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MSH6 G39E Polymorphism and CpG Island Methylator Phenotype in Colon Cancer

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Abstract

The *MSH6* G39E germline polymorphism is not associated with an increased risk of either microsatellite stable or unstable sporadic colorectal cancer. Other than microsatellite instability, however, most genetic and epigenetic changes of tumors associated with this common variant have not been studied. The objective of our investigation was to evaluate associations between the *MSH6* G39E (116G>A) polymorphism and CpG island methylator phenotype (CIMP) and *BRAF* V600E mutations in tumors from a sample of 1048 individuals with colon cancer and 1964 controls from Utah, Northern California, and Minnesota. The G39E polymorphism (rs1042821) was determined by the five prime nuclease assay. CIMP was determined by methylation-specific polymerase chain reaction (PCR) of CpG islands in *MLH1*, methylated in tumors (MINT)1, MINT2, MINT31, and *CDKN2A*. The *BRAF* V600E mutation was determined by sequencing exon 15. In microsatellite stable tumors, homozygous carriers of the G39E polymorphism had an increased risk of CIMP+ colon cancer (odds ratio (OR) 2.2, 95% confidence interval (CI) 1.1, 4.2) and *BRAF* V600E mutation (OR 3.1, 95% CI 1.01, 9.7) in a case–control comparison. This finding was not observed in unstable tumors; however, power may have been low to detect an association. Age at diagnosis, family history, and alcohol use did not interact with *MSH6* G39E and CIMP. The *MSH6* G39E germline polymorphism may be associated with CIMP+ colon cancer.

Keywords

MSH6; CIMP; DNA mismatch repair; microsatellite instability; colon cancer

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), the inherited form of microsatellite instability (MSI), is due to germline mutations in one of four DNA mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6*, or *PMS2*. Lynch-associated tumors typically do not have CpG island methylator phenotype (CIMP), *MLH1* methylation, or *BRAF* mutations [1]. MSI, the expansion or contraction of short nucleotide repeats, occurs in 10–15% of colorectal cancers (CRC) [2]. The more common sporadic form of MSI is usually secondary

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to acquired hypermethylation of the promoter of one of the MMR genes, *MLH1* [3], and is commonly associated with the CIMP and activating mutations in the oncogene *BRAF* [4]. Germline polymorphisms in MMR genes, including *MSH6*, have been well studied in Lynch-associated cancers [5]; however, reports are limited regarding the association of common MMR variants in CRC as defined by genetic and epigenetic changes.

We previously reported that a common missense polymorphism in exon 1 of *MSH6*, G39E (116G>A, rs1042821), was not associated with colon cancer overall; however, we observed a modest increased risk of colon cancer in men who were heterozygous or homozygous carriers of the variant allele [6]. We found no significant associations between *MSH6* G39E and microsatellite stable (MSS) or unstable tumors in colon cancer, but reported that among men, risk was slightly stronger for MSI+ tumors than MSI− tumors [6]. Other than MSI, most genetic and epigenetic changes of tumors associated with this polymorphism have not been studied. We therefore evaluated the *MSH6* G39E polymorphism located in exon 1 and associated tumor characteristics in a sample of 1048 individuals with colon cancer evaluated for CIMP status and 1964 controls from Utah, Northern California, and Minnesota.

METHODS

Study Population

Study participants were predominantly non-Hispanic white and were from either the Kaiser Permanente Medical Care Program (KPMCP) of Northern California, an eight county area in Utah (Davis, Salt Lake, Utah, Weber, Wasatch, Tooele, Morgan, and Summit counties), or the Twin Cities Metropolitan area in Minnesota (Table 1). Eligibility criteria for cases included diagnosis with first-primary incident colon cancer (ICD-O, 2nd edition codes 18.0, 18.2–18.9) between October 1, 1991, and September 30, 1994, between 30 and 79 yr of age at time of diagnosis, and mentally competent to complete the interview. Cases with adenocarcinoma or carcinoma of the rectosigmoid junction or rectum (defined as the first 15 cm from the anal opening), or with known familial adenomatous polyposis, ulcerative colitis, or Crohn's disease were not eligible. Of all cases asked to participate, 75.6% cooperated. Seven cases previously identified as probable HNPCC were included [7], although excluding them did not impact the results; all seven individuals were wild type for the common *MSH6* G39E allele.

In addition to the eligibility criteria for case subjects, control subjects could never have been diagnosed with a colorectal tumor and those who reported a diagnosis of FAP, ulcertive colitis, or Crohn's. Controls were selected from eligibility lists for KPMCP and random-digit-dialing, driver's license lists for Minnesota, and random-digit-dialing, driver's license lists, or Health Care Finance Administration (HCFA) lists for Utah, using the same eligibility criteria as was used for cases. These methods have been described in detail [8]. Of all controls contacted, 63.7% participated. All aspects of this study were approved by the University of Utah and KPMCP Institutional Review Boards.

MSH6 G39E Genotyping

DNA was extracted from blood drawn on study controls and cases during the study interview. Genotyping of the *MSH6* G39E polymorphism has been described previously [6]. Briefly, polymerase chain reaction (PCR) amplification of the genomic DNA in this region was followed by allelic discrimination using the 5′ nuclease assay on a 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Validation of the 5′ nuclease assay and quality control procedures have been previously detailed [6].

Microsatellite Instability (MSI)

The MSI status of the tumors had been determined in a previous study that preceded the development of the Bethesda consensus panel; the MSI markers used were BAT-26 (a mononucleotide repeat which by itself is a very good measure of generalized instability), *TGFβRII* (a coding mononucleotide repeat which is unstable in most CRC with MSI), and a panel of 10 tetranucleotide repeats which show a high correlation with the Bethesda consensus panel and BAT-26 [9]. A hierarchical approach was then used for MSI determination. A majority of tumors (95%) were classified as either stable (MSI−) or unstable (MSI+) for BAT-26. The remaining 5% of tumors (which did not have a result for BAT-26) were classified using *TGFβRII*. In four tumors for which BAT-26 and *TGFβRII* were inconclusive, if 30% or more of the 10 tetranucleotide repeats were unstable, the tumor was classified as unstable.

CpG Island Methylator Phenotype (CIMP)

The CIMP status of the tumors was determined in a previous study [4]. Briefly, sodium bisulfite modification was performed on DNA extracted from tumors microdissected from formalin-fixed, paraffin-embedded blocks. Methylation-specific PCR was then performed as described previously for the following CpG islands: methylated in tumors (MINT)1, MINT2, MINT31, *CDKN2A* (*p16*), and *MLH1*, and the criterion for CIMP+ was methylation of two or more of these CpG islands. CIMP− was defined as less than two of five markers methylated. The use of established assays, selection of genetic loci, and criterion for CIMP+or CIMP− was based on the pioneering work of other groups that previously defined the CIMP phenotype [10,11]. We have previously demonstrated associations between CIMP defined in this way and numerous clinicopathologic variables, including the very strong relationship to *BRAF* V600E mutations seen by others using different CpG panels and detection techniques [12]. Sufficient DNA was available for CIMP determination for 1048 tumors.

BRAF V600E Mutation Detection

The *BRAF* V600E mutation status of the tumors was determined in a previous study [4]. Briefly, exon 15 of *BRAF* was PCR amplified from DNA previously extracted from tumors microdissected from formalin-fixed, paraffin-embedded blocks as described [13]. Mutations were verified by bidirectional sequencing. Sufficient DNA was available for *BRAF* V600E determination for 976 tumors.

Statistical Analysis

A χ^2 test was used to determine if the observed genotype frequencies differed from Hardy–Weinberg equilibrium. Odds ratios (ORs) and 95% confidence intervals (CIs) for risk of CIMP or *BRAF* mutation in colon cancers associated with *MSH6* G39E were analyzed using polytomous unconditional multivariate logistic regression models comparing cases with and without CIMP or *BRAF* mutation to controls using SAS® (release 9.1.3). Adjustment for potential confounding variables and variables previously reported to be associated with colon cancer or CIMP and *BRAF* included age at diagnosis or selection, sex, race/ethnicity, study center, regular cigarette smoking (within 2 yr of the referent period), dietary fiber and calcium intake, body mass index (BMI), and physical activity [8,12,14]. A case–case comparison comparing cases with CIMP+ phenotype to cases with CIMP− tumors was also performed. Assessment of interactions between *MSH6* genotype and tumor characteristics, age, and family history were based on a multiplicative scale. Effect modification was determined by evaluating the improvement in fit (difference in −2 log likelihood values) of a model that included a multiplicative interaction term compared to a restricted model with no interaction term.

RESULTS

Characteristics of colon cancer cases and controls are shown in Table 1. The *MSH6* G39E (116A>G) genotypes were in Hardy–Weinberg equilibrium for both controls and cases analyzed separately [6].

The relationship between the *MSH6* G39E polymorphism and risk of CIMP and *BRAF* colon cancer is shown in Table 2. Overall, as compared to controls, the AA genotype appeared to confer an increased risk of CIMP+ and *BRAF*-mutated tumors; however, 95% CIs for the ORs contained one. In MSS colon cancer as compared to controls, the AA genotype was associated with a statistically significant twofold increased risk of CIMP+ and a threefold increased risk of *BRAF* mutation. Although an association with the AA genotype and CIMP+ or *BRAF* was not observed in MSI+ tumors, the study may have lacked sufficient power to detect one given the small sample size in this subgroup. There were no significant interactions between age, gender, family history of CRC in first-degree relatives, smoking or long-term alcohol consumption, and the *MSH6* G39E polymorphism in modifying risk of CIMP or *BRAF* in colon cancer (data not shown).

In a case–case comparison (data not shown), the AA genotype was associated with CIMP+ compared to CIMP− (OR 2.9, 95% CI 1.4, 6.3) and with BRAF mutation compared to no mutation (OR 2.8, 95% CI 1.1, 8.0). In stable tumors, the magnitude of the OR was a fourfold increased risk of a CIMP+ to CIMP− or *BRAF* mutation to no *BRAF* mutation comparison; this association was not observed in unstable tumors. An increased risk was not observed in heterozygote carriers of the variant allele in either a case–control or case–case comparison, indicating a recessive mode of inheritance of the disease allele. Under a recessive model, the P-heterogeneity was significant for the CIMP+ to CIMP− comparison in all tumors and in MSS tumors (0.02 and 0.003, respectively).

DISCUSSION

The findings we present are from a large, population-based case–control study of sporadic colon cancer. As far as we could determine, this represents the first examination of a common polymorphism in *MSH6* and CIMP and *BRAF* in colon tumors. We previously reported that a MMR polymorphism, *MLH1* −93 G>A, had an association in MSI+ cancers with these genetic and epigenetic changes [15]. In the current study, we describe a polymorphism in *MSH6* (G39E) that increases the risk of CIMP+/*BRAF* mutation in stable colon cancers. An increased risk was not evident in microsatellite unstable colon cancers; the variant genotype occurred infrequently (~3%) in study subjects, and power may have been too low to detect an association.

The associations we detected were limited to MSS tumors. While our power to detect such associations in unstable tumors was limited, it is of interest that the associations with stable tumors mirrors to some extent what has been reported in Lynch syndrome-associated cancers occurring in individuals with germline *MSH6* mutations. These tumors have not consistently been associated with an MSI-high phenotype [5,16,17]. This is in contrast to tumors occurring in individuals with the more common *MLH1* or *MSH2* germline mutations, as these tumors are typically MSI high [18].

Our group and others have shown that the *MLH1* −93 G>A polymorphism is associated with an increased risk of MSI+, but not MSS tumors [6,19]; however, this may not be the case for *MSH6* polymorphisms. Parc et al. [20] reported *MSH6* polymorphisms, including G39E, do not play a significant role in the development of sporadic CRC with an MSI-low phenotype, and in a case–control study that examined promoter polymorphisms in MMR genes, Mrkonjic et al. [21] found no association with *MSH6*-159C>T and MSI. As mentioned above, highly penetrant HNPCC-associated germline *MSH6* mutations do not necessarily lead to an unstable

tumor. Thus, it is not surprising that tumors associated with a presumably less deleterious, less penetrant germline change, which is not associated with the Lynch syndrome phenotype, would also not exhibit high levels of MSI. Based on our earlier finding that the *MLH1* −93G>A MMR polymorphism was associated with CIMP+, *BRAF* mutation, and *MLH1* methylation in microsatellite unstable tumors, we hypothesized that CIMP/*BRAF* mutations in tumors occur as an intermediate step, in which a tumor either becomes *MLH1* methylated and unstable (as in the case of the *MLH1* −93G>A), or if methylation of *MLH1* does not occur, a stable tumor is the end result. The *MSH6* G39E variant may also be acting at the CIMP/*BRAF* stage in tumors, in this case promoting the development of a non-*MLH1* methylated, MSS tumor [15].

A limitation of studying DNA methylation in colon tumors is that there is presently no "consensus" as to the appropriate CpG island panel or method of detection to determine CIMP [22]. We have demonstrated significant relationships between CIMP and numerous clinicopathologic variables using out panel, including cigarette smoking and the *BRAF* V600E mutation, which were independent of MSI [4,12]; this work has helped to support the legitimacy of the CIMP concept [23]. Our previous report of very strong correlations of *BRAF* V600E mutations and CIMP+ tumors, CIMP+ and MSI+ in tumors, and *BRAF* mutations and MSI+ in tumors [4], is in agreement with results using different panels and techniques [22,24]. We also reported associations between CIMP+ and *BRAF* or K-*ras* mutations which approximately correspond to CIMP high and CIMP low tumors defined by Ogino et al. [25] and Shen et al. [26]. Thus, our definition of CIMP+ based on our established CIMP panel of markers likely combines CIMP high and CIMP low tumors as defined by these other investigators.

In summary, homozygous carriers of the G39E polymorphism in the *MSH6* MMR gene may have an increased risk of CIMP+ tumor and *BRAF* V600E mutation in stable colon cancers. As the number of cases with the AA genotype and CIMP+ or *BRAF* mutation is small, these results are exploratory in nature and should be verified in another population. It is unclear from our study if the polymorphism is associated with CIMP and *BRAF* in unstable colon cancers, as power may have been too low to detect an association with this infrequently occurring genetic variant.

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Abbreviations

HNPCC	hereditary nonpolyposis colon cancer
MSI	microsatellite instability
MMR	mismatch repair

CIMP	CpG island methylator phenotype
CRC	colorectal cancers
MSS	micro-satellite stable
KPMCP	Kaiser Permanente Medical Care Program
PCR	polymerase chain reaction
OR	odds ratio
CI	confidence interval

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Table 1

Description of Study Subjects With *MSH6* G39E Genotype and CIMP Status

	Controls		Cases	
Characteristic	**Number**	**%**	**Number**	**%**
Total	1964	100.0	1048	100.0
Age, mean (SD)	65.0	10.2	65.1	9.5
Center				
KPMCP	816	41.6	561	53.5
Minnesota	802	40.8	296	28.2
Utah	346	17.6	191	18.2
Sex				
Male	1036	52.8	590	56.3
Female	928	47.3	458	43.7
Race				
White, non-Hispanic	1828	93.1	940	89.7
White, Hispanic	79	4.0	49	4.7
Black	55	2.8	57	5.4
Other	2	0.1	2	0.2
First-degree family history of CRC				
None	1781	90.7	870	83.0
≥1 relative	183	9.3	178	17.0
MSH6 G39E (116G>A)				
GG	1343	68.4	685	65.4
GA	559	28.5	333	31.8
AA	62	3.2	30	2.9
Allele frequency		17.4		18.8
CIMP status				
CIMP−	—	—	765	73.0
CIMP+	—	—	283	27.0
BRAF V600E (n = 976)				
No mutation	—	—	883	90.6
Mutation	—	—	93	9.4
MSI status (n = 1035)				
MSI−	—	—	873	84.4
MSI+	—	—	162	15.7

CIMP, CpG island methylator phenotype; MSI, microsatellite instability.

CIMP− was defined as 0 or 1 of 5 CpG islands methylated, CIMP+ was defined as 2 or more of 5 CpG islands methylated; MSI− (stable) and MSI+ (unstable) were defined according to BAT-26 and *TGFβRII* analysis (see Methods Section).

Table 2

Association of CIMP and *BRAF* V600E Mutations With *MSH6* G39E (116G>A) Genotype and MSI Status

		All tumors						Microsatellite stable tumors						Microsatellite unstable tumors					
		CIMP−			CIMP+			CIMP−			CIMP+			CIMP−			CIMP+		
Genotype	**Controls (N)**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**
GG	1337	498	1.0	Reference	184	1.0	Reference	453	1.0	Reference	114	1.0	Reference	36	1.0	Reference	68	1.0	Reference
GA	558	247	1.2	(1.0, 1.4)	82	1.1	(0.8, 1.4)	234	1.2	(1.0, 1.5)	41	0.9	(0.6, 1.3)	11	0.7	(0.4, 1.5)	41	1.4	(0.9, 2.1)
AA	62	16	0.7	(0.4, 1.2)	14	1.7	(0.9, 3.0)	13	0.6	(0.3, 1.1)	12	2.2	(1.1, 4.2)	3	1.9	(0.5, 6.3)	2	0.6	(0.1, 2.7)

		All tumors						Microsatellite stable tumors						Microsatellite unstable tumors					
		$BRAF_{WT}$			$BRAF_{MUT}$			$BRAF_{WT}$			$BRAF_{MUT}$			$BRAF_{WT}$			$BRAF_{MUT}$		
Genotype	**Controls (N)**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**	**N**	**OR**	**95% CI**
GG	1337	579	1.0	Reference	57	1.0	Reference	514	1.0	Reference	23	1.0	Reference	60	1.0	Reference	33	1.0	Reference
GA	558	274	1.1	(0.9, 1.3)	30	1.3	(0.8, 2.0)	248	1.1	(1.0, 1.4)	9	1.0	(0.5, 2.2)	25	1.0	(0.6, 1.6)	21	1.5	(0.8, 2.6)
AA	62	23	0.8	(0.5, 1.3)	6	2.0	(0.8, 5.3)	21	0.8	(0.5, 1.4)	4	3.1	(1.01, 9.7)	2	0.7	(0.2, 3.1)	2	1.3	(0.3, 5.7)

Adjusted for age, sex, race, center, cigarette smoking, BMI, physical activity, energy-adjusted dietary fiber, and calcium intake. Number of subjects varies slightly from Table 1 due to missing data for one or more adjustment variables.