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The Role of Modifier Genes in Lynch Syndrome

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1. Introduction

There are a number of inherited predispositions to colorectal cancer (CRC) which can be broadly categorized into two groups; those with associated polyposis, such as familial adenomatous polyposis and the hamartomatous polyposis syndromes; and those that are linked to the non-polyposis syndromes, such as hereditary non polyposis colorectal cancer (HNPCC). The genetic basis of both the polyposis and non-polyposis syndromes are reflected in the CRC population who have no apparent family history of disease. Approximately 80% of all cases of CRC are associated with chromosomal instability [1] and are likely to have mutations in the Adenomatous Polyposis Coli (APC) gene whereas the remaining 20% with microsatellite instability appears to be due primarily to epigenetic inactivation of the DNA mismatch repair (MMR) gene *MLH1* [2].

The disease HNPCC accounts for somewhere between 2% and 5% of all CRCs diagnosed and is associated with a younger age of disease onset compared to the general population [3,4]. HNPCC is a disease by definition based on the Amsterdam Criteria where there need to be three cases of CRC, one of which must be diagnosed under the age of 50 years, one patient must be a first degree relative of the other two, span two generations and familial adenomatous polyposis should be excluded [5]. Modification of the Amsterdam Criteria has been ongoing since its original inception due to an increasing awareness of what constitutes this disease. HNPCC used to be known as either the Cancer Family Syndrome or Lynch Syndrome [6]. It is now accepted that families where a mutation in the DNA mismatch repair genes (MMR) *MSH2, MLH1, MSH6* or *PMS2* has been identified are now termed Lynch Syndrome families whereas those with no mutation are termed HNPCC [7]. The primary function of MMR genes is to eliminate base-base mismatches and insertion-deletion loops which arise as a consequence of DNA polymerase slippage during DNA replication [8]. MMR confers several genetic stabilisation functions; it corrects DNA biosynthesis errors, ensures the fidelity of genetic recombination and participates in the earliest steps of cell cycle checkpoint control and apoptotic responses [9,10]. MMR gene defects increases the risk of malignant transformation of cells, which ultimately results in the disruption of one or

several genes associated with epithelial integrity. The identification of germline mutations in families with Lynch Syndrome accounts for only ~50% of all families that fulfil the clinical diagnosis defined by the Amsterdam criteria [11]. The remaining families have no identifiable genetic predisposition yet fulfil the diagnostic criteria for the disease and are referred to as HNPCC families.

DNA MMR is a housekeeping function of all nucleated cells and as such any breakdown in the fidelity of this process is likely to result in disease irrespective of which gene is affected. Unlike other predispositions to colorectal cancer such as familial adenomatous polyposis, there are no obvious genotype/phenotype correlations in Lynch syndrome. Mutations that result in the loss of MSH2 or MLH1 irrespective of where they occur in the respective gene alter the risk of developing malignancy. Furthermore, mutations in DNA MMR genes do not predict a phenotype since any breakdown in the fidelity of this process results in a "mutator phenotype". It has been obvious from the first MSH2 and MLH1 mutation reports that differences in the ages of cancer diagnosis in patients harbouring germline mutations in DNA MMR genes do occur both within and between families. Furthermore, unrelated families harbouring the same mutation present with different disease profiles as do patients from within the same family [12-14]. The differences in disease expression both within and between families harbouring the same mutation are most likely a result of environmental, genetic or a mixture of both influences.

Identification of environmental factors that could account for differences in the age of colorectal cancer diagnosis of Lynch Syndrome is almost intractable when undertaken as a retrospective study and is best undertaken prospectively to include as many environmental variables as possible. Notwithstanding, knowledge about environmental factors and disease risk in Lynch Syndrome is important and studies are required to identify those which protect or promote disease.

Conversely, as genetic factors can be assessed after the fact they lend themselves more readily to retrospective interrogation and consequently identification. Identifying genetic factors that could explain differential disease expression in Lynch syndrome is now achievable due to the development of appropriate technology that allows for the rapid screening of large numbers of patients in conjunction with the accumulation of large cohorts of patients that allow for robust statistical analysis.

The search for modifier genes has been ongoing ever since the first groups of Lynch syndrome families were identified. Initial studies focused on genes associated with xenobiotic metabolism which have been followed by genes involved in the immune response, DNA repair, cell cycle control and as yet undefined genomic regions identified as a result of large genome wide association studies searching for genetic risk factors for colorectal cancer. This review will focus on "modifiable" (those that can be altered by manipulation) candidate modifier genes and those that have been chosen as a result of biological plausibility (which may or may not be modifiable), as shown in Table 1.

Biological plausibility and pathways of published "positive" results have been questioned [15], indicating that the functional significance of single nucleotide polymorphisms (SNPs) should be known before they are linked to disease [16]. A few published reports linking SNPs without known functional significance [17,18] or studies have failed to confirm a reported associations [19]. Known genetic variation has significantly impacted on the early detection and diagnosis of inherited cancer [20, 21], indicating that the search for genetic variation in cancer should continue.

Modifier Genes and polymorphisms studied in Lynch Syndrome			
Candidate Genes	Type of Polymorphisms	Effect	Publication indicating association or not
IGF1	CA-repeat	promoter function	[22, 23]
MTHFR	SNP	enzyme activity	[24]
HFE	SNP	protein function	[25]
NAT2	SNP	enzyme activity	[26, 27]
GSTM1	null allele	enzyme activity	[26, 27]
GSTT1	null allele	enzyme activity	[26, 27]
ATM	SNP	protein function	[28]
IL6	SNP	cytokine activity	[29]
IL4	SNP	cytokine activity	[29]
IL1β	SNP	receptor binding	[29]
IL10	SNP	cytokine activity	[29]
IL1Rn	SNP	null receptor	[29]
TNF-α	SNP	cytokine activity	[29]
IFN-γ	SNP	cytokine activity	[29]
TP53	SNP	protein function	[32, 39, 40]
MDM2	SNP	promoter function	[42]
Aurora-A	SNP	protein function	[44, 45, 48]
Cyclin D1	SNP	protein function	[44, 45, 48]

Table. 1. Candidate modifier genes and their respective types of polymorphism that have been studied in cohorts of Lynch syndrome patients.

2. Cell cycle control gene polymorphisms: TP53. MDM2, Aurora-A and CyclinD1

The TP53 gene isa tumour suppressor gene that regulates the transcription of genes necessary for the maintenance of genomic integrity by blocking cell proliferation after DNA damage and initiating apoptosis if it is too extensive [30, 31]. In 2004 the R72P polymorphism in TP53 was found to be associated with age of diagnosis of colorectal cancer (CRC) in an American Lynch syndrome study [32]. The R72P SNP in TP53 has been shown to result in two forms of the protein, which are not functionally equivalent [33, 34], and has been widely studied in a variety of malignancies [35 - 38]. Subsequent studies, including one Finnish and a collaborative Australian and Polish study, of the TP53 polymorphism and age of diagnosis of CRC in Lynch syndrome failed to confirm the reported association [39, 40]. The lack of an association was suggested to be related to a polymorphism in MDM2, which results in increased levels of MDM2 that culminates in the inability to properly stabilise TP53's response to cellular stress [40]. Evidence supporting this notion in HNPCC however, could not be found in other studies [39, 41]. The failure to corroborate the role of TP53 as a modifier gene between the different studies could be due to differences in the mutation spectrum of the various study populations; number of relatives included, population stratification and/or type 1 statistical error. Population stratification is unlikely to account for differences between the study populations as it has been shown that for most of the common disease associated polymorphisms, ethnicity is likely to be a poor predictor of an

individuals' genotype [43]. Type 1 statistical error seems to be the most likely explanation since the population sizes differ significantly in size with a range between 86 cases through to a maximum number of 220. In the larger studies reported to date (encompassing 193 and 220 patients, respectively), no association was observed thereby providing evidence against an association.

Aurora-A and Cyclin D1, genes both involved in cell cycle control, have also been associated with the age of onset of CRC in Lynch syndrome patients from North America [44, 45]. After the initial studies suggesting Aurora-A polymorphisms were linked to the average age of disease diagnosis follow-up reports in larger patient populations consistently failed to replicate this finding. In contrast, studies of Cyclin D1 polymorphisms and their association with the age of disease onset in Lynch syndrome resulted in contradictory results when studied in populations from North America, Germany, Finland and a combined study of Australian and Polish patients [44, 46 – 48]. A potential explanation for the association between Cyclin D1 and hMSH2 mutation carriers observed in the Australian and Polish Lynch syndrome patients was the relative paucity of MSH2 mutation carriers in the German and Finnish populations [47]. With the expansion of the study population from the Australian/Polish patient cohort the original report of an association with Cyclin D1 could not be replicated (See Fig. 1). In conclusion, the evidence now suggests that there is no association between Cyclin D1, MSH2 and disease risk in Lynch syndrome, such that overall Cyclin D1 does not appear to be associated with the age of disease diagnosis.

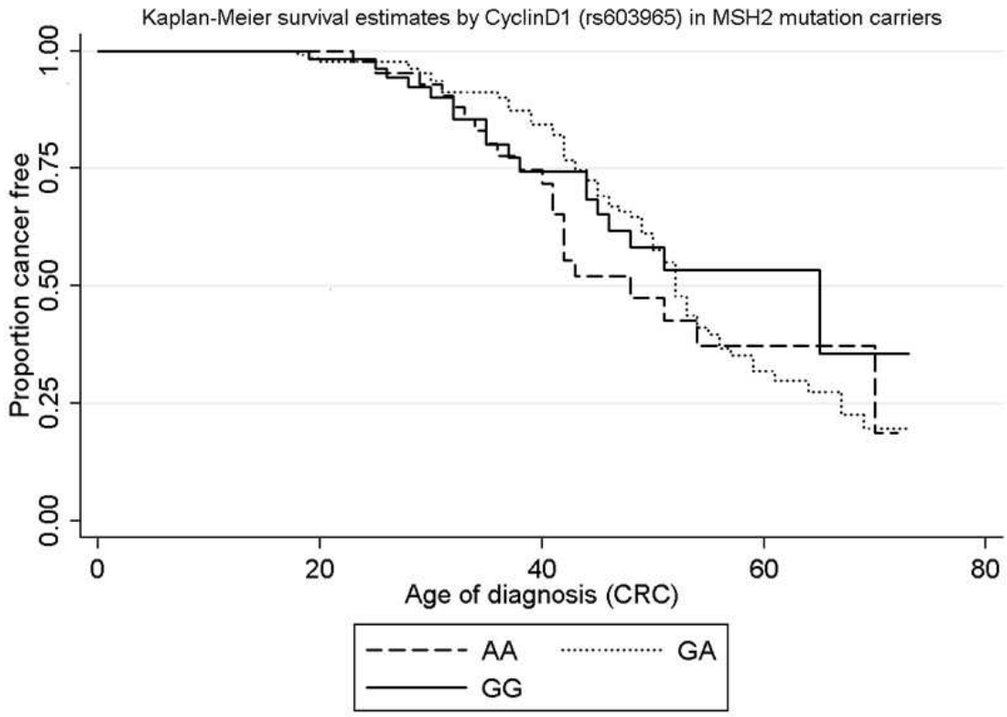

Fig. 1. Kaplan-Meier analysis of Cyclin D1 polymorphism and the age of disease onset in Australian and Polish Lynch syndrome patients. 276 MSH2 mutation positive patients were included in this study of which 107 were diagnosed with colorectal cancer. Log-rank, Wilcoxon and Tarone-Ware tests were not significant.

3. Xenobiotic clearance gene polymorphisms: NAT1, NAT2, GST, CYP1A1

Genes involved in xenobiotic metabolism, which include N-acetyl transferase 1 (NAT1), N-acetyl transferase 2 (NAT2), glutathione-S-transferase (GST) and cytochrome P450, have the ability to influence an individual's susceptibility to environmental and occupational carcinogens and predisposition to cancer [49]. The detoxification and elimination of foreign chemicals is controlled by complex mechanisms involving phase I enzymes that include cytochrome P450, and phase II enzymes such as GSTs and NATs [50]. Because of the significance of xenobiotics in the environment, perturbations in the ability to remove them are likely to alter disease risk. Polymorphisms in the genes mentioned above have been associated with colorectal cancer but the roles that the different SNPs have on cancer risk are controversial [26, 27, 51 – 61].

In 1999 an association between polymorphisms in NAT2 and the age of diagnosis of CRC in Lynch syndrome patients was reported, and the association was later replicated in a second independent report [26, 54]. Both studies had relatively small sample sizes (78 and 86 cases). Re-investigation of the association in a smaller study (69 cases) and a more appropriately sized one (220 cases) failed to confirm the association [58, 26]. The failure to confirm the association could be due to population stratification, but this is unlikely since if there is a functional difference in the gene in question,so its effects should be observed in all subjects, although not necessarily statistically significant in all populations. The most likely explanation for the failure to replicate initial findings is the small study population sizes that were used in assessing the potential association. This is further confirmed in a review by Brockton *et al.* 2000 [62] concluding that in 10 of 11 studies of invasive CRC and NAT2 acetylator genotype, no association was observed.

Similar results are reported for the polymorphisms in GST and cytochrome p450 genes and Lynch syndrome. Several research groups reported an association, while others failed to confirm them [25, 26, 51, 52, 53, 53, 63]. In on study the Msp1 wildtype allele of cytochrome P450 1A1 gene (CYP1A1) was associated with a decreased risk of CRC [26] which could have been due to it not being in Hardy-Weinberg equilibrium. The identification of an allele that is not in Hardy-Weinberg equilibrium suggests that either a genotyping error has occurred thereby skewing the results or it can be taken as supporting evidence for a correlation with disease [64]. The CYP1A1 gene has previously been associated with CRC and two SNPs in the CYP1A1 gene have been associated with CRC [65], which taken together with the report of Talseth et al. 2006 [26] supports the notion that variation in this gene is involved in the some aspect of CRC development.

Studies examining variation in xenobiotic clearance are likely to be subject to strong environmental influence and this is supported by findings from different countries. Studies examining patients of European descent for polymorphisms in GST genes seems to find no obvious relationship between the SNPs and cancer risk, while a study from Korea reports an association [25, 26, 63]. Taken together, these results suggest a complex relationship between the environment and individual genotypes that add to other more obvious problems associated with searching for modifier genes. Additional studies are required to determine the relationship between GST and CYP1A1 polymorphisms and disease risk in Lynch syndrome.

4. Immune response gene polymorphisms: IL6, IL4, IL1β, IL10, IL1Rn, TNF-α, IFN-γ

Cytokine mediated events may play a role in tumour development within inflammatory cells by producing an environment that supports tumour growth by promoting angiogenesis and facilitating genomic instability. The quintessential example is that of Crohn's disease where there is an increased risk of developing CRC if left untreated [66]. Inflammatory responses can also increase DNA damage, growth stimulation and enhanced survival of damaged cells [66, 67]. SNPs in cytokine genes can have an effect on the transcription levels of the respective genes and resulting in differences in both pro- and anti-inflammatory response activity. A series of polymorphisms in a number of cytokines has been investigated in relation to CRC risk and other cancer types but not for Lynch syndrome [68 – 77]. In addition, genetic variation in pro- and anti-inflammatory cytokine genes has been shown to influence individual response to carcinogen exposure [69], but no association has been identified in the one report focusing on a series of SNPs in cytokine genes and disease expression in Lynch syndrome [28]. Given the complexity of the inflammatory response and the limited number of SNPs utilised in that study, it cannot be ruled out that a relationship between SNPs influencing the immune response and Lynch syndrome exits.

5. Insulin like Growth Factor IGF-1 Gene polymorphisms

The *IGF-1* gene was first reported as a potential modifying gene in Lynch syndrome disease expression in 2006. The CA-repeat polymorphism located near the *IGF-1* promoter region was described as having an association with the age of disease onset in a cohort of 121 Lynch syndrome patients originating from the United States [22]. Certainly this is not the first time that a repeat region has been implicated in disease; with numerous studies reporting a link between DNA repeat regions significantly altering risk of prostate cancer [78 – 80] breast cancer, squamous cell carcinoma, bladder and lung cancers [81 – 84]. DNA microsatellite repeat regions are also strongly associated with Lynch Syndrome by virtue of their instability in tumours which is a consequence of the loss in the fidelity of DNA MMR [8].

IGF-1 is important for cellular proliferation and differentiation however, elevated levels of IGF-1 have been reported to have significant links to diseases such as CRC which is thought to be a result of the mitogenic and anti-apoptotic effects elicited by this protein [22, 85]. Several environmental and physiological reasons have been proposed that influence IGF-1 expression; however there is now evidence to suggest that a genetic role is significant. Rosen *et al.* was the first to report that the length of the CA repeat region in *IGF-1* may be associated with circulating IGF-1 levels [86]. In a similar growth factor related gene, Epidermal Growth Factor Receptor (EGFR), a CA repeat region is located in intron 1. A study of this *EGFR* polymorphic repeat region revealed lower transcriptional activity with increasing numbers of polymorphic CA repeats coinciding with lower levels of gene expression [87]. In 2007, a similar result was reported for the *IGF-1* gene in swine where the length of the CA repeat region was clearly associated with circulating levels of IGF-1 [88]. More recently, additional human data has been published which supports the notion that this polymorphism is linked to serum levels of IGF-1 [89]. From this data a trend is emerging that CA repeat polymorphisms in growth factor related genes, such as *IGF-1,* are related to overall gene expression, which is reflected in the circulating serum levels of the respective proteins. Accumulating evidence suggests that serum IGF-1 levels appear to be

linked to disease with recent reports indicating that elevated levels of IGF-1 are observed in breast, prostate and CRC [90 – 93]. There have been estimates that higher circulating levels of IGF-1 result in a 15% increase in the risk of developing disease, insinuating the importance of circulating IGF-1 in disease progression [94].

As CRC involves the accrual of a number of specific molecular alterations [95, 96], consistently high IGF-1 serum levels may increase cellular proliferation, thereby enhancing the rate by which genetic alterations accumulate. Both normal colonic epithelial and transformed cells are IGF-1 responsive; thus, IGF-1 can influence not only the likelihood of disease initiation but also disease progression. This overall process provides some insight into how intracellular serum levels of IGF-1 may have a significant influence in accelerating the accumulation of genetic errors leading to disease, especially in persons who have inherited a predisposition to develop malignancy characterized by a mutator phenotype as observed in Lynch syndrome.

An equally important facet to disease risk as a result of increased levels of IGF-1 is its link with obesity. Obesity and physical inactivity are strong independent determinants of insulin resistance and hyperinsulinaemia [97 – 104] and this is associated with an increased risk of CRC [101, 102]. Increased blood insulin lowers IGF-1 binding protein levels, which often results in an increase of free IGF-1 [105]. As IGF-1 is associated with both percentage body fat and general overall obesity [106], an increased level of IGF-1 expression as a result of shorter CA repeat lengths may have an enhanced effect in persons who are obese where IGF-1 serum levels are already elevated.

In addition to the IGF-1 effect, CRC risk is also increased in obese patients through oxidative stress in adipose tissue. This is caused by increased lipid peroxidation leading to the production of reactive oxygen species. In regards to cancer, reactive oxygen species can damage DNA by several methods including DNA base modification, deletions, frame shifts, strand breaks, DNA-protein cross-links, and chromosomal rearrangements [107]. Both lipid peroxidation and increased DNA damage are likely to promote tumour development by generating reactive oxygen species, increasing hormone production/bioavailability of IGF-1 and providing an energy-rich environment. This combined mechanism is potentially a risk factor for all types of CRC, however in Lynch syndrome this may be of greater significance in a deficient DNA repair environment where enhanced levels of IGF-1 inhibit cell death and encourage cellular proliferation. Together, the relationship between obesity and *IGF-1* CA repeat length may be of particular importance in obese Lynch syndrome cases as these may be at greatest risk of developing disease at a younger age.

The role of inherited factors in circulating IGF1 serum levels is likely to be substantial with estimates of the proportion of variance in IGF-1 that is genetically determined varying somewhere between 38% to over 80% [108]. A substantial amount of data has been reported revealing differences in IGF levels across ethnic groups [109 – 111], however this is suggestive of dietary and lifestyle factors having a more modifiable effect on serum levels when combined with genetic ancestry. One such study has shown that the impact of several nutritional factors such as calcium, dairy products and vegetables on IGF1 levels is quite different in racially stratified models as reported between African-American and European American males [112]. This is strongly suggestive of there being population differences that differentially modify the effect of several nutrients on IGF levels. Together this information is suggestive that environmental factors such as calorific intake, lifestyle and demographic factors are probably playing a substantial role in ethnic variation in disease risk in regards to

serum IGF levels. This is intriguing as it may also be contributing to the differences in relative disease risk observed between the Polish and Australian cohorts as reported [22].
The data reported to date [21] indicate a significant interaction between the CA repeat polymorphism length and disease expression in Lynch syndrome which is likely to be linked to circulating levels of IGF-1. The data suggest a significant correlation for earlier onset CRC in participants who carry 17 or less IGF-1 CA repeats in over 400 Lynch syndrome patients. An encouraging aspect of the results of this study is that significance is retained across two different populations where variance in IGF-1 allele size frequencies occur [22]. A limitation however in defining the exact relationship between IGF-1 expression and cancer incidence in Lynch syndrome patients is the genotype–phenotype correlation between the *IGF-1* CA-repeat number and the corresponding serum levels. Assessment of serum IGF-1 concentration, however, has the inherent problem of serum IGF-I measurement, which is typically assessed at only one time point yet for accurate analysis should be performed multiple times from any single patient. Whether it would be feasible to monitor IGF-1 serum levels in families with Lynch syndrome is an area which needs further investigation. Future work should also include additional candidate polymorphisms located within *IGF-1* or *IGFBP-3* that interact with the IGF-1 pathway and may provide further insight into the overall IGF-1 effect. At present, however the IGF-1 pathway remains largely under-investigated, and there is now a requirement for further work to develop a more thorough understanding of the relationship between *IGF-1* genotype, expression and its implication in disease risk.

6. Methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms

There have been tantalizing reports in the literature that polymorphisms in the *MTHFR* gene are associated with altered CRC risk. These polymorphisms occur in relatively high frequency in the general population and the two that promote special attention are both associated with altered enzymatic function. MTHFR is a key folate-metabolizing enzyme involved in both DNA methylation and DNA synthesis. The enzyme catalyses the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF), needed for purine and thymidine synthesis, to 5-methyltetrahydrofolate (5-MTHF), which is necessary for methionine production. Insufficient thymidine results in uracil misincorporation into DNA, leading to single-strand and double-strand breaks. This can increase the incidence of DNA damage, thereby increasing the risk of genetic instability. The understanding that folate metabolism can both equally influence DNA synthesis and methylation has made the study of environmental and genetic variants associated with MTHFR particularly attractive as a candidate genetic factor that influences cancer susceptibility. Two common polymorphisms, *C677T* and *A1298C* are located within the MTHFR gene and have been linked to altering the function of the encoded protein. This has lead to these variants being the focus of numerous studies into CRC risk outside the context of an inherited predisposition to disease. Both polymorphisms result in a substitution of an amino acid and have previously been shown to significantly influence MTHFR enzyme activity [113]. *C677T* is located within the coding region for the catalytic domain, resulting in an amino acid substitution from alanine to valine that is associated with a reduction of enzyme activity. The *A1298C* polymorphism, located in the regulatory region of MTHFR, substitutes an amino acid change from glutamine to alanine. Evidence suggests that *A1298C* also reduces MTHFR activity, however it is reported to be less influential than *C677T* [114]. This modifying effect incurred by the

presence of one or both polymorphisms in a pivotal folate metabolism pathway and its association with sporadic disease suggests that these polymorphisms are of particular interest with respect to modifying disease risk in Lynch syndrome.

Both *A1298C* and *C677T* are in strong linkage disequilibrium with no evidence of the existence of a *MTHFR* allele that carries both the homozygote (*C1298C/T677T*) variants of these polymorphisms [115 – 117]. Owing to this linkage disequilibrium, no studies have been reported where patients have inherited both homozygote variants. Nevertheless, heterozygote carriers of 1298C and 677T have been reported. The effect of inheriting both alleles in trans (i.e. one allele with the 677T polymorphism and the other with the 1298C polymorphism) effectively reduces overall MTHFR activity, thereby significantly altering the kinetics of folate metabolism. Data reported from an Australian and Polish study on the effects of MTHFR variants and disease expression in Lynch syndrome revealed that heterozygote forms of the *MTHFR* variants were required for a significant protective effect to occur [23]. The Kaplan-Meier survival estimates reported in this study predicted a median age gap of 10 years later for CRC onset in patients carrying the combined heterozygote *MTHFR* genotype which was supported by multi variable regression modelling statistics. The data also suggested this effect was significant in both *hMLH1* and *hMSH2* carriers, where previously only a significant association had been described in *hMLH1* for *C677T* only [118]. The most likely cause for this discrepancy between the Australian/Polish study and those by reported by Pande *et al* (2007) [119] is likely to be due to a type 1 statistical error as the reported association in *hMLH1* carriers were in a considerable smaller sample size, although differences in the ethnicity of Lynch syndrome cohorts cannot be ruled out as a contributing factor.

The mechanism by which *C677T* and *A1298C* appears to influence disease risk can be explained by the functional effects that these polymorphisms have on MTHFR and consequently folate metabolism. Previous reports have demonstrated a reduction of up to 60% in the activity of MTHFR when both *C677T* and *A1298C* heterozygote alleles were present in the gene. The reduction of *MTHFR* activity leads to an increased concentration of its substrate 5,10-MTHF. The increased pool of 5,10-MTHF pushes folate metabolism towards DNA synthesis, in turn reducing the pool of uracil. A reduced quantity of uracil potentially reduces the overall risk of uracil misincorporation as a result of its limited availability. For individuals with a MMR deficiency, the effect of reduced MTHFR enzyme activity may be advantageous since uracil misincorporation could be particularly deleterious in conjunction with an impaired DNA repair pathway. The subsequent lower levels of 5-MTHF may also be beneficial due to a potential reduction in DNA methylation. Hypermethylation of the promoter of tumour suppressor or MMR genes may lead to gene silencing, therefore a reduction in methylation through decreased MTHFR activity could lead to lower probability of this type of gene silencing occurring.

Numerous case control and cohort studies have investigated the relationship between folate intake and CRC risk with the majority reporting a reduction in CRC incidence with higher levels of folate [116]. The outcome of one meta-analysis suggested that CRC risk could be reduced by up to 25% with a high level of dietary folate compared to a low level one [117]. Further studies are required to clarify to what extent total folate has on disease risk; however it is generally accepted that there is an association and that a number of common genetic variants alter either the cellular levels or functioning of folate metabolism enzymes and are likely to have an important role in determining an individual's response to changes in dietary folate. With this in mind further studies into functional polymorphisms in the

folate metabolism pathway would benefit significantly by including total folate levels so that a more exact assessment its role could be made. Using this approach a more precise view of the relationship between folate intake and disease risk may become apparent where Lynch syndrome patients could be stratified by *MTHFR* genotype. Accurately estimating dietary folate intake however may prove difficult and therefore the analysis of plasma folate levels may be a more viable alternative. Future studies would benefit by including other dietary factors including alcohol, choline, and methionine intake which are known to effect folate metabolism besides folate and folic acid [119]. An accurate level of plasma folate combined with *MTHFR C677T* and *A1298C* genotypes is an interesting prospect and may provide an indicator of individual risk of developing a Lynch syndrome related CRC.

The identification of *MTHFR* polymorphisms being associated with divergence in disease risk in Lynch syndrome provides the basis for targeted intervention measures that could be used to reduce the risk of disease development. Dietary supplementation of folate/folic acid in Lynch syndrome families may prove to be beneficial in decreasing disease risk or prolonging the time before the diagnosis of malignancy. Dietary supplementation and a change in disease risk however, are more complex than previously thought. Folic acid supplementation has been proven to be beneficial in decreasing neural tube defects (NTD's), [120] and was the catalyst for the United States and Canada introducing the compulsory supplementation of folic acid in flour in 1996 with the aim to reduce the incidence of NTD's. Despite proving successful for this purpose an unexpected trend was observed in both countries as described by Mason et al. (2007) [121] who investigated the relationship between the onset of folic acid fortification and rises in the incidence of CRC. This analysis indicated that in the early part of the 1990's the age-adjusted incidence of CRC had declined gradually in both countries. Between 1995 and 1996 however, the incidence rate in the United States showed a slight increase followed by more marked increases in 1997 and 1998. A similar finding was observed in the Canadian population, which also corresponded to the mandatory supplementation of folic acid. In both populations the increase in CRC incidence was highly significant when compared to pre-existing trends in both men and women. These observations have lead to a hypothesis that mandatory folic acid supplementation was responsible for the spike in CRC rates which after peaking approximately 2-3 years after its introduction have begun to decline once again [121].

The association of increased CRC incidence with folate supplementation has been supported by the results of two large-scale studies which have recently emerged from both the United States and United Kingdom. In both these phase III studies a common trend was observed in participants who supplemented their diets for three years with a daily dose of 1000ug and 500ug folic acid respectively, and an increased risk of developing a colorectal adenoma, with the greater risk in those participants consuming the higher 1000ug dose [122, 123]. Studies in mismatch repair or tumour suppressor gene deficient mice have demonstrated that the timing of folate supplementation is important in the association it may have on disease risk. In the first few months of folate supplementation a threefold decrease in colorectal adenomas has been observed when compared to mice with a moderate folate deficient diet. Dietary folate treatment after the development of carcinomas had the opposite effect however, with folate deficiency significantly decreasing the number of adenomas compared with supplementation [124, 125]. Together, this evidence suggests that as long as an individual is healthy, folate supplementation is protective whereas if a tumour has been initiated folate restriction is more important. This dual modulatory role of folate may be of even greater influence in an impaired DNA mismatch repair pathway as found in Lynch

syndrome patients. In this case folate supplementation may be particularly beneficial or deleterious depending upon any early tumour development.

7. Haemochromatosis HFE gene polymorphisms

The role of high body iron levels in modifying the risk of colorectal cancer has been investigated by several groups but remains unclear [126 – 130]. The genetic iron overload disorder hereditary haemochromatosis (HH) is characterised by high iron indices and progressive parenchymal iron overload and occurs due to a problem in restricting iron uptake (reviewed in [131- 133]. While clear associations have been established between haemochromatosis and liver disease, studies investigating the correlation between haemochromatosis and other pathologies have yielded conflicting results [134 – 137].

The primary cause of classical HH has been ascribed to SNPs in the *HFE* gene, in particular the 845G>A SNP which results in the substitution of a tyrosine residue for a cysteine at position 282 (C282Y) and is present in 10-15% of individuals of northern European descent. The more common but less penetrant 163C>G SNP (H63D) is present in 15-30% of individuals [131, 136 – 142]. A longitudinal also study has demonstrated that up to 30% of men and 1% of women homozygous for the C282Y polymorphism develop iron overload that subsequently manifests as a disease phenotype [143]. The risk of developing colorectal cancer increased 3-fold in C282Y homozygotes when compared to matched controls without the mutation [144].

A number of other epidemiological studies have also investigated the impact of *HFE* genotype on colorectal cancer risk, with mixed results [145 -148]. Most studies exploring the link between *HFE* genotype and the risk of developing colorectal cancer have approached the problem by selecting subjects diagnosed with colorectal cancer and comparing the frequency of *HFE* polymorphisms to matched controls.

In regards to Lynch syndrome and the potential influence of disease risk one study has been reported suggesting that homozygosity of the *HFE* H63D mutation may act as a modifier, increasing the risk of developing CRC. In addition, there was evidence for earlier CRC onset age in H63D homozygotes [24]. The results of this study suggest that the median age of disease onset could be as much as 6 years earlier in H63D homozygotes (who represent around 2.5% of the Australian and Polish general populations).

While these findings will require substantiation in other populations, they support a possible relationship between iron dysregulation and colorectal cancer risk. While mechanisms cannot be established by a genetic epidemiological study of this nature, it appears likely that iron is involved, in view of the roles of the HFE gene in iron metabolism, the previously reported effects of H63D homozygosity on iron status [149] and existing evidence that iron status can modify CRC. Since iron levels in haemochromatosis patients can usually be maintained at normal levels through phlebotomy and regulating factors such as diet, this might have the potential to substantially reduce colorectal cancer risks or delay onset by several years in people with HNPCC-associated MMR gene mutations.

However the possibility of other mechanisms not directly reflecting abnormal body iron status cannot be ruled out. Homozygosity of the H63D polymorphism increases the risk of the neurodegenerative brain disease amyotrophic lateral sclerosis in the absence of apparent effects of C282Y polymorphism [150 – 152], suggesting that in some tissues the H63D mutation might have pathological consequences that are not directly related to whole body iron status. It will be important to validate the findings on H63D and also to investigate the

effects of C282Y homozygosity in larger HNPCC samples, preferably in conjunction with information on patient iron status, to determine the mechanisms involved and the role of iron.

While this is the first time that the H63D polymorphism has been specifically associated with HNPCC, there is some previous evidence for association of both the H63D and the C282Y polymorphism with colorectal cancer in general [147, 148]. Power has limited past studies, as the homozygous and compound heterozygous mutations that have been associated with the greatest increases in iron loading and potentially the highest disease risks, are relatively rare. For this reason, some studies have analysed all *HFE* mutation genotypes as a single group, which may dilute observed effects. Although past epidemiological studies of *HFE* genotype and colorectal cancer risk have had mixed results, an American study of 475 colorectal cancer case patients and 833 control subjects found an odds ratio of 1.4 for participants with any *HFE* mutation after adjustment for a range of factors including age, gender and total iron intake [148]. The increased risk predominantly occurred in the quartile with greatest dietary iron intakes. In addition, a recent study of a large Australian sample has found that homozygosity for the C282Y SNP is associated with a three-fold increase in the risk of developing colorectal cancer in men [144]. This suggests that the effects of *HFE* on colorectal cancer may not be limited only to MMR gene mutation carriers, although such effects may be stronger when both types of mutation are present simultaneously.

Heterozygosity for either the H63D or C282Y SNP does not appear to have any modifying effect in either the Australian or Polish samples, although it is possible that small effects may be detectable with very large samples. While heterozygosity for C282Y or H63D has been reported to have a range of effects in other diseases, reviewed in [153], these genotypes are not usually associated with significant changes in iron parameters [26, 154 – 156]. For these reasons, for our final analyses it was considered more appropriate to compare mutant homozygotes to combined heterozygotes and wildtype homozygotes, as is usually done in most studies of *HFE* gene SNPs. However, while this was effective in revealing the potential modifying effect of H63D homozygosity on HNPCC development, we were not able to do this for the C282Y SNP, due to its relative rarity and the lack of C282Y homozygotes in the samples. Stronger modifying effects may occur in C282Y homozygotes or C282Y/H63D compound heterozygotes, as it is well established that iron indices are increased most in individuals with these genotypes, reviewed in [131, 132, 157].

Gender affects both the onset age and site of first tumour manifestation in HNPCC. In females, the age of onset of colorectal cancer is delayed 5 to 10 years when compared to males [158]. Gender is also a factor in the manifestation of iron loading as a result of *HFE* genotype, affecting males much earlier in life than females [159]. In a larger sample it is possible that *HFE* genotype may show a contribution to the earlier onset of CRC in males when compared to females.

8. Candidate polymorphisms not associated with disease risk

Not all polymorphisms which have been associated with hereditary disease have remained consistently significant across cohorts. An example is the delta *DNMT3b* SNP which was reported to have a significant association in a cohort of participants in the United States [160]. *DNMT3B* has been identified as a candidate in disease modifying risk due to its role in methylation. DNA methylation is regulated by a family of DNA

methyltransferases (DNMTs), of which three active forms (DNMT1, DNMT3A and DNMT3B) have been identified in mammalian cells [161]. It has been reported that an increase in DNA methyltransferase enzyme activity of the DNA methyltransferases DNMT1, and DNMT3A and DNMT3B, is elevated in several types of disease including leukemia, prostate, lung, breast and endometrial cancers [162 -165]. A polymorphism located within *DNMT3b* has been reported to influence enzyme expression through altering promoter activity. It has been suggested that in *in vitro* assays the C>T variant could lead to an increase of promoter activity of up to 30% [161]. Using a study group of over 400 individuals, no association was observed between age of onset and *DNMT3b* genotype in an Australian and Polish Lynch syndrome cohort. The failure to confirm the potential modifying influence of a polymorphism in one population compared to another could be simply due to insufficient numbers of test subjects. If a polymorphism is an effect modifier its response should be similar no matter what population is examined even though it may not reach statistical significance. In the case of the delta *DNMT3b* SNP no such trend was observed. The Australian/Polish study group was approximately three times larger than the participants of a previous study [160] and the most likely explanation for the difference in results is a type 1 error. Notwithstanding, it is worth noting that it does not rule out the possibility that *DNMT3b* expression may be associated with Lynch syndrome disease expression. Different isoforms of DNMT3b exist therefore expression levels of these may vary influencing disease risk. Numerous other polymorphisms have also been reported in the functional domains of DNMT3b which could also alter methylation status and thereby alter disease risk.

Genes involved in DNA repair have also been prime candidates in the search for modifying effects due to their important role in the cell cycle. Polymorphisms located within genes involved in this process have been widely reported to be associated with cancer susceptibility in an extensive range of malignancies that include CRC. For one combined cohort (Australian and Polish Lynch syndrome patients), eight common polymorphisms were selected across several genes involved in the DNA repair pathway including *BRCA2, hMSH3*, *Lig4*, *hOGG1* and *XRCC* 1, 2 and 3, which had not previously been assessed for disease risk in Lynch syndrome. When considered separately conflicting data were identified in the two populations. Cox regression modelling indicated a significant protective effect in Polish participants for both polymorphisms *hMSH3* A>G (rs26279) and *XRCC2* G>A (rs1799793). This finding was somewhat contradictory as the homozygote form of both rs26279 and rs1799793 have been previously weakly associated with an increased risk of CRC and bladder cancer respectively [166, 167]. Two points need to be taken into account in interpreting this data. First, since multiple tests were undertaken in evaluating the possible influence of DNA repair gene polymorphisms a correction for multiple testing must be undertaken to ensure that any observed result is not due to a chance association; second, population stratification may adversely affect result outcome but is less likely (Reeves et al. 2011 [168]. Differences in the probabilities of an association with the age of disease onset in relation to DNA repair gene polymorphisms occurring in small study groups is more likely to be a result of a type 1 or 2 statistical error and can be overcome by undertaking an appropriate power calculation to determine the expected power to detect an association. Furthermore, statistical correction (such as Bonferroni) is required especially where multiple testing is undertaken although some types of correction are somewhat conservative and could remove an association where one exists.

9. Summary

There have been a number of studies that demonstrate the role of modifying genes that influence disease risk in Lynch Syndrome. Many studies have been undertaken that have failed to identify a range of candidate modifying genes as a result of studies being too small in size to provide robust statistical results. Nevertheless, there is a growing body of evidence that suggests modifying genes do influence disease risk in Lynch Syndrome and some of these are of particular interest as they suggest potential avenues by which disease risk can be modulated.

The role of genome wide association studies in identifying new agnostic modifier genes is currently generating special interest and at this point two studies have reported intriguing associations that correlate well with disease risk. It remains to be seen if such associations can be verified in larger populations. The use of genome wide data or even target assessment of several thousand potential modifiers is fraught with difficulties not the least of which is the available population size and the number of individual SNPs analysed.

Despite the difficulties encountered in identifying polymorphic modifier genes, their role in improving disease risk assessment is becoming clearer and the search for those that can make for individualised patient care will continue.

10. References

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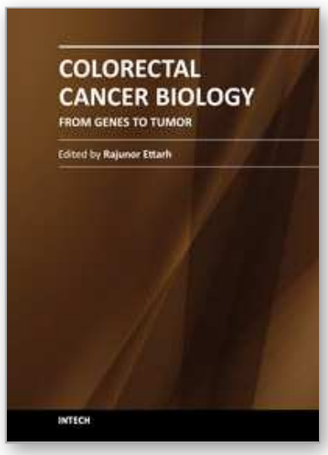

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Colorectal cancer is a common disease, affecting millions worldwide and represents a global health problem. Effective therapeutic solutions and control measures for the disease will come from the collective research efforts of clinicians and scientists worldwide. This book presents the current status of the strides being made to understand the fundamental scientific basis of colorectal cancer. It provides contributions from scientists, clinicians and investigators from 20 different countries. The four sections of this volume examine the evidence and data in relation to genes and various polymorphisms, tumor microenvironment and infections associated with colorectal cancer. An increasingly better appreciation of the complex inter-connected basic biology of colorectal cancer will translate into effective measures for management and treatment of the disease. Research scientists and investigators as well as clinicians searching for a good understanding of the disease will find this book useful.

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