RESEARCH ARTICLE

Role of *MYH* Polymorphisms in Sporadic Colorectal Cancer in China: A Case-control, Population-based Study

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Abstract

Purpose: Biallelic germline variants of the 8-hydroxyguanine (8-OG) repair gene *MYH* have been associated with colorectal neoplasms that display somatic G:C→T:A transversions. However, the effect of single germline variants has not been widely studied, prompting the present investigation of monoallelic *MYH* variants and susceptibility to sporadic colorectal cancer (CRC) in a Chinese population. Patients and Methods: Between January 2006 and December 2012, 400 cases of sporadic CRC and 600 age- and sex-matched normal blood donors were screened randomly for 7 potentially pathogenic germline *MYH* exons using genetic testing technology. Variants of heterozygosity at the *MYH* locus were assessed in both sporadic cancer patients and healthy controls. Univariate and multivariate analyses were performed to determine risk factors for cancer onset. Results: Five monoallelic single nucleotide polymorphisms (SNPs) were identified in the 7 exon regions of *MYH*, which were detected in 75 (18.75%) of 400 CRC patients as well as 42 (7%) of 600 normal controls. The region of exon 1 proved to be a linked polymorphic region for the first time, a triple linked variant including exon 1-316 G→A, exon 1-292 G→A and intron 1+11 C→T, being identified in 13 CRC patients and 2 normal blood donors. A variant of base replacement, intron 10-2 A→G, was identified in the exon 10 region in 21 cases and 7 controls, while a similar type of variant in the exon 13 region, intron 13+12 C→T, was identified in 8 cases and 6 controls. Not the only but a newly missense variant in the present study, p. V463E (Exon 14+74 T→A), was identified in exon 14 in 6 patients and 1 normal control. In exon 16, nt. 1678-80 del GTT with loss of heterozygosity (LOH) was identified in 27 CRC cases and 26 controls. There was no Y165C in exon 7 or G382D in exon 14, the hot-spot variants which have been reported most frequently in Caucasian studies. After univariate analysis and multivariate analysis, the linked variant in exon 1 region (p=0.002), intron 10-2 A→G (p=0.004) and p. V463E (p=0.036) in the *MYH* gene were selected as 3 independent risk factors for CRC. Conclusions: According to these results, the linked variant in Exon 1 region, Intron 10-2 A→G of base replacement and p. V463E of missense variant, the 3 heterozygosity variants of *MYH* gene in a Chinese population, may relate to the susceptibility to sporadic CRC. Lack of the hot-spot variants of Caucasians in the present study may due to the ethnic difference in *MYH* gene.

Keywords: Colorectal cancer - MutY homologue - monoallelic variant - heterozygosity - Chinese population

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Introduction

The accumulation of multiple variations in the oncogenes and tumor-suppressor genes (TSGs) that sustain regular growth and differentiation in the colon, which gives rise to CRC (Fearon et al., 1990). While it is conjectured that amount to 25% of CRC cases develop as a result of inherited genetic factors, known genes predisposing to this malignancy account for less than 5% (Burt et al., 2005). As well as the APC gene causing familial adenomatous polyposis and the mismatch repair genes responsible for hereditary nonpolyposis colorectal cancer (HNPCC), recent studies have identified a new gene implicated in hereditary CRC, the human homologue of the base excision repair (BER) gene MutY (*MYH*), which encodes a member of the base excision repair system and has been involved in the occurrence of *MYH* associated polyposis (MAP) that is a new adenoma-prone phenotype (Al-Tassan et al., 2002; Dickson et al., 2008; Aretz et al., 2006; Nielsen et al., 2011).

MYH is a DNA glycosylase that acts at a third level of defense, in charge for removing the mispaired 8-OG, one of the most mutagenic DNA products of oxidative DNA damage, form DNA of adenines (Slupska et al., 1996;

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Hayashi et al., 2002). Failure to rectify these mispairs leads to G:C→T:A transversions in target genes. Thus far, pathogenic variants in the BER systems have been limited to the *MYH* gene, with no distinct variations identified in the other two BER genes, MTH1 and OGG1 (Al-Tassan et al., 2002; Jones et al., 2002; Sieber et al., 2003).

It is known to all, *MYH* has been localized to 1p32-34 (Slupska et al., 1996), where LOH has been observed in a small group of CRC and in early lesions, including hyperplastic polyps and adenomas (Bardi et al., 1993; Bomme et al., 1994; Lothe et al., 1995; Rashid et al., 2000). By way of physical association with the MSH2/MSH6 mismatch repair heterodimer, as well as with proliferative cell nuclear antigen (PCNA) and replication protein A (RPA), *MYH* boosts the allegiance of DNA replication and genetic recombination (Parker et al., 2001; Gu et al., 2002; Hayashi et al., 2002). These protein-protein interactions, DNA repair may possibly be hindered by a fraction of SNPs in *MYH*, which furthering colorectal carcinogenesis at last.

The first presentation of association between the inherited compound heterozygotes of two nonconservative missense variants, not Y165C but G382D in the *MYH* gene, and multiple colorectal adenomas were found in white European British families. Subsequently, two biallelic variants of Y165C and G382D in *MYH* have been coincidentally identified to predispose to an attenuated form of familial adenomatous polyposis (Al-Tassan et al., 2002; Jones et al., 2002). Those two most usual variants in the *MYH* gene mentioned above, both accounting for more than 80% of all *MYH* variants reported in white populations; however other pathogenic variants have been found in different ethnic populations, thus being in line with founder effects (Al-Tassan et al., 2002; Cheadle et al., 2003; Gismondi et al., 2004; Jones et al., 2002; Kim et al., 2007; Sieber et al., 2003).

In reality, nearing 40% of attenuated familial adenomatous polyposis (AFAP) without variations in the APC gene lie behind germline *MYH* variations, peculiarly in the cases with a recessive family history (Sieber et al., 2003; Sampson et al., 2003). These adenomas displayed excess somatic G:C→T:A transversions. Thus, MAP is an autosomal recessive disorder, and a SNP in the *MYH* gene that resulted in alternative splicing and decreased translation efficiency of its transcripts, was also identified in patients with CRC. Although those who are *MYH* variant homozygotes carring an increased risk for multiple adenomas and CRC (Sieber et al., 2003), it is unknown if a single copy of an *MYH* variant results in increased susceptibility to CRC (Slupska et al., 1996; Cheadle et al., 2003; Sampson et al., 2003).

An autosomal recessive pattern of biallelic *MYH* gene variations predisposing to CRC has been demonstrated recently, accounting for equivalent to 1% of these neoplasms. As no single research has found statistically significant evidence, a meta-analysis of published data has demonstrated an tendency significant heterozygous gene effect. In accordance with this assumption, the largest population-based case-control study assessing CRC risk in *MYH* variation carriers, has proposed that monoallelic *MYH* variation carriers could be at increased risk in their advanced age (55 years). To sum up, larger sample sizes, population-based studies, specific-variation analysis and proper methodologic approaches are needed to conform the weak gene effect (Farrington et al., 2005; Peterlongo et al., 2005; Jenkins et al., 2006; Tenesa et al., 2006).

In this setting, we present a case-control, population-based study, of which the aims were establishing the sporadic CRC risk associated with single germline *MYH* variations.

Materials and Methods

Between January 2006 and December 2012, 400 cases of sporadic CRC and 600 age- and sex-matched normal blood donors were screened randomly for 7 most potentially pathogenic germline *MYH* exons using genetic testing technology at Colorectal Cancer Center, the Affiliated Jiangsu Cancer Hospital of Nanjing Medical University & Jiangsu Institute of Cancer Research, Nanjing, China. The medical notes of all patients and healthy donors were assessed in detail.

Eligibility criteria of cases included primary CRC, phrase I to III of TNM stage, histologically proven adenocarcinoma, and antibiotics using for 7 postoperative days, with no personal history of cancer at the time of ascertainment. Exclusion criteria of cases was as follow: phrase IV of TNM stage, information on family history of colorectal neoplasia or previous colonoscopy reports was not available for these subjects. Eligibility and Exclusion criteria of controls were listed: present any type of cancer, personal and family history of cancer. The study was approved by the Ethics Committee of Science of our hospital, and written informed consent was obtained from all patients and volunteers.

Demographic, clinical, and tumor-related characteristics of patients, as well as a detail information on each donor, were obtained. Age at cancer diagnosis, gender, pathological type, tumor site, and tumor stage of the neoplasm, and variants of *MYH* gene were registered for each affected patient or participant.

A stage by approach was performed to identify individuals arrying heterozygous variants in the *MYH* gene. Initially, DNA samples extracting from blood of sufferers and volunteers were obtained from each study object, then immediately frozen in liquid nitrogen, and stored at -80°C until use. Genomic DNA was isolated by using the QIAamp DNA Blood Mini Kit (Qiagen, Duesseldorf, Germany). Afterwards, *MYH* was screened for somatic variations in the variation cluster region of 7 exon regions, and linked variants at Exon 1 region in particular was mapped by Linkage Mapping Set v2.5 (Applied Biosystems, Branchburg, NJ). Furthermore, to ensure maximum amplification specificity, a primer sets were designed and a PCR protocol was employed using QIAquick PCR Purification Kit (Qiagen, Duesseldorf, Germany). The *MYH* Variant-specific primer sets were listed in Table 1. Next, denaturing high-pressure liquid chromatography (dHPLC) was performed for each amplicon using the The WAVE Systerm 3500 (Transgenomic, Inc., Glasgow, UK). Running conditions were optimized based on the Navigator software

Table 1. ***MYH*** **Variant-specific Primer Sets**

Exon regions	Variant	Forward primer sequences	Reverse primer sequences	Product size
1	the linked variant in Exon 1 region†	GCTCAATCCACTCCACTG	TGGGTCGAACTTCCGTTC	467
7	None	CAGCTTCTCAAACCGTCATC	CCTCGACACCCTCTGAGATC	231
9	None	GACACATCTTTGCTCTGC	GGGACAAACGTGAAGAGC	231
11	Intron 10-2 A→G	GAAGGGGCAGTGAGAAG	CCCATTCCAGTTCTTCCTC	200
13	Intron 13+12 C→T	CTGGATACTGGGCGTGGAG	CTATTTGAACCCCTTGACCC	313
14	p. V463E	CTATTTGAACCCCTTGACCC	GTGTTTCCTACATGTTCTAC	235
16	nt. 1678-80 del GTT	TCAGTAGAGTCGGGGAAAG	CGCTATGTTGCTCAGACTG	342

†Containing Exon 1-316 G→A, Exon 1-292 G→A and Intron 1+11 C→T

Table 2. Demographic Data and Clinical Characteristics of the Randomly Selected Sample Stratified by Group

Variable categories	CRC patients (n=400)	Normal blood donors (n=600)	*p* value
Age (mean) y	55.97±5.1	56.11+4.9	*p*=0.673
Gender			
Male	223	318	*p*=0.393
Female	177	282	
Tumor site			
Right hemicolon cancer	96		
Left hemicolon cancer	47		
Sigmoid colon cancer	58		
Rectal cancer	199		
Pathological pattern			
Tubular adenocarcinoma	219		
Villous adenocarcinoma	61		
Tubulovillous adenocarcinoma	89		
Signet ring cell carcinoma	31		
Degree of differentiation			
Poor	64		
Moderate	269		
Well	67		
TNM stage			
I~II	187		
III	213		
the linked variants in Exon 1 region			
Yes	13	2	*p*<0.001
No	387	598	
Intron 10-2 A→G			
Yes	21	7	*p*=0.003
No	379	593	
Intron 13+12 C→T			
Yes	8	6	*p*=0.187
No	392	594	
p. V463E			
Yes	6	1	*p*=0.013
No	394	594	
nt. 1678-80 del GTT			
Yes	27	26	*p*=0.309
No	373	574	

(Transgenomic, Inc., Glasgow, UK). For the samples, in which an aberrant peak was identified. Finally, the PCR product was purified and automatically sequenced using PTC-200DNA Sequencer, (Applied Biosystems, Branchburg, US) to verify the varitants.

To test the association between the heterozygous variants of *MYH* gene and CRC risk, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for each variant. Statistical analyses were performed using IBM SPSS statistics 19.0 for Windows (SPSS Inc; IBM, Chicago, IL). All continuous variables were dichotomized. Chi-squared or Fisher's exact test for categorical variables was used for statistical comparisons of those variables between the cases and controls. Multivariate analysis to detect the variants as risk factors for sporadic CRC was conducted with a logistic regression model. Difference in each variant has been analyzed using one-way analysis of variance (ANOVA) before multivariate analysis was performed.

We have enough experience in conducting medical researches, and have published some results elsewhere (Huang et al., 2011; Li et al., 2011; Li et al., 2011; Li et al., 2011; Xu et al., 2011; Xu et al., 2011; Xu et al., 2011; Yan et al., 2011; Zhang et al., 2011; Gong et al., 2012; Liu et al., 2012; Li et al., 2012; Shu et al., 2012; Zhan et al., 2012; Zhan et al., 2012; Xu et al., 2012; Xu et al., 2012; Yu et al., 2012; Zhang, et al., 2012; Zhang et al., 2012; Chen et al., 2013; Dai et al., 2013; Deng et al., 2013; Gu et al., 2013; Huang et al., 2013; Liu et al., 2013; Liu et al., 2013; Liu et al., 2013; Lu et al., 2013; Sun et al., 2013; Wei et al., 2013; Wu et al., 2013; Yang et al., 2013; Yin et al., 2013; Yin et al., 2013).

Results

The demographic data and clinical characteristics of the selected sample stratified by goup are detailed in Table 2. A total of 400 patients [223 male patients (55.8%)] with a mean age of 55.97±5.1 years, as well as a total of 600 normal blood donors [318 male patients (53%)] with a mean age of 56.11±4.9 years, were included at the time of assessed. Nearing half of the patients suffered from rectal cancer (49.8%), the others suffered from right hemicolon cancer (24%), left hemicolon cancer (11.8%) and sigmoid colon (14.5%), respectively. Over half of the (54.80%) patients were affected by tubular adenocarcinoma, the rest of them were villous (15.3%), tubulovillous (22.3%) and signet ring cell carcinoma (7.8%). Among all the patients, 67.3% (219) of whom with a morderate degree of differentiation, and the number of patients with poor differentiation was almost equal to the well (16% vs. 16.8%). Two hundred and thirteen (53.30%) sufferers were diagnosed with phrase III CRC pathologically and postoperatively, the remaining patients were detected with phraseI~II CRC.

For the purposes of this study, the following 5 SNPs with potential pathogenic significance were assayed (Figure 1): (1) the triple linked variant included Exon 1-316 G→A, Exon 1-292 G→A and Intron 1+11 C→T, of which result is still unknown to this day; (2) Intron 10-2 A→G, caused a splicing abnormality that result in

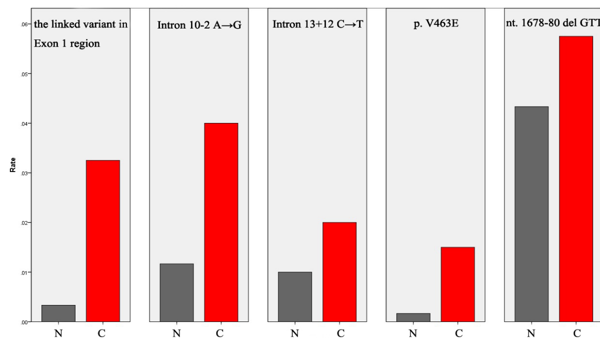

Figure 1. The Histogram of the 5 Variants Identified in Both the Cases and the Controls. N stands for normal blood donors, while C represents CRC patients

Table 3. Multivariate Analysis of the Variants

Variants	*p* value	OR	95% CI of OR	
			lower limit	upper limit
The linked variants in Exon 1 region	0.002	10.507	2.358	46.826
Intron 10-2 A→G	0.004	3.695	1.506	9.067
p. V463E	0.036	9.699	1.163	80.883

OR, odds ratio; CI, confidence interval

the production of an aberrant mRNA transcript encoding a truncated *MYH* protein (Tao H et al., 2004); (3) Intron 13+12 C→T; and (4) p. V463E, which may shift the polarity of *MYH* protein; (5) nt. 1678-80 del GTT, which is a LOH.

As mentioned above, the five monoallelic variants were identified in the 7 exon-regions of *MYH*, which were detected in 75 (18.75%) cases as well as 42 (7%) controls. The region of exon 1 proved to be a linked polymorphic region using a linkage mapping technique for the first time, a triple linked variant included Exon 1-316 G→A, Exon 1-292 G→A and Intron 1+11 C→T, was identified in 13 CRC patients and 2 normal donors. A variant of base replacement, Intron 10-2 A→G, was identified in Exon 10 region in 21 cases and 7 controls, while similar type of variant in Exon 13 region, Intron 13+12 C→T, was identified in 8 cases and 6 controls. Not the only but a newly missense variant in present study, p. V463E (Exon 14+74 T→A), was identified in the region of Exon 14 in 6 patients and 1 normal control. In the region of Exon 16, nt. 1678-80 del GTT of a LOH was identified in 27 CRC cases and 26 controls. There was no Y165C in Exon 7 or G382D in Exon 14, the hot-spot variant, which had been reported most frequently in Caucasia studies.

The variants as risk factors for CRC of patients and normal blood donors are also summarized in Table 2. Univariate analysis demonstrated that the linked variant at Exon 1 region might be a risk factor for CRC ($p<0.001$), to which Intron 10-2 A→G ($p=0.003$) and p. V463E ($p=0.013$) might be similar. Age, gender, Intron 13+12 C→T and nt. 1678-80 del GTT were not significant risk factors for CRC. The variants with p values less than 0.20 then were subjected to multivariate analysis using a forward stepwise logistic regression model. Results of multivariate analyses are detailed in Table 3.

After multivariate analysis, the linked variant in Exon 1 region ($p=0.002$; odds ratio, 10.507; 95%CI, 2.358-46.826), Intron 10-2 A→G ($p=0.004$; odds ratio, 3.695; 95%CI, 1.506-9.067) and p. V463E ($p=0.036$; odds ratio, 9.699; 95%CI, 1.163-80.883) in the *MYH* gene were selected as 3 independent risk factors for CRC.

Discussion

The *MYH* variations are prevailing in patients with multiple colorectal adenomas, which is classified according to their cumulative number of adenomas, and is comparable to that found in other continuities of patients from different geographic origins (Sieber et al., 2003; Gismondi et al., 2004; Isidro et al., 2004; Jo et al., 2005; Kairupan et al., 2005; Miyaki et al., 2005). The prevalence of monoallelic and biallelic variations according to the number of adenomatous polyps is also comparable with previous studies (Croituru et al., 2004; Nielsen et al., 2005).

In the light of the description in the literatures, hot-spot, the Y165C and G382D variations on the *MYH* gene take up more than 80% of all *MYH* variants were documented hitherto in Caucasian populations (Croitoru et al., 2004). Research has also interpreted that the spectrum and abundance of *MYH* transcripts as well as the related products, which was produced by *MYH* still unknown (Plotz et al., 2012). Thus as far as current situation is concerned, more variations on the gene still need to be identified or described.

Theoretically, inchoate lesions are distinguished by restraint of apoptosis, and hence the pro-apoptotic effects of backlogging DNA mismatches would be eased (Jass et al., 2002). Eventually, a variation may lead to further neoplastic progression. monoallelic *MYH* variants may confer only a small overall susceptibility because of the increased risk of being confined to a relatively infrequent pathway of colorectal tumorigenesis. However, the net effect would be the overlap of an autosomal dominant effect on an already well-documented autosomal recessive effect to achieve this level so that some examples of apparent autosomal dominant CRC or multiple adenomas could in nature be explained by this mechanism. Genetic instability is a likely mechanism for accelerated colorectal tumorigenesis, and *MYH* variants could account for such instances of high-risk adenomas (Almendingen et al., 2003).

As proved in present and previous studies, it is clear that monoallelic variations in the *MYH* gene carry a true risk for colorectal polyposis and cancer. In present series, we proposed the 5 monoallelic SNPs as 5 potential pathogenic factors for CRC, and they are listed as follows, the triple linked variant in Exon 1 region, Intron 10-2 A→G that was reported as a promotor of splicing abnormality, Intron 13+12 C→T, p. V463E and nt. 1678-80 del GTT that is a LOH. It is noteworthy that there was no Y165C in Exon 7 or G382D in Exon 14, the hot-spot variant, which had been reported most frequently in Caucasia studies, and in addition, the frequency of monoallelic variants, however, was significantly higher in our study group (11.7% compared with that of 3.9% as reported by Sieber et al. (2003)), therefore, those outcomes may

reflect ethnic and geographic differences between the populations studied.

Based on the assumption above, we found that our investigation are similar to some other Asian studies, especially the east Asian ones. It was confirmed that the variant, Intron 10-2 A→G, led to a splicing abnormality that resulted in the production of an aberrant mRNA transcript encoding a truncated *MYH* protein. Contrast with the wild-type *MYH* protein, immunofluorescence analysis revealed that the variant protein was localized in the cytoplasm, not in the nucleus, indicating that the ability of the variant protein to repair nuclear DNA was impaired (Tao et al., 2004). In this study the remaining wild-type allele by somatic variation or LOH was not detected in the CRC patients with the Intron 10-2 A→G genotype, and the distribution of the Intron 10-2 A→G variants significantly differ between the cases and controls. The heterozygote for the Intron 10-2 A→G variant was found in 21 CRC cases, and in 7 non-cancer controls who were post-adolescents with mean age of 36.2±5.7 years, which implied that the 7 normal carriers would probably suffer from CRC someday. These results indicate that 8-OG repair activity may impact Chinese individuals due to Intron 10-2 A→G variant, and that the heterozygote for the Intron 10-2 A→G may be responsible for the occurrence of CRC.

The Intron 10-2 A→G variant was detected at a frequency of 1.2% in controls in the Chinese CRC case-control study. As documented in the Japanese research, the truncated *MYH* protein as a result of the Intron 10-2 A→G variant lacks an nuclear localization signal (NLS) and binding sites for apurinic/apyrimidinic endonuclease1 (APE1) and PCNA proteins. The NLS is possible for the necessity of the repair function of *MYH* protein for nuclear DNA. Additionally, the APE1 protein is required for the BER pathways, and the PCNA protein serves on a molecular adaptor integrating and adjusting the proceedings of DNA replication, DNA repair and cell cycle control (Kelman et al., 1998; Krokan et al., 2000). According to the previous study, the Intron 10-2 A→G variant abridged *MYH* protein had been experimentally shown to localized in the cytoplasm, which fits in with the loss of NLS. This suggested that the Intron 10-2 A→G variant damaged the BER function of the *MYH* protein for 8-OG in the chromosomal DNA in the nucleus. A low level of 8-OG repair capacity in the cells with the Intron 10-2 A→G variant may result in an increase in their variation rate, and the effect would be essential in organs exposed to severe oxidative stress, such as the colorectum. Therefore, a definite association between this variant and CRC risk was detected in our series, which corroborated the involvement of the *MYH* gene in human carcinogenesis.

In present study, both of the variant p. V463E and the linked variant at Exon 1 region are the newly found variations. Interestingly, as these variants have not been reported in previous papers and have not been registered in the Entrez SNP homepage of the NCBI web site, they are novel SNPs. The p. V463E (Exon 14+74 T→A) was identified at the region of Exon 14 in 6 patients and 1 normal control, and the mean age of 6 cases was 59.3±6.1years while the only only control was a 31-year-old female. Consequently, in this variation, a electroneutral valine was replaced by a electropositive glutamic, which might be a functional change of *MYH* protein due to the transformation of molecular polarity and structure, and then cancer progression is under way. Eventually, it is assumed that the young woman would suffer from CRC when she is growing old. The linked variant in Exon 1 region is also a brand new variation that contains 3 variation site, Exon 1-316 G→A, Exon 1-292 G→A and Intron 1+11 C→T, was found in 13 CRC patients and 2 normal donors. Surprisingly, the age difference of the carriers between the two groups is similar to the variants aforementioned. The molecular mechanism of the above two variants in producing cancerization will be investigated in our follow-up study. However, the other two variants, Intron 13+12 C→T and 1678-80 del GTT, did not significantly differ between the patients and normal blood donors, although they were the novel findings too. In brief, our findings are similar to Asian studies, especially those east Asian ones, which implied the ethnic difference in *MYH* gene.

To clarify further, a strong dominance component of *MYH* variation may confer CRC risk because of the similarity of monoallelic and biallelic *MYH* variation carriers. Cumulatively, monoallelic *MYH* variations may also be the CRC risk factors. It appears to be one the most frequent major predispositions to CRC. The present study corroborates recent reports highlighting the increased risk of CRC in monoallelic *MYH* gene carriers (Croituru et al., 2004; Jenkins et al., 2006), suggesting an autosomal dominant mode of inheritance. It is recommended that taking *MYH* identification as a health screening procedure should be a proposed as a strategy of preventing CRC. Furthermore, monoallelic as well as biallelic variation *MYH* carriers are equally entitled to participate in screening procedures.

From the above, the linked variant in Exon 1 region, Intron 10-2 A→G of base replacement and p. V463E of missense variant, the 3 heterozygosity variants of *MYH* gene in a Chinese population, may relate to the susceptibility to sporadic CRC. Lack of the hot-spot variants of Caucasians in present study may due to the ethnic difference in *MYH* gene.

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