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

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

by

© Aihua Ma

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

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AFAP</td>
<td>attenuated familial adenomatous polyposis</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CHRPE</td>
<td>congenital hypertrophy of retinal pigment epithelium</td>
</tr>
<tr>
<td>CIN</td>
<td>chromosomal instability</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>EB1</td>
<td>end-binding protein 1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ECMs</td>
<td>extracolonic manifestations</td>
</tr>
<tr>
<td>ESE</td>
<td>exonic splicing enhancer</td>
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<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
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<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>hDLG</td>
<td>human discs large tumor suppressor protein</td>
</tr>
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<td>HNPCC</td>
<td>hereditary non-polyposis colorectal cancer</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
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<td>MAP</td>
<td>MYH associated polyposis</td>
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<td>MAPH</td>
<td>multiplex amplification and probe hybridization</td>
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<td>MCR</td>
<td>mutation cluster region</td>
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<td>MLPA</td>
<td>multiple ligation-dependent probe amplification</td>
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<td>Abbreviation</td>
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<tr>
<td>MYH</td>
<td>Mut Y Homolog</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PTT</td>
<td>protein truncation test</td>
</tr>
<tr>
<td>SAP</td>
<td>shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SLS</td>
<td>sample loading solution</td>
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<td>UC</td>
<td>ulcerative colitis</td>
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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).
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.

Table 1. Benign and malignant lesions associated with familial adenomatous polyposis (Nandakumar et al. 2004).

<table>
<thead>
<tr>
<th>Malignant tumours (lifetime risk)</th>
<th>Other lesions</th>
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<tr>
<td>Duodenal (5-11%)</td>
<td>Osteomas</td>
</tr>
<tr>
<td>Pancreatic (2%)</td>
<td>Radiopaque jaw lesions</td>
</tr>
<tr>
<td>Thyroid (2%)</td>
<td>Supernumerary teeth</td>
</tr>
<tr>
<td>Brain (medulloblastoma) (&lt;1%)</td>
<td>Lipomas, fibromas, epidermoid cysts</td>
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<tr>
<td>Hepatoblastoma (0.7% of children &lt;5 yrs)</td>
<td>Desmoid tumours</td>
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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 \textit{APC}, \textit{p53} and \textit{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 \textit{p53} and \textit{APC} (27.1\%), whereas, mutations in \textit{K-ras} and \textit{p53} co-occurred less frequently than expected by chance.

Lamlum et al (1999) found that in patients carrying an \textit{APC} germline mutation, tumor development starts in the polyps when the remaining wild-type \textit{APC} allele is mutated by somatic mutation which is consistent with Knudson's "two hit" hypothesis. However, some contradictory 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 \textit{APC} gene product can influence the function of the wild-type gene product, resulting in the total loss of \textit{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.
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.

![Diagram of the structure of the APC gene](image)

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 \textit{APC} protein

The \textit{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 \textit{APC} function contributes to cancer progression. \textit{APC} also has roles in cell migration, adhesion, chromosome segregation, spindle assembly, apoptosis, and neuronal differentiation.

i) \textit{APC} protein plays a role in the control of cell proliferation

The \textit{APC} protein plays an integral role in the Wnt-signalling pathway, especially in regard to the degradation of $\beta$-catenin within the cell cytoplasm (Bienz. 1999; Willert et al. 1998). The \textit{APC} protein binds $\beta$-catenin and regulates intracellular levels of $\beta$-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.
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).
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 \textit{APC}

In approximately 80\% of individuals with FAP, germline mutations can be identified within the \textit{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 \textit{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 \textit{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 \textit{APC} allele is suggested to underly the genetic susceptibility for FAP/AFAP/multiple adenomas. The reported germline mutations in \textit{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 \textit{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).

![Graph showing distribution of APC mutations](image)

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 Hahnlooser 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).

![Diagram of *APC* gene regions](image)

**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 \textit{APC} gene

Because germline mutations of \textit{APC} gene are well known to cause genetic susceptibility to FAP and AFAP, detection of \textit{APC} germline mutations could be a powerful tool for clinical diagnosis or predictive testing for FAP/AFAP/multiple adenomas. Testing for known mutations in \textit{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 \textit{APC}-associated polyposis conditions.

Mutation scanning of the entire \textit{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 \textit{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

<table>
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<th>Patient No</th>
<th># of polyps/age at diagnosis of polyps</th>
<th>CRC/age at diagnosis of CRC</th>
<th>Extracolonic Manifestation</th>
<th>Family History</th>
<th>Phenotype</th>
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</table>
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% Ig-pal (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 10 ml of TKM$_1$ buffer, re-suspended in 0.8 ml of TKM$_2$ buffer (10 mM Tris HCl, 10 mM KCl, 10 mM MgCl$_2$, 4M NaCl, 2 mM 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 °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 Na₂EDTA, 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

<table>
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<tr>
<th>Exon</th>
<th>Forward primer 5'-3'</th>
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</table>

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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 (50 mM) MgCl₂, 2.5 μl (10 mM KCl, 10 mM (NH₄)₂SO₄, Tris-HCl, 2 mM MgSO₄, 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 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 50 °C -58 °C for 1 min, 72 °C for 1 min, plus a final extension of 72 °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 (Qigian) 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 °C for 15 min, and terminated by heating at 80 °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 °C for 4 min, as an initial denaturing step, followed by 40 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °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 °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 °C) 200 μl of 70% ethanol to remove the unincorporated dye-terminators and the residual
salts. For each rinse, cold (-20 °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 (1 fmol 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 MgCl₂, 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

<table>
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<th>Patient</th>
<th>Clinical</th>
<th>Mutations</th>
<th>Polymorphisms</th>
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<tbody>
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<td>c.3067dupA</td>
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<td>c.3183_3187delACAAA at codon 1061</td>
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<td>63</td>
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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

<table>
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<td>c.1458 T&gt;C (p.Y486Y)</td>
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<tr>
<td>c.1635 G&gt;A (p.A545A)</td>
<td>18.8</td>
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<tr>
<td>c.2946G&gt;A (p.S982S)*</td>
<td>2.1</td>
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<tr>
<td>c.3165A&gt;T (p.I1055I)*</td>
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<tr>
<td>c.4479G&gt;A (p.T1493T)*</td>
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<td></td>
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<tr>
<td>c.5034 A&gt;G (p.G1678G)</td>
<td>33.3</td>
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<tr>
<td>c.5880 G&gt;A (p.P1960P)</td>
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<tr>
<td>c.7201C&gt;T (p.L2401L)*</td>
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<tr>
<td>c.5465T&gt;A (p.V1822D)</td>
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</tr>
<tr>
<td>c.7504G&gt;A (p.G2502S)</td>
<td>2.1</td>
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</tr>
</tbody>
</table>

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.
Figure 12 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.
Figure 12b. 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 predicted 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**

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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 \( \beta \)-catenin binding and down-regulation (Bright-Thomas and Hargest. 2003). The \( \beta \)-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 \( \beta \)-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 β-catenin down-regulation domain, and therefore, is unlikely to crucially effect β-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
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 \textit{APC} causing FAP. This hypothesis is suggested based on the observations that approximately 20\% of clinically typical FAP kindreds fail to show any \textit{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 \(\alpha\)-catenin gene (Mcpherson et al. 1994), \(\beta\)-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 (\textit{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


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