RESEARCH ARTICLE

Contribution of the *MLH1* -93G>A Promoter Polymorphism in Modulating Susceptibility Risk in Malaysian Colorectal Cancer Patients

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Abstract

Background: Colorectal cancer (CRC) exists in a more common sporadic form and less common hereditary forms, associated with the Lynch syndrome, familial adenomatous polyposis (FAP) and other rare syndromes. Sporadic CRC is believed to arise as a result of close interaction between environmental factors, including dietary and lifestyle habits, and genetic predisposition factors. In contrast, hereditary forms such as those related to the Lynch syndrome result from inheritance of germline mutations of mismatch repair (MMR) genes. However, in certain cases, the influence of low penetrance alleles in familial colorectal cancer susceptibility is also undeniable. Aim: To investigate the genotype frequencies of *MLH1* promoter polymorphism -93G>A and to determine whether it could play any role in modulating familial and sporadic CRC susceptibility risk. Methods: A case-control study comprising of 104 histopathologically confirmed CRC patients as cases (52 sporadic CRC and 52 Lynch syndrome patients) and 104 normal healthy individuals as controls was undertaken. DNA was extracted from peripheral blood and the polymorphism was genotyped employing PCR-RFLP methods. The genotypes were categorized into homozygous wild type, heterozygous and homozygous variants. The risk association between these polymorphisms and CRC susceptibility risk was calculated using binary logistic regression analysis and deriving odds ratios (ORs). Results: When risk association was investigated for all CRC patients as a single group, the heterozygous (G/A) genotype showed a significantly higher risk for CRC susceptibility with an OR of 2.273, (95% CI: 1.133-4.558 and p-value=0.021). When analyzed specifically for the 2 types of CRC, the heterozygous (G/A) genotype showed significantly higher risk for sporadic CRC susceptibility with and OR of 3.714, (95% CI: 1.416-9.740 and p-value=0.008). Despite high OR value was observed for Lynch syndrome (OR: 1.600, 95% CI: 0.715-3.581), the risk was not statistically significant (P=0.253). Conclusion: Our results suggest an influence of *MLH1* promoter polymorphism -93G>A in modulating susceptibility risk in Malaysian CRC patients, especially those with sporadic disease.

Keywords: Colorectal cancer - Lynch syndrome - *MLH1* -93G>A - sporadic cases - Malaysia

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Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide and the incidence is increasing in developed as well as developing countries including Malaysia. According to the latest Malaysian Cancer Statistics, CRC ranks as the most common gastrointestinal cancer in Malaysia and also the first most common cancer in men and second in women (Goh et al., 2005; Malaysia Cancer Statistics, 2006). CRC exists in a more common sporadic form and less common hereditary form, such as the Lynch syndrome, familial adenomatous polyposis (FAP) and other rare syndromes. Sporadic CRC is believed to arise as a result of close interaction of environmental factors such as diet, tobacco smoke and alcohol consumption with the genetic predisposition factors (Giovannucci, 2001; Terry et al., 2001; Neagoe et al., 2004). Lynch syndrome also known as hereditary nonpolyposis colorectal cancer (HNPCC) accounts for approximately 1-5% of all colorectal cancers (Aaltonen et al., 1998; Salovaara et al., 2000). It is characterized by an autosomal dominant inheritance with multiple members

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affected in families, inheritance of susceptibility gene (mismatch repair genes) with incomplete penetrance (80-90%), early onset of colorectal cancer and/or extracolonic cancers such as cancers of endometrium, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain and skin (Jass, 2006; Lagerstedt et al., 2007).

Germline mutations in a group of DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, *PMS1*, *PMS2*) are responsible for inherited susceptibility to Lynch syndrome (Jacob and Praz, 2002; Dionigi et al., 2007; Lagerstedt et al., 2007) where the mutations in *MLH1* and *MSH2* genes alone account for more than 90% of identifiable mutations (Dionigi et al., 2007). However, a small portion of sporadic CRC cases also arise due to defective DNA MMR system, mainly caused by the hypermethylation of the promoter region of *MLH1* gene resulting in the transcriptional silencing of *MLH1* gene (Boland and Goel, 2010). Human *MLH1* gene which is located on chromosome 3p21.3, encodes *MLH1* protein which play a role as part of protein complex involved in the initiation phase of MMR process to repair the replication error that occurs spontaneously during DNA replication (Hampel et al., 2006; Plotz et al., 2006).

MLH1 promoter polymorphism -93G>A (rs1800734) is located in the core promoter region of *MLH1* gene, 93 nucleotides upstream of the transcription start site and the polymorphic site is also located in potential transcription factor binding sites (Ito et al., 1999). Functional study conducted by Ito et al. (1999) have already demonstrated that the *MLH1* promoter region from nucleotide position -184 to the transcription start site, is essential for the transcription of *MLH1* gene (Ito et al., 1999). The chances of interrupting *MLH1* transcription and expression has been reported to be further enhanced by the location of *MLH1* promoter polymorphism -93G>A which is in the region of two potential transcription factor binding sites GT-IIB (GT-motif 2B) and NF-IL6 (interleukin-6-regulated nuclear factor) (Chen et al., 2007; Campbell et al., 2009). Few studies have already investigated the influence of the *MLH1* promoter polymorphism -93G>A with CRC susceptibility risk (Raptis et al., 2007; Allan et al., 2008; Muniz-Mendoza et al., 2012) as well as the risk association with the other types of cancer such as ovarian cancer (Harley et al., 2008), endometrial cancer (Beiner et al., 2006) and lung cancer (Park et al., 2004). Previously our group had reported the predominance of *MLH1* promoter polymorphism -93G>A in Malaysian Lynch syndrome patients (Mohd et al., 2011). But no reports are available from Malaysia on the risk association of this polymorphism with CRC susceptibility. Therefore, this case-control study was undertaken to determine the genotype frequencies of *MLH1* promoter polymorphism -93G>A and to investigate whether polymorphism in the promoter region of *MLH1* gene could play any role in modulating familial and sporadic CRC susceptibility risk.

Materials and Methods

Study subjects

This case-control study was approved by Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia and National Institutes of Health for conducting research under Ministry of Health, Malaysia. Fifty two (52) histopathologically confirmed sporadic CRC patients, 52 suspected Lynch syndrome patients and 104 healthy normal controls were recruited as study subjects. The subjects were recruited from different hospital all over Malaysia such as Hospital Universiti Sains Malaysia (HUSM), Hospital Sultanah Bahiyah, Alor Star, Kedah, Hospital Raja Perempuan Zainab II, Kota Bharu, Kelantan and Hospital Queen Elizabeth, Kota Kinabalu, Sabah. For the recruitment of sporadic CRC patients, cases with known familial adenomatous polyposis, ulcerative colitis or Crohn's disease or any other previous malignancy as stated in the pathology reports were excluded.

Fifty two (52) suspected Lynch syndrome patients who fulfilled any of the following revised Bethesda Guidelines were recruited: (i) CRC with age less than 50 years old, (ii) presence of synchronous or metachronous colorectal or other HNPCC-associated tumors regardless of age, (iii) CRC with MSI-positive morphology with age less than 60 years old, (iv) CRC with one or more first-degree relatives with CRC or other HNPCC-related tumor, with one of the cancers with age less than 50 years old, (v) CRC with two or more first- or second-degree relatives with CRC or other HNPCC-related tumor (regardless of age), including cancers (endometrial, stomach, ovarian, cervical, esophageal, leukemia, thyroid, bladder, ureter and renal pelvis, biliary tract, small bowel, breast, pancreas, liver, larynx, bronchus, lung and brain (glioblastoma), sebaceous gland adenomas and keratoacanthomas. Personal and demographic details of the patients including history of CRC in the family were collected and recorded. CRC patients with strong family history of CRC among first or second degree relatives and who met the selection criteria were subjected to detailed pedigree analysis. Detailed 3 generation pedigrees of these suspected cases of Lynch syndrome were prepared.

Controls were normal healthy individuals, individuals who visited HUSM for other problems unrelated to colorectal cancer and were selected by using the same eligibility criteria as those used for cases. Controls were biologically unrelated to the patients and were cancer free participants. Epidemiological data was collected from patients using pre-structured questionnaire which consisted of information such as socio-demographic status, physical status, dietary factors, occupation, tobacco/alcohol habits, previous illness, radiation exposure etc.

Genotyping of MLH1 promoter polymorphism -93G>A

Three (3) ml of blood sample was collected from study subjects after obtaining informed consent. Genomic DNA was extracted from blood using commercially available DNA extraction kit, QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). The *MLH1* promoter polymorphism -93G>A was genotyped employing Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The promoter region of *MLH1* gene containing the polymorphic site was amplified using appropriate primers, *MLH1* ex1 FW: 5' CAG AGT TGA GAA ATTTGA CTG G 3', *MLH1* ex1 RV: 5' TAA

Table 1. Genotype and allele Frequencies of *MLH1* Promoter Polymorphism -93G>A in Colorectal Cancer Cases and Normal Controls

Polymorphism		Cases (n=104)	Controls (n=104)	p-value	Sporadic CRC (n=52)	p-value	Lynch syndrome (n=52)	p-value
Genotype	G/G	22 (21.1%)	33 (31.7%)	0.084	7 (13.5%)	0.014*	15 (28.8%)	0.713
	G/A	50 (48.1%)	33 (31.7%)	0.016*	26 (50%)	0.027*	24 (46.2%)	0.078
	A/A	32 (30.8%)	38 (36.6%)	0.379	19 (36.5%)	1	13 (25%)	0.148
Allele	G allele	0.452	0.475		0.385		0.519	
	A allele	0.548	0.524		0.615		0.481	

*p-value<0.05, statistically significant

GTC GTA GCC CTT AAG TGA G 3' which produced a 339 bp fragment. PCR reactions were carried out in 20 μl of volume consisting of 1.875 mM $MgCl_2$ (Applied Biosystems, California, USA), 1 X GeneAmp PCR Buffer II (Applied Biosystems, California, USA), 0.375 mM dNTPs (Applied Biosystems, California, USA), 0.4 μM of each forward and reverse specific primers, 4 ng/μl of template DNA and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems, California, USA). The PCR conditions were as follows: 96°C of pre-denaturation for 5 min, 95°C of denaturation for 1 min, 50°C for 1 min and 72°C of extension (1 min) for 40 cycles followed by 72°C of final extension for 7 min in Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Amplicons were then detected by gel electrophoresis in a 2% agarose gel.

Following amplification, PCR products containing the polymorphic site were digested using *Pvu*II restriction enzyme (New England Biolabs Inc., Ipswich, MA, USA) and incubated at 37°C for 1 hour in IPP 400 incubator (Memmert GmbH +Co.KG, Schwabach, Germany). The *Pvu*II cleaves wild type allele (G allele) and yield two fragments (254 bp and 85 bp) where as *Pvu*II does not cleave the variant allele (A allele) and yield a 339 bp fragment. Accordingly, the heterozygous genotype contains three fragments (339, 254 and 85 bp). Based on the bands, the genotypes was categorized as homozygous wild type (G/G), heterozygous (G/A) and homozygous variant (A/A) as shown in Figure 1.

Statistical analysis

The Chi square test was used to compare the distribution of genotype frequencies of *MLH1* promoter polymorphism -93G>A in sporadic CRC, Lynch syndrome cases and normal controls. The Odds Ratios (ORs) and 95% Confidence Interval (CI) were calculated using binary logistic regression (SPSS version 18) to evaluate the risk association of variant genotype of *MLH1* -93G>A with sporadic CRC and Lynch syndrome cases. All statistical tests were two sided and p-value <0.05 was considered as statistically significant.

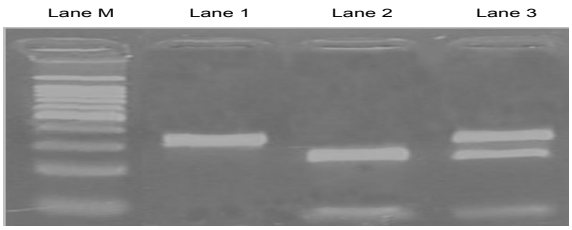

Figure 1. Representative Gel Electrophoresis of PCR-RFLP Analysis of the *MLH1* Promoter Polymorphism -93G>A. Lane M: 100 bp marker; Lane 1: homozygous variant genotype (339 bp); Lane 2: homozygous wild type genotype (254 and 85 bp); Lane 3: heterozygous genotype (339, 254 and 85 bp)

Results

A total of 104 CRC patients (52 sporadic CRCs and 52 suspected Lynch syndrome patients) and 104 normal controls were recruited into this case-control study. Out of 104 CRC patients, 58 were males and 46 were females with a mean age of 52.96±13.17 years. The normal controls comprised of 50 males and 54 females with a mean age of 49.62±10.78 years. The frequencies of the *MLH1* -93G>A genotypes observed in the study subjects are shown in Table 1. The genotype frequencies in 104 CRC patients were 21.1% (G/G), 48.1% (G/A) and 30.8% (A/A) where as in 104 normal controls, the frequencies were 31.7% (G/G), 31.7% (G/A) and 36.6% (A/A). Moreover, the frequency of heterozygous genotype (G/A) was significantly higher in cases (p=0.016) compared to controls. When patients were stratified into sporadic CRC cases and Lynch syndrome cases, out of 52 sporadic CRC patients, 7 (13.5%) showed G/G genotype, 26 (50%) showed G/A genotype and 19 (36.5%) showed A/A genotype. On the other hand, out of the 52 Lynch syndrome patients, 15 (28.8%) showed G/G genotype, 24 (46.2%) showed G/A genotype and 13 (25%) showed A/A genotype. In the case of sporadic CRC, the frequencies of homozygous wild type (G/G) was significantly higher in controls (p=0.014) where as the frequencies of heterozygous (G/A) genotype was significantly higher among cases (p=0.027)

The risk association of variant genotype of *MLH1* promoter polymorphism -93G>A with CRC susceptibility was calculated using binary logistic regression analysis and the results are shown in Table 2 and Table 3. The Odd Ratios (ORs) were calculated relative to subjects and the wild type GG genotype was used as a reference. When analyzed considering the whole group of 104

Table 2. Association of *MLH1* Promoter Polymorphism -93G>A with CRC Susceptibility Risk

Polymorphism	Cases (n=104)	Controls (n=104)	OR (95%CI)	p-value
MLH1 -93G>A				
G/G	22	33	1 (Ref)*	
G/A	50	33	2.273 (1.133-4.558)	0.021**
A/A	32	38	1.263 (0.618-2.584)	0.522

*The genotype served as reference category, **p-value < 0.05, statistically significant

Table 3. Association of *MLH1* Promoter Polymorphism -93G>A with CRC Susceptibility Risk

Polymorphism		Sporadic CRC (n=52)	Controls (n=104)	OR (95%CI)	p-value	Lynch syndrome (n=52)	Controls (n=104)	OR (95%CI)	p-value
MLH1 -93G>A	G/G	7	33	1 (Ref)*	-	15	33	1 (Ref)*	-
	G/A	26	33	3.714 (1.416-9.740)	0.008**	24	33	1.600 (0.715-3.581)	0.253
	A/A	19	38	2.357 (0.881-6.306)	0.088	13	38	0.753 (0.313-1.809)	0.525

*The genotype served as reference category, **p-value < 0.05, statistically significant

CRC patients, the heterozygous (G/A) genotype showed significantly higher risk for CRC susceptibility with OR: 2.273, (95%CI: 1.133-4.558 and p-value=0.021). Risk association was evaluated separately for sporadic CRC and Lynch syndrome patients. In this analysis, the heterozygous (G/A) genotype showed significantly higher risk for sporadic CRC susceptibility with OR: 3.714, (95%CI: 1.416-9.740 and p-value=0.008) (Table 3). Even though homozygous variant (A/A) also showed high OR value of 2.357 for sporadic CRC risk, it was not statistically insignificant. Furthermore, the heterozygous (G/A) genotype showed high OR value of 1.600 (95%CI: 0.715-3.581) for Lynch syndrome risk, but however, the value was statistically insignificant.

Discussion

Familial aggregation of CRC such as Lynch syndrome is mainly due to the inheritance of germline mutations in high penetrant DNA MMR genes. However, it is also believed that a substantial proportion of genetic variants in low penetrance genes as well can contribute to the incidence of familial CRC. Therefore, several molecular genetic epidemiological studies have been conducted in order to investigate the potential association between high and low penetrance alleles of known associated genes and CRC susceptibility. *MLH1* gene is one of the human DNA MMR genes which encode *MLH1* protein; a key component in human DNA MMR system. DNA MMR system is a crucial post-replication repair process because of its function to maintain the fidelity of the genome during replication. Germline mutation in one of the MMR genes will cause the inactivation of MMR system where the MMR proteins would no longer be expressed, resulting in failure to repair the replication error that occur spontaneously during DNA replication. Defective MMR system leads to the accumulation of errors in repetitive DNA sequences (microsatellites) throughout the genome of tumors resulting in the condition known as microsatellite instability.

Due to its critical location in the core promoter region, the *MLH1* promoter polymorphism -93G>A could possibly be interrupting the process of *MLH1* transcription and expression and ultimately might be reducing the overall DNA mismatch repair capability. Functional study conducted by Perera et al. (2011) have already demonstrated that *MLH1* -93G>A polymorphism was associated with higher promoter activity by changing the affinity of nuclear factors that bind to the promoter region of *MLH1* gene (Perera et al., 2011). The location of polymorphism within the CpG islands (Deng et al., 2001) also prompted us to postulate that it might be susceptible to hypermethylation and gene silencing. Tumor-specific *MLH1* methylation might also be associated with *MLH1* -93G>A promoter polymorphism (Chen et al., 2007). In fact, the polymorphism was found to be associated with CpG island methylator phenotype (CIMP) and *MLH1* methylation in CRC patients with microsatellite unstable tumors (Samowitz et al., 2008). They also suggested that the polymorphism may play a role at late stage of colorectal tumorigenesis by influencing the CIMP positive tumors towards the microsatellite instability pathway. In contrast, lack of association was observed between *MLH1* methylation and *MLH1* promoter polymorphism -93G>A in Australian sporadic CRC patients (Wong et al., 2011). It is also possible that *MLH1* promoter polymorphism -93G>A may alternatively be in linkage disequilibrium with the other coding region or intronic polymorphism of *MLH1* gene or with the other low penetrance alleles in the other genes which may affect the level of *MLH1* mRNA expression required for certain cell type to perform their normal function.

Often, the polymorphism frequencies vary by ethnic background of the study subjects. Considerable difference in the frequencies has been reported between Asian and Caucasian populations. The frequency of variant A allele of *MLH1* -93G>A in the Ontario general population was 21.4% and that in the Newfoundland general population was 19.3% (Raptis et al., 2007). On the contrary, a higher percentage of 44% and 50% were reported in the Japanese and Korean population respectively (Ito et al., 1999, Shin et al., 2002). The frequency of variant A allele in our population (54.8%), is also in concordance with the frequency reported in Japanese and Korean population.

In this case control study, we investigated the genotype frequencies of *MLH1* promoter polymorphism -93G>A and its association with sporadic and familial CRC susceptibility risk in Malaysian population. We observed that the heterozygous (G/A) genotype was significantly associated with higher risk for CRC susceptibility with OR: 2.273, 95%CI: 1.133-4.558 and p value=0.021. When stratified into sporadic and Lynch syndrome groups and the risk association was evaluated, the heterozygous (G/A) genotype showed significantly higher risk for sporadic CRC susceptibility with OR: 3.714, (95%CI: 1.416-9.740 and p value=0.008). The heterozygous (G/A) genotype also showed higher OR value for Lynch syndrome (OR: 1.600, 95%CI: 0.715-3.581) but however, was not statistically significant (p=0.253). Based on our results, subjects with *MLH1* -93 G/A genotype have an almost four-fold higher risk for sporadic CRC development compared to individuals with G/G genotype.

MLH1 promoter polymorphism -93G>A has been reported in a large scale case-control study conducted involving Ontario and Newfoundland population in Canada and it was found to be associated with an increased

risk of CRC with high microsatellite instability (MSI-H) (Raptis et al., 2007). Similar findings also have been observed in a study conducted by Campbell et al. (2008) in American population where the *MLH1* promoter polymorphism -93G>A was found to be associated with MSI-positive colon cancers with OR: 2.47, 95%CI: 1.48-4.11, p value=0.01. Study conducted by Allan et al. (2008) found that the variant allele of *MLH1* promoter polymorphism -93G>A was associated with significantly higher risk of CRC with absence expression of *MLH1* protein (OR: 3.30, 95%CI: 1.46-7.47, p-value=0.004) (Allan et al., 2008). They even suggested that *MLH1* promoter polymorphism -93G>A might be defined as a low penetrance risk allele for CRC. Despite high OR values for risk association was obtained for Lynch syndrome susceptibility in our study, the statistical insignificance does not allow us to consider this risk association. This could be due to low sample size, a potential limitation of this present study. Based on results from case-control study conducted in United States, the variant genotype of *MLH1* promoter polymorphism -93G>A also has been found to modulate the risk association of CRC with smoking (Yu et al., 2006). On the contrary, recent study conducted by Muniz-Mendoza et al. (2012) showed that *MLH1* -93G>A polymorphism was found to be significantly associated with a reduced risk of CRC (OR: 0.60, 95%CI: 0.40-0.89, p-value=0.01) among Mexican colorectal cancer patients (Muniz-Mendoza et al., 2012).

However, those evidences and the current study results also favor the *MLH1* promoter polymorphism -93G>A as a functional polymorphism which may have a modifying effects in CRC susceptibility risk especially sporadic CRC. As a conclusion, it is reasonable to suggest that the *MLH1* promoter polymorphism -93G>A may contribute to the etiology of sporadic CRC susceptibility in Malaysian population and individuals with variant allele may have a higher risk for CRC susceptibility. Relatively small sample size used in the present genotyping analysis could have resulted in insufficient statistical power and more susceptible to the fluctuation of risk estimation. Therefore, further study with larger sample size may derive a statistical significance of *MLH1* promoter polymorphism -93G>A with risk association for Lynch syndrome susceptibility. Thus, further study on the combination of SNPs involving other low penetrance alleles is also warranted in order to unravel the contribution of low penetrance alleles on CRC incidence, disease progression and risk association with CRC susceptibility.

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