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Frequency of the Common *MYH* Mutations (G382D and Y165C) in MMR Mutation Positive and Negative HNPCC Patients

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Abstract

Recently mutations in the *MYH* gene have been associated with a milder form of adenomatous polyposis which is characterized by a variable level of colonic polyps ranging from a few to several hundred. In the context of HNPCC it is not unusual to identify patients with a smattering of polyps. The *MYH* gene product is involved in DNA repair and indeed the hMSH2/hMSH6 complex (both genes being essential elements of the DNA mismatch repair pathway) is required to stimulate *MYH* activity. We reasoned that because of the clinical similarity of a subset of HNPCC patients to those described with *MYH* mutations and the role of the hMSH2/hMSH6 complex in the activation of MYH protein that *MYH* mutations may account for a small proportion of HNPCC patients. In a study of 442 HNPCC patients we identified *MYH* mutations at the same frequency as that expected in the general population. Nevertheless, two HNPCC families were identified harbouring biallelic changes in *MYH*.

Introduction

Hereditary Non Polyposis Colorectal Cancer (HNPCC) is an autosomal dominant inherited disorder associated with a familial predisposition to colorectal cancer (CRC). It is characterised by early age of disease onset, neoplastic lesions with microsatellite instability (MSI) and an increased incidence of extracolonic malignancies such as cancers of the endometrium, ovary, stomach, small bowel, ureter and renal pelvis [1]. Approximately 20% of all CRC cases display familial inheritance [2] however familial colorectal cancer syndromes, that include HNPCC and FAP account for around 3% of all colorectal cancer patients [3]. A significant number of cases that appear to be inherited therefore do not have mutations in known genes involved in familial cancer syndromes. HNPCC has been found to be associated with *hMLH1* and *hMSH2* genes involved in the mismatch repair (MMR) pathway. Germline mutations in *hMLH1* and *hMSH2*, the main causes of mismatch repair deficiency, have been linked with a significantly increased risk for developing HNPCC [4, 5]. Among all HNPCC patients only 80% of men and 40% of women that have germline mutations in MMR genes develop CRC [6, 7] and a further 25-50% of women develop endometrial cancer [8]. MMR gene mutations do not account for all CRC cases in HNPCC since some patients fit the clinical criteria for HNPCC yet they do not harbour a MMR gene mutation. Therefore,

it is highly likely that there are other genes involved in the aetiology of HNPCC.

MYH has recently been identified as an autosomal recessive inherited form of colonic polyposis. It functions to repair DNA damage caused by reactive oxygen species through the DNA repair process known as base excision repair (BER). *MYH* removes adenines misincorporated into DNA opposite guanine or 7,8-dihydro-8-oxo--guanine to prevent G:C to T:A mutations (for review see [9]). Al-Tassan et al [10] were the first to describe the role of *MYH* in colorectal polyposis and since then a number of studies have confirmed this association [11-17]. The results obtained in these studies suggest that colorectal cancer and in particular HNPCC might not only be confined to mutations in MMR genes but also to other genes involved in DNA repair such as *MYH*.

Two known polymorphisms in *MYH*, Y165C and G382D, are functionally compromised in the variant form. Al-Tassan et al [10] demonstrated that the variant forms of these proteins reduced the glycosylase ability of the proteins to remove DNA damage caused by reactive oxygen species (8-oxoG:A). A study by Jones et al [15] showed that biallelic nonsense and missense mutations in *MYH* were found in colorectal cancer patients that were unrelated and in addition they identified that sequence variants are found in different ethnic groups that include the Caucasian (Y165C, G382D), Indian (E466X), Pakistani (Y90X) and the Italian (1395delGGA). It is not currently known whether *MYH* mutations in the heterozygous form cause CRC or if they modify the effects of genes already associated with CRC such as *APC*, *hMLH1* or *hMSH2*. The study performed by Kairupan et al [11] demonstrated that *MYH* mutations Y165C, G382D and 1391delAGG were not present in the *APC* mutation positive FAP population which suggests that MYH is not a modifier gene in FAP.

It has recently been shown that the *hMSH2/hMSH6* heterodimer formed as part of the MMR process functions to stimulate the activation of *MYH* thereby linking two DNA repair processes and implying a connection of *MYH* to HNPCC [18]. Gu et al [18] suggest that hMSH proteins and *MYH* have a combined effect to remove any DNA damage that occurs. They have shown a direct interaction between the *hMSH2/hMSH6* heterodimer between *hMSH6* and *MYH* at residue 232-254 of *MYH*. Mutant *MYH* has also been shown to have a decreased ability to recognize and repair mismatches that occur due to reactive oxygen species (ROS). Additionally, Gu et al [18] showed that MSH2 defective cells have an impaired function in oxidative damage. This shows that MSH2 and *MYH* both have roles in the removal of oxidative damage. They suggest that this interaction must be studied further to determine whether the mutant forms of *MYH* disrupt the MMR and BER pathways in colorectal tumourigenesis. Single nucleotid polymorphisms (SNPs) found in *MYH* could potentially interfere with these protein-protein interactions thereby hindering the ability of MMR and BER pathways to remove DNA damage and consequently promoting colorectal cancer [12].

Kambara et al [13] suggest that there are a number of other tumour suppressors on chromosome 1p since microsatellite stable (MSS) colorectal tumours often show loss of heterozygosity (LOH) on chromosome 1, however LOH of chromosome 1 is not usually evident within patients that have high microsatellite instability (MSI-H) tumours. MSI-H tumours are usually associated with HNPCC. This suggests that dysfunction of tumour suppressors on chromosome 1 might display a different phenotype to that of HNPCC (microsatellite stabile [MSS] but a small number of adenomas).

In the current study, we have investigated a series of patients that have been extensively screened for *hMLH1* and *hMSH2* germline mutations by denaturing high performance liquid chromatography (dHPLC) analysis, denaturing gradient gel electrophoresis (DGGE), direct sequencing and multiplex ligation probe amplification (MLPA) analysis. Using this screening strategy, two groups of patients were identified: patients with a confirmed germline mutation in *hMLH1* or *hMSH2*, and patients that were mutation negative. These two groups were assessed for the presence of the two common *MYH* mutations (Y165C and G382D) to determine whether patients that manifest the clinical characteristics of HNPCC harbour these mutations.

Methods

Patients

Patients were selected from across the State of New South Wales because they fulfilled the clinical criteria of Hereditary Non Polyposis Colorectal Cancer (HNPCC). All patients enrolled in this study had given informed consent for their anonymous DNA to be used for research into genetic predispositions to colorectal cancer. This study was carried out with the approval of the Hunter Area and the University of Newcastle's Research Ethics Committees.

Table 1. Genetic alterations identified in the *MYH* gene and disease characteristics of the patients

Patient Number	HNPCC MMR Mutation	First Alteration	Second Alteration	Sex	Age of Diagnosis of Adenomas	Number of Adenomas	CRC (Age of Diagnosis)	Other Cancers
1	NO	Y165C	NO	F	43	1	N-AF	
2	NO	G382D	V22M	F	>30	?	AF (36)	
3	*hMSH2* (P)*	G382D	NO	M	46	3	AF (46)	Melanoma (42)
4	NO	G382D	NO	M	>70	?	N-AF	Gastric (78)
5	*hMSH2*	G382D	NO	M	>30	?	N-AF	
6	NO	G382D	V22M	F	>40	?	AF (47)	
7	NO	G382D	NO	F	45	1	N-AF	

* hMSH2 polymorphism with an unknown functional consequence

The patients involved in this study fulfilled either the Amsterdam or Bethesda HNPCC criteria. This study included patients from families that fit the clinical criteria for HNPCC even though they might not have developed disease nor had a mutation in *hMLH1* and *hMSH2*. Three groups of patients were used in this study. Group 1 consisted of 233 patients of which 162 patients were affected with CRC who were shown not to harbour germline mutations in the *hMLH1* and *hMSH2* genes by denaturing high performance liquid chromatography (dHPLC) analysis followed by direct sequencing, the multiplex ligation probe amplification (MLPA) assay and denaturing gradient gel electrophoresis (DGGE). This group of patients was collected from 1997 to 2004. Group 2 consisted of 209 patients that had a confirmed mutation in either *hMLH1* or *hMSH2*. This group of patients was collected from 1997 to 2004. Group 3 (control group) consisted of a series of 296 anonymous control DNA samples collected between the years of 1993 and 1997 with a mean age of 51 years (range 30-94).

DNA isolation

Genomic DNA was isolated from Na_2EDTA blood according to the method previously described by Miller et al [19].

Real Time PCR (RT-PCR) SNP genotyping

DNA samples were SNP genotyped to determine the allele frequency of the two common *MYH* mutations, Y165C and G382D. Allelic discrimination was performed on an ABI PRISM® 7900HT sequencing detection system (PE Applied Biosystems, Foster City, USA). Assay-by-Design℠, a service that is offered by Applied Biosystems (PE Applied Biosystems, Foster City, USA), was used to design primers and probes. The assay functions under universal reactions and conditions with each reaction containing: 50ng DNA, 0.125μl 40x Assay Mix, 2.5 μl Taqman® Universal PCR master mix made up to 5 μl with sterile MilliQ water. The thermal cycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. Post PCR, the plate was scanned to allow discrimination between the different genotypes.

Primer and probe sequences

The primers and probes used for genotyping both *MYH* mutations are listed below:

Y165C

Forward	5'–CCACAGGAGGTGAATCAACTCT
Reverse	5'–CCTTACCTTCCGAGCTCCCT
Wild type probe	VIC–CTGGGCTACTATTCT
Mutant probe	FAM–TGGGCTGCTATTCT

G382D

Forward	5' – GACCCCTGCCTGGCT
Reverse	5' – GACGGGAACTCCCACAGT
Wild type probe	VIC – CCTCTCAGGTCTGCTG
Mutant probe	FAM – CCTCTCAGATCTGCTG

DNA sequencing of the *MYH* gene

All patients who were determined to be heterozygous for either Y165C or G382D polymorphisms were further investigated for additional mutations in the *MYH* gene. Primers designed by Kairupan et al [11] were used for this analysis.

DNA analysis was performed using a semi-automated sequencing unit (model 310, Perkin-Elmer Applied Biosystems Division, Foster City, USA) according to the manufacturer's instructions. All germline mutations identified were confirmed by reverse sequencing. The sequence variant position in the *MYH* gene was compared to that on the NCBI database, accession number BC003178.1.

Statistics

Differences in allele frequency between groups were determined by chi-squared analysis. Statistical differences were assessed using the STATA statistical package (STATA Corporation, College Station TX); p values less than 0.05 were considered significant.

Results

In total, 442 HNPCC patients were assessed for the presence of two *MYH* polymorphisms, Y165C and G382D. The frequency of the mutations in the entire HNPCC group compared to the controls was not significant (p=0.798).

In the control group of 296 patients, the G382D mutation was found at a frequency of 1.35% and the Y165C mutation was not found in any of the controls. The frequency observed in the control population is consistent with the observed frequency in other populations studied.

In group 1 (mutation negative), of the 233 patients, 1 was heterozygous for the Y165C mutation and 4 were heterozygous for the G382D mutation. The frequency of the mutations in this group was not significantly different as compared to group 2 (p=0.317) or the control population (p=0.483).

In group 2 (mutation positive), of the 209 patients, 2 were heterozygous for the G382D mutation. One patient had a confirmed causative mutation in *hMSH2* and the other had 2 polymorphisms, one of which was non-causative as judged by information on the Insight mutation database (www.insight-group.org) and the other was an unknown functional variant (1661+12A>G and 1551-9T>A, respectively). The polymorphism in intron 9 is close to the intron-exon boundary of intron 9 and exon 10 and therefore could possibly have a detrimental functional consequence. The frequency of the mutations in this group was not significantly different as compared to the controls (p=0.687).

The DNA of all patients that were heterozygous for Y165C and G382D was further analysed to determine whether any other mutations were present. Two of the seven patients were heterozygous for the G382D polymorphism, harboured the causative mutation V22M. No other changes were found.

Discussion

Identifying genes involved in colorectal cancer remains a challenge since known mutations in inherited disorders only account for approximately 2-5% of all CRC cases [5]. A large proportion of CRC cases that appear to have Mendelian dominant inheritance, however, have an unknown genetic cause of disease. It has been suggested that inherited predispositions to CRC are possibly due to low-penetrance genetic variants clustered at many loci [20].

In our study, we evaluated the two common *MYH* polymorphisms in the Caucasian population, Y165C and G382D, which are associated with an increased risk for developing CRC, in an Australian series of HNPCC patients. The *MYH* polymorphisms studied previously have been found in 1% of the general population and the findings in the current study confirm this result since 1.35% of the control population was heterozygous for *MYH* mutations, all were heterozygous for G382D.

In total 7 HNPCC patients were heterozygous for 1 of the 2 polymorphisms in *MYH* (1.6%). Five of the patients were from the sub-population of HNPCC patients who were shown not to harbour mutations in the DNA mismatch repair genes *hMSH2* or *hMLH1*. This accounts for approximately 2% of the HNPCC mutation negative population. Of the five *MYH* mutation carriers, one patient was heterozygous for Y165C and four patients were heterozygous for G382D. Direct sequencing of the *MYH* gene revealed that two of them harboured the V22M mutation, which has been previously identified [11, 13, 21, 22]. Kairupan et al [11] showed that 1 patient heterozygous for the Y165C mutation also harboured the V22M polymorphism. Together, the evidence suggests that the V22M polymorphism could be an additional common *MYH* polymorphism associated with colorectal cancer. The suggestion that there are other common *MYH* polymorphisms associated with colorectal cancer needs to be verified in a much larger study. In the remaining 5 patients no evidence of a second mutation was forthcoming but the possibility remains that mutations could be present in the

promoter region of *MYH*. Alternatively, exon deletions cannot be ruled out as these were not investigated.

Approximately 1% of the HNPCC mutation positive population carried the *MYH* mutations. Two patients that were heterozygous for the G382D mutation harboured *hMSH2* mutations, one of which has not been functionally evaluated. This is the first study to describe the existence of *MYH* polymorphisms in association with MMR mutation positive patients. This is an interesting finding since the MMR heterodimer *hMSH2/hMSH6* interacts directly with *MYH* through *hMSH6* to stimulate DNA binding and glycosylase activity to remove mismatches caused by reactive oxygen species (ROS) which are the most prevalent source of DNA damage in aerobic organisms [12, 18]. A previous study suggests that *MYH* does not initiate progression of cancer but it acts as a promoter [13].

In a previous study, Kairupan et al [11] found that the *MYH* mutations, Y165C and G382D, were not represented in FAP *APC* mutation positive patients suggesting that *MYH* does not play a modifying role in APC driven FAP. Interestingly, this study shows that *MYH* mutations could possibly modify the functionality of the heterodimer *hMSH2/hMSH6* since two patients with mutations in *hMSH2* were also heterozygous for *MYH* (G382D) mutations.

In addition, *MYH* mutations were found in the HNPCC MMR mutation negative group. This result suggests that *MYH* may account for some of the cases of CRC where no genetic predisposition has been identified. In a previous study we have shown that the clinical features of some of the patients harbouring *MYH* mutations are similar to HNPCC patients [11]. In this study, we found that from the clinical information available on each patient only a few adenomas were identified in patients harbouring mutations in *MYH*.

Similar to Kairupan et al [11], in the HNPCC population studied herein there was no overrepresentation of extracolonic cancers apart from one patient diagnosed with melanoma and another with gastric cancer. Together the evidence suggests that upper gastrointestinal malignancies might not be a feature of this type of colorectal cancer predisposition.

In conclusion, the results of our study show that *MYH* is associated with colorectal cancer risk and can possibly be associated with the clinical features of HNPCC. This finding may aid in screening individuals at risk of developing CRC. In addition, there remains the possibility that other *MYH* mutations common in the Australian population may indeed modify colorectal cancer risk in an otherwise high-risk population.

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