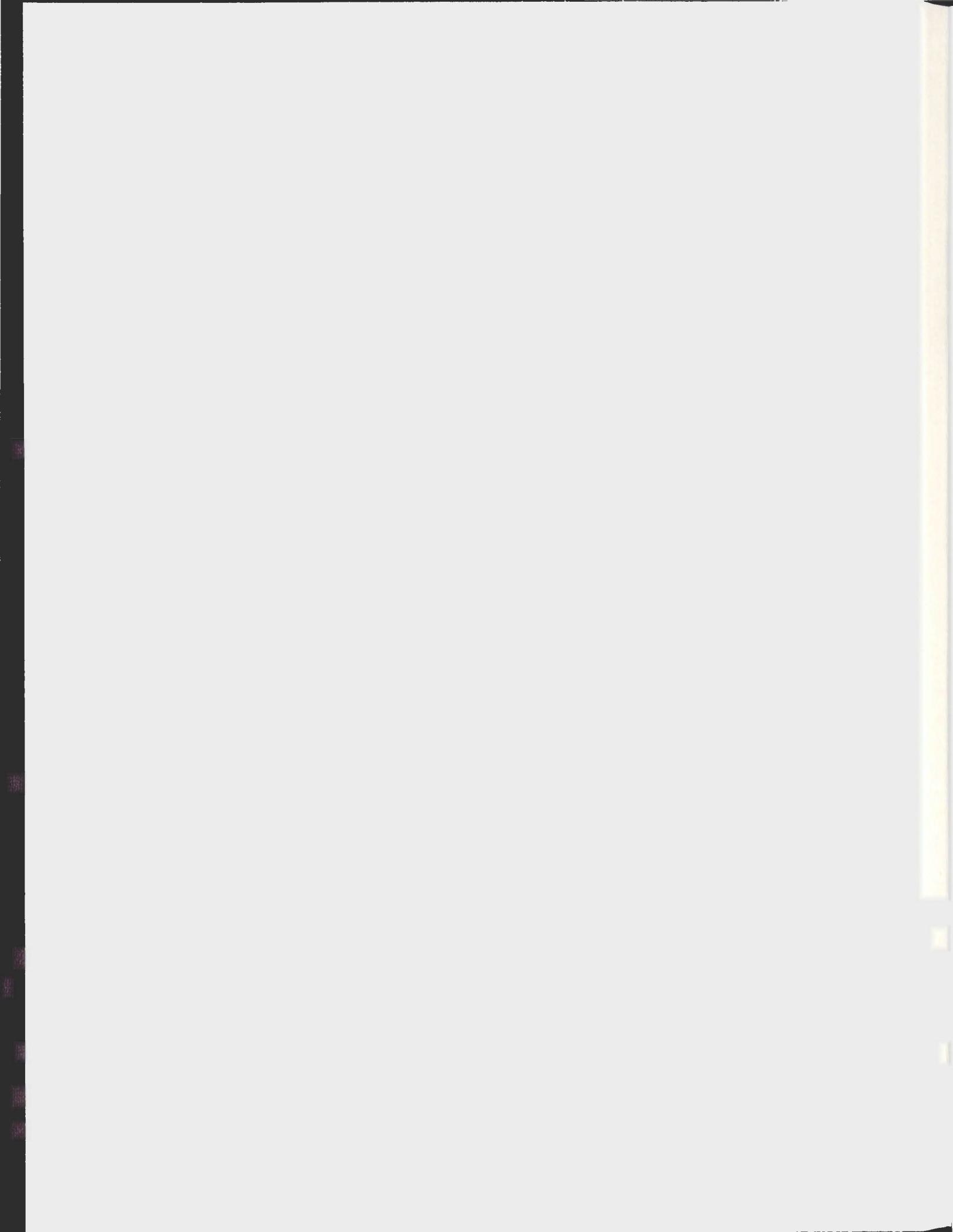# THE INVESTIGATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN GENES OF THE CELL CYCLE AND RELATED PATHWAYS AS CANDIDATE MODIFIERS OF THE AGE OF DISEASE ONSET IN HEREDITARY NON-POLYPOSIS COLORECTAL CANCER

AIMÉE DAWN ADAMS

THE INVESTIGATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN GENES OF THE CELL CYCLE AND RELATED PATHWAYS AS CANDIDATE MODIFIERS OF THE AGE OF DISEASE ONSET IN HEREDITARY NON-POLYPOSIS COLORECTAL CANCER

by

Aimée Dawn Adams

A thesis submitted to the

School of Graduate Studies

In partial fulfilment of the

Requirements for the degree of

Master of Science –Medicine

Discipline of Genetics/Faculty of Medicine
Memorial University of Newfoundland

April 2009

St. John's   Newfoundland and Labrador

Abstract

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is the most common type of inherited colorectal cancer. Eighty to ninety percent of identified mutations in HNPCC families involve *MSH2 or MLH1* genes. However, a great degree of variability has been observed within and between families carrying the same mutation. Therefore, other factors such as modifying genes may be involved in the presentation of this disease. The cell cycle, the mismatch repair pathway, and folate metabolism have been associated with cancer. Therefore, I studied 31 single nucleotide polymorphisms (SNPs) from genes in these pathways to determine if they had a modifying effect on the disease penetrance. Two *MSH2* kindreds were used in this study, one from Newfoundland and one from the Lower North Shore of Quebec. They included 135 mutation carriers. I identified 3 SNPs *CCND1* ^{1722} GC, *CCNA2* GA, and *CDKN1B (p27^{KIP1})* TG, which had significant effect on the age of disease onset.

Acknowledgements

with me on numerous occasions to provide me with the materials I needed to support my ideas that eventually made this project and thesis a reality –Thank-you Des you are sadly missed.

To everyone at Memorial University, especially in the Faculty of Medicine and the Discipline of Genetics including the University of Toronto group, it's been a pleasure working with all of you. I have learned very valuable lessons during my time with you. Especially to the people in Ban's lab (and Michelle Simms), thank-you so much for being a part of my life and being so good to my children. Sure going to miss the birthday rituals. A special mention to Deborah Quinlan is offered for all of her administrative and emotional support. I would also like to thank Dr. Sue Moore for introducing me to Ban, encouraging me to do this project, and for her statistical assistance at times when I was in a "crunch".

I want to thank the staff at the Health Sciences Library for being so helpful in finding resources, keeping the photocopiers and computers running smoothly, and being such a pleasure to chat with. I also wish to acknowledge Dr. Debrah Wirtzfeld, Dr. Mary O'Brien, Dr. Brendon Barrett, Dr. Adrien Lear, Dr.Phil Davis, Betty-Ann Lewis, Cecile Stares, Dr.Kathy Hodgkinson, Dr.Nancy Whalen, Dr. Simon Kirby, Vickie Jamieson (RN), Nicky and Sam Allan, Becky Paluch, Tonia and John Sheridan, Mary O'Driscoll, Dr. Robert Dean and the late Peggy Dean (RN), Donna and Dereck Lee, and Rhonda and Danny Squires who each have helped in their own special way at various points throughout my time at Memorial University. I am truly thankful for your help with my

appreciate what HNPCC patients actually go through. She made the importance of this research project very, very real to me.

Iliasse, thank-you for our beautiful daughter, although her arrival more than doubled the duration of this degree, I do not regret a single moment.

To my children Nathan, Melanie, and Amina, you truly are the sunshine of my life. Nathan and Melanie, although I pursued my education with the optimism of bettering our lives, sacrifices were made. Your patience with me throughout this journey goes far beyond what any parent could have ever dreamed of let alone obtained. You are absolutely amazing children and it is to you that I dedicate this thesis.

Sincerely,
Aimée Dawn Adams

Dedication
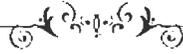
This thesis is dedicated to the HNPCC families of

Newfoundland and the Lower North Shore of Quebec, to my

late Uncle Donald Pilgrim, to my Aunt Charlotte Ash, and

most importantly, to my wonderful children Nathan,

Melanie, and Amina.
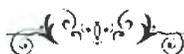
"Nature is all that a man brings with himself into the world; nurture is every influence that affects him after his birth"

Francis Galton,
English Men of Science,
1874 (p. 12).

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List of Abbreviations

AFAP	Attenuated Familial Adenomatous Polyposis
AJCC	American Joint Committee on Cancer
Amp_len	Amplicon Length
APC	Adenomatous Polyposis Coli
APC/C	Anaphase Promoting Complex or Cyclosome
ASP	Affected Sib Pairs
BRCA1	Breast Cancer Susceptibility Protein1
BWS	Beckwith-Wiedemann Syndrome
CAK	Cdk-Activating Kinase
CCN	Cyclins
CCNA	Cyclin A
CCNB	Cyclin B
CCNC	Cyclin C
CCND	Cyclin D
CCNE	Cyclin E
CCNF	Cyclin F
Cdc2	Cell Division Cycle2
CDKs	Cyclin Dependent Kinases
CDK1	Cyclin Dependent Kinase 1 also known as cdc2
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
Chk1	Casein Kinase-1
Chk2	Casein Kinase-2
CHRPE	Congenital Hypertrophy of the Retinal Pigment Epithilium
CI	Confidence Interval
CKIs	Cdk Inhibitors
C-Mad2	Closed Mad2
CRC	Colorectal cancer
DFNM1	Deafness Nonsyndromic Modifier 1
DNA dbsb	DNA double strand breaks
DP	DRTF1 polypeptide molecule
DSP	Discordant Sib Pairs
Family C	A large HNPCC family from the Northeast coast of NL
Family 11	An HNPCC family from the Lower North Shore of Quebec that traditionally received their healthcare from the hospital in St. Anthony, NL
FAP	Familial Adenomatous Polyposis
FCC-X	Familial Colorectal Cancer Syndrome X
FDR	First-Degree Relatives
FH	Familial Hypercholesterolemia
FOBT	Fecal Occult Blood Test
hME	Homogenous Mass Extend

HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IBD	Identity-By-Decent
ICG-HNPCC	International Collaborative Group on HNPCC
INSIGHT	International Society of Gastro Hereditary Tumours
IVP	Intravenous Plyelogram
kD	Kilodaltons
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MCC	Mitotic Checkpoint Complex
MIN	Multiple Intestinal Neoplastic
MLH1	MutL Homolog1
MLH3	MutL Homolog3
MMR	Mismatch Repair Pathway
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI	Microsatellite Instability
MSS	Microsatellite Stability
MTHFR	Methylenetetrahydrofolate Reductase
MUN	Memorial University of Newfoundland
NBS1	Nibrin Protein
NCBI	National Centre for Biotechnology Information
NFCCR	Newfoundland Colon Cancer Registry
NL	Newfoundland and Labrador
NSAID	Non-Steroidal Anti-Inflammatory Drugs
NSCLC	Non-Small Cell Lung Cancer
ng	Nanogram (10^9 grams)
O-Mad2	Open Mad2
PcG	Polycomb Protein
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PMS1	Post Meiotic Segregation 1
PMS2	Post Meiotic Segregation 2
pRb	Retinoblastoma gene
p27^{KIP1}	*CDKN1B gene*
p53BP1	p53 binding protein1
QTL	Quantitative-Trait-Locus
RFLP	Restricted Fragment Length Polymorphism
SAC	Spindle Assembly Checkpoint
SAP	Shrimp Alkaline Phosphatase
SCCHN	Squamous Cell Carcinoma of the Head and Neck
SNPs	Single Nucleotide Polymorphisms
SSCP	Single Strand Conformation Polymorphism
TDT	Threonine 160
TNM	Tumor Node Metastasis
Tsq	Thermosequenase

Tyr15	Tyrosine 15
U	Units
UEP	Unextended Primer

Chapter 1 Introduction and Overview

1.1 **Introduction**

Colorectal cancer (CRC) is a disease affecting both women and men. The age of onset varies from youth to the elderly population depending on the type of CRC and the genetic or environmental influences that are involved. This disease is curable provided it is detected in an early stage.

Determining what stage the cancer is in is based on a specific set of criteria that has evolved over time. In 1932, C.E.Dukes first introduced the Dukes staging system. This system was pathologically based on tumour resection that measured the depth of invasion through the mucosa and bowel wall. It was categorized as follows: Dukes A—tumour confined to mucosa, Dukes B——tumour invading through the intestinal wall (no lymph node involvement), Dukes C—positive lymph nodes identified, Dukes D—distant metastases has occurred. The specifics of this system have been revised several times since its first use because it did not provide detailed information about the tumour size, nodal involvement, or spread throughout the body. Finally, in 1990, the American Joint Committee on Cancer (AJCC) replaced Dukes staging with a more descriptive and detailed model called the TNM staging system.

According to the TNM staging system T represents the primary thickness of the tumour, N describes nodal involvement, and M indicates the presence or absence of distant metastases. Each of these letters contains numerical subcategories that describe the cancer stage. For example, the T is subdivided from 1-4 based on the level of tumour invasion (Table 1.1). The N is described as 0, 1, or 2 where 0 refers to no nodal involvement, 1

includes 1-3 positive nodes, and 2 means that >3 positive nodes have been detected.

(Table 1.1). M is either 0 for no distant metastases or 1 for distant metastases (Table 1.1).

Table 1.1: TNM Staging System for Colorectal Cancer*

Primary Tumour (T)	Description
T1	Tumour invades submucosa lining
T2	Tumour invades muscularis propria
T3	Tumour invades through muscularis propria into submucoas, or non-peritonealized pericolonic, or perirectal tissue
T4	Invades other organs/structures, and/or perforates visceral peritoneum
Regional Lymph Nodes (N)	**Description**
N0	No regional nodal metastasis
N1	1-3 regional lymph nodes
N2	>3 regional lymph nodes
Distant Metastasis (M)	**Description**
M0	No distant metastasis detected
M1	Distant metastasis

*American Joint Committee on Cancer (AJCC) Cancer Staging Manual 6th ed. (2002) Springer-Verlag New York Inc.

The levels of TNM staging are (I) through (IV) where (I) describes the earliest form of a carcinoma while (IV) represents the latest. These stages are defined based on the numerical subcategories given to depict the tumour, node, and metastasis status (Table 1.2). The five-year survival rates for each of these stages in colorectal cancer are as follows: (1) TNM Stage I (Dukes A) is 93.2%, (2) TNM Stage IIA (Dukes B1) is 84.7%, TNM Stage IIB (Dukes B2) is 72.2%, (3) TNM Stage IIIA, (Dukes C1) is 83.4%, TNM

Stage IIIB (Dukes C2) is 64.1%, TNM Stage IIIC is 44.3%, and (4) TNM Stage IV,

(Dukes D) is 8.1%.

Table 1.2: Comparison of TNM staging to Duke's Staging System and the 5-year survival rate for colorectal cancer*.

Dukes Staging	Cancer Stage	TNM staging	5-Year Survival
Dukes A	Stage I	T1/T2, N0, M0	93.2%
Dukes B	Stage IIA	T3, N0, M0	84.7%
	Stage IIB	T4, N0, M0	72.2%
Dukes C	Stage IIIA	T1/T2, N1, M0	83.4%
	Stage IIIB	T3/T4, N1, M0	64.1%
	Stage IIIC	Any T, N2, M0	44.3%
Dukes D	Stage IV	Any T, Any N, M1	8.1%

* American Joint Committee on Cancer (AJCC) Cancer Staging Manual 6th ed. (2002) Springer-Verlag New York Inc

Surgical removal of the colorectal cancer and resection of the bowel cures this disease provided lymph nodes and other organs have not been invaded. Colorectal cancer is also preventable by polyp or pre-cancerous cell removal during regular screening through a colonoscopy.

The Canadian Cancer Society recommends that individuals without a known family history of colorectal cancer who are 50 years or older should have at least one colonoscopy every 10 years and one fecal occult blood test (FOBT) every two years.[1] FOBT is used to screen for traces of blood in the feces.[1] If a positive result is found further investigation by colonoscopy should follow to determine the source of the bleeding as it may suggest the presence of a polyp or cancer.[1] The frequency of screening after the age of 50 ranges anywhere from 1 to 10 years based upon health, lifestyle, and family history. In higher risk populations such as those with a family history of CRC at an

early age, colonoscopies are offered at ages <50 years based on the age prevalence within that family. The general rule of initiating screening in such families is 10 years younger than the youngest age of malignant diagnosis within a given family.[1]

The preventative purpose of the colonoscopy procedure is to detect and remove any polyps that may be present. Since polyps are believed to be pre-cursors to cancer, removing them eliminates their potential of evolving into a malignancy. Due to the common asymptomatic nature of CRC in the earlier stages, if the individual is not undergoing regular routine colonoscopy screening, it is often late stage III or stage IV before it is diagnosed. During these later stages the symptoms tend to become more prevalent and severe. Therefore, the prognosis is often poor for unscreened individuals when CRC is finally detected.

Once CRC is detected and family history taken, the type is determined as one of the following three categories: hereditary, familial or sporadic. Sporadic CRC occurs in approximately 6-7% of the general population.[2] It does not have any obvious family history or mode of inheritance and there are very few to virtually no family occurrences of CRC at all. Familial CRC presents in family clusters more than would be expected in the general population but no genetic factors are clearly identified. Hereditary CRC is a single gene disorder and its risk can be identified in families as long as the gene(s) involved are known.

The two most common hereditary forms of CRC are Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC). FAP involves mutations in the APC gene and some HNPCC involves mutations in genes of the Mismatch Repair Pathway (MMR) following a dominant mode of inheritance.

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In Newfoundland and Labrador and the Lower North Shore of Quebec, Canada there have been large families or founder clusters of families identified with HNPCC. These families serve as very informative models for studying this type of genetic disease due to geographical isolation, ethnic homogeneity, and a large number of individuals within each of the families remaining in the ancestral region.

Mutations in an MMR gene *MSH2* were identified in three of these Newfoundland and Quebec families and family clusters. In the Newfoundland and Labrador families from Bonavista North, referred to as Family C, there was a mutation found in the (A→T942+3) 3'splice region of intron 5; a group of families from Trinity/Conception Bay were identified to have a deletion of exon 8; while the Quebec family (Family 11) has a deletion in exons 4-16.[3] Each family has multiple mutation carriers identified, but there is variation in the phenotype presentation. For example, there is a variation in the age of onset, location of cancer, the number of primary cancers between individuals within each family as well as between families. Therefore, other factors must be involved in determining phenotype expression besides the identified *MSH2* mutation. This study included two of these three families, Family C and Family 11 for the purpose of investigating these variations.

1.2 Colorectal Cancer

Colorectal cancer (CRC) is the second leading cause of cancer death for men and women in North America. The Canadian Cancer Society estimates that ~ 21,500 Canadians will be diagnosed with CRC and about 8,900 will die from their disease in 2008. In Newfoundland and Labrador (NL) approximately 480 people will be diagnosed in 2008

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with CRC and >50% of them will eventually die from their disease. Women and men are at unequal risk of developing CRC with men having a slightly higher incidence rate than women. After the age of 50 years, the risk of developing this form of cancer increases significantly.[3] The incidence rate of CRC in NL is 27% higher than the Canadian average.[2] This demonstrates the importance to the people of Newfoundland and Labrador of investigating this disease.

Because CRC is usually asymptomatic until it is in a late stage, it generally has a poor prognosis at the time of diagnosis. Even if symptoms are present, many people are too embarrassed to talk about their "bowel problems" thus delaying diagnosis until their CRC is advanced and difficult to treat. For these reasons, it is important that the public be educated about the seriousness and prevalence of CRC in the general population.

The Canadian Cancer Society recommends that screening with colonoscopy after age 50, and earlier if there is a family history, is the most important preventative method to date. The current treatments available for those with colon cancer are surgery (total colectomy, partial colectomy, and tumour resection), chemotherapy, and radiation. The extent of surgery depends on the location and stage of cancer.

In 2007, the cost of treatment of Canadians with CRC was estimated to be approximately $500,000,000.[2] Therefore; research on the cause, treatment, and prevention of this disease is of importance to those involved both provincially and federally in managing health care expenses.

The actual cause of the different types of CRC has not yet been fully determined. Therefore, CRC may or may not follow different mechanisms of disease formation.

However, all cancers do involve changes in genes in somatic cells and sometimes in conjunction with germline cell mutations.[4, 5]

There are three categories of mutated genes that are commonly associated with CRC: [6, 7] oncogenes, tumour suppressor genes, and DNA mismatch repair genes. The cellular proto-oncogenes are up-regulators of cell proliferation.[8] Hence, a mutation in one of these genes can result in it being transformed into an oncogene. Once this occurs the oncogene can cause the cell to proliferate uncontrollably. For instance, mutations in the *B-raf* and *K-ras* oncogenes have been associated with an increased risk of colorectal cancer.[9] Tumour suppressor genes, predicted in Knudson's model of the "two-hit theory" in retinoblastoma, function as negative regulators of cell proliferation.[10] Thus, an effect is seen only when both alleles are mutated.[10, 11] The DNA mismatch repair (MMR) genes repair errors that occur during DNA replication.[12, 13] As a result, a loss of MMR function leads to an accumulation of mutations throughout the genome including mutations in growth regulatory genes.[14]

As the above description indicates, both inherited and sporadic cancer involves mutational alterations of genes. It is believed that all colorectal carcinomas develop from adenomas.[15] Together, hereditary and environmental factors contribute to the development of these neoplasms.

In 1990, Fearon and Vogelstein developed a model that describes the development of a tumour from polyp to carcinoma.[2, 4] First, a pocket of colonic epithelial stem cells[16] containing a coupled mutation between oncogenes and tumour suppressor genes cause these mutated cells to propagate. As more of these mutated cells divide and make cloned copies, a growth advantage of mutated cells over surrounding normal cells is believed to

occur. The genes most frequently found to be involved in this process are the oncogene *K-ras*[17, 18] and the tumour suppressor gene *p53*.[19, 20] Approximately 50% of colorectal adenomas >1 cm in diameter, as well as carcinomas, have *K-ras* mutations.[21-23] However, only ~10% of adenomas <1 cm in diameter have a mutation in this gene regardless of whether the CRC arose sporadically or in the context of an inherited predisposition.[23, 24]

In adenomas only one *p53* allele is mutated. The mutated p53 protein functions in a dominant negative pattern by binding to the wild type p53. Such binding prevents normal function of this prtoein. This was observed in mice,[25-28] rats,[29] and humans.[20, 30] In cells that have an impaired wild type p53, the adenoma is able to continue to grow. Since these adenomas also grow and divide at a faster rate than normal cells, they are at an increased growth advantage to the normal cells. This in turn, increases the likelihood for the wild type *p53* gene to also be mutated as more copies of the cells containing both a mutated and wildtype allele proliferate. Once both of the *p53* alleles are mutated the tumour suppressor function is completely knocked out. It is at this stage that adenomas often progress further to become carcinomas.[20, 30]

An accumulation of 4 or more mutations in these oncogenes, tumour suppressor genes, or other genes must take place in the cell before it forms a malignant tumour. If there are < 4 accumulated mutations, the adenoma will remain a benign tumour.[30, 31] It is the accumulation of mutations rather than the sequence of events that determine the tumour's biological properties. Finally, although tumour suppressor genes generally act in a recessive pattern, some behave in a dominant negative fashion at the cellular level as in the case described above for p53.[29]

1.2.1 Sporadic Colorectal Cancer

People with sporadic colorectal cancer have very few to only one family member affected with the disease. This category of CRC has a late age of onset usually starting over 50 years of age and includes 70-75% of all CRCs.[3] The cause is believed to be a multi-factorial result of dietary, environmental, and genetic factors that have yet to be defined.[5] The sporadic incidence of CRC has been identified with mutations in somatic cells rather than in the germline cells that are noted in hereditary forms of this disease.[4] Apart from these gene mutations, recent studies have also associated a possible epigenetic cause such as the hypermethylation of the CpG islands and microsatellite instability (MSI).[32, 33] Hypermethylation of CpG islands in promoter regions of genes are an epigenetic process that inhibits transcription of specific genes throughout the genome. Microsatellite instability is the result of a genetic mutation that causes the number of microsatellite repeats in a particular region of the cell's genome to vary from that of a normal cell. Together, hypermethylation of *p16* and MMR deficiency of *MLH1*, relates to loss of cell cycle control.[32, 33] This phenomenon is found at very high frequencies in MSI tumors.[34-36] Hong-Zhi et al (2002) related *p16* hypermethylation to MSI tumours and suggested it be used as a marker in serum analysis for the prediction of colorectal cancer prognosis.[37]

Since Sporadic CRC includes the vast majority of CRC, a better understanding is necessary in order to determine who is at risk of developing it. As it is only apparent in population history, individuals are at a lower risk of developing this disease when compared to familial and hereditary forms of CRC. Therefore, regular screening is recommended for anyone over the age of 50 as previously described.

1.2.2 Hereditary Colorectal Cancer

Individuals with relatives that have hereditary colorectal cancer are at the highest risk of developing the disease in comparison to those at risk of sporadic or familial colorectal cancer. It is in this group where screening programs are generally implemented. One of the main differences between hereditary and familial CRC is its earlier age of onset. Hereditary CRC almost always develops at <50 years of age rather than the later age of onset (60+ years) observed in the familial groups. This earlier age of onset that is observed in hereditary cancers results from a germline mutation in one wild type tumour suppressor allele. Therefore, only one more "hit" in the tumour suppressor gene is required for a cell to lose its tumour suppressor function.

Out of all of the colorectal cancers approximately 5-10% of them demonstrate a pattern of dominant inheritance.[38-40] These dominantly inherited CRCs are referred to as hereditary colon cancers. The two best-known forms are HNPCC and FAP. Together, both of these types are inherited autosomal dominantly and account for approximately 5-6% of all CRCs.

FAP occurs in <1% of all colorectal cancers.[41] In this form of the disease the colon can develop several hundred to thousands of adenomatous polyps, typically beginning in the second or third decade of life. These polyps are initiated at a greatly increased rate compared to the general population, but once formed, their rate of progression to carcinoma is remarbably fast.[42, 43] Inevitably, one or more of the polyps will progress into an invasive carcinoma.

Extracolonic manifestations are also common in families with FAP. These include polyps in the stomach or duodenum, which may also progress to cancer, desmoids, osteomas, or

epidermal (sebaceous) cysts. Papillary thyroid cancer and brain tumours, usually meduloblastoma, occur rarely in these families but at an increased rate in comparison to the general public.

The FAP locus was mapped to chromosome 5q21 after the discovery of an interstitial deletion of chromosome 5q21 in an individual with FAP and mental retardation.[44-46] The adenomatous polyposis coli (*APC*) gatekeeper gene was identified in this region[47] and extends 120 kilobases with 15 exons. The protein product of the *APC* gene has 2843 amino acids whose function is to negatively regulate the Wnt signaling pathway. It does this by binding to and destroying beta-catenin. Loss of this function causes an unregulated intracellular build-up of beta-catenin.[48] The biological significance of this build-up is that elevated levels of beta-catenin are associated with an increased growth advantage of tumour cells.

Inherited APC mutations co-segregate with FAP in affected families.[49] Other studies showed that the location of the mutation influenced the phenotype of this disease. For example, truncating mutations between codons 463 and 1387 are associated with congenital hypertrophy of the retinal pigment epithilium (CHRPE) [50] while mutations between codons 1403 and 1578 are linked to desmoid and osteoma cancers but not CHRPE.[51] However, there is considerable variation of disease presentation even with the same mutation and non-penetrance has been observed.[49] It is yet unknown whether these varying phenotypes are caused by environmental or genetic modifiers. Having said that, the *MOM1* gene on chromosome 4 in multiple intestinal neoplastic (MIN) mice has demonstrated a genetic modifying effect on the number of polyps lining the colon of mice with germline *APC* mutations.[52] The environmental effects on these phenotypes have also

been shown through both chemo-preventive non-steroidal anti-inflammatory drugs (NSAID) and dietary factors such as arachidonic acid lipids.[53-55] Together these environmental findings correlate with the genetic *MOM1* modification described above since *MOM1* normally expresses an enzyme that metabolizes arachidonic acids.[56] If there is an abnormality in the *MOM1* gene then its enzymatic activity may also be altered and could result in a change in the way that these acids are metabolized Thus, environmental influences may enhance the effects of the *MOM1* mutation in an epigenetic manner.

Apart from this classical form of FAP, there is an atypical phenotype referred to as attenuated familial adenomatous polyposis (AFAP). In AFAP, the number of polyps varies and they are smaller in size than those observed in FAP phenotypes. Individuals present with AFAP later in life and have a reduced penetrance of the disease.[9, 57] Quite often AFAP can be mistaken for HNPCC in terms of the clinical features.[9, 57]

HNPCC accounts for ~3-5% of all colorectal cancers with a high penetrance of 80-85%.[42, 58-63] Unlike FAP, usually no polyps or a small number (1-5) form in the colon of those with this syndrome. These adenomas form at approximately the same rate as in the general population.[42, 43] Once formed, however, they progress 2-3 times more rapidly.[42, 43] The age of onset of HNPCC is variable from the third to the seventh decade with a median age of 42 years.

HNPCC has recently been re-named and divided into two groups: Families with mutations in the mismatch repair (MMR) genes are considered to have Lynch Syndrome, while families without evidence of MMR gene mutations are referred to as having Familial Colorectal Cancer Syndrome X (FCC-X). The average age at onset of CRC is typically earlier in Lynch Syndrome than FCC-X.

Lynch Syndrome commonly has extracolonic cancers that occur either exclusively or in addition to CRC. They include cancers of the endometrium, ovaries, stomach, small bowel, transitional cells in the uretergenital system, skin (sebaceous cell cancer), and less commonly glioblastomas.[64-66] The MMR genes that have been identified in association with this syndrome are *MSH2, MLH1, MSH6, MSH3, PMS1,* and *PMS2.*

To date, no genes have been found associated with FCC-X. Unlike Lynch Syndrome, the presentation of extracolonic cancers in this particular group is rare. It is also uncommon in FCC-X to find the synchronous or metachronous CRC that are observed in other forms of CRC.

Identification of HNPCC is done through family history. The International Society of Gastro Hereditary Tumours (INSIGHT) previously known as the International Collaborative Group on HNPCC (ICG-HNPCC) defined the "Amsterdam criteria" used mainly for research purposes in order to identify this disease.[67-69] The criteria includes the following: (1) three or more first degree relatives with CRC, one must be a first degree relative of the other two, (2) at least 2 successive generations affected, (3) one or more of these cases identified before age 50, (4) Familial adenomatous polyposis should be excluded, and (5) tumours should be verified by pathological examination.[68] After this guideline was published Lynch et al. (1993) stated that these criteria were too stringent because extracolonic cancers such as endometrial cancer were not considered.[70] As a result "Amsterdam 2 criteria" were defined.[71] These new criteria took into account extra-colonic cancers as follows: (1) three or more first degree relatives with an HNPCC-related cancer (CRC, endometrial, small bowel, ureter, or renal pelvis); and one must be a first degree relative of the other two, (2) at least 2 successive generations should be

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affected, (3) and one or more of these cases identified before age 50, (4) Familial adenomatous polyposis should be excluded, and (5) tumours should be verified by pathological examination.[71]

Once a kindred has been identified with HNPCC, at-risk family members are offered a clinical screening program to identify and treat early cancers or precancerous lesions.[71] There are two main ways of doing this. First, if no mutation has yet been found in an HNPCC kindred all first degree relatives are assumed to be at 50% risk and recommended to have a colonoscopy every 1-2 years starting at 10 years prior to the age of the youngest HNPCC cancer diagnosed in that family.[1] Screening for extracolonic cancers may also be recommended based on family occurrence. Secondly, when a mutation has been identified in a family, only those who test positive for the mutation are screened aggressively through colonoscopy, and screening for extracolonic HNPCC-related cancers is also applied.[64, 72-74] These extracolonic screening protocols include endometrial biopsy, transvaginal ultrasound to view the endometrium and ovaries, and IVP (intravenous plyelogram) cystoscopy monitoring of the genitourinary tract.[58, 75-77] Preventative treatments are also an option for those with a strong history of HNPCC, and are usually offered following genetic counselling along with the advice of a surgeon or oncologist. These options include prophylactic surgeries, such as hysterectomy, oophrectomy, and subtotal or total colectomy with ileorectal anastomosis followed by regular screening of the rectum.[58, 60, 75, and 78] Other organs at risk may also require screening and prophylactic surgery.[58, 60, 75, and 78]

1.2.3 Familial Colorectal Cancer

Familial colorectal cancer is a cluster group of CRC in families that do not meet the Amsterdam criteria and the disease causing or contributing genes have not yet been identified. This group of CRC has a slightly higher risk than that of the general population noted in sporadic CRC.

Approximately 20% of all CRCs are Familial. The number of individuals diagnosed within a family is higher than what would be expected by random chance in the general population of those without any obvious family history. However, an inheritance pattern is less obvious for these familial colon cancers in comparison to hereditary forms. For example, the family history may include more distantly related affected relatives such as the individual affected and his/her aunt/uncle (primary degree relative), and/or cousin (second degree relative). In addition, the cancers in these families usually do not present until later in life such as age 60 or older.

The risk for relatives within these families increases based on the number of cancers that are present within the family. Therefore, if a person has two first-degree relatives (FDR) with CRC, that person's risk is increased two-fold compared to the general population and ten-fold if there are three FDRs.[79] Also, if there are relatives in the family that had a polyp or CRC before the age of 60, the individual is at an even higher risk of developing CRC.[79] If there are also second- or third-degree relatives with this type of cancer the risk is increased by >50% compared to the general population.[79] As in the sporadic group, the cause for Familial CRC is unknown. It has been hypothesized that familial CRCs may result from a combination of both genetic and environmental factors such as radiation exposure and/or smoking.[3]

Recently, it has been observed that the province of Newfoundland and Labrador has 43% of all its CRC cases to be of a familial nature[80]and 31% have at least one first-degree relative that has been affected with CRC.[80] The reason Newfoundland and Labrador has a higher proportion of familial CRC than found in other populations is currently under investigation.[80]

1.3 HNPCC Mutations

Although mutations in >50% of HNPCC families have not yet been identified, to date all of the mutated genes that were found to be associated with HNPCC are part of the mismatch repair (MMR) pathway.

The mismatch repair pathway is a process in the cell cycle where DNA is checked for nucleotide mismatches after DNA replication.[81] This pathway occurs in the S-phase checkpoint of the cell cycle and is a necessary process for progression to the next phase.[82, 83] If there is a base-base mismatch or an insertion deletion loop in the DNA, the MMR pathway will repair it.[84-86] Otherwise, a different cell signal will direct the cell to die via apoptosis.[84-86] These replication errors generally are caused by slippage of repeated sequences of the DNA polymerase.[87] Some of the genes known to be involved in the mismatch repair pathway are *MLH1, MLH3, MSH2, MSH3, MSH4, MSH5, MSH6, PMS1, PMS2, PMS2L3,* and *PMS2L4.*[88]

In brief, the MSH2 protein forms a heterodimer with either MSH3 or MSH6. If there is a base-base mismatch in the DNA, MSH2 will bind with MSH6, whereas for insertion deletion loop repair, it will bind with both MSH3 and MSH6.[89, 90] MLH1 also forms heterodimers in this pathway by binding with PMS2 to operate the mismatch recognition

complex. It may also bind with other MMR proteins such as proliferating cell nuclear antigen, DNA polymerase δ and ε, single stranded DNA-binding proteins, and helicases. MLH1 may also form a heterodimer with MLH3 for insertion deletion loop repair and with PMS1 where the function has not yet been clearly defined.[91-93]

To date, mutations in six mismatch repair genes have been identified in HNPCC families around the world. These genes are *MSH2* (mutS homolog 2) located on chromosome 2p16,[94] *MLH1* (mutL homolog1) on 3p21,[95, 96] *MSH6* (mutS homolog 6) on 2p16,[97] *MLH3* (mutL homolog3) on 14q24,[98] *PMS1* (postmeiotic segregation 1) on 2q31,[99] and *PMS2* (postmeiotic segregation 2) on 7p22.[99] More than 80% of HNPCC mutations have been identified in either *MSH2* or *MLH1*.[87, 100, and 101] The remaining MMR mutations are mostly in *MSH6* with less then 10% of families having an alteration in *MLH3, PMS1*, or *PMS2*.[87] The ICG-HNPCC database (www.nfdht.nl) has reported more then 1000 different mutations in these MMR genes in over 500 kindreds globally.[87] A new database developed at Memorial University of Newfoundland documents >2400 variants in these genes (http: //www.med.mun.ca/mmrvariants/).[102]

A feature observed in those with HNPCC and 15% of sporadic colon cancer cases involves short repeating nucleotide fragments of the DNA called microsatellites.[103] Changes in the number of repeated nucleotides in microsatellites either through deletion or addition in tumour cells with an MMR defect, is called microsatellite instability (MSI). During testing for MSI, the number of repeats or deletions for a particular marker is compared between tumour cells and normal cells. The number of repeated nucleotides fluctuates after a biallelic loss of the *MSH2* or *MLH1* gene or a combination of other minor MMR genes.[104]

Three categories of microsatellite instability (MSI) are recognized: MSI-high if more than 30% of the microsatellites tested for a particular marker are unstable, MSI-low if 20-29% of the tested microsatellite markers present with instability, and microsatellite stability (MSS) when no instability is noted. Guidelines known as the "Bethesda criteria" were compiled in 1997 to identify individuals that should be tested for MSI.[103-105]

To improve the specificity the guidelines were revised as follows: (1) colorectal cancer diagnosed in a patient who is less than 50 years of age, (2) presence of synchronous, or metachronous colorectal or other HNPCC-associated tumours, regardless of age, (3) colorectal cancers with the MSI-high histology diagnosed in a patient who is less than 60 years of age, (4) colorectal cancer or HNPCC-related tumour diagnosed in one or more first-degree relatives, with one of the cancers being diagnosed under age 50 years, and (5) colorectal cancer or HNPCC-related tumours diagnosed in two or more first- or second-degree relatives, regardless of age.[106]

Phenotypic variations have been observed in HNPCC families depending on which MMR gene carries the mutation.[64, 87] There is some evidence that *MSH2* mutations present with more extracolonic cancers than do the *MLH1* mutations.[64, 87] *MSH6* mutation carriers generally have a later age at onset, a higher incidence of endometrial cancer, and have tumours that are typically MSS or MSI-low.[97, 107-111] *MLH3* mutations result in tumours with MSI variations ranging from low, intermediate, to high numbers of short nucleotide sequence repeats.[112]

1.3.1 Family C

A large kindred in Newfoundland, Canada, referred to as Family C, was involved in the identification of *MSH2* as a susceptibility gene for HNPCC.[113] This was the first human

mismatch repair gene linked to HNPCC. The Family C mutation was identified as a founder mutation at the splice-site of codon 942+3 A>T.[114] This mutationt is also recurrent in other parts of the world. Since these findings, more then 1000 mismatch repair gene mutations have been identified with ~40% *MSH2*, 41% *MLH1*, 12% *MSH6*, and 7% *PMS2*. [102, 115]

Family C has now been extended to include 16 Newfoundland sub-families that trace back to a common ancestor originating from Bonavista Bay, which is on the northeast coast of Newfoundland, Canada. Three North American families and four English kindreds were identified with the same mutation that disrupts the 3' splice site of intron 5 thus leading to deletion of the exon 5 sequence from MSH2 mRNA.[116, 117] The result was an inframe deletion of amino acids 265-314 of the protein.[113, 118] Eight percent of CRC families in eastern England have this mutation.[113, 118] The HNPCC phenotype expressed in this family includes endometrial, ovarian, skin, colon, and rectum.[119] Less frequent are cancers of stomach, ureter, small intestine, upper biliary tract, and brain.[119] The number of people in Family C is >1000 with >200 carriers of the *MSH2* mutation.

1.3.2 Family 11

Another *MSH2* mutation identified in an HNPCC kindred used in this study was a multi-exonic deletion from exon 4 to 16.[120] This mutation was identified in a kindred from the lower north shore of Quebec, Canada referred to as Family 11. This large family is of English background living in an isolated area with a stable population. The phenotypic expression of this mutation is mainly colon, rectal, ureter, and endometrial cancers.[121, 122] Less frequent are skin, stomach, and brain. Interestingly, no ovarian cancers have been observed in this kindred.[121, 122]

There are >577 people in Family 11. Of these 577, 125 were tested by haplotype analysis for the *MSH2* multi exonic deletion. Sixty-eight out of the 125 tested were mutation positive carriers and 57 were negative. However, 11 of the 57 who were negative developed a polyp or presented with HNPCC/HNPCC-related characteristics. Outside of the 125 individuals that were tested in this family, 34 people that did not have testing were assumed to be at 50% risk of having the mutation based on their family history.

1.4 Modifier Genes

1.4.1 Modifier Gene Definition

Phenotypic variation of disease within families with the same disease predisposing mutation has been attributed to several different causes. Some of these causes include genetic imprinting, environmental influences, X-inactivation, and modifier genes.

The concept of modifier genes was first proposed approximately 100 years ago.[123] However, it is only in recent years, through molecular and genetic technological advances; that this phenomenon can be investigated.

Starting with the most widely accepted definition, Nadeau (2001) suggested the following: a modifier gene is a locus that alters the phenotypic output of a major effect locus referred to as the target gene.[124] It can either reduce or increase the penetrance, expressivity, pleiotropy, or severity of a phenotype giving it either protective or susceptibility effects, respectively.[124-126] Nadeau also defined modification as an epistatic process where a genetic interaction of one allele with another could mask the phenotype caused by a mutation or sequence alteration in one of those genes.[124]

A less accepted definition comes from Weatherall (2001) who recognized three categories of modifiers: (1) primary modifiers (i.e. Nadeau's target genes), (2) secondary modifiers (Naudeau's modifier genes) and (3) tertiary modifiers, being genes that alter pathologic processes that are not related directly to the function of primary modifiers.[127]

Finally, in 2003, Slavotinek stated in her review of modifier genes that both of these definitions have imperfections because no two loci equally interact to influence a phenotype.[128] In other words, there may not always be a major locus, such as with the case of digenic inheritance where two loci each have necessary and equal effect. Slavotinek also points out that genes and gene products interact in networks rather than linearly. Therefore, a continuous spectrum of the degree of allele impact on many phenotypes caused by alleles at different loci should be expected.[128] Based on these criticisms, Slavotinek stated that the most common effects of modifier genes could be described as either additive or multiplicative.[128] That is, the relative risk of a disease phenotype resulting from mutations at two different loci was either the sum or multiple of the risks from each individual allele, separately.[128]

There are numerous types of effects that modifiers can mediate. It can be cell-autonomous, tissue specific or systemic action; qualitative or quantitative, both at the level of the protein and the disease phenotype; specific or non-specific action on the disease pathways; direct action, or requiring activation through somatic mutation or a specific environment.[129] Modification could involve any aspect of a trait from the primary action of the target gene, as in transcription, through to the intermediate phenotypes at the molecular or cellular levels, or the organ, system or whole body levels.[124]

1.4.2 Methods For Identifying Modifiers

Identifying genetic modifiers is more complex and time consuming than searching for disease-causing mutations. The reason is that they occur more frequently in the population and have a higher susceptibility to environmental influences than the pathogenic mutation. The two main methods used for identifying modifiers are linkage analysis and association (candidate gene) analysis.

In situations where the pathology of the disease is understood, any other gene(s) involved in the primary process are considered to be candidates for modifying the phenotype of the disease. Under such conditions, association analysis is the method commonly used.

The association of different alleles of these candidate genes is measured relative to the severity of the disease or other clinical variables such as age of onset or rate of disease progression. These studies are usually done in a case-control setting or through a transmission disequilibrium test (TDT) when DNA of both parents is available.[130]

If the pathological process of the disease has not yet been clearly defined, it becomes less obvious which genes to choose as candidate modifiers. Therefore, a linkage analysis is required.

Linkage analysis of modifier genes is most commonly conducted in a mouse model or in human families. Quite often when the pathology of the disease is not completely understood mouse models can have advantages. For example, mouse models permit large numbers of matings in shorter periods of time due to their short gestation periods.[131] Also, it is easier to control environmental influences that might cause variability of the disease phenotype.[131] However, there are also disadvantages to studying human diseases by using animal models. For instance, in Mendelian diseases there may be a difference in the

phenotypic expression between mice and humans with the same mutation.[131] At other times, there is a different genetic pathway involved even though the same type of disease is present.[131] Therefore, finding a disease locus or modifying effect in the mouse model does not mean there will be the same result when studying humans.

Another problem with mouse models versus human disease is with selective pressures and the effects of chance on the type and extent of genetic variability that can change from strain to strain in mice, as well as the transfer of data between human and mice studies[131]Thus, finding a genetic locus in an animal model does not imply that it will exist in humans.

Sometimes a knockout mouse model may be required to test a hypothesis. Unfortunately, inbred laboratory mice may disrupt linkage disequilibria making it difficult, if not impossible, to identify a modifying gene in the pure strain because the wild type genes may have been "bred out" from inbreeding.[131]

Mainly for these reasons, it is better to use human families whenever possible. Linkage analysis of modifiers in human diseases is based on human pedigrees. Since identifying modifier genes is not as straightforward as identifying primary disease loci, sib pairs are often used. The benefits of using sib pairs are that the relative risk of disease between siblings is higher than it would be in distant relatives.[131] Also, the frequency of the modifying allele must be considered in determining co-segregation with the primary disease.[131] Therefore, siblings would have an increased likelihood of a shared modifier allele when compared to distant relatives or the general population.[131] In cases where environmental influences on the disease phenotype are a concern, nuclear families become more informative as they generally share a similar environment.[131] Finally, sib

pairs are usually close in age thus reducing age-dependent factors that may contribute to the phenotypic variation of the disease of interest.[131]

In sib pair analyses, two methods can be used: affected sib pairs (ASP) and discordant sib pairs (DSP). ASP is generally the method of choice for identifying the primary disease locus.[131] However, for determining genetic modifiers, DSP are more useful.[131]

Discordant sib pairs consist of one affected and one unaffected sib.[131] Since ASP tend to have a higher incident rate and decreased survival because both are affected, it is more difficult to obtain clinical information on living subjects.[131] DSP are less likely to have these issues and therefore such comparisons provide the strongest evidence of any genetic modifying effects they may possess. Therefore, both quantitatively and qualitatively, DSP make the best model for studying modifiers of human genetic diseases.[131] It is expected that if both individuals have the primary disease causing mutation, the affected individual will also have the modifier allele that the unaffected sib does not have.

1.4.3 Identified Modifiers

Despite the preference for human systems outlined above, animal models have provided the most information regarding modifier genes so far. More is known of the biological systems of animal models, and their enviro-genetic status is controllable. One example involves a strain of mice known to have a dominant mutation in the *Apc* mouse gene at codon 850 in exon 15.[132] This strain of mice is referred to as the multiple intestinal neoplasia (MIN) mouse model, which is analogous to Familial Adenomatous Polyposis (FAP) in humans. Despite the common primary *Apc* mutation, in the mouse model, the number of colonic polyps varies with genetic background. This implies that other genes must be involved which affects the phenotypic presentation of the disease. Through

studies using a backcross with inbred MIN mice, a Modifier of MIN (*MOM1*) locus has been identified in the ~4cM region of mouse chromosome 14 syntenic to human chromosome 1p35-p36.[133] It accounts for ~40% of the genetic variance observed in the number of polyps in the *Apc*^min mice.[134] Genes found in this region that are candidate modifiers are *Pla2g2a, Egfr, Dnmt1,* and *Mmp7*. Of these candidates, the strongest data to date involves *Pla2g2a*.[135, 136] Mice with the mutated *Apc*^min, along with a null *Pla2g2a* gene had more polyps at a younger age than strains with the *Pla2g2a* wild type allele.[131] One study in humans found a significant correlation with the *Pla2g2a* modifier and patient survival.[137]

It has been more difficult to confirm modifying genetic effects in human disease. Some of the difficulties in studying modifiers in humans include genetic complexity, a longer life span compared to animal models, and ethical issues regarding study designs. Despite these obstacles, some modifier loci and genes have been identified in humans for diseases such as cystic fibrosis, familial hypercholesterolemia, and hereditary deafness.

Through linkage analysis, a penetrance modifier was found in the homozygous recessive form of a deafness gene linked to the *DFNB26* locus on chromosome 4q31.[138] A modifying locus within a 5.6cM region of chromosome 1q24 has been identified and named 'deafness nonsyndromic modifier 1' (*DFNM1*).[138] This modifier suppresses deafness for those homozygous for the primary mutation in the *DFNB26* gene.[138]

Another human genetic modifier affects the expression of mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene responsible for the autosomal recessive disease cystic fibrosis. CFTR, a gene of 230 kilobases on chromosome 7q31.3,[139] encodes a 1480 amino acid protein that is expressed in the apical membrane of

epithelial cells lining the lungs, sinuses, pancreas, intestines, sweat ducts, and vas deferens.[140] The *CFTR* gene significantly influences sodium transport and water balance since it functions as a cAMP-dependent chloride channel that interacts with numerous other channels.[141] There are a number of different phenotypes expressed in cystic fibrosis, such as lung disease, pancreatic enzyme insufficiency, and meconium ileus. Differences in the more than 800 mutations in *CFTR* cannot completely account for the clinical variations of this disease.[142] Through multipoint and haplotype analysis of sib pairs and parents in nuclear CF families, an approximate 7.65Mb locus on chromomosome 19q13.2-q13.4 was identified as a modifier of the penetrance of meconium ileus in those with *CFTR* mutations.[142] This CF modifier locus includes 141 candidate modifier genes.

Finally, a cholesterol-lowering gene on chromosome 13q has been proposed as a human disease modifier for the familial form of hypercholesterolemia (FH). FH is an autosomal dominant disorder, which affects cholesterol metabolism causing higher than normal serum cholesterol levels. Knoblauch et al. (2000) used linkage analysis, multipoint quantitative-trait-locus (QTL) linkage analysis, an independent study of monozygotic and dizygotic twins, and an identity-by-decent (IBD) linkage analysis of an Arab family in Armenia to confirm a locus on chromosome 13q as a modifier locus.[149] Those with the modifier have decreased cholesterol levels because of lower LDL concentrations and do not present with FH.[138] Further studies are being conducted to identify the exact gene(s) involved in this protective effect.

1.4.4 Modifier Genes and Cancer

The number of cancer modifier genes identified is limited. However, several candidate modifiers continue to be evaluated. Two examples include *RAD51* (135C/G) and *CyclinD1*.

Cyclin D1 (CCND1) is found on chromosome 11q13. It is a cell cycle checkpoint gene critical for the G1/ S phase transition of the cell cycle (see cell cycle: cyclins and cyclin complexes section below). [8, 143] A single nucleotide polymorphism (SNP), *CCND1* (870A/G), has been studied as a candidate modifier for phenotypic variation of squamous cell carcinoma of the head and neck (SCCHN), [144] non-small cell lung cancer (NSCLC), [145] and hereditary non-polyposis colorectal cancer (HNPCC). [146-148] In 1995 Betticher et al. reported that NSCLC patients with the AG and AA genotypes had a shorter relapse-free survival time when compared to the homozygous G group. Matthias et al., (1998) found the opposite to be true for SCCHN. [144] They concluded that the GG genotype of SCCHN correlated with a shorter time to tumour recurrence and with poor differentiation. In another study, Kong et al. (2000) reported an 11-year earlier age of onset of HNPCC for those with either the homozygous A or heterozygous AG alleles compared to the homozygous G genotype. [146] However, McKay et al. (2000) did not find any clinical significance for either genotype in those with HNPCC. [148] To date, no definitive study has implicated *CCND1* as a diagnostic/prognostic indicator for cancer treatment.

A second example, *RAD51* has also been studied for its role in cancer. This gene is responsible for maintaining genomic stability during recombination[150] and is essential for recombination repair of breaks in double stranded DNA.[150] *RAD51* is known to interact with the two breast cancer genes *BRCA1* and *BRCA2*.[151, 152] One study found that a

RAD51 (135G/C) allele was associated with an increased risk of breast cancer in both *BRCA1* and *BRCA2* mutation carriers.[153] They concluded that *BRCA2* mutation carriers with a *RAD51* (135G/C) C allele should be considered modifier candidates for an increased risk of breast cancer and a decreased risk of ovarian cancer.[153] Another study found no association between breast cancer and the *RAD51* G/C SNP, and concluded that it may not be a reliable candidate for modifying breast cancer risk.[154] Two other studies could only find a correlation between the penetrance of the *BRCA2* (6174delT) and the C allele of the *RAD51* (135G/C) SNP.[155, 156] No association was found with *BRCA1* (185delAG) penetrance.[155, 156] If further studies demonstrate significance of this modifier in relation to increased risk, then the *RAD51* (135G/C) SNP could be used to indicate which individuals might require more aggressive screening or preventative measures for breast cancer.

It is common to find discordance in the data for candidate modifiers from one study to another. Different cancers and even different studies with the same cancer are yielding different results despite studying the same candidate modifier gene polymorphism. This demonstrates the complexity involved in cancer development, its multigenetic attributions, as well as non-genetic influences or the limitations in the design of the studies. Therefore, when comparing studies, it is important to consider the population size, ethnicity, allele frequency, age, as well as how the data are collected and analyzed to rule out any underlying factors that may be associated with disease expression.

1.5 The Cell Cycle

1.5.1 Overview

The cell cycle is a process a cell undergoes in order to reproduce itself.[157-159] This complex pathway, although still not completely understood, requires the expression and interaction of numerous genes and mitogens. First, a cell will duplicate its DNA and cell mass. Following this step it will divide into two cells after appropriate signaling. The new cells are referred to as daughter cells while the original is called the parent cell.

The steps involved in cell replication have been categorized into four main phases: Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M). When the cell is not replicating it is in a resting state and referred to as Quiescent Gap 0 (G0) phase. The phases are a strictly regulated process as the integrity of the DNA and replication machinery is a vital component to the cell's viability. The length of time it takes to replicate is variable. However, a typical somatic cell is in the S-phase for approximately 10 hours, G2 for 4.5 hours, and M-phase for about 30 minutes. The variation in cell cycle duration depends on the transition time from G0 to G1.

Within these phases there are also sub-phases, as well as slight alterations pending on which type of cell is undergoing division. For instance, a mammalian germ cell will have additional phases know as meiosis that include a second cell division without going through the S phase. An Amphibian cell has rapid cell division that alternates between the S and M phases without any cell growth. The end result is 4000 cells with a full complement chromosomes at the end of 12 cleavage cycles.[160] As for the mammalian

somatic cell cycle, it begins when a cell starts to increase in size and duplicate its contents. It ends when the daughter cells resulting from this process do the same.

Cancer has a genetic etiology resulting in uncontrolled cell growth. Proto-oncogenes becoming activated while tumor suppressor genes are deactivated causing genetic instability leading to tumorigenesis. Since DNA damage and errors in DNA replication play such an important role in the malfunction of the cell resulting in the development of cancer, the normal progression of the cell cycle will be reviewed. The following sections refer to the mammalian somatic cell pathway. They describe how various genes, proteins, and mitogens are involved in the reproduction of a cell.

1.5.2 Cyclin Dependent Kinase (CDK) Regulation

The regulation of cyclin dependent kinases (cdks) involves a series of activations and inhibitions depending on where they are in the cell cycle and what signals are being given. To date, 9 cdks have been identified in mammals. Each of these protein kinases are between 30 and 40 kilodaltons (kD) in size with > 40% sequence identity to one another. Only those involved in the cell cycle will be discussed here.[161-163] These include cdk1, 2, 4, 6, and 7. The cdk2, 4, and 6 are all required for G1 progression whereas only cdk2 is known to be active in the S-phase. The cdk7 forms the cdk-activating kinase (CAK) trimeric complex. This cdk is involved in the cell cycle transition from G1→S phase. Finally, cdk1 also known as cell division cycle2 (cdc2) is active both in the S-phase and mitotic entry and exit of the cell cycle.[164, 165]

1.5.2.1 Activation

Cyclin dependent kinases (cdks) are protein kinases that are inactive as monomers. To become active cdks bind to protein subunits called cyclins (CCN) forming heterodimers. They become further activated through the phosphorylation of the threonine 160 (Thr160) residues[166] or through the dephosphorylation of tyrosine 15 (Tyr15) residues. In more detail, the cdk protein has a T-loop that prevents ATP from binding to its active site when the cdk is in its monomeric state.[167, 168] However, binding of a cyclin subunit causes a conformational change that removes the T-loop from the activation site of the cdk. In its heterodimeric state, cyclin-cdk can be phosphorylated at the Thr160 residue of the cdk to further enhance the enzymatic activity of this complex.[166] Phosphorylation of this residue is done by the CAK trimeric complex.[169-172]

Apart from the above, cdks can also be activated through the dephosphorylation of their Tyr15 inhibitory site. The cell division cycle (cdc) 25 phosphatase removes a phosphate group from the Tyr15 residue of the cdk in the cdk-cyclin complex to make it active.[173-177] There are three isoforms of cdc25: cdc25a, cdc25b, and cdc25c and each has its own role in the cell cycle pathway although the details are still only vaguely understood.[173, 178-180] In general, cdc25a functions in the nucleus throughout the cell cycle by activating the CCN-cdk complexes involved in the G1→S transition, S-phase, and the G2→M phase transition.[178, 179, 182] These CCN-cdk complexes include CCNE-cdk2 and CCNB-cdk1. Next, the cdc25b is hyperphosphorylated during the S phase and early G2 thus activating CCNB-cdk1, CCNA-cdk1, and CCNA-cdk2. Cdc25b is involved in the G2→M transition and is localized in the cell's cytosol.[182-186] Finally, hyperphosphorylated cdc25c is also a regulator of the M-phase by maintaining CCNB-cdk1 activity.[187, 188] Otherwise, cdc25c is

not active throughout any other part of the cell cycle and exactly where it is localized is controversial.[189-191]

1.5.2.2 Inhibition

Cyclin-dependent kinases are inhibited through phosphorylation by either wee1 and mik1 kinases or by subunits called cdk inhibitors (CKIs). When these inhibitors are over expressed, the result is an arrest of the G1 phase. This occurs in response to any DNA damage or antiproliferative agents that may be present in the cellular environment. There are two mammalian classes of CKIs, (1) the INK4 proteins and (2) the Cip/Kip family.[192] The INK4 proteins are $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$. They specifically bind to the inhibitory subunit of cdk4 and cdk6 enzymes when they are complexed with cyclin D to make them inactive.[192] These proteins can also bind to monomeric cdk4 and cdk6 to prevent formation of a heterodimer complex with cyclin D.[193]

The p15 protein is also known as INK4B. Its gene is on chromosome 9p21.[192] An antiproliferative agent, TGF-β, stimulates an increase in p15. As p15 levels increase they replace any p27KIP1 inhibitors that may be bound to CCND1-cdk4 or CCND3-cdk6.[192] The p27 inhibitors then travel to the CCNE-cdk2 complexes. p16, also referred to as INK4A, is another INK4 protein whose gene is on chromosome 9p21.[192] p16 directly inhibits CCND1-cdk4 complexes. Several different mutations of p16 have been associated with various cancers such as melanomas, leukemia, gliomas, non-small cell lung carcinoma, and esophageal cancers [194-202]. As with p15 and p16, a lot is still unknown about the complete involvement of p18 and p19 in the cell cycle other than their association with inhibiting CCND1-cdk4 complexes.

The second class of inhibitors is the Cip/Kip CKIs. They are universal cdk inhibitors that can inhibit all CCN-cdk complexes in the G1 and S-phases of the cell cycle. [192] There are three types of these universal CKIs, which include p21[Cip1], p27 [Kip1], and p57[Kip2]. The p21[Cip1], located on chromosome 6p21, inhibits G1 cdk activity when DNA damage has been detected.[203] Transcriptional activation is the main form of regulation of this enzyme. [193, 204] However, post-translational modification, as well as the stability of mRNA can also influence p21[Cip1]levels. [193, 204] In the promoter region of p21[Cip1] there are two binding sites for p53. The p53 protein detects any damage and then signals for the transcription of p21. Following this, p21[Cip1] binds to the cdks in the G1 phase of the cell cycle and causes G1 arrest. [205,390] Apart from the p53-binding site, p21[Cip1] has an amino terminal site that binds with cdk and a carboxy terminal site for PCNA (proliferating cell nuclear antigen; a DNA- replicating promoting protein) binding.[206-209] The purpose of these binding sites is to stop the G1 phase of the cell cycle when DNA damage has occurred and to prevent the cell from completing the cell cycle once it has been registered for programmed cell death.[166, 210]

Another type of CKI, the p27[Kip1] inhibitor, is located on chromosome 12p13[211-213] with a 42% homology to the cdk binding domain of p21[Cip1] [214] It is very similar to the p21[Cip1] inhibitor except that it is not regulated by p53.[211, 212] Instead, p27[Kip1] is stimulated through extra cellular mitogenic and antimitogenic signalling[192] It binds to the catalytic cleft of cdk so that ATP cannot bind the cdk-cyclin complex for activation.[215] One example of a cyclin-cdk-p27[Kip1] complex is with cyclin E- cdk2. This particular type of binding inactivates the kinase and causes G1 arrest of the cell cycle.[216] When p27[Kip1] is complexed

with CCNE-cdk2, it blocks the phosphorylation site that is normally phosphorylated by CAK enzymes.[217]

Apart from the function just described, p27^{Kip1} is also involved in the cell-to-cell contact inhibition of the cell cycle. During such an event, the p27^{Kip1} expression levels increase thus acting as a mediator for this form of inhibition to take place.[218] On the contrary, when the oncogene Ras is activated it causes a decrease in the synthesis of p27^{Kip1} as well as an increase in its degradation. This function has lead to the postulation of Ras being a late G1 regulator that decreases p27^{Kip1} resulting in G1 restriction point passage and entry into S-phase entry.[219] Without this function, increased p27^{Kip1} levels will lead to a G1 arrest of the cell cycle. Therefore, p27^{Kip1} is an inhibitory mediator of cell cycle progression in response to external stimuli. Interestingly, p27^{Kip1} has been observed to be in a methylated state in various tumors such as in the pituitary gland.[220]

Finally, the last CIP/KIP CKI to be discussed is p57^{Kip2}. Although this CKI is known to be restricted to terminally differentiated tissue, little is known of its exact function. It shares homology with the p27^{Kip1} inhibitor[195, 221] and is located on chromosome 11p15.5.[222-225] Notably, this particular locus contains several imprinted genes including the Beckwith-Wiedemann Syndrome (BWS) gene.[224] The expression of p57^{Kip2} is maternal since the paternal allele is methylated and transcriptionally silent.[226-229] The paternal allele only represents 5% of the expressed protein in various human tissues excluding fetal brain where both parental alleles are expressed almost equally.[226-229] As for the function of p57^{Kip2} it inhibits the cdks involved in the G1 to S-phase of the cell cycle and has also been associated with inhibiting the cyclin B-cdk1 complex of mitosis.[221]

Apart from these two classes of cdk inhibitors there is another known method of CCN-cdk inhibition. This method involves phosphorylation of the tyrosine 15 residue (Tyr15) of cdks. When phosphorylated, Tyr15 can stop cyclin-cdk activity regardless of whether the Thr160 residue is phosphorylated or not.[170, 172, 230-232] The kinases involved in phosphorylating this residue are wee1 and mik1. Activation of either of these two kinases sets the baseline for cdk activity in order to determine mitotic phase entry of the cell cycle.[181, 190, 233-236]

1.5.3 Cyclins and Cyclin Complexes

As was discussed in the previous section, cyclins form heterodimers with cdks in order to activate them. Their expression is rate limiting for the activation of cdks and their levels are controlled by transcription and regulated proteolysis by the ubiquitin-proteasome system. The subunits of cyclins determine cdk activity at different times throughout the cell cycle based on which cyclin and cdk is present. For example, during the G1 phase in the mammalian cell cycle, the D and E cyclins associate with cdk 4/6 or cdk2, respectively.

All 16 mammalian cyclins have a conserved homologous region known as the cyclin box. They are as follows: cyclin A, cyclin B1, cyclin B2, cyclin C, cyclin D1, cyclin D2, cyclin D3, cyclin E, cyclin F, cyclin G1, cyclin G2, cyclin H, cyclin I, cyclin K, cyclin T1, and cyclin T2. Only those involved in cell cycle regulation will be discussed here.

Cyclin D (CCND) is the best understood of the cyclins. This cyclin initiates the cell cycle as its expression begins early in the G1 phase. Unlike the other cyclins, CCND level is directly influenced by the extracellular stimuli of mitogens.[162] As long as there are mitogenic stimuli, CCND will remain at high levels. If there is an over expression of

CCND the G1 phase will be shortened and less mitogenic influence will be required.[237, 238] On the contrary, if CCND is inhibited the cell will not proceed through the G1 phase.[238, 239] The RAS/RAF/MAPK pathway controls the mitogenic stimuli and results in the activation of CCND transcription.[162, 240] Three isoforms of CCND have been identified, CCND1, CCND2, and CCND3. Each of these isoforms is expressed in a cell type-specific fashion [161, 162, 241] and each associates with either cdk 4 or cdk 6.

CCNE is activated in the G1 phase after CCND peaks at the G1-S phase boundary. Without CCNE kinase activity the cell will not enter the S-phase. As the S-phase progresses the level of CCNE decreases.[242-244] The level of CCNE is transcriptionally controlled by E2F through the phosphorylation of the pRb active site by the CCNE-cdk2 complex itself. After pRb has been activated it releases the transcriptional inhibitory E2F factor allowing further gene transcription of cyclin E. Increased levels of cyclin E activate the CCNE-cdk2 complex through autophosphorylation.[244] CCNE-cdk2 is also regulated through proteolysis. If CCNE is over expressed it will shorten the G1 phase and therefore, less mitogenic influence is required to activate the progression through to the next phase. If CCNE-cdk2 is inhibited, the mitotic phase will not begin.[245]

Another cyclin that forms a complex with cdk1 is cyclin B (CCNB). During the S and G2-phases of the cell cycle this cyclin forms a cdk complex in the cytoplasm. However, this is inactive due to phosphorylation of the Thr14 and Tyr15 residues of cdk1 by the inhibitory kinases myt1 and wee1. The CCNA-cdk1 complex first phosphorylates the cdc25b phosphatase and in the activated state removes the wee1 kinase from cdk1.[246-249] This in turn activates CCNB-cdk1 formation. After critical levels of CCNB-cdk1 are formed this complex is transferred to the nucleus to allow the initiation of mitosis.[248, 250]

In doing so CCNB-cdk1 regulates both mitotic entry and exit.[247] CCNB-cdk1 is also important in the expression of the protein Survivin. This protein functions in sustaining the life of the cell and the regulation of mitotic spindle formation.[251-253]

Cyclin A (CCNA) can form a heterodimer with either cdk2 or cdk1 (cdc2) subunits. The CCNA-cdk2 complex is required for starting the S-phase once this heterodimer has been actively phosphorylated by CAK. This active CCNA-cdk2 complex directly inactivates the E2F factor thus allowing the S-phase to begin. When CCNA forms a heterodimer with cdk1, it is involved with the transition from the G2 to the mitotic phase.[254-256]

Another cyclin indirectly contributing to cell cycle progression is cyclin H which when complexed into a trimeric form becomes an essential component of cell cycle continuity. This trimeric complex consisting of CCNH-cdk7-Mat1 also called p35, is known as the cdk-activating kinase (CAK). [257-259] The role of CAK is to phosphorylate cdk1, 2, 4, and 6 at their Thr160 residue when they are complexed with their cyclin unit. This phosphorylation step is necessary for activation of the cyclin-cdk complex and progression of the cell cycle.[172, 260-265]

The C cyclin (CCNC) is of unknown function, however, it has been hypothesized that CCNC-cdk8 complexes are involved in the transcriptional machinery and therefore contribute to the cell cycle indirectly via regulation of transcription.[266, 267]

Cyclin F (CCNF) is the largest cyclin identified with a molecular weight of 87 kD and its gene sequence is closely related to that of CCNA and CCNB. The exact function of CCNF is unknown but it may have some involvement in the G2$\rightarrow$M transition.

Although much is yet to be explored about cyclins their importance is clear. They appear to act as the "starters" and "extinguishers" of the cell cycle. However, as more is

discovered it is becoming evident that they not only influence cell cycle progression and arrest, but also the regulation of transcription, DNA repair, cell differentiation, and apoptosis.

1.5.4 Cell Cycle Regulation

Regulation of the cell cycle is not completely understood. What is known thus far involves such things as restriction points, checkpoints, and cyclin dependent kinase substrates. These processes work together to permit a cell to replicate, repair, or destroy itself based on circumstances of both the cell and its environment.

The restriction point is the point at which the cell no longer requires stimulation by external growth factors.[268-271] This is when the cell is transferring from a quiescent G0 state to a proliferative G1 phase. Once the cell passes its restriction point it cannot revert. Instead, it is committed to either proliferate or destroy itself based on details within the cell itself. Such events involve mechanisms referred to as checkpoints.

Checkpoints of the cell cycle are mechanisms that monitor the quality and viability of DNA. They also follow mitosis and cytokinesis to ensure that it is carried through correctly.[271, 272] Two main types of checkpoints are: (1) DNA damage checkpoints and (2) Mitotic phase (M-phase) checkpoints. However, they can be subdivided into four groups (1) G1-phase checkpoints, (2) S-phase checkpoints, (3) G2-phase checkpoints, and (4) Spindle checkpoints (also known as Mitotic phase checkpoints).

After a cell is committed to proliferation the G1 checkpoint monitors the integrity of the cell's DNA. If damage such as double stranded breaks occur, or hypoxia, or oncoprotein activation is detected this checkpoint prevents further proliferation until the defect has

been corrected.[273-276] If it cannot be corrected the cell will be directed into apoptosis and cell death.

Some of the main players in the G1 checkpoint are: the tumor suppressor p53, the oncoprotein mdm-2, the cyclin dependent kinase inhibitor p21[CIP1/WAF1], casein kinase-1 (chk1) and –2 (chk2), and the ATM and ATR pathways.

When the cell encounters damage, activation of the G1 checkpoint takes over. In response to a defect, the levels of p53 increase rapidly and p53 becomes active through a series of phosphorylations and dephosphorylations. Under normal cell circumstances p53 binds to the oncoprotein MDM-2, which acts as a negative regulator of p53.[277-284] Such binding acts as a shuttle for transferring p53 from the cytosol to the nucleus.[285-287] However, when there is a disruption in the G1 phase the ATM-dependent pathway mediates the phosphorylation of p53 both directly and indirectly.[288, 289]

First, ATM phosphorylates the threonine 68 residue of chk2, which in turn phosphorylates p53 at its serine 20 site.[290, 291] This causes interference with MDM-2 binding. [290, 291] A second phosphorylation of p53 at threonine 18 follows ATM activation of chk1.[292] Before this can occur, the serine 15 residue of p53 needs to receive a phosphate group.[292] Finally, ATM also phosphorylates MDM-2 at its serine 395 residue.[293] The purpose of phosphorylating p53 and MDM-2 is to prevent the two from interacting with each other. [293] When MDM-2 and p53 do not form a complex, the p53 accumulates and is activated by hyperphosphorylation. Once p53 is activated it signals for the transcription of the cyclin dependent kinase inhibitor p21[WAF1/CIP1].[294] This cdkI suppresses CCNE-cdk2 and CCNA-cdk2 thus preventing G1→S transition. [294]

Apart from this chain of events, p53 can also trigger the cell into apoptosis depending on the degree of damage involved.[295] Another pathway called the ATR pathway is also involved in the G1 phase checkpoint. Exactly how it participates is unclear but it is believed to help in serine 20 phosphorylation by chk1 activation.[294]

Entering the next phase of the cell cycle involves the S-phase checkpoint, which delays synthesis in the event of DNA double strand breaks (DNA dbsb) or impaired progression of the replication fork. This checkpoint inhibits synthesis in order to give the repair mechanisms a chance to repair these errors. Two main pathways involved include the ATM and ATR pathways.

When there is a breakage in the DNA double strand that requires repair, ATM responds through a series of phosphorylations. This process begins by phosphorylation of the nibrin protein NBS1 at its serine 343, 397, and 615 residues.[296-299] The NBS1 protein forms a complex with MreII and Rad50, which also work to maintain the genome.[300, 301] This trimer plays a role in recombinational repair of DNA dbsb, [300] and is also an upstream regulator of the phosphatase cdc25a discussed earlier.[301-303]

Another player involved in this form of DNA repair is the p53 binding protein1 (p53BP1). This nuclear protein responds to the damage immediately following ATM phosphorylation.[304, 305] Its mode of action is not well understood other than having many serine/threonine-glutamine sites and involvement in DNA damage checkpoints throughout the cell cycle.[306]

Coinciding with the DNA repair is the inhibition of DNA synthesis. To do this ATM phosphorylates the chk2 protein to activate it. Once chk2 has been activated it phosphorylates the serine 123 site of cdc25a in preparation for ubiquitin-proteasome

degradation.[307] Without cdc25a, CCNA-cdk2 and CCNE-cdk2 cannot be activated thus preventing DNA synthesis from continuing.[307-309]

The second player of the S-phase checkpoint involves the ATR pathway. ATR responds to fork replication errors during synthesis. The details of this pathway are still being worked out. However, what is known is that a polo/primase complex function initiates the ATR pathway. ATR then phosphorylates a group of hus1 and Rad proteins. These proteins somehow work together with ATR to activate chk1 and breast cancer susceptibility protein1 (BRCA1) to delay the S-phase.[310-312] Hus1, Rad, and ATR also are involved in the activation of BRCA1 and NSB1 for homologous recombination replication fork repair and restart. [310-312]

Following along the cell cycle, after the successful completion of the S-phase is the second gap phase (G2). This phase also involves both the ATM and ATR pathways. The purpose of G2 is to ensure DNA viability and prevent further progression into mitosis if damage is detected. Once repairs have been made the cell cycle is re-started to proceed into the next phase. Otherwise, if the damage cannot be repaired the cell is signaled to go through apoptosis for destruction.

The G2 checkpoint begins with the ATR pathway during the S-phase. DNA damaging agents activate this pathway through Hus1 and various Rad proteins.[313, 314] When signaled by these proteins ATR activates BRCA1, which goes on to regulate both chk1 and chk2 through phosphorylating the p53BP1.[315-317] Activation of ATR also leads to the phosphorylation of the serine 345 residues on chk1 that then bind with cdc25. [315-317] This process initiates the G2 phase checkpoint by enabling the binding of cdc25 to the 14-3-3 family proteins that normally sequester cdc25 and CCNB-cdk1 in the cytoplasm.[315-317]

When bound together with chk1, cdc25 is unable to bind to CCNB-cdk1, thus inhibiting G2 from going on to mitosis. Once the G2 checkpoint has been activated by ATR the ATM pathway then takes over.

ATM activates MDC1 which also upregulates p53BP1. The role of ATM in G2 checkpoint control involves the threonine 68 phosphorylation of chk2.[318] Chk2 then goes on to phosphorylate serine residue 216 of the cdc25 bound 14-3-3 complex. [318] This phosphorylation step stops the G2$\rightarrow$M transition by inhibiting the phosphorylation of CCNB bound cdk1. Apart from this event chk2 phosphorylation also activates the wee1 cdk1 inhibitor. [318] This inhibitor once upregulated stops the activation of CCNB-cdk1. Therefore, the cycle cannot progress to mitosis until signaled otherwise.

The final checkpoint to be discussed in this review is the mitotic phase checkpoint also known as the spindle assembly checkpoint (SAC). It has been hypothesized that during the M-phase unattached kinetochores somehow signal these checkpoints to stop the cell cycle in mitosis.[319, 320] Proteins associated with this pathway are Bub1, Bub2, Bub3, Mad1, Mad2, and Mad3.[321-325] Of these proteins, Bub1 and Mad2 have been found on kinetochores that are unattached to spindles. In response to a lack of tension in the kinetochores, these proteins stop the destruction of mitotic CCNB-cdk2 complexes thus preventing entry into anaphase. Mad2 is first sequestered into the kinetochore region of the DNA. Then Bub1 and Mad2 bind together to trigger the MAP-dependent kinase pathway.[326-336] This causes an increase in CCNB-cdk2 activity and inhibition of the anaphase promoting complex or cyclosome (APC/C), which breaks these cyclin complexes down.

The function of Mad2 involves a newly investigated pathway currently under study. Somehow, Mad1 acts as a delivery unit carrying Mad2 from the cytosol to the nuclear kinetochores of the microtubule spindles.[337] There are two forms of Mad2; open Mad2 (O-Mad2) and closed Mad2 (C-Mad2). [338-342]The open form is found mostly in the cytosol.[338-341, 343] Once it binds to Mad1 it takes on a closed conformation as it is moved into the kinetochore region where the Mad1 transfers the Mad2 to cdc20.[338-341, 343] By doing this the spindle assembly checkpoint (SAC) is activated. Sufficient binding of the kinetochores to the spindles is necessary for adequate formation of the mitotic checkpoint complex (MCC) otherwise anaphase will be delayed.[337]

Finally, substrates of CDKs include E2F transcription factors and pocket proteins p107, p130, and retinoblastoma (pRb). In order to mediate positive and negative feedback loops of the cell cycle, CDKs use these substrates. Within recent years much has been discovered about pocket proteins and E2Fs. Previously, these substrates were believed to be involved in a vaguely understood process with the G1/S transition and S-phase of the cell cycle. However, the pocket proteins are now recognized to be involved in numerous stages of the cell cycle including G0/G1, G1/S, G2/M, DNA repair and recombination, apoptosis, cell differentiation and development.[344-346]

E2F is a heterodimeric protein consisting of an E2F molecule and a DRTF1 polypeptide (DP) molecule. There are eight related E2Fs labeled E2F1, E2F2, E2F3a, E2F3b, E2F4, E2F5, E2F6, and E2F7 and two DPs, DP1 and DP2. Together, these heterodimers act as an essential factor for the transcription of genes that are necessary for the initiation, progression, and regulation of the S-phase.[347-349] Apart from this, the heterodimer(s) play numerous roles throughout the cell cycle depending on which E2F is involved. These

roles vary from being a player in tumorigenesis and oncogenesis, a regulator of cell death, cell proliferation and differentiation, to acting as either an activator or repressor of transcription.[350]

E2F1, E2F2, and E2F3a are transcription activators that are involved in the progression of the cell cycle. However, E2F3b, E2F4, E2F5, and E2F6 are transcription repressors that assist in cell cycle exit and differentiation. E2F7 is also a transcription repressor but its exact mode of action is unknown.

Three subgroups of E2Fs that form complexes are as follows: (1) E2F/DP and pRb dependent complexes which involve E2F 1-4, (2) E2F/DP dependent complexes without pRb that involve E2F 4, 5, and 6, and (3) E2F7, which is independent of pRb and DP and appears to be a homodimer.[350] More specifically, E2F1, 2, 3a, and 3b can only form a complex with pRb. The E2F4 can complex with any of the following three pocket proteins pRb, p107, or p130. From the second group, E2F5 only binds with p130 while E2F6 forms a complex outside of these pocket proteins. Instead, E2F6 forms a complex with the polycomb protein PcG, which is also DP-dependent.

As described above these cdk substrates work together to either activate or repress the transcription of genes that are involved at various points throughout the cell cycle. First, the pocket proteins bind to the E2F factor in a hypophosphorylated state. Once they have been hyperphosphorylated by various CCN-cdk complexes through a series of steps, the E2F transcription factor is released and becomes transcriptionally active.[351]

The next group of substrates to be discussed is referred to as the "pocket proteins". They include p107, p130, and pRb. As mentioned these proteins work in conjunction with E2F transcription factor proteins and cdks of CCN-cdk complexes.[352, 353] They have multiple

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CCN-cdk binding sites and are genetically similar to one another because of sharing sequence homology.[354-357] This homology has been conserved throughout evolution and across species.[358-360] Therefore, although they are not yet completely understood, their significance must be important. Of the three pocket proteins, p107 and p130 are most genetically similar to one another.[358-361]

The pRb protein is expressed in both proliferating and non-proliferating cells.[362] It is a nuclear protein that is hypophosphorylated during the G0 phase of the cell cycle.[363] As the cell cycle progresses to the G1 phase pRb becomes continuously phosphorylated until it reaches a hyperphosphorylated state during the mitotic phase where it remains until late mitosis.[363] When pRb is hypophosphorylated it inhibits cell cycle proliferation through its association with proteins such as E2F transcription factor1, 2, 3, and 4.[364] The pRb pocket protein responds to mitogenic stimulation of the cell cycle during the early to mid phase of G1 in conjunction with the CCND-cdk4 and CCND-cdk6 complexes. It also associates with CCNE-cdk2 during late G1.[364]

The remaining two pocket proteins, p107 and p130 although described as somewhat similar to pRb also have differences. Unlike pRb, which maintains relatively constant levels throughout the cell cycle, p107 and p130 fluctuate antagonistically to one another in each of the phases of cell replication.[365-367] Starting with the G0 phase, p130 is abundant and involved with cells being exited from mitosis and maintained at a quiescent state.[368, 369] As the cell enters G1 and progresses through each phase of the cell cycle the levels of p130 drop dramatically.[368, 369] The opposite is true for p107.[370-372]

Another difference from pRb is that p107 and p130 have a high affinity for CCNA-cdk2 or CCNE-cdk2.[355, 357, 373-375] Also, even though they too bind with E2F, p107 and p130

function more as co repressors acting as CDKIs rather than as activators as discussed above.[376, 377] The E2F transcription factors that they mainly interact with are E2F4 and E2F5. However, they can also bind with the E2F1, 2, and 3 factors when they are over expressed.[378-380]

1.5.5 Conclusion

In conclusion the cell cycle is a very sophisticated process that involves, directly and indirectly, several pathways, key players, and influences from extra and intra-cellular sources. Although only a portion of cell replication has been elucidated it is clear that there is much to be discovered and completely understood. However, as more discoveries are being accumulated the complex intricacy of the cell cycle become evident. Therefore, a more complete understanding of cancer, cell differentiation, and development will follow based on knowledge gained from cell cycle discoveries.

The relevance of the cell cycle to this study is to obtain a clearer understanding of how a cell replicates, divides, and destroys itself. As previously mentioned, cancer is the result of an uncontrolled cell. Knowing the process involved in the cell cycle helps coordinate an organized investigation for determining where or how a malfunction in this pathway can lead to a cancerous cell. The fact that the MMR pathway of the families in this study has an identified *MSH2* mutation, suggests that cell cycle malfunctions are involved in the development and possibly penetrance variations of HNPCC.

1.6 Background and Rationale For Study

1.6.1 Overview

HNPCC (hereditary non-polyposis colorectal cancer) has been linked to mutations in mismatch repair (MMR) genes in some families. However, not all mutation carriers develop cancer and there is a wide variation in phenotypic presentation and age of onset within these carriers. Therefore, other factors must be involved.

Modifier genes may contribute to the phenotypic variation of HNPCC. Thus, it is hypothesized that the genes of pathways such as the cell cycle and DNA mismatch repair may act as modifiers in individuals carrying a known *MSH2* HNPCC mutation.

Of particular interest to this study are i) the *MSH2* A→T point mutation at nucleotide 942+3A→T in the 3' splice site of intron 5 and ii) an *MSH2* deletion of exons 4-16.[114, 120] In two HNPCC families from Newfoundland and Labrador and the lower north shore of Quebec with either of these two identified *MSH2* mutations, there is a variation within each family in the age at onset or severity of disease despite similar environmental backgrounds. Other environmental factors such as nutrition, smoking, alcohol, or chemicals may play a modifying role. Given our knowledge of modifier genes in animal models and some human diseases, it is also possible that other genes may independently modify the phenotype of the *MSH2* mutations in these kindreds. For example, it has been shown in diseases such as cystic fibrosis, deafness, and other forms of colon cancer that a modifying gene affects the penetrance of their phenotypes. These modifiers can provide either protection from or enhancement of the disease depending on the allelotype that the individual has. Considering the amount of phenotypic variations within HNPCC family

members carrying the same mutation, it is possible that other genetic influences such as modifiers may be playing a role. This modification can be either direct or indirect in a metabolic, physiological, or disease pathway.[124] Determining the risk due to such modifiers may more clearly define the risk and severity of expression in individual mutation carriers. In other words, identifying modifiers should aid in more precise diagnosis, prognosis, and treatment of HNPCC in these two families.

It is known that carcinogenesis involves a malfunction in the control of cell division. Based on that fact, I focused my research on genes directly involved in the cell cycle pathway. In my project I studied frequency and distribution of SNPs within genes of the cell cycle in carriers of *MSH2* mutations with different disease expression. Since MMR genes are mutated in this disease, I also analyzed SNPs within MMR genes. In addition, there have been a number of studies involving SNPs in the methylenetetrahydrofolate reductase (MTHFR) enzyme in association with colon cancer. It was observed in these studies that folate status and allele type for the C677T *MTHFR* gene might be involved with expression of colorectal adenomatous and hyperplastic polyps.[391, 392] Therefore, some MTHFR SNPs were also included in this analysis.

With the collected data I analyzed how the allele types of such genes were related to the age of onset of HNPCC. The purpose of doing this analysis was to study genetic factors that could modify the age-dependent penetrance of HNPCC. Confirmed results from this data would be used to alter the current screening methods because of genetic identification of those within these mutation positive carriers who might be more likely to develop a specific form of HNPCC–related cancer (colon, rectal, endometrial, or other forms). Such information would also be applied to identifying who in this group was

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more likely to develop the disease earlier than other family members with the same *MSH2* mutation.

Choosing the Newfoundland population for this study was based on their genetically isolated homogeneous nature. The people of Newfoundland mainly came from the southwest coast of England and south east coast of Ireland starting in the 1700's. Fishing was the livelihood of these people explaining their coastal habitat patterns. Since there were few roads, many towns remained isolated from one another except by sea. This type of setting contributed to a series of homogenous genetic isolates since little in or out migration took place for approximately 250 years. The majority of families in Newfoundland were large with the average family having 6 or more children. Also, the community churches and later the provincial archives kept records of births and deaths of everyone in the community. Combining family size and history records with isolation makes Newfoundland a genetically rich resource for studying inherited diseases such as HNPCC.

1.6.2 Specific Objectives

1. To identify candidate genes by a comprehensive literature and database search.
2. To genotype candidate modifier loci in HNPCC or polyp patients carrying one of the two defined *MSH2* mutations.
3. To determine if there is a polymorphic difference in the age of onset of those who get HNPCC, an HNPCC-related cancer, or polyp and those who do not.

Chapter 2 Materials and Methods

2.1 Subjects

Two large HNPCC kindred's have been ascertained through studies at Memorial University of Newfoundland (MUN) and the Charles S. Curtis Memorial Hospital, in St. Anthony, Newfoundland and Labrador (NL). This includes a large NL family from the Northeast coast of NL (called Family C), and a family from the Lower North Shore of Quebec who traditionally received their healthcare from the hospital in St. Anthony, NL (called Family 11). For each family, family history and medical records had been collected to extend the pedigrees and to determine the type and age of onset of CRC and extracolonic cancers in affected individuals.

MSH2 mutations were identified in both of these families: (i) Family C carries a c.942+3 A→T point mutation of intron 5 resulting in a deletion of exon 5 from the mRNA at the 3' splice site[114] and (ii) Family 11 carries a multi-exonic deletion extending from exon 4-16.[120]

Family C includes twelve sub-families with >1000 family members. Ancestors of all sub-families came from within a 40 km radius of each other along a stretch of north eastern coastline. A couple who settled in Newfoundland more than 200 years ago was identified through archival searches as ancestral to four sub-families of Family C. All sub-families have a common haplotype of linked markers in the immediate region of *MSH2* of chromosome 2 suggesting a common ancestor for all the sub-families.

Family 11 has a pedigree size of >575 family members over 7 generations. The family is Anglo-Saxon origin living in an isolated area of the Lower North Shore of the St. Lawrence River, Quebec and their clinical status regarding colorectal cancer has been followed for over 30 years.

Only those who tested positive for the relevant mutation in these two families and gave research consent were included in the present study. In total 135 genomic DNA samples including 94 from Family C and 41 from Family 11 were used for this study.

2.2 Phenotype Presentation

The HNPCC cancers occuring in Family C included colorectal, endometrium, ovarian, stomach, small bowel, and skin (sebaceous cell cancer and keratocanthomas).

In Family 11 HNPCC cancers included colorectal, endometrial, stomach, ureter, bladder, kidney, and sebaceous cells but ovarian cancer was not observed. Both families had skin lesions considered to be Muir-Torre features including keratocanthoma and sebaceous adenoma.[120]

2.3 Screening

When these families were identified and characterised a screening program was offered to affected and at risk family members based on the type and frequency of the cancers present in their family. Results of all screening investigations have been recorded.

2.4 DNA Samples

DNA samples were collected at the time of initial mutation testing of members of these families. The DNA extraction method followed standard procedures.[393] Family members consented to the use of an aliquot of DNA for research studies. DNA samples were grouped according to age of onset of an HNPCC related cancer or polyp.

Thirty-one SNPs from 17 genes were chosen for study (1 *CCNA2*, 3 *CCND1*, 1 *CCND2*, 2 *CCND3*, 5 *CCNH*, 1 *CDK2*, 1 *CDKNIA*, 2 *CDKN1B*, 3 *CDKN2A* (p16), 3 *E2F2*, 1 *RAD51*, 1 *PMS2*, 2 *MLH1*, 2 *MSH2*, 1 *MSH6*, 1 *TFD-P1*, and 1 *MTHFR*) (Table 2.1). The allele frequency for each SNP was determined and analyzed according to the age of onset categories.

2.5 **MassArray**

The MassArray is a homogeneous massExtend (hME) assay that can be used for SNP analysis. The analysis is done through the use of MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), which permits high throughput genotyping. This form of mass spectrometry allows for processing of numerous samples simultaneously. Fewer reactions are required to obtain large quantities of data, when compared to traditional procedures such as SSCP and RFLP. It is a useful method when DNA supplies are limited, as each reaction only requires 2.5 ng of DNA.

In general, the MassArray involves a PCR step, a SNP region, and then an hME reaction. The hME step is a process of adding hME nucleotides by using specific primers that are adjacent to a SNP location on a gene. Since different alleles vary in mass a SNP is differentiated by the mass of the allele. Thus, adding hME nucleotides to the PCR product allows tagging of each SNP of interest for identification by its mass so that the laser MALDI-TOF-MS can identify it.

A multiplex reaction is also incorporated into this protocol. Multiplex is a reaction where more than one SNP primer is added to the same tube of multiple PCR and hME products and then dispensed into a single well on a sample plate. A computer program called SpectroDESIGNER (Sequenom, Newtown, MA, USA) determines which SNPs can be

multiplexed together into one reaction tube. It also designs the PCR and MassEXTEND primers required (Table 2.2 and 2.4), as well as the multiplex conditions for the appropriate termination mix to be used for each reaction (Table 2.5).

Following the multiplex protocol the samples are transferred onto a 384 well chip (~1 x 1.5 inches). The 384 wells are ~1/4 mm in diameter. The samples are dotted onto the chip by a robot. Once the chip has been spotted it is placed into the MassArray apparatus, which does MALDI-TOF-MS analysis by reading each spot 3 times and then averaging the peaks. The machine-called results must be reviewed manually before being considered final.

2.6 SNPs Analyzed

SNPs were chosen using the National Center for Biotechnology Information (NCBI) website as a guide. Initial criteria for choosing these SNPS were: (i) a minor allele frequency of 5% or higher (ii) involvement in the cell cycle either directly or indirectly, (iii) location in a MMR gene, and/or (iv) causing a non-synonymous amino acid change. However, many of these SNPs were recently discovered and allele frequencies were either unknown or less than 5%. (Table 2.1)

Table 2.1: List of SNPs used for RFLP & MassARRAY Analysis.

Gene	SNP	Amino Acid Change	Hz	Genbank SNP Id
$CCND1^{870}$	A→G	N/A	N/A	rs603965
$CCND1^{1722}$	G→C	N/A	N/A	rs678653
$MTHFR^{677}$	C→T	VAL→ALA	0.409	rs1801133
$CCND1$	G→T	N/A	N/A	ss3163670
$CCND2$	A→G	GLY→ARG	0.01	rs3217921
$CCND3$	T→G	ALA→SER	0.49	rs1051130
$CCND3$	C→T	PRO→SER	0.02	rs3218089
$CCNH$	T→C	VAL→ALA	0.169	rs2266690
$CCNH$	A→G	LYS→ARG	0.051	rs226669
$CCNH$	G→T	ARG→LEU	0.011	rs2234942
$CCNH$	T→C	VAL→ARG	0.144	rs2266690
$CCNH$	A→G	LYS→ARG	0.045	rs2266691
$CCNA2$	G→A	VAL→ILE	0.067	rs769242
$CDK2$	A→C	TYR→SER	0.00	rs3087335
$CDKN1A(p21^{Cip1})$	C→T	SER→ARG	0.29	rs1801270
$CDKN1B(p27^{Kip1})$	G→T	VAL→GLY	0.28	rs2066827
$CDKN1B(p27^{Kip1})$	C→T	ARG→TRP	0.00	rs206682
$CDKN2A(p16)$	G→A	ALA→THR	0.056	rs3731249
$CDKN2A(p16)$	G→A	GLY→ARG	0.012	rs4987127
$CDKN2A(p16)$	C→T	PRO→SER	0.011	rs3731190
$E2F2$	A→G	ASP→ASN	0.02	rs4134982
$E2F2$	G→A	GLY→ARG	0.011	rs4134973
$E2F2$	G→T	GLN→HIS	0.39	rs2075995
$E2F2$	G→A	GLY→ARG	0.01	rs2229287
$RAD51$	A→C	LYS→GLN	0.00	rs1056742
$MLH1$	A→G	ILE→VAL	0.11	rs2229023
$MLH1$	G→A	VAL→MET	0.00	rs2308317
$MSH2$	G→A	GLY→ASP	0.01	rs4987188
$MSH2$	T→G	HIS→GLN	0.00	rs1800152
$MSH6$	A→G	GLY→VAL	N/A	rs1042821
$PMS2$	A→G	LYS→GLU	0.125	rs2228006
$TFD-P1$	G→A	ASP→ASN	0.011	rs4150823

*N/A=not available from resources at time of selection. Hz= Heterozygosity

Data was collected by either PCR-RFLP analysis or through MassArray. PCR-RFLP involves using the polymerase chain reaction to amplify a sample of DNA including the SNP to be tested and then digesting the amplified DNA with a restriction enzyme. The restriction enzymes cut the amplified DNA into fragments based on their ability to recognize a specific DNA sequence (usually in the range of 6-10 basepairs in length). If a DNA sample has the sequence recognized by a particular restriction enzyme it will be cleaved at that specific region. If the SNP allele alters the restriction rate, cleavage will not occur. Thus, the samples will be of different lengths depending on the SNP allele enabling identification of the presence of a specific sequence. This method can only be used to genotype SNPs that occur in restriction enzyme recognition sequences.

The initial 3 SNPs (*CCND1*A870G, *CCND1* G1722C, and *MTHFR* C677T) were analyzed using PCR-RFLP analysis. MassArray^{TM}, was used (see below) for the remaining SNPs (*CCNA, CCND1, CCND2, CCND3, CCNH, CDK2, CDKN1A (p21^{Cip1}), CDKN1B (p27^{Kip1}), CDKN2A (p16), E2F2, RAD51, MLH1, MSH2, MSH6, PMS2 and TFD-P1*).

2.7 PCR-RFLP analysis was conducted on SNPs in *CCND1^{870}*, *CCND1^{1722}*, and *MTHFR^{677}* as follows:

2.7.1 Genotyping of CCND1 A870G Polymorphism

The 168-bp fragment containing the A/G polymorphism in exon 4 of *CCND1* was analyzed using the PCR-RFLP method. The PCR reaction was performed using the 5'-GTGAAGTTCATTTCCAATCCGC-3' and 5'-GGGACATCACCCTCACTTAC-3' primers (Qiagen, Operon Technologies, Alameda, CA, USA). The PCR reactions contained the following: 1x PCR buffer, 0.2 mM/dNTP, 1.5 mM MgCl$_2$, 2.5 ul /primer,

1U Taq, 20 ng DNA in a final volume of 25 ul. Amplification conditions were: 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s with a final extension of 72°C for 7 min. The PCR product was digested overnight at 37°C using 10 ul of PCR product, 2.0 ul of 10x RE buffer, 0.5 ul (5 units) of ScrFI restriction enzyme (New England Biolabs Inc., Beverly, MA, USA), and 7.5 ul ddH$_2$O to make a final volume of 20 ul. The presence of the G allele resulted in two fragments of 146 and 22 bp, whereas the undigested product of 168 bp represented the A allele. RFLP products were visualized on a 3% agarose gel.

2.7.2 Genotyping of CCND1 G1722C Polymorphism

The *CCND1* G/C SNP was genotyped using the 5'-AAGTAGAAGAGGGTTTTAGG-3' and 5'-TCGTAGGAGTGGGACAG-3' primers (Qiagen, Operon Technologies, Alameda, CA, USA). The PCR reactions contained the following: 1x PCR buffer, 0.2 mM/dNTP, 1.5 mM MgCl$_2$, 2.5 ul /primer, 1U Taq polymerase, 20 ng DNA in a final volume of 25 ul. The amplification conditions were an initial denaturing step at 94°C/ 2 min followed by 34 cycles of 94°C/1 min, 57°C/ 1 min, 72°C/ 1 min with a final extension of 72°C/7 min. Ten micro-litres of PCR product were digested at 37°C overnight with 0.5 ul (10 U/ul) of HaeIII enzyme, 2.0 ul of 10xRE buffer, and 7.5 ul of dH$_2$O to make a 20 ul volume (New England Biolabs Inc., Beverly, MA). The products were visualized on a 3% agarose gel. The restriction cut site was at the G allele and was 22 base pairs in length.

2.7.3 Genotyping of MTHFR C677T Polymorphism

The *MTHFR* C/T SNP was analyzed using the 5'-TGAAGGAGAA GGTGTCTGCG GGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3' primers (Qiagen, Operon

Technologies, Alameda, CA, USA). The 15 ul reaction mixture for PCR contained 1.5 ul 10X buffer, 0.45 ul/primer, 0.75 ul dNTPs, 0.20 ul Taq polymerase, 1 ul DNA, and 0.45 ul $MgCl_2$. The amplification conditions had an initial denaturing step at 95°C/3 min followed by 30 cycles of 94°C/30 s, 64°C/30 s, 72°C/45 s, and a final extension at 72°C/7 min. RFLP analysis was conducted by mixing 10 ul of PCR product with 0.5 ul of *HinfI* enzyme (New England Biolabs, Inc., Beverly, MA, USA) with a digestion period of 37°C/2 h. Alleles were visualized on a 3% agarose gel. The restriction cut site was at the T allele.

2.8 MassARRAY analysis was conducted on SNPs in *CCNA, CCND1, CCND2, CCND3, CCNH, CDK2, CDKN1A (p21^{Cip1}), CDKN1B (p27^{Kip1}), CDKN2A (p16), E2F2, RAD51, MLH1, MSH2, MSH6, PMS2* and *TFD-P1*. Some of the data was collected from the samples used in the uniplex test runs (a uniplex reaction means that only one primer is used per reaction tube). The purpose of such a test run was to determine if the protocol for each primer, reagent, and DNA samples was appropriate and working for this analysis. The remaining data was collected in multiplex reactions (a multiplex reaction has more than one primer used per reaction tube).

    2.8.1 The following protocol was used for the MassARRAY analysis:
First, a PCR reaction was performed for each SNP on the genomic DNA samples provided for this study (Tables 2.3 and 2.4.). In this reaction, an additional 10 nucleotides, 5'-ACGTTGGATG-3', were added to the 5' end of the forward and reverse primers (Table 2.2). These extensions were referred to as a hME-10-mer tag and are used to improve the hME reaction that followed (see below). This additional sequence tagged unused primers from the amplified PCR product so that they would have a greater mass.

The larger mass of these tagged primers was outside of the parameters set for calling a SNP sequence during the mass array analysis. Therefore, the tagging process screened out unused primers.

Following the PCR reaction, SAP (shrimp alkaline phosphatase) was added to each of the PCR reaction tubes, which were then incubated at 37°C for 20 minutes followed by the hME reaction (Table 2.5). The SAP dephosphorylates dNTPs that were not incorporated into the amplified DNA sequence.

Next, hME termination (Table 2.6) and primer mixes (Table 2.7 and 2.8) were added to the SAP treated PCR product, which then underwent another series of thermocycling (Table 2.9 and 2.10). A termination mixture was used to allow differentiation between the SNP alleles. A single primer is used which anneals to the target sequence immediately adjacent to the SNP. The ME reaction mix contains only 3 types of dNTPs which will allow for the addition of only 1 or 2 bases, depending on the allele in the test sample. One additional nucleotide was added immediately after the polymorphic site of one allele while adding two additional nucleotides after the polymorphic site of the other allele. The difference in nucleotide numbers created a difference in mass between the two alleles that was identifiable by a later step using MALDI-TOF mass spectrometry (Table 2.7). However, before proceeding to the mass spectrometry, resin was added to desalt the final product (Table 2.11). This removed any ions or other unwanted materials that could interfere with the final result of the massarray analysis.

Finally, after resin treatment, the hME reaction products were spotted onto a silicon 384-well SpectroCHIP. The data was then collected using Spectra (MALDI-TOF MS) by

SEQUENOM, Newtown, MA, USA using Typer 3.0.1 software program for displaying spectra and calculating the relative allele frequencies.

The same procedure was followed for both the multi – and uniplex reactions with the exception of variances in chemical concentrations and number of SNPs per reaction tube.

2.9 Statistical Analysis

Statistical analysis was conducted using SPSS for Windows 95 version 10.0. The association between the age of onset and allele genotype was compared using Kaplan-Meier survival curves, the log rank test hazardous ratios and Cox regression. Events were characterized as age of first HNPCC cancer (colonic or extracolonic) or age of first colorectal polyp. In other words, a single event was defined as having a minimum of one of the following: an HNPCC cancer (colonic or extracolonic) or a polyp. Once a patient presented for the first time with either of these criteria no further follow-up information was included in this analysis. Furthermore, the specific site(s) and age at which the first event occurred was the age and anatomical location that was entered into this study's analysis. Comparisons were then made between the age of first HNPCC cancer (colonic or extracolonic) or colorectal polyp and SNP genotype.

Table 2.2: The primers used for MassARRAY PCR reactions for each SNP.

SNP	1st PCR Primer	2nd PCR Primer	AmpLen
CCND1 G/T	ACGTTGGATGATGCCAACCTCCTCAACGAC	ACGTTGGATGTTAAAGTAGCACACCGAGGG	90
CCND2 A/G	ACGTTGGATGCTGGCTTGGTCCAGTTCATC	ACGTTGGATGTCAATAGCCTGCAGCAGTAC	89
CCND3 C/T	ACGTTGGATGAGTTACACACGCACCCGCAA	ACGTTGGATGCCCTGACCATCGAAAAACTG	93
CCND3 T/G	ACGTTGGATGTTTGGGCGCTGGGCTGGAG	ACGTTGGATGCTGTCAGGAGCAGATCGAAG	119
CCNH A/G	ACGTTGGATGTTGGAAACCTCCGGGAGAGT	ACGTTGGATGAGGTGGAAATTAAGTTGCTG	102
CCNH T/C	ACGTTGGATGAAGAAGTATGAACCACCCAG	ACGTTGGATGTGCAAGCTCAGCAGAATGAC	92
CCNH T/C	ACGTTGGATGAAGAAGTATGAACCACCCAG	ACGTTGGATGTGCAAGCTCAGCAGAATGAC	102
CCNH G/T	ACGTTGGATGTTGGCCACGGCTTTGCATCT	ACGTTGGATGACAGTAGTCAGAAGCGGCAC	99
CCNH A/G	ACGTTGGATGAGGTGGAAATTAAGTTGCTG	ACGTTGGATGTTGGAAACCTCCGGGAGAGT	117
CCNA2 G/A	ACGTTGGATGTGAATTGTCCCAGAGTCACC	ACGTTGGATGCTTCATTAACACTCACTGGC	120
CDK2 A/C	ACGTTGGATGAGGTGGAAAAGATCGGAGAG	ACGTTGGATGCTCTCCCGTCAACTTGTTTC	94
CDKN1A p21^{Cip1} C/T	ACGTTGGATGCCCGCCATTAGCGCATCACA	ACGTTGGATGATGTCCGTCAGAACCCATGC	96
CDKN1B p27^{Kip1} C/T	ACGTTGGATGATGTCAAACGTGCGAGTGTC	ACGTTGGATGAAGAGGTTCCTGCAGGCCGA	113
CDKN1B p27^{Kip1} G/T	ACGTTGGATGAGCCGGAGCCCCAATTAAAG	ACGTTGGATGCCAAAGGTGCCTGCAAGGTG	101
CDKN2A p16 G/A	ACGTTGGATGCTCAGATCATCAGTCCTCAC	ACGTTGGATGCACCAGAGGCAGTAACCATG	119
CDKN2A p16 G/A	ACGTTGGATGAGGCATCGCGCACGTCCAG	ACGTTGGATGACTCTCACCCGACCCGTGCA	119
CDKN2A p16 C/T	ACGTTGGATGAACCACGAAAACCCTCACTC	ACGTTGGATGGAACATGGTGCGCAGGTTCT	118
E2F2 G/A	ACGTTGGATGGGAATGTTTGAAGACCCCAC	ACGTTGGATGGTGTTCATCAGCTCCTTCAG	106
E2F2 A/G	ACGTTGGATGAGTCTGCTGTAAGAGGTTGG	ACGTTGGATGTTGGCTTCAACCAACTCAGG	120
E2F2 G/T	ACGTTGGATGATCTCTTGTTGGCCTTGTCC	ACGTTGGATGTGAAGGAGCTGATGAACACG	96
E2F2 G/A	ACGTTGGATGTGTGTTTCAGTCTCTTCTGG	ACGTTGGATGCCACTTCTGTTCATAGAGCC	101
RAD51 A/C	ACGTTGGATGATCTGAGGAAAGGAAGAGGG	ACGTTGGATGGCTTAGCTTCAGGAAGACAG	120
MLH1 G/A	ACGTTGGATGTCGACATACCGACTAACAGC	ACGTTGGATGCTGATGTTAGGACACTACCC	99
MLH1 A/G	ACGTTGGATGCTGATGTTAGGACACTACCC	ACGTTGGATGTCGACATACCGACTAACAGC	120
MSH2 T/G	ACGTTGGATGTTATTCAGCAAGGCAGCCAG	ACGTTGGATGCACTAATGAGCTTGCCATTC	110
MSH2 G/A	ACGTTGGATGTTATTCAGCAAGGCAGCCAG	ACGTTGGATGCACTAATGAGCTTGCCATTC	116
MSH6 A/G	ACGTTGGATGGCATCCCCGCCTGGGGAAG	ACGTTGGATGGCTGAGTGATGCCAACAAGG	117
PMS2 A/G	ACGTTGGATGTGGTTTGAATGGCAGTCCAC	ACGTTGGATGAGGAACATGTGGACTCTCAG	102
TFD-P1 G/A	ACGTTGGATGCGTCCTCGTCATTCTCGTTG	ACGTTGGATGAATGGGTCTCAGTACAGCGG	99

*Amp_len = amplicon length underlined sequence = hME-10-mer tag**see *Appendix*

Table 2.3: PCR contents per tube for Multiplex Analysis. Each tube contained 5 ul of PCR product.

Multiplex PCR	ul/Rx
dH20	0.920
Buffer(10x)	0.625
dNTPs(25mM)	0.100
Forward Primer(0.5 uM)	1.00
Reverse Primer (0.5uM)	1.00
MgCl (25 mM)	0.325
Hot Star Taq	0.030
DNA 2 ng/ul	1.00
Total Volume	**5.00**
Thermocycle	**3.35hrs**

Thermocycle conditions: 95°C 15 mins, (95°C 20 sec, 56°C 30 sec, 72°C 1 min) repeat 100 cycles, 72°C 3 min. **see Appendix*

Table 2.4: PCR per tube for Uniplex Analysis. Each tube contained 5 ul of PCR product.

Uniplex PCR	ul/Rx
dH2O	2.24
Buffer (10X)	0.50
MgCl (25 mM)	0.20
dNTPs (25 mM)	0.04
Forward Primer (1 uM)	1.00
Reverse Primer (1 uM)	1.00
Taq	0.02
DNA (2 ng/ul)	1.00
Total Volume	**5.00**
Thermocycle Time	**1.52 hrs.**

Thermocycle conditions: 95°C 15 mins, (95°C 20 sec, 56°C 30 sec, 72°C 1 min) repeat 40 cycles, 72°C 3 min.**see Appendix*

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Table 2.5: SAP addition to PCR Product per tube for Multiplex and Uniplex Analysis. Each tube contained 5 ul of PCR product and 2 ul of SAP mixture with a final volume of 7 ul/tube.

SAP addition	*ul/Rx*
dH20	1.53
hME Buffer(10x)	0.17
SAP	0.30
Total Volume	**2.00**
Thermocycle	**25min.**

Thermocycle conditions: 37°C for 20 mins
followed by 85°C for 5 mins. **see Appendix*,
SAP: *shrimp alkaline phosphate*

Table 2.6: SNP contents of each Termination Mix tube used for each MassARRAY reaction. Mixes 1-5 are multiplex and Mixes 6 and 7 are uniplex reactions.

	dNTPs ADDED TO EACH TERMINATION MIX						
	ACT Mix1	ACG Mix2	ACT Mix3	ACT Mix4	ACT Mix5	CGT Mix6	ACG Mix7
SNPs ADDED TO EACH TERMINATION MIX	CDKN2A G/A Ala→Thr	MLH1 A/G Ile→Val	CDKN1B G/T Val→Gly	CCND3 T/G Ala→Ser	CCNH A/G Lys→Arg	CCND1 G/T	CDKN2A C/T
	CDK2 A/C Tyr→Ser	CCNA2 G/A Val→Ile	CDKN1B C/T Arg→Trp	CCNH G/T Arg→Leu	MSH6 A/G Gly→Val		
	CCND2 A/G Gly→Arg	CCNH A/G Lys→Arg	E2F2 G/T Gln→His	CCNH T/C Val→Arg	E2F2 G/A Gly→Arg		
	MSH2 T/G His→Gln	CDKN1A C/T Ser→Arg	MSH2 G/A Gly→Asp	PMS2 A/G Lys→Glu	CDKN2A G/A Gly→Arg		
	MLH1 G/A Val→Met	E2F2 G/A Gly→Arg	CCNH T/C Val→Ala	TFD-P1 G/A Asp→Asn			
	RAD51 A/C Lys→Gln	CCND3 C/T Pro→Ser	E2F2 A/G Asp→Asn				

Termination Mix: The 3 dNTPs added to the reaction after SAP addition in order to allow for differentiation between the SNP alleles during MALDI-TOF-MS.

Table 2.7: The primers used for the MassExtend hME reaction for each SNP.

SNP	hME Primer UEP_Sequence
CCND1 G/T	CTCCGCCTTCAGCATGG
CCND2 A/G	GTCAGGACCAACGTGAC
CCND3 C/T	CCGACCACGCTGTCTCT
CCND3 T/G	GAGAGCCTCAGGGAAGCC
CCNH A/G (Lys/Arg)	GTATCTGTTCAAGTGCC
CCNH T/C Val/Ala	ACTTCTGTTTCAGAACAGCA
CCNH T/C Val/Arg	ACTTCTGTTTCAGAACAGCA
CCNH G/T	GCGGGCTGACGCCAACC
CCNH A/G	AGAGTCCTCTTGGACAGGAGA
CCNA2 G/A	CACTGGCTTTTCATCTTCTAATA
CDK2 A/C	TTTGTACACAACTCCGT
CDKN1A p21^{Cip1}C/T	GACAGCGAGCAGCTGAG
CDKN1B p27^{Kip1}C/T	CCTGCCTGGCGTCCATCC
CDKN1B p27^{Kip1}G/T	GCGCAGGAGAGCCAGGATG
CDKN2A p16 G/A Ala/Thr	ACCATGCCCGCATAGATGCC
CDKN2A p16 G/A Gly/Arg	GCCCGGGAGGGCTTCCT
CDKN2A p16 C/T	TTCGGCGCGCGTGCGGC
E2F2 G/A Gly/Arg	CCCCAGCTGTTGCTGCTTCC
E2F2 A/G Asp/Asn	CCAACTCAGGACATAGC
E2F2 G/T	GGAGCAGGCCTTGGACCA
E2F2 G/A Gly/Arg	ATACATTTGGCAAGTACCCAA
RAD51 A/C	ACAGGGAGAGTCGTAGATTT
MLH1 G/A	CTACCCAATGCCTCAACC
MLH1 A/G	GACTAACAGCATTTCCAAAGA
MSH2 T/G	GTTCTGTTGAAGATACCACTG
MSH2 G/A	GTTCTGTTGAAGATACCACTG
MSH6 A/G	TGCCGCCGCTGCCCCCG
PMS2 A/G	GACTCTCAGGAGAAAGCGCCT
TFD-P1 G/A	AGGACGACGAGGAGGAC

**see Appendix
hME (homogeneous massextend): addition of nucleotides adjacent to SNP of interest to allow allele differentiation by mass.
UEP=unextended primer

Table 2.8: 500 uL stock of hME primer mixes for each multiplex hME reaction. Primers that could be combined into one multiplex reaction were mixed into a 500 ul stock solution for easier handling and equal dispensing.

SNPs for Mix1	Mix 1 Primer ul per SNP	SNPs for Mix2	Mix 2 Primer ul per SNP	SNPs for Mix3	Mix 3 Primer ul per SNP	SNPs for Mix4	Mix 4 Primer ul per SNP	SNPs for Mix5	Mix 5 Primer ul per SNP
CDKN2A G/A Ala→Thr	50.89 88.41uM	MLH1 A/G Ile→Val	52.4 85.89uM	CDKN1B G/T Val→Gly	46.95 95.86uM	CCND3 T/G Ala→Ser	126.22 35.65uM	CCNH A/G Lys→Arg	58.40 77.05uM
CDK2 A/C Tyr→Ser	52.64 85.48uM	CCNA2 G/A Val→Ile	50.4 89.22uM	CDKN1B C/T Arg→Trp	56.06 80.27uM	CCNH G/T Arg→Leu	53.10 84.75uM	MSH6 A/G Gly→Val	56.77 79.27uM
CCND2 A/G Gly→Arg	54.92 81.93uM	CCNH A/G Lys→Arg	42.2 106.59uM	E2F2 G/T Gln→His	50.90 88.43uM	CCNH T/C Val→Arg	55.49 81.10uM	E2F2 G/A Gly→Arg	49.96 90.08uM
MSH2 T/G His→Gln	51.64 87.15uM	CDKN1A C/T Ser→Arg	48.8 92.18uM	MSH2 G/A Gly→Asp	50.82 88.54uM	PMS2 A/G Lys→Glu	55.84 80.59uM	CDKN2A G/A Gly→Arg	46.22 97.35uM
MLH1 G/A Val→Met	51.98 86.58uM	E2F2 G/A Gly→Arg	49.4 91.12uM	CCNH T/C Val→Ala	52.47 85.76uM	TFD-P1 G/A Asp→Asn	48.64 92.51uM		
RAD51 lA/C Lys→Gln	58.31 77.17uM	CCND3 C/T Pro→Ser	50.7 88.72uM	E2F2 A/G Asp→Asn	49.83 90.31uM				
dH2O	179.62		206.10		192.97		251.28		288.65
Total Volume	500		500		500		500		500

Table 2.9: Multiplex hME reaction/tube. Each tube contained 5ul PCR product, 2 ul SAP addition, and 2 ul of hME mix with a final volume of 9 ul/tube.

Multiplex hME reaction	ul/Rx
dH$_2$0	0.76
Termination Mix	0.20
hME Primer Mix	1.00
TSQ	0.04
Total Volume	**2.00**
Thermocycle	**27min.**

Thermocycle conditions: 94°C 2 mins, (94°C 5 sec, 52°C 5 sec, 72°C 5 sec) repeat 100 cycles, **see Appendix*
Tsq: Thermosequenase

Table 2.10: Uniplex hME cocktail recipe per reaction.

SNP	dH$_2$0 ul	Termination mix	hME primer ul	Tsq (32 U/ul)	Total Vol. ul	Thermocycle Minutes
CCND1 G/T	1.716	0.200	0.066	0.018	2.00	27
CCND2 A/G	1.716	0.200	0.066	0.018	2.00	27
CCND3 C/T	1.721	0.200	0.061	0.018	2.00	27
CCND3 T/G	1.739	0.200	0.043	0.018	2.00	27
CCNH A/G Lys/Arg	1.731	0.200	0.051	0.018	2.00	27
CCNH T/C Val/Ala	1.719	0.200	0.063	0.018	2.00	27
CCNH T/C Val/Arg	1.716	0.200	0.066	0.018	2.00	27
CCNH G/T	1.718	0.200	0.064	0.018	2.00	27
CCNH A/G	1.712	0.200	0.070	0.018	2.00	27
CCNA2 G/A	1.721	0.200	0.061	0.018	2.00	27
CDK2 A/C	1.719	0.200	0.063	0.018	2.00	27
CDKN1A p21^{Cip1}C/T	1.723	0.200	0.059	0.018	2.00	27
CDKN1B p27^{Kip1}C/T	1.715	0.200	0.067	0.018	2.00	27
CDKN1B p27^{Kip1}G/T	1.726	0.200	0.056	0.018	2.00	27
CDKN2A p16 G/A Ala/Thr	1.721	0.200	0.061	0.018	2.00	27
CDKN2A p16 G/A Gly/Arg	1.727	0.200	0.055	0.018	2.00	27
CDKN2A p16 C/T	1.719	0.200	0.063	0.018	2.00	27
E2F2 G/A Gly/Arg	1.723	0.200	0.059	0.018	2.00	27
E2F2 A/G Asp/Asn	1.721	0.200	0.060	0.018	2.00	27
E2F2 G/T	1.721	0.200	0.061	0.018	2.00	27
E2F2 G/A Gly/Arg	1.722	0.200	0.060	0.018	2.00	27
RAD51 A/C	1.712	0.200	0.070	0.018	2.00	27
MLH1 G/A	1.720	0.200	0.062	0.018	2.00	27
MLH1 A/G	1.719	0.200	0.063	0.018	2.00	27
MSH2 T/G	1.720	0.200	0.062	0.018	2.00	27
MSH2 G/A	1.721	0.200	0.061	0.018	2.00	27
MSH6 A/G	1.714	0.200	0.068	0.018	2.00	27
PMS2 A/G	1.715	0.200	0.067	0.018	2.00	27
TFD-P1 G/A	1.724	0.200	0.058	0.018	2.00	27

Thermocycle conditions: 94°C 2 mins, (94°C 5 sec, 52°C 5 sec, 72°C 5 sec) repeat 100 cycles

**see Appendix*, Tsq: Thermosequenase

Table 2.11: Desalting of hME product following PCR in preparation for MALDI-TOF-MS analysis. The hME product plate was rotated for 20 minutes following this addition.

Desalting Agent	Amount added to product of each hME reaction well
Resin	6mg/well
dH$_2$0	16ul/well

**see Appendix

Sources of Materials for Genotyping

Software: MassARRAY™ Typer 3.0.1 or higher (Sequenom, Newtown, MA, USA) and MassARRAY™ Assay Design 2.0 (Sequenom, Newtown, MA, USA).

Equipment: MassARRAY™ Liquid Handler (Sequenom, Newtown, MA, USA), MassARRAY™ Nanodispenser (Sequenom, Newtown, MA, USA), and MassARRAY™ Analyzer (Sequenom, Newtown, MA, USA).

Consumables for PCR: Hot Star Taq (Qiagen Operon Technologies, Alameda, CA, USA,), Hot Star Taq PCR Buffer (Qiagen, Operon Technologies, Alameda, CA, USA), dNTPs, PCR primers.

Consumables for hME: MassExtend™ Starter Kit (Sequenom, Newtown, MA, USA), Homogenous MassExtend™ Mix (Sequenom, Newtown, MA, USA), MassExtend™ Primers, Thermo Sequenase™ (Sequenom, Newtown, MA, USA), Clean Resin (Sequenom, Newtown, MA, USA), Clean Kit (Sequenom Newtown, MA, USA,), and Shrimp Alkaline Phosphatase (SAP) (Sequenom, Newtown, MA, USA).

SpectroCHIP Bioarrays™: 384-well silicon SpectroCHIP (Sequenom, Newtown, MA, USA).

Clean Resin Dimple Plate: 6mg Dimple Plate (Sequenom, Newtown, MA, USA).

Chapter 3 Results

3.1 RESULTS

Thirty-one SNPs from 17 different genes were chosen for this study. They were: 1 *CCNA2*, 3 *CCND1*, 1 *CCND2*, 2 *CCND3*, 5 *CCNH*, 1 *CDK2*, 1 *CDKN1A*, 2 *CDKN1B*, 3 *CDKN2A* (*p16*), 3 *E2F2*, 1 *RAD51*, 1 *PMS2*, 2 *MLH1*, 2 *MSH2*, 1 *MSH6*, 1 *TFD-P1*, and 1 *MTHFR* (Table 3.1.1 & 3.1.2). Details of these SNPs were outlined in the Materials and Methods section above.

Two of the 31 SNPs (1 *CCNH* and 1 *PMS2*) had to be eliminated as a result of invalid primers either from contamination or sequence inaccuracies. There were two other SNPs that yielded limited results because the sample size for their reaction was relatively small (*CCND3* T/G Ala→Ser and *CCNH* T/C Val→Ala). For this reason, both were eliminated from the final analysis. Therefore, 27/31 of the SNPs that were selected for this study were analyzed completely. However, *CCND3* T/G Ala→Ser and *CCNH* T/C Val→Ala were analyzed using the limited data that was available before being eliminated because a polymorphism was observed. No significant data in either of these two SNPs was found regarding allele types in relation to the age of onset of an HNPCC-related cancer (either CRC or an extra-colonic cancer) or colorectal polyps in males, females, or males and females combined in either of these two SNPs (Table 3.2, 3.3, and 3.4).

In total, 17 of these 29 SNPs were not polymorphic in our HNPCC subjects. The other 12/29 SNPs (including *CCND3* T/G Ala→Ser and *CCNH* T/C Val→Ala) were polymorphic, and were analyzed based on the set parameters discussed in the materials and methods above.

After *CCND3* T/G Ala→Ser and *CCNH* TC Val→Ala were removed from the study, 10 polymorphic SNPs remained. Of those 10, *CDKN2A (p16)* A/G Ala→Thr and *E2F2* A/G Asp→Asn SNPs were only observed to be polymorphic in Family C but not in Family 11 (Table 3.1.1 and 3.1.2). Three other SNPs from the polymorphic group were of statistically significant value in the set parameters for this study. These three SNPs included *CCNA2* G/A (Figure 3.1 and 3.2), *CDKN1B (p27KIP1)* T/G (Figure 3.3), and *CCND1* G/C (Figure 3.4). Thus, altogether 5/10 or 50% of the polymorphic SNPs that were completely analyzed displayed results that may be either candidates for further investigation or have significance in relation to HNPCC.

For each polymorphic SNP, the age to first HNPCC-related cancer or colorectal polyp and allele type was analyzed by Kaplan-Meier analysis under three categories: (i) Males and Females combined (Table 3.2), (ii) Females only (Table 3), and (iii) Males only (Table 3.4).

3.2 CCNA2 G/A

In the Males and Females combined group, those with a *CCNA2* A allele were found to have a significantly earlier age of onset of an HNPCC-related cancer, or colorectal polyp than those with the homozygous G allele (p=0.0094; Mean age: 37; 95% CI: 32, 42; Cox Forward Confidence Interval (CI): 1.29-14.9) (Figure 3.1). The frequency for the homozygous G/G allele type was 107/112 (46 males; 61 females) with 50/107 (22 males;

28 females) of these subjects having had an event (Table 3.1.1). The heterozygous G/A genotype was present in 5/112 (3 males; 2 females) with events having occurred in 3/5 (3 males; 0 females) (Table 3.1.1). There were no subjects in this analysis that had the homozygous A/A alleles. When the males and females were separated and then re-analyzed the male only group with the heterozygous G/A allele type remained statistically significant for having a modifying effect for an earlier age of onset (p = 0.0239; Mean age: 35; 95% CI: 29, 42; Cox Forwarding CI: 4.3{1.29-14.9} (Figure 3.2 and Table 3.1.1).

3.3 CDKN1B (p27kip1) T/G

The second SNP to demonstrate a significant modifying effect was *CDKN1B (p27kip1)* T/G where there was an earlier age of onset of an HNPCC-related cancer or a colorectal polyp in those with the homozygous G/G allele type in the female only group (p = <0.0001; Mean age: 34; CI {28,40}) (Figure 3.3 and Table 3.1.1). There were 63 females in this group and 35/63 were homozygous T/T with 14/35 events, 24/63 were heterozygous T/G with 9/24 events, and 4/63 were homozygous G/G with 4/4 events (Table 3.1.1).

3.4 CCND1 G/C

Finally, in the male only group the *CCND1* GC was found to be associated with the age of onset of an HNPCC-related cancer or a colorectal polyp. There were 46 males in this group and 21/46 had had an event (Table 3.1.1). The homozygous G/G was observed in 4/46 with 3/21events, heterozygous G/C was in 19/46 with 8/21 events, and 23/46 were homozygous C/C with 10/21 events (Table 3.1.1). The homozygous C/C allele type of

70

this SNP was observed to be present in those with an earlier age of onset (p=0.0137;

Mean age: 32; 95% CI: {25, 40}) (Figure 3.4).

Table 3.1.1: Summary of allele frequencies for 31 SNPs`chosen for analysis by RFLP or MassARRAY™. The minor allele frequency was taken from NCBI (National Centre for Biotechnology Information) database while the remaining allele frequencies were the results from this study.

Gene	SNP	Amino Acid Substitution	NCBI minor Allele Frequency	Genotype Frequency Males & Females	Genotype Frequency Males Only	Genotype Frequency Females Only
CCND1	A/G	Modulates mRNA splicing producing 2 transcripts: a and b	N/A	AA=37;AG =67;GG=15	AA=17;AG =27;GG=7	AA=20;AG=40; GG=8
CCND1	G/C	3'UTR region	N/A	GG=11;GC =43;CC=47	GG=4;GC= 19;CC=23	GG=7;GC=24;C C=24
MTHFR	C/T	Ala→Val	0.409	CC=62;CT= 44;TT=12	CC=28;CT= 14;CT=6	CC=34;CT=30;T T=6
MLH1	A/G	Ile→Val	0.11	AA=56;AG =45;GG=7	AA=23;AG =19;GG=5	AA=33;AG=26; GG=2
CDKN2A (P16) INK4a	A/G	Ala→Thr	0.056	AA=n/a; AG=10; GG=100	AA=n/a; A/G=6; GG=41	AA=n/a; AG=4;GG=59
CDKN1B (P27) Kip1	T/G	Val→Gly	0.28	TT=66;TG= 37;GG=6	TT=31;TG= 13;GG=2	TT=35;TG=24;G G=4
E2F2	TG	Gln→His	0.39	TT=33;TG= 47;GG=22	TT=12;TG= 16;GG=14	TT=21;TG=31;G G=8
MSH6	A/G	Gly→Val	N/A	AA=n/a; AG=17;GG =85	AA=n/a; AG=8;GG= 35	AA=n/a; AG=9;GG=50
CCNA2	G/A	Val→Ile	0.067	GG=107; GA=5;AA= n/a	GG=46;GA =3;AA=n/a	GG=61;GA=2;A A=n/a
E2F2	A/G	Asp→Asn	0.02	AA=n/a; AG=12; GG=80	AA=n/a; A/G=2;GG= 35	AA=n/a; AG=10; GG=45
**CCND3*	T/G	Ala→Ser	0.49	TT=4;TG=6 ;GG=2	TT=1;TG=3 ;GG=1	TT=3;TG=3;GG =1
**CCNH*	T/C	Val→Ala	0.169	TT=24;TC= 3;CC=1	TT=12;TC= 2;CC=1	TT=12;TC=1;CC =N/A
CCNH	AG	Lys→Arg	0.051	Non-polymorphic	Non-polymorphic	Non-polymorphic
CDKN2A (p16)	A/G	Gly→Arg	0.011	Non-polymorphic	Non-polymorphic	Non-polymorphic
TFD-P1	A/G	Asp→Asn	0.011	Non-	Non-	Non-polymorphic

				polymorphic	polymorphic	
CCND1	G/T	N/A	N/A	Non-polymorphic	Non-polymorphic	Non-polymorphic
CCNH	T/G	Arg→Leu	0.011	Non-polymorphic	Non-polymorphic	Non-polymorphic
CDKN2A (P16)	C/T	Pro→Ser	0.011	Non-polymorphic	Non-polymorphic	Non-polymorphic
MSH2	A/G	Gly→Asp	0.01	Non-polymorphic	Non-polymorphic	Non-polymorphic
CDKN1B	T/C	Arg→Trp	0.00	Non-polymorphic	Non-polymorphic	Non-polymorphic
CDK2	A/C	Tyr→Ser	0.00	Non-polymorphic	Non-polymorphic	Non-polymorphic
CCND2	A/G	Gly→Arg	0.01	Non-polymorphic	Non-polymorphic	Non-polymorphic
MLH1	A/G	Val→Met	0.00	Non-polymorphic	Non-polymorphic	Non-polymorphic
RAD51	A/G	Lys—Gln	0.00	Non-polymorphic	Non-polymorphic	Non-polymorphic
MSH2	A/G	His→Gln	0.00	Non-polymorphic	Non-polymorphic	Non-polymorphic
CCNH	G/A	Lys→Arg	0.045	Non-polymorphic	Non-polymorphic	Non-polymorphic
CDKN1A	C/T	Ser→Arg	0.29	Non-polymorphic	Non-polymorphic	Non-polymorphic
E2F2	G/A	Gly→Arg	0.011	Non-polymorphic	Non-polymorphic	Non-polymorphic
CCND3	C/T	Pro→Ser	0.02	Non-polymorphic	Non-polymorphic	Non-polymorphic
CCNH	T/C	Val→Arg	0.144	Non-polymorphic	Non-polymorphic	Non-polymorphic
PMS2	A/G	Lys→Glu	0.125	Non-polymorphic	Non-polymorphic	Non-polymorphic

*Small subset from family 11 was found polymorphic before contamination occurred.

Table 3.1.2: Summary of 31 SNPs chosen for analysis by RFLP or MassARRAY™.

POLYMORPHIC SNPs	NON-POLYMORPHIC SNPs	ELIMINATED SNPs
CCND1 A/G	*CCND3* C/T	*PMS2* A/G Lys→Glu
CCND1 G/C	*CDKN1B* T/C	**CCNH* T/C Val→Ala
MTHFR	*E2F2* G/A Gly→Arg	*CCNH* T/C Val→Arg
MLH1 G/A Ile→Val	*RAD51* A/C	**CCND3* T/G Ala→Ser
# *CDKN2A* (p16) A/G Ala→ Thr	CCNH A/G Lys→Arg	
CDKN1B (p27KIP1) T/G	*MLH1* A/G Val→Met	
E2F2 T/G	*MSH2* A/G	
MSH6 A/G	*CCND1* G/T	
CCNA2 G/A	*CCND2* A/G	
# *E2F2* AG Asp→Asn	CDK2 A/C	
*CCND3 T/G Ala→Ser	*CDKN1A* C/T	
*CCNH T/C Val→Ala	*E2F2* A/G Gly→Arg	
	TFD-P1 A/G	
	CDKN2A (p16) A/G Gly→ Arg	
	CDKN2A (p16) C/T	
	CCNH T/G Arg→Leu	
	MSH2 A/G Gly→Asp	

* small subset from Family 11 was found polymorphic before contamination occurred; # only polymorphic in Family C

Table 3.2: Polymorphism summary of Kaplan-Meier Survival Analysis of the age of first HNPCC cancer or colorectal polyp and allele type for males and females in Family C and Family 11 *MSH2* mutation positive carriers.

SNP ID	Total Patients Analyzed	Events (n)	P-value (log rank)
CCND1 A/G	119	57	0.9617
CCND1 G/C	101	51	0.5263
MTHFR	118	60	0.6507
MLH1 IV	108	57	0.9497
P16 Ala→ Thr	110	59	0.1419
CDKN1B (P27KIP1) T/G	109	56	0.0667
E2F2 T/G	102	54	0.2622
MSH6 A/G	102	52	0.7867
CCNA2 G/A	112	50	0.0094
E2F2 A/G Asp→Asn	93	44	0.9160
*CCND3 T/G Ala→Ser	12	5	0.7801
*CCNH T/C Val→Ala	28	17	0.3316

* small subset from Family 11 was found polymorphic before contamination occurred.
Note: number of patients analyzed varies due to depletion in DNA supply & nil result for some samples.

Table 3.3: Polymorphism summary of Kaplan-Meier Survival Analysis of the age of first HNPCC cancer or colorectal polyp and allele type for females in Family C and Family 11 *MSH2* mutation positive carriers.

SNP ID	Total Analyzed	Events	P-value (log rank)
CCND1 A/G	68	33	0.7112
CCND1 G/C	55	30	0.6651
MTHFR	70	36	0.7638
MLH1 IV	61	32	0.9694
P16 Ala→ Thr	63	34	0.7234
CDKN1B (P27KIP1) T/G	63	31	<0.00001
E2F2 T/G	60	30	0.8266
MSH6 A/G	59	28	0.7406
CCNA2 G/A	63	28	0.7140
E2F2 A/G Asp→Asn	55	24	0.2700
*CCND3 T/G Ala→Ser	7	2	NA
*CCNH T/C Val→Ala	13	6	0.5264

* small subset from Family 11 was found polymorphic before contamination occurred.

Table 3.4: Polymorphism summary of Kaplan-Meier Survival Analysis of the age of first HNPCC cancer or colorectal polyp and allele type for males in Family C and Family 11 *MSH2* mutation positive carriers.

SNP ID	Total Analyzed	Events	P-value (log rank)
***CCND1* A/G**	31	24	0.7322
***CCND1* G/C**	46	21	0.0137
MTHFR	48	24	0.5614
***MLH1* IV**	47	25	0.9934
***P16* Ala→ Thr**	47	25	0.0932
***CDKN1B (P27KIP1)* T/G**	46	25	0.3450
***E2F2* T/G**	42	24	0.3517
***MSH6* A/G**	43	24	0.9953
***CCNA2* G/A**	49	22	0.0239
E2F2 A/G Asp→Asn	38	20	0.5948
*CCND3 T/G Ala→Ser	5	3	0.5890
*CCNH T/C Val→Ala	15	11	0.6934

* small subset from Family 11 was found polymorphic before contamination occurred.

CCNA2 GA

Figure 3.1: Kaplan-Meier survival curve of age of onset of first HNPCC-related cancer or first colorectal polyp in males and females in the *CCNA2* G/A SNP (p=0.0094; Mean age: 37; 95% CI: {32, 42}). Individuals with heterozygous G/A alleles had earlier age of onset than those who were homozygous G/G. No one had a homozygous A/A allele in this analysis.

Figure 3.2: Kaplan-Meier survival curve of age of onset of first HNPCC-related cancer or first colorectal polyp in males and the *CCNA2* G/A SNP (p=0.0239; Mean age: 35; 95% CI: {29,42}). Individuals with a heterozygous G/A allele type had an earlier age of onset than those with a homozygous G/G. No one in this analysis had the A/A allele type.
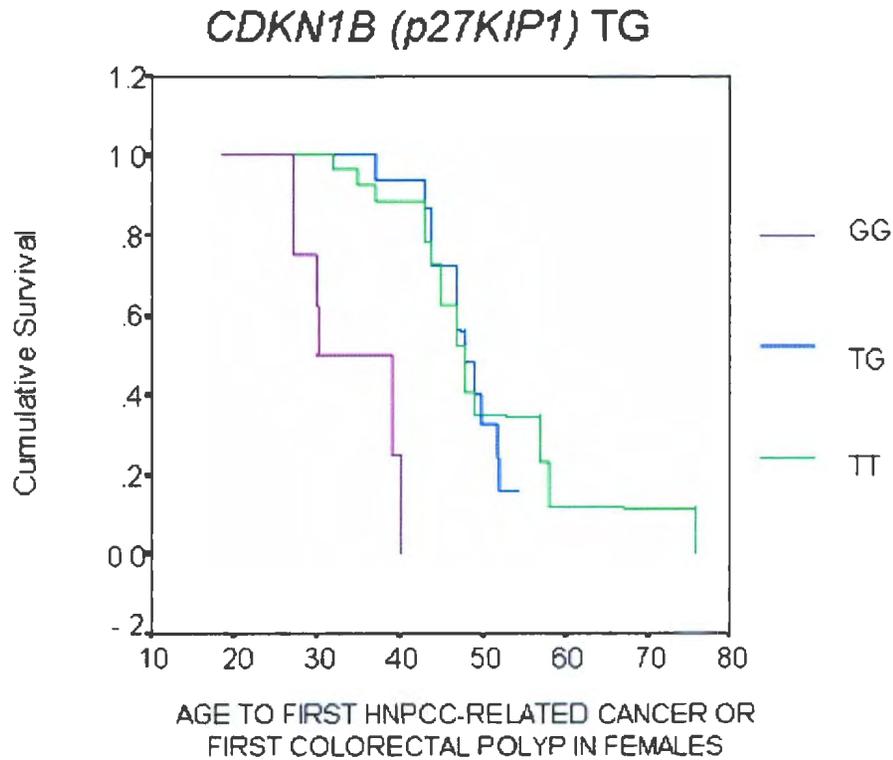
Figure 3.3: Kaplan-Meier survival curve of age of onset of first HNPCC-related cancer or first colorectal polyp in females and the *CDKN1B (p27kip1)* T/G SNP (p<0.0001; Mean age: 34; 95% CI: {28, 40}). Individuals with homozygous G/G allele had earlier age of onset than those with a T allele.

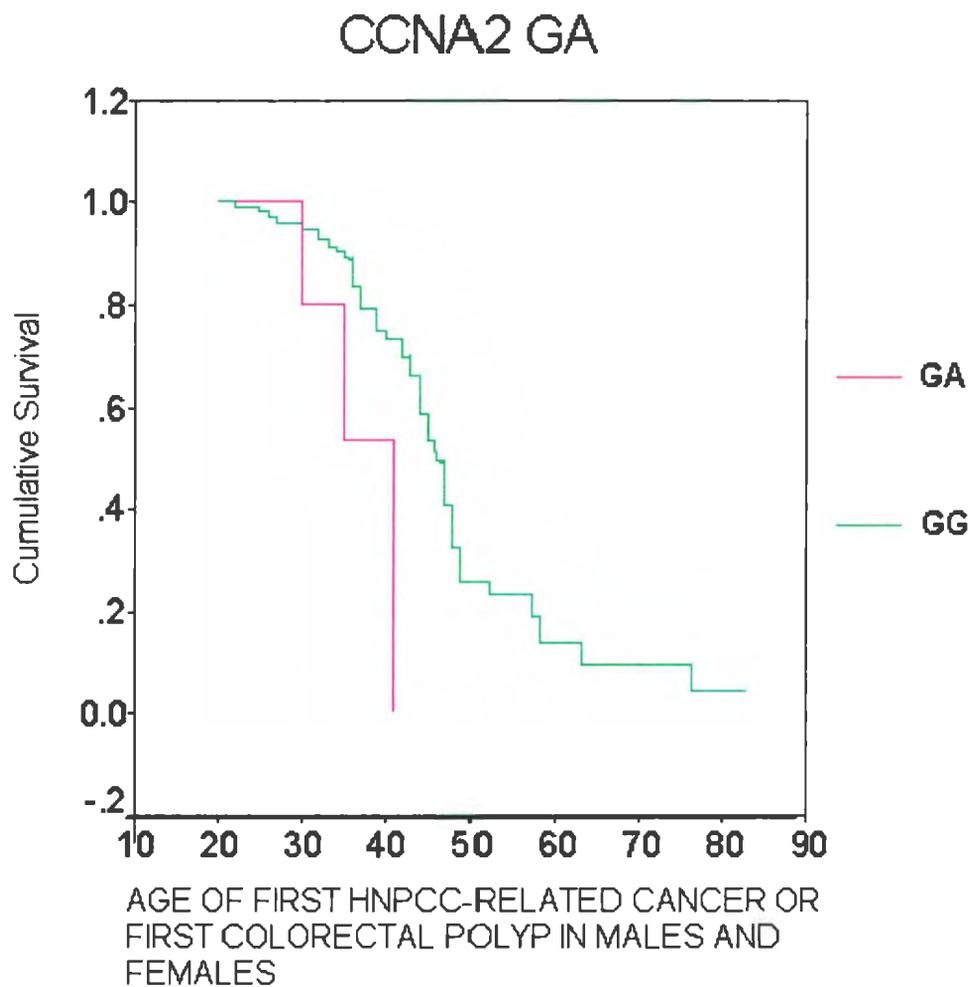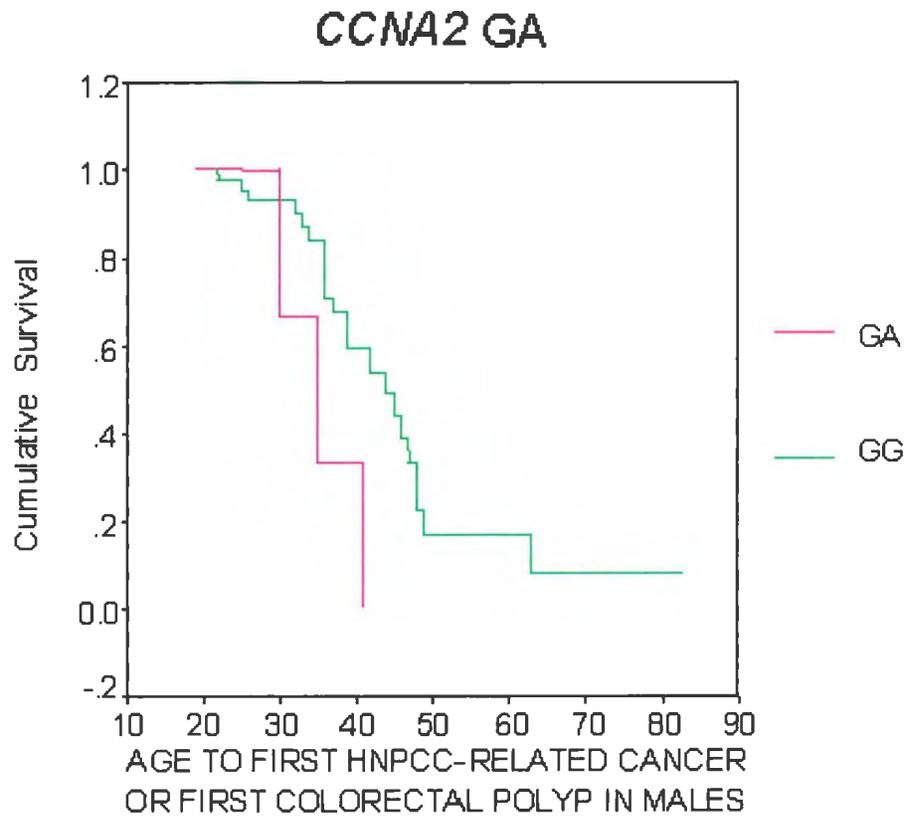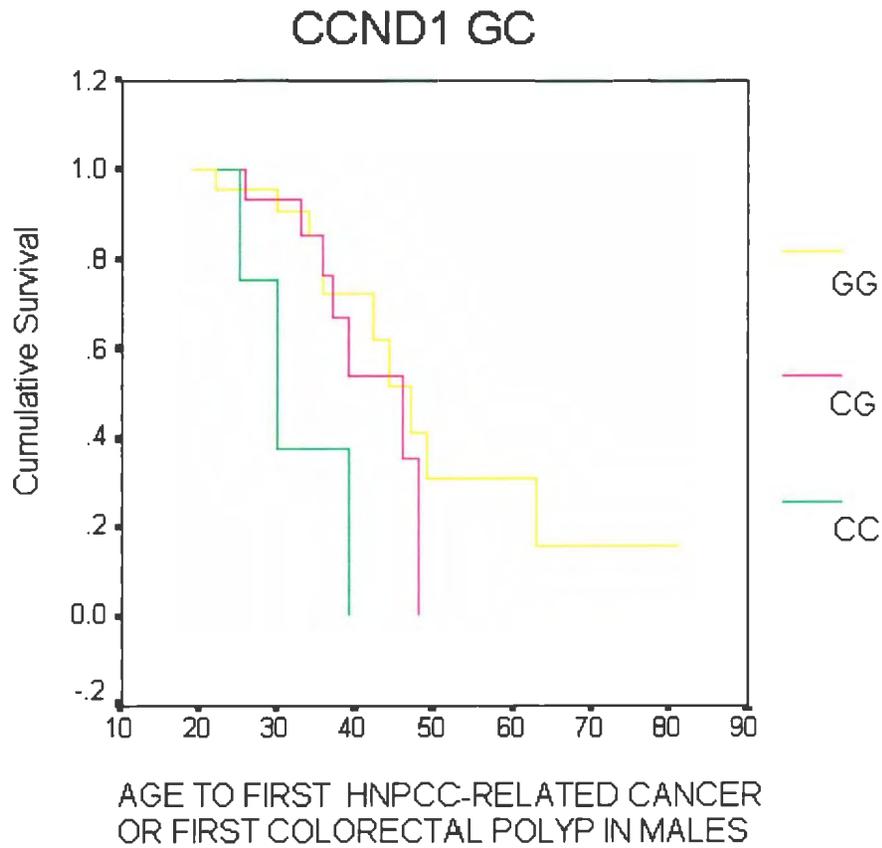**Figure 3.4:** Kaplan-Meier survival curve of age of onset of first HNPCC-related cancer or first colorectal polyp in males and the *CCND1* G/C SNP (p=0.0137; Mean age: 32; 95% CI: {25, 40}). Individuals with homozygous C/C had an earlier age of onset than those with a G allele.

Chapter 4 Discussion and Conclusion

4.1 DISCUSSION

Mutations in the MMR genes cause Hereditary Non-Polyposis colorectal cancer (HNPCC), however, there is still a great deal about this disease, which is not understood. One area that has not been extensively studied is variations in the penetrance of pathogenic mutations. Areas of interest surrounding these variations include: how to predict who in the general population is at a higher risk than others for developing forms of CRC, how to identify these people before they get cancer, why some members within the same high risk family with the same predisposing mutation get CRC and related cancers earlier in life than other members, or why some of these family members get multiple primary tumours whereas others in their family never develop cancer.

Past studies have centered on the examination of variants of single candidate genes for their ability to modify the penetrance or expressivity of the MMR mutation. In this study, I have examined a group of potential modifiers of HNPCC. I looked at how candidate modifiers affected the age of onset of HNPCC cancers (and number) in those with the same HNPCC mutation The candidate modifiers included single nucleotide polymorphisms (SNPs) in the cell cycle and related pathways (such as the mismatch repair (MMR) pathway), and an epigenetic pathway involving folate metabolism via methylenetetrahydrofolate reducatase (MTHFR)). I analysed these potential modifiers in order to determine whether they correlate with age of onset of HNPCC in males and/or females.

The cell cycle genes and genes in the related functions were examined through SNP analysis to determine their modifying impact on the penetrance of HNPCC. I focused upon the cell cycle because carcinogenesis involves a malfunction in the control of cell division, thus, implying that there is uncontrollable cell division in the presence of cancer or the precursors leading to it. Therefore, it is possible that a variant that might increase the rate of cell division could also include the potential for developing into cancer, or *vice versa*.

Through the use of genetic analysis, some forms of CRC have been identified as resulting from gene mutations that are inherited within families. The families used in this HNPCC study were from that category. They were described as being carriers of a mutation in one of the MMR genes, *MSH2*.[120] There were two *MSH2* mutations described in that study, including a c.942+3A>T point mutation (Newfoundland and Labrador, Family C), and an exon 4-16 deletion (Lower North Shore of Quebec, Family 11). [120] Historically, mutation carriers presented symptomatically with one or more HNPCC-related cancers. Penetrance in hereditary cancer syndromes is traditionally calculated based on the percentage of mutation carriers developing cancer. Age-related penetrance uses age at onset of cancer in the calculation. However, some forms of screening for members of families with hereditary cancer result in identification and removal of the pre-cancerous lesions such as polyps. Thus, these individuals may never develop cancer. The definition of penetrance therefore has to be revised to include age at development of pre-cancerous lesions such as polyps, which may be markers for cancer development. Since genetic testing became available in these families, screening interventions were offered to those who tested positive for either of these two mutations.

This has had a significant impact in decreasing the number of mutation positive carriers that developed an HNPCC-related cancer in these two families. As polyps are removed during colonoscopy screening, the occurrence of CRC is altered, and fewer subjects with cancer are available. As a result, this study included age of onset of first colorectal polyp identified during screening procedures, as well as age of onset of HNPCC. Therefore, although the age at polyp identification may be younger than the age that the cancer would have occurred, there is less bias than having eliminated the subjects as not having had an event at all. As polyp studies provide more information on how long it takes for a polyp to change into a carcinoma and which type of polyp becomes cancerous, more accurate age to event data will be available for future studies such as this one. In fact, as more mutations predisposing to hereditary CRC are identified, and increasing numbers of subjects are having screening interventions, polyp history will be a more important feature of hereditary CRC analysis and intervention.

At the start of my project, cell cycle SNP discovery was still in its preliminary stages with limited information available. However, based on what was available and what is known thus far about the cell cycle, a group of SNPs was chosen and analyzed (Table 3.1.1 in Results). Ideally, the greater the allele frequency of the SNP the more likely it would provide definitive data. Due to the fact that many cell cycle genes are still being discovered and that SNPs within these genes are currently being determined, allele frequencies were not yet known for most of the ones chosen (Table 3.1.2 in Results). Therefore, the criterion for choosing SNPs with unavailable frequencies was that the SNP had to cause a non-synonomous amino acid change in a coding region of one of the cell cycle genes. Taking into consideration this limitation, encouraging results were still

obtained from 7 of the 29 SNPs (includes *CCND3* T/G and *CCNH* T/C before

elimination) that were investigated. That is, approximately one quarter (24%) of the

analyzed SNPs demonstrated possible association with the penetrance of HNPCC.

The most significant observation noted in this study involved three SNPs that correlated

with the age of onset of an HNPCC-related cancer or first colorectal polyp, and are

potentially modifiers. These SNPs include: *CCND1*[1722] G/C, *CCNA2* G/A, and *CDKN1B*

(p27[KIP1]*)* Val/Gly T/G. With the *CDKN1B (p27*[KIP1]*)* SNP it was found that females with

the homozygous G/G allele type had an earlier age of onset of HNPCC or first colorectal

polyp compared to other family members having either a homozygous or heterozygous T

allele type (n = 63; events31; p= <0.00001) (Figure 3.3 from Results). Therefore, the

homozygous G allele in these two families demonstrated a possible association as a risk

factor for developing an HNPCC-related cancer or colorectal polyp. Women with

homozygous G/G had incidence of cancer/polyp in the third and fourth decade of life with

a mean age of diagnosis at 34 years. However, those possessing a T allele did not develop

an event until later in life with a mean age of 47. This coincides with a similar study in

oral squamous cell carcinoma of the head and neck (SCCHN), which found that the

homozygous G/G allele of the *CDKN1B* T/G variant might be associated with an

increased risk in those already in an at-risk subset for SCCHN.[381]

Opposite results have been found in other studies. For example, the *CDKN1B* T/G SNP

has been found to be associated with tumorigenesis in advanced prostate carcinoma

patients.[382] It was concluded that those with the homozygous T/T allele that expresses for

the valine amino acid were associated with advanced prostate cancer progression.[382]

Another study also observed a penetrance effect with the T allele in hereditary prostate

cancer families.[383] However, their findings were not of significant value and they suggested that this SNP might be involved in sporadic prostate cancers rather than in hereditary cases with high-risk factors.[383]

The second potential modifier identified involved the *CCNA2* gene with the G/A polymorphism. In the male and female combined group, those with the heterozygous G/A alleles developed an event earlier than those with homozygous G/G (n=112; events=50; p=0.0094) (Figure 3.1 in Results). No homozygous A/A individuals were observed. The same result was also found in the male only group. In this subgroup, the age to event was earlier in those with the heterozygous G/A alleles compared with those with the homozygous G/G. The mean age of onset for the heterozygous group was 35 years compared with 42 years for the homozygous G/G. The A allele appears to be a risk factor for these mutation positive carriers (n=49; events=22; p=0.0239) (Figure 3.2 in Results). To my knowledge there have not been any other studies reported that investigate SNPs in the *CCNA2* gene and their association with cancer. However, other investigators have reported an over expression of the CCNA2 protein in association with various cancers such as breast, lung, and liver.[384-388] It has been suggested that CCNA2 could potentially be used as a predictive marker for tumorigenesis and prognosis of those already with cancer.[384-388]

Finally, for the *CCND1*[1722] G/C SNP, males with the homozygous C/C alleles had an event earlier than males with either G/C or homozygous G/G genotypes (n=46; events=21; p=0.0137) (Figure 3.4 in Results). The mean age of onset for those with the C/C alleles onset was 32 years compared with the mean age of 40 years for those with

either a heterozygous or homozygous G allele. Therefore, having a C allele is associated

with an earlier age of onset of either a colorectal polyp or an HNPCC-related cancer.

In the literature only one study was found regarding this particular $CCND1^{1722}$ G/C SNP.

The study was conducted on the polymorphism and expression in patients with squamous

cell carcinoma of the head and neck (SCCHN). [389] It was because of this study that I

chose to investigate this SNP using our *MSH2* mutation positive carriers and the

incidence of HNPCC. The results from the SCCHN study found that the homozygous C/C

SNP was associated with poorly differentiated tumours and decreased disease-free

interval.[389] This coincides with my study in that the C/C genotype was correlated with an

earlier age of onset of HNPCC-related cancers. However, the study on the SCCHN from

the literature noted that their results had a higher association of the C/C allele type in

females whereas in my study there was a higher association in males.

It has also been observed that there are minor phenotypic differences between Family C

and Family 11. For instance ovarian cancer has only been found in Family C and not in

Family 11 and urothelial cancers were present in Family 11 but not Familt C. [120]

Therefore, although both families have an *MSH2* mutation that leads to a higher risk for

HNPCC -related cancers compared to the general population, the effect of the location of

the primary mutation may be a factor. There also might be other undetermined underlying

modifiers that contribute to these phenotypic differences. This study detected a difference

between these two families in the presence of polymorphisms for SNPs in 2 genes:

CDKN2A (p16) A/G Ala/Thr and *E2F2* A/G Asp/Asn. These SNPs were polymorphic in

Family C but not in Family 11 (Table 3.1.2 in Results). However, no significant

difference was found with regards to the age to event and allele type for either of these

two SNPs. Considering the phenotypic differences between these two families, further analysis of the *CDKN2A* and *E2F2* SNPs including various forms of penetrance variables should be investigated in larger studies. Although there was no specific allele type associated with family members having an HNPCC-related event compared to those who did not in Family C, it might be part of a multi-genetic polymorphism that either protects or predisposes for the occurrence of HNPCC. Therefore, investigations of polymorphisms and polymorphic combinations within these two families should be considered.

A final observation in this study that warrants further investigation involves the *CCND3* T/G Ala/Ser and *CCNH* Val/Ala SNPs. As described in the results section, these were only partially analyzed in a small sample from Family 11 (n=12 *CCND3*; n=28 *CCNH*) because of technical difficulties (Table 3.2). However, both SNPs were polymorphic (Table.3.1.2 in Results). Thus, it would be interesting to investigate these two SNPs in a larger sample to determine whether or not they contribute to a modifying effect on HNPCC penetrance.

4.2 Conclusion

In conclusion, based on the data gathered in this study, SNPs in cell cycle genes should be considered as potential modifiers of HNPCC expression. This is especially true for the families in this study with either the *MSH2* c.942+3A>T point mutation or the *MSH2* exon 4-16 multi-exonic deletion. As new cell cycle genes and their SNPs are identified a better choice of candidate SNPs with higher allele frequencies will become available and be more informative for future studies. The other pathways investigated such as the one involving folate metabolism using *MTHFR* was not significant in this study due to the fact that other factors outside of genetics such as diet and smoking need to be considered

in conjunction with allele types. This type of information was not available for this project.

The SNPs that did not have any significant value in this analysis may be a result of the study being underpowered. Increasing the sample size would present more significant data. This would help determine if the SNPs found to be insignificant in this analysis could be screened out and considered less important or not involved in the modifying effects of penetrance of HNPCC.

The cell cycle genes that did present as candidates for modifying the age of onset of HNPCC are significant players in the cell cycle. For instance, Cyclin A is a crucial cyclin for transferring the cell cycle from G1 to S-phase, as well as the G2 to M -phase. It also inhibits the E2F factors thus allowing the S-phase to begin. CyclinD1 brings a cell through each restriction point so that it can be committed to completing G1. This cyclin is also influenced by external stimuli and can prevent or initiate the start of the G1 phase based on the stimuli that it receives. CDKN1B (p27^{KIP1}) is a cyclin dependent kinase inhibitor of the phosphorylation of the cyclinE-cdk2 complex. Once phosphorylation of the CCNE-cdk2 complex is inhibited the cell goes into G1 arrest. It is also influenced by external stimuli such as cell-to cell contact inhibition, which prevents the cell from proceeding through the G1 phase and puts the cell cycle in arrest.

As for the other potentially modifying genes mentioned in this discussion, the CCNH prtoein is a major player in the CAK complex, which activates the cyclin-cdk complexes. Without this step the cell cycle cannot proceed. CCND3 has the same function as CCND1 discussed above. E2F2 are transcription activators involved in cell cycle progression. Finally, CDKN2A (p16^{INK4A}) inhibits the cyclinD1-cdk4 complexes. Therefore, given

their roles in the cell cycle and their noted polymorphisms despite being non-significant in this study possibly because of the small sample size used, the potential for a larger sample to yield significant results in these candidates should be investigated further. In addition, the results from this study were not corrected for multiple test analysis. Therefore, to solidify the value of this data further corrections should be done to confirm these findings.

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