. Fam Cancer. 2006;5(2):205-8.

McGarrity TJ, Ruggiero FM, Chey WY, Bajaj R, Kelly JE, Kauffman GL Jr. Giant fundic polyp complicating attenuated familial adenomatous polyposis. Am J Gastroenterol. 2000; 95(7):1824-8.

McPherson JD, Morton RA, Ewing CM, Wasmuth JJ, Overhauser J, Nagafuchi A, Tsukita S, Isaacs WB : Assignment of the human α-catenin gene (CTNNA1) to chromosome 5q21-q22. Genomics. 1994; 19: 188-190.

Michils G, Tejpar S, Thoelen R, van Cutsem E, Vermeesch JR, Fryns JP, Legius E, Matthijs G : Large deletions of the APC gene in 15% of mutation-negative patients with classical polyposis (FAP): a Belgian study. Hum Mutat. 2005; 25:125-34.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16(3):1215.

Miyoshi Y, Ando H, Nagase H, Nishisho I, Hori T, Utsunomiya J, Baba S, Petersen G, Hamilton SR, Kinzler KW, Vogelstein B and Nakamura Y. Germ-Line mutations of the APC gene in 53 familial adenomatous polyposis patients. Proc Natl Acad Sci USA. 1992; 89:4452-4456.

Moisio AL, Jarvinen H, Peltomaki P: Genetic and clinical characterisation of familial adenomatous polyposis: A population-based study. Gut. 2002; 50:845-850.

Montera M, Piaggio F, Marchese C, Gismondi V, Stella A, Resta N, Varesco L, Guanti G, Mareni C. A silent mutation in exon 14 of the APC gene is associated with exon skipping in a FAP family. J Med Genet. 2001; 38:863-867.

Morin P.J, Vogelstein B, and. Kinzler K.W. Apoptosis and APC in colorectal tumorigenesis Proc Natl Acad Sci U S A. 1996; 93: 7950-7954.

Moss SF, Liu TC, Petrotos A, Hsu TM, Gold LI, Holt PR: Inward growth of colonic adenomatous polyps. Gastroenterology. 1996;111:1425-1432.

Muto T, Bussey HJ, Morson BC: The evolution of cancer of the colon and rectum.Cancer. 1995 ; 36:2251-2270.

Nagase H, Miyoshi Y, Horii A, et al. Correlation between the location of germ-line mutations in the APC gene and the number of colorectal polyps in familial adenomatous polyposis patients. Cancer Res. 1992; 52:4055-4057.

Nagase, H.; Nakamura, Y. Mutations of the APC (adenomatous polyposis coli) gene. Hum. Mutat. 1993; 2: 425-434.

Nandakumar G, Morgan JA, Silverberg D, Steinhagen RM. Familial polyposis coli: clinical manifestations, evaluation, management and treatment. Mt Sinai J Med. 2004 Nov;71(6):384-91

Narayan S and Deodutta . Role of *APC* and DNA mismatch repair genes in the development of colorectal cancers. Mol Cancer. 2003; 2: 41.

Nishisho, Y. Nakamura, and Y. Miyoshi, et al. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science. 1991; 253: 665-669.

Nowak MA, Komarova NL, Sengupta A, Jallepalli PV, Shih IeM, Vogelstein B, Lengauer C. The role of chromosomal instability in tumor initiation. Proc Natl Acad Sci U S A. 2002;99(25):16226-31.

Nugent K, Phillips R, Hodgson S, et al. Phenotypic expression in familial adenomatous polyposis: partial prediction by mutation analysis. Gut. 1994; 35:1622-1623.

Nystrom-Lahti M, Holmberg M, Fildalgo P, Salovaaro R, de la Chapelle A, Jiricny J, Peltomaki P. Missense and nonsense mutations in codon 659 of *MLH1* cause aberrant splicing of messenger RNA in HNPCC kindreds. Genes Chrom Cancer. 1999; 26:372-375.

O' Brien A, Hibberd M. Clinical efficacy and safety of a new leuprorelin acetate depot formulation in patients with advanced prostatic cancer. J Int Med Res. 1990;18 Suppl 1:57-68.

Offerhaus, G. J. A.; Giardiello, F. M.; Krush, A. J.; Booker, S. V.; Tersmette, A. C.; Kelley, N. C.; Hamilton, S. R : The risk of upper gastrointestinal cancer in familial adenomatous polyposis. Gastroenterology. 1992;102: 1980-1982.

Okkels H, Sunde L, Lindorff-Larsen K, Thorlacius-Ussing O, Gandrup P, Lindebjerg J, Stubbeteglbjaerg P,Oestergaard JR, Nielsen FC, Krarup HB. Polyposis and early cancer in a patient with low penetrant mutations in MSH6 and APC: hereditary colorectal cancer as a polygenic trait. Int J Colorectal Dis. 2006; 21:847-850.

Olschwang, S.; Tiret, A.; Laurent-Puig, P.; Muleris, M.; Parc, R.; Thomas, G. : Restriction of ocular fundus lesions to a specific subgroup of APC mutations in adenomatous polyposis coli patients. Cell. 1993; 75: 959-968.

Paffenholz R, Mandl M, Caspari R, Sengteller M, Propping P, Friedl W. Eleven novel germline mutations in the adenomatous polyposis coli (APC) gene. Hum Mol Genet. 1994;3(9):1703-4.

Pang CP, Fan DSP. Congenital hypertrophy of the retinal pigment epithelium and APC mutations in Chinese with familial adenomatous polyposis. Ophthalmologica. 2001; 215: 408-411.

Park JG, Vasen HF, Park KJ, Peltomaki P, Ponz de Leon M, Rodriguez-Bigas MA, Lubinski J, Beck NE, Bisgaard ML, Miyaki M, Wijnen JT, Baba S, Lynch HT. Suspected hereditary nonpolyposis colorectal cancer: International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC) criteria and results of genetic diagnosis. Dis Colon Rectum. 1999;42(6):710-5; discussion 715-6.

Peifer M. Regulating cell proliferation: as easy as APC. Science. 1996;272(5264):974-5.

Pilarski RT, Brothman AR, Benn P, Shulman Rosengren S.Attenuated familial adenomatous polyposis in a man with an interstitial deletion of chromosome arm 5q. Am J Med Genet. 1999; 86(4):321-4.

Plawski A, Nowakowska D, Podralska M, Lipinski D, Steffen J, Slomski R. The AAPC case, with an early onset of colorectal cancer. Int J Colorectal Dis. 2007; 22(4):449-51.

Polakis P: The adenomatous polyposis coli (APC) tumor suppressor. Biochim Biophys Acta. 1997; 1332 (3): F127-47.

Polakis P: Wnt signaling and cancer. Genes Dev. 2000;14:1837-1851.

Popat S, Stone J, Coleman G, Marshall G, Peto J, Frayling I, Houlston R. Prevalence of the APC E1317Q variant in colorectal cancer patients. Cancer Lett. 2000;149:203-6.

Potter JD. Colorectal cancer: molecules and populations. J Natl Cancer Inst. 1999;91(11):916-32.

Powell SM, N. Zilz, and Y. Beazer-Barclay, et al. APC mutations occur early during colorectal tumorigenesis. Nature. 1992 359: 235-237.

Powell SM, Peterson GM, Krush AJ, Booker S, Jen J, Giardiello FM, Hamilton SR, Vogelstein B, Kinzler K. Molecular diagnosis of familial adenomatous polyposis. N Engl J Med. 1993; 329:1982-1987.

Ripa R, Bisgaard ML, Bulow S, Nielsen FC. De novo mutations in familial adenomatous polyposis (FAP). Eur J Hum Genet. 2002; 10: 631-7.

Rozen P, Samuel Z, Shomrat R, Legum C. Notable intrafamilial phenotypic variability in a kindred with familial adenomatous polyposis and an APC mutation in exon 9. Gut. 1999; 45(6): 829-33

Rubinfeld, B, Souza B, Albert I, Muller O, Chamberlain SH, Masiarz FR, Munemitsu S and Polakis P. Association of the APC gene product with beta catenin. Science. 1993; 262: 1731-1734.

Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S and Polakis P. Binding of GSK3B to the APC-B-catenin complex and regulation of complex assembly. Science. 1996; 272: 1023-1026.

Rubinfeld B, Albert I, Porfiri E, Munemitsu S, Polakis P. Loss of ß-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene. Cancer Res. 1997; 57:4624–30.

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G: Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002; 30:e57.

Scott RJ, Froggatt NJ, Trembath RC, Evans DG, Hodgson SV, Maher ER. Familial infiltrative fibromatosis (desmoid tumours) (MIM135290) caused by a recurrent 3' APC gene mutation. Hum Mol Genet. 1996; 5(12):1921-4.

Scott R. J., Meldrum C., Crooks R., Spigelman A. D., Kirk J., Tucker K., Koorey D. Familial adenomatous polyposis: more evidence for disease diversity and genetic heterogeneity. Gut. 2001; 48: 508-514.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW: Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. 1997; 88:593-602.

Sieber, O. M.; Lamlum, H.; Crabtree, M. D.; Rowan, A. J.; Barclay, E.; Lipton, L.; Hodgson, S.; Thomas, H. J. W.; Neale, K.; Phillips, R. K. S.; Farrington, S. M.; Dunlop, M. G.; and 9 others : Whole-gene APC deletions cause classical familial adenomatous polyposis, but not attenuated polyposis or 'multiple' colorectal adenomas. Proc. Nat. Acad. Sci. 2002; 99: 2954-2958.

Sieber OM, Lipton L, Crabtree M, et al. Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in MYH. N Engl J Med. 2003; 348:791-9.

Sieber O , Segditsas S , Knudsen A , Zhang J , Luz J , Rowan A , Spain S , Thirlwell C , Howarth K et al. Disease severity and genetic pathways in attenuated familial adenomatous polyposis vary greatly, but depend on the site of the germline mutation. Gut. 2006;119(1-2): 9-22.

Slattery ML, Samowitz W, Ballard L, Schaffer D, Leppert M, Potter JD. A molecular variant of the APC gene at codon 1822: its association with diet, lifestyle, and risk of colon cancer. Cancer Res. 2001; 61:1000–4.

Smith, G.; Carey, F. A.; Beattie, J.; Wilkie, M. J. V.; Lightfoot, T. J.; Coxhead, J.; Garner, R. C.; Steele, R. J. C.; Wolf, C. R. Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. Proc. Nat. Acad. Sci. 2002; 99: 9433-9438.

Solomon E, Voss R, Hall V, Bodmer WF, Jass JR, Jeffreys AJ, Lucibello FC, Patel I, Rider SH. Chromosome 5 allele loss in human colorectal carcinomas. Nature. 1987;328(6131):616-9.

Soravia C, Berk T, Madlensky L, Mitri A, Cheng H, Gallinger S, Cohen Z, Bapat B : Genotype-phenotype correlations in attenuated adenomatous polyposis coli. Am J Hum Genet. 1998; 62:1290-301.

Spirio L., Olschwang S., and. Groden J, et al. Alleles of the APC gene: an attenuated form of familial polyposis. Cell. 1993; 75: 951-957.

Spirio L, Green J, Robertson J, Robertson M, Otterud B, Sheldon J, Howse E, Green R, Groden J, White R, Leppert M. The identical 5' splice-site acceptor mutation in five attenuated APC families from Newfoundland demonstrates a founder effect. Hum Genet. 1999;105(5):388-98.

Su, L.-K.; Vogelstein, B.; Kinzler, K. W. Association of the APC tumor suppressor protein with catenins. Science. 1993; 262: 1734-1737.

Su, L.; Steinbach, G.; Sawyer, J.C.; Hindi, M.; Ward, P.A.; Lynch, P.M.: Genomic rearrangements of the APC tumor-suppressor gene in familial adenomatous polyposis. Hum. Genet. 2000; 106:101-107.

Su, L.-K.; Kohlmann, W.; Ward, P. A.; Lynch, P. M. Different familial adenomatous polyposis phenotypes resulting from deletions of the entire APC exon 15. Hum. Genet. 2002; 111:88-95.

Sulekova Z, Reina-Sanchez J, Ballhausen WG. Multiple APC messenger RNA isoforms encoding exon 15 short open reading frames are expressed in the context of a novel exon 10A-derived sequence. : Int J Cancer. 1995;63(3):435-41.

Tranah GJ, Giovannucci E, Ma J, Fuchs C, Hunter J. APC Asp1822Val and Gly2502Ser polymorphisms and risk of colorectal cancer and adenoma. Cancer Epidemiol Biomarkers Prev. 2005;14(4):863-70.

Valanzano R, Cama A, Volpe R, Curia MC, Mencucci R, Palmirotta R, Baslista P, Ficari F, Mariani-Costantini R, Tonelli F. Congenital hypertrophy of the retinal pigment epithelium in familial adenomatous polyposis. Cancer. 1996; 78:2400-2410.

Van der Luijt, R. B.; Khan, P. M.; Vasen, H. F. A.; Breukel, C.; Tops, C. M. J.; Scott, R. J.; Fodde, R. : Germline mutations in the 3-prime part of APC exon 15 do not result in truncated proteins and are associated with attenuated adenomatous polyposis coli. Hum. Genet. 1996; 98: 727-734.

Van der Luijt, R. B.; Khan, P. M.; Vasen, H. F. A.; Tops, C. M. J.; Van Leeuwen-Cornelisse, I. S. J.; Wijnen, J. T.; van der Klift, H. M.; Plug, R. J.; Griffioen, G.; Fodde, R. : Molecular analysis of the APC gene in 105 Dutch kindreds with familial adenomatous polyposis: 67 germline mutations identified by DGGE, PTT, and Southern analysis. Hum. Mutat. 1997; 9: 7-16.

Van Es JH, Giles RH, Clevers HC. The many faces of the tumor suppressor gene APC. Exp Cell Res. 2001;264(1):126-34.

Vasen HF: Clinical diagnosis and management of hereditary colorectal cancer syndromes. J Clin Oncol. 2000; 18:81S-92S.

Wallis YL, Morton DG, McKeown CM, Macdonald F: Molecular analysis of the APC gene in 205 families: extended genotype–phenotype correlations in FAP and evidence for the role of APC amino acid changes in colorectal cancer predisposition. J Med Genet. 1999; 36: 14– 20.

Walter F, Bodmer. Cancer genetics: colorectal cancer as a model. J Hum Genet. 2006: 51: 391-396.

Wei SC, Yu CY, Tsai-Wu JJ, Su YN, Sheu JC, Wu CH, Wang CY, Wong JM. Low mutation rate of hMSH2 and hMLH1 in Taiwanese hereditary non-polyposis colorectal cancer. Clin Genet. 2003;64(3):243-51.

Willert K. and. Nusse R. Beta-catenin: a key mediator of Wnt signaling Curr Opin Genet Dev. 1998; 8: 95-102.

Winawer SJ, Fletcher RH, Miller L, Godlee F, Stolar MH, Mulrow CD, Woolf SH, Glick SN, Ganiats TG, Bond JH, Rosen L, Zapka JG, Olsen SJ, Giardiello FM, Sisk JE, Van Antwerp R, Brown-Davis C, Marciniak DA, Mayer RJ. Colorectal cancer screening: clinical guidelines and rationale. Gastroenterology. 1997;112(2):594-642.

Woods MO, Hyde AJ, Curtis FK, Stuckless S, Green JS, et al. High Frequency of Hereditary Colorectal Cancer in Newfoundland Likely Involves Novel Susceptibility Genes . Clinical Cancer Research. 2005: 11: 6853-6861.

Young J, Simms LA, Tarish J, Buttenshaw R, Knight N, Anderson GJ, Bell A, Leggett B. A family with attenuated familial adenomatous polyposis due to a mutation in the alternatively spliced region of APC exon 9. Hum Mutat. 1998; 11(6): 450-5.