Author's response to reviews

Title: A haplotype variation affecting the mitochondrial transportation of hMYH protein could be a risk factor for colorectal cancer in Chinese

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Author's response to reviews: see over
Dear Editor,

We are submitting a revised manuscript for the consideration of publication in the BMC Cancer.

In the revised version, we have added details relating to the population of patients and controls. As suggested by the reviewers, the manuscript has been greatly modified and the conclusions have been thoroughly rephrased following the presented data. The revised vision is formatted with EndNote X1. In addition, we asked a scientist who has spent the last 20 years studying and working in major American university and research institutes for English editing and improvement.

I would like to take this opportunity to express my great appreciation to the editor and reviewers for their helpful and constructive comments. We believe the quality of the revised manuscript has been significantly improved and would like to ask the editor for consideration of publication. We understand that the processing charge will be paid in the required time, if the manuscript is kindly accepted.

The followings are point by point response to the reviewers’ comments and suggestions.

Sincerely yours,
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Response to reviewer Pavel Vodicka:

1. The manuscript by Chen et al. represents a potentially interesting contribution, consisting of two unequal parts: an experimental one, being stronger and a case-control study, posing substantial weaknesses. Given the number of investigated individuals, the study should be considered as a preliminary, pilot one. Additionally, there are several conclusions and/or summaries, which are not based on the generated data or the literature.
We appreciate the encouraging comments. The revision has been modified according to the following detail comments. All the minor points about editing mistakes and language problems have been correctly revised. We thank this reviewer for spending previous time to help us making this manuscript more presentable.

2. Abstract:

1) The abstract generally needs a language correction (e.g. line 10 from the top-sentence is missing the verb). A revision of the Methods part is necessary as the reader gets the impression that it is a simple case-control study, while at the end there is also a functional study on cell cultures.

Thank you very much for your suggestions. We have added the detail information of the functional study on cell cultures in the Method of Abstract. (Page 2)

2) The population included in the case-control study is not sufficient for this kind of study. Such a high OR and huge confidence intervals are doubtful considering the low frequency of variant alleles found.

We are very sorry for a mistake in the primary manuscript. In fact, there were 343 healthy subjects enrolled as controls in this study. The OR and confidence intervals were calculated between 138 cases and 343 controls. (Page 21 Table 3, which is as same as the primary Table 2) Unfortunately, “343” was mistaken as “243” in writing of the main text. It has been corrected in the whole revision. (Page 2, the method of abstract; Page 6, line 3; Page 7, the last line; Page 8, line 9 of the Results; et al)

3) The conclusions in the abstract are too strong and a bit speculative in light of the results.

According to the comments, the conclusion of the study has been modified based on the results. These results suggest that: the haplotype variant allele of hMYH leads to a missense protein, which is partly affected by mitochondrial transportation and shows nuclear localization, and might be responsible for the increased susceptibility to develop cancer in Chinese, including CRC.
The hypothesis of the relationship between the haplotype variant and impaired repair function in mtDNA oxidative damage has been deleted. *(Page 3, the conclusion of abstract)*

3. Background part:

1) There are several editing mistakes and language problems which require appropriate revision for English (e.g. reference formatting, missing verb „been“ in line 15, extensively instead of greatly, „which is“ may be omitted on p. 5, line 6, etc.).

   Thank you very much for your help. The references have been formatted using EndNote X1. In addition, we try to correct all the language problems under the aid of a scientist who has spent the last 20 years studying and working in major American university and research institutes.

2) In general, there is not a proper use of the term "haplotype".

   A haplotype has been defined as a set of closely linked genetic markers, which present on one chromosome and tend to be inherited together. Haplotype may refer to as few as two loci or to an entire chromosome depending on the number of recombination events that have occurred between a given set of loci. Sometimes, the genetic markers are located in one gene.

   The two base pair substitutions, identified in our previous study, occurred at a same allele and could not been separable by recombination. Therefore, although “haplotype” in this manuscript is a bit of difference from its common definition, we think it is acceptable to define the variation.

3) At the end of the first paragraph it is stated that "the biallelic germline mutations .......lead to the autosomal recessive colorectal adenomatous polyposis and cancer": this statement is not completely true because the mutations are not causative of CRC, they create a situation in the affected individual (extreme high number of polyps) which increases the susceptibility to develop CRC.

   Thank you very much for your suggestion. It is recently discovered that the germline mutations of the human MutY homolog (*hMYH*) increase the susceptibility to develop colorectal cancers (CRC) associated with adenomatous polyposis. *(Page 4, the line 8, 9, 10 and 11)*
4) The previous study cited (ref 10) is not correctly described in the text. Same problems may be observed for other studies (ref 11 and 12). It is not clear what the authors wanted to demonstrate by quoting ref. 12. The connection between sporadic colorectal cancer (object of the present study) and FAP is not sufficiently clarified.

We have reviewed the three papers once more carefully before modification. The studied published suggested that the two base mutations might specially occur in East Asian region rather than in Western countries. It is the reason why we cite the three papers in the manuscript. *(Page 4, the second paragraph)*

Actually, it has been well studied that the individual affected FAP (extreme high number of polyps) has high risk for developing CRC. In this manuscript, we do not discuss the relationship between sporadic CRC and FAP, whereas we illustrate the potential association between MYH variants and susceptibility to the diseases. Then, we explore the effect of the haplotype variant on Chinese population and the underlying mechanism.

5) In the third paragraph it is assumed the same pathogenesis for both colon and rectal cancer, but there is scarce evidence for this.

Although there is obvious difference between the two kinds of cancers, colon cancer and rectal cancer have many features in common. Then, they are often referred to together as “colorectal cancer,” and in some sections, discussed together. The distribution of CRC was categorized into three segments: the proximal colon (cecum, ascending colon, hepatic flexure, transverse colon), the distal colon (splenic flexure, descending colon, sigmoid colon), and the rectum above the anal canal.

On the other hand, a series of studies have investigated the genetic factors associated with colon cancer and rectal cancer. In some studies, the two kinds of cancers have been considered as one object, colorectal cancer. Therefore, we illustrated the genetic basis for sporadic colorectal cancers in the present study. The detail has been discussed in the revision. *(Page 10, the last paragraph)*
6) First sentence of the 4th paragraph (In general, mutations...) has no meaning, please reshape it. Page 5, lines 6 and 7: I wonder whether the authors did not rather mean genome „stability” instead of „instability“. The sentence „Increasing evidences have suggested that…” is rather vague and needs focusing. Last sentence on page 5: the aim of the study is not correctly clarified. There is an apparent overinterpretation, as a multistage process of colorectal cancer development was not investigated in relation to hMYH gene.

According to the reviewer’s suggestion, the background has been greatly modified in the revision. (Page 5 and 6) The detail evidences of the effect of mitochondrial oxidative damage on human diseases have been added. (Page 4, the last paragraph) The purpose of the study is to investigate whether the haplotype variation of hMYH gene is associated with the susceptibility to colorectal cancer and underlying mechanism. Thank you very much for help us making the manuscript more presentable.

4. Methods part:

1) Obviously, the weakest part is the population characteristic, which lacks important information (e.g. age, sex, life-style, tumor localization or fundamental clinical data). I assume that the group of CRC patients (by the way, how the sporadicity was ensured?) and controls (not characterized at all) are insufficient in size for this kind of studies. There is a high risk of by-chance findings and bias!

A total of 138 Chinese patients with sporadic CRC had been enrolled from Jiangsu, China. CRC was diagnosed by histopathological examination using established clinical criteria and the clinical stage was evaluated on the basis of the TNM classification system of the UICC. The distribution of CRC was categorized into three segments: the proximal colon (cecum, ascending colon, hepatic flexure, transverse colon), the distal colon (splenic flexure, descending colon, sigmoid colon), and the rectum above the anal canal. We had confirmed that these patients were not familial cases after the structured assessment including documentation of family history. The demographic features and clinical manifestations of two groups were added in Table 1.

We have explained the error that “343” was mistaken as “243” in the primary manuscript. All 343 healthy individuals without any apparent cancer phenotype or history in the same geographic origin were taken as the control group. We did not observe any characteristic difference in association between the cases and controls. (Table 1)
2) Page 7, line 9: If the authors set up above methodology, it has to be provided by details. Otherwise a proper reference should be included.

According to the reviewer’s suggestion, the paper by Ho et al. has been cited in the revision, which described the detail information of the methodology about overlapping PCR site-directed mutagenesis. *(Page 7, the first paragraph)*

3) Authors should have provided some details on the strenght of the statistics, carried out on 138 cases...

In the study, the SPSS 11.0 program was used to conduct the statistical analysis. For testing of significance of differences between 138 CRC patients and 343 healthy controls, nonparametrical Mann–Whitney Utest (unpaired) was applied. The observed genotype frequencies were compared with a chi-square ‘goodness-of-fit’ test to determine whether they were in Hardy–Weinberg equilibrium. The Fisher’s exact tests were used to assess the genotype and allele distribution between two groups. The genotypic-specific risks were estimated as odds ratio (OR) with associated 95% confidence intervals (CI) by unconditional logistic regression and the ORs were adjusted for age and sex. For the statistical calculations, wild-type genotypes were assigned as “0” and heterozygous variant genotypes as “1”. A \( P \) value < 0.05 was considered statistically significant. *(Page 7, the last paragraph)*

4) In this part there are few editing mistakes, such as: page 7, line 1 „nucleartide“, line 2 „preformed“.

Thank you very much for your help. The correct spelling should be “nucleotide” and “performed”. All the mistakes in the Method have been revised. We are very sorry for the previous errors.

5. Results part:

1) First paragraph: It is not clear, what kind of DNA was used for sequencing.

Firstly, the PCR products were sequenced directly, if an abnormal DHPLC peak was detected in the samples. It was showed in Figure 1B. Secondly, the repeated PCR products would be cloned into the PMD-18-T vector (Takara), and then amplified in *E. Coli* top10 for further sequencing analysis, if two variations were detected in the direct sequencing. Thirdly,
ten randomly picked clones for each sample were subjected to sequence. It was showed in Figure 1C. *(Page 8, the second paragraph)*

2) The population of controls was not tested for the Hardy-Weinberg equilibrium.

Thank you very much for your suggestion. The allele and genotype frequencies among controls were consistent with the Hardy-Weinberg equilibrium ($P>0.05$). The detail information has been added in the revision. *(Page 8, the third paragraph)*

3) The variant allele frequencies represent very weak point in relation to the population size! CI ranging from 1 to 20 suggests high degree of randomness. The heterozygous variant configuration was discovered in about 4% of cases and 2% of controls (i.e. in 6 and 2 persons, respectively!), but none bore homozygous variant configuration! Considering such frequency, one wonders about the relevance for sporadic CRC.

We are very sorry for the confusion. In fact, there are 138 CRC patients and 343 healthy controls enrolled in the study. The “343” was mistaken as “243” in writing in the previous manuscript. The error has been explained and corrected in the revision.

But, the previous table 2 is correct. *(Page 21, Table 3)* It illustrated that the heterozygous variant configuration was discovered in about 4.35% of cases and 0.87% of controls. The frequency the variant allele was 2.17% in cases and 0.44% in controls. The allele frequency of this haplotype variation detected in CRC patients is significantly higher than that in healthy individuals ($P=0.020$). Therefore, these results allow us to rationally make a proposition that the variation is associated with sporadic CRC.

4) Surprisingly, out of 6 cases with the heterozygous variant configuration, 5 suffered from rectal cancer! Do the authors think that there is a same etiology for colon and rectal cancer? No hint about it was found in Discussion.

In this study, the percentage of cancer was 11.6% in the proximal colon, 31.9% in the distal colon, and 56.5% in the rectum, as showed in table 1. *(Page 19)* The genotype of heterozygous haplotype T/A variation was not associated with any clinical characteristics presented by CRC patients ($P>0.05$). And, 5/6 patients carrying this variation had a cancer at rectum, while 1/6 cases had a cancer at proximal colon.

Actually, there is obvious difference between the two kinds of cancers. However, the colon cancer and rectal cancer have many features in common. Then, they are often referred to
together as “colorectal cancer;” and in some sections, discussed together. A series of studies have investigated the genetic factors associated with colon cancer and rectal cancer. In some studies, the two cancers have been considered as one object, colorectal cancer (CRC). The discussion about it has been added in the revision. *(Page 10, the last paragraph)*

5) The last sentence of this section: apparently an exaggeration, as the excision repair function has not directly been investigated.

Thank you very much for your suggestion. As the excision repair ability of oh8G has not been directly investigated in the study, the results show that the effect of the variant is narrowed on the protein transfer. This section has been modified. *(Page 10, the first paragraph)*

6) Random examples for language/editing problems: page 9, line 3 „exon 2“, page 9, line 8: „243 healthy controls“, page 9, line 9: „heterozygous“, page 9, line 19: „resulting in“, page 9, line 20: „was just“, page 9, line 23: „affected its“, page 10, line 6: „results previously“, page 10, line 8: „rather present both in“.

All the minor points have been correctly revised: Page 8, line 12 “exon 2”, line 18 “343 healthy controls”, line 20 “heterozygous”, Page 9 line 8 “resulting in”, line 9 “was just”, line 12 “affected its”, line 20 “results previously”, line 23 “rather presented both in”. We thank this reviewer very much for helping us modified the paper more presentable.

6. Discussion section:

1) First paragraph: It should be smoothed down. On the basis of the given results, I doubt whether the authors may claim the role of hMYH variant in CR carcinogenesis. The discussion of the data (and their strength) on Chinese patients and controls is not sufficient.

According to this comment, the first paragraph of the discussion in the revision has been greatly modified. The prevalence of the variant allele has been highlighted. It suggested a correlation between such hMYH variation and sporadic CRC susceptibility in Chinese. *(Page 11, the first paragraph)* Actually, we could not formally exclude the possibility that selection bias contributed to the observed differences between the two groups, because of the low frequency of variant alleles found. The potential limitation has been added to the discussion and further studies are processing. *(Page 11, the first paragraph)*
On the other hand, we have added the section regarding the relationship between colon cancer and rectal cancer. Although the issue whether colon and rectal cancer should be considered as a single or two distinct entities is still debated, cancers of the colon and rectum were discussed together in this study, because they share many features and show little different in our results. (Page 10, the last paragraph)

2) It is not clear for the reader, what is the link between ROS in mitochondria and colorectal carcinogenesis. The same applies for the last sentence on page 13. At least more moderate expressions should be used.

According to this comments, the link between ROS in mitochondria and colorectal carcinogenesis has been interpreted in the last paragraph in the Discussion. (Page 12, the last paragraph) Mitochondrial oxidative energy metabolism is the major intracellular source of ROS and mitochondrial biomolecules including mitochondrial DNA are constantly exposed to a high ROS. Almost all the studies performed to date have found increases in oxidative damage in mitochondrial DNA and it is consistent with the observation that mitochondrial DNA mutations accumulate. Mitochondrial DNA mutations have been reported in 10% to 70% of CRC and the presence of tumour mitochondrial DNA mutations seems to be a prognostic marker and a relevant predictive factor of CRC.

On the other hand, mitochondrial DNA repair enzymes involved in BER system play an important role in mitochondrial genome stability and hMYH protein is the requisite enzymes in this system. Thus, if the haplotype variant allele increased risk for mitochondrial genome instability, we would suggest that oxidative mitochondrial DNA damage might be responsible for the carcinogenesis, including CRC.

3) Page 12, references should be cited: e.g. sentences ending on lines 10, 14 and 19.

Language editing: page 11, line 20: „have shown“, page 12, line 16: „influences“.

Thank you very much for your help. The minor points have been corrected: Page 11, line 19 “have shown” and page 12 line 15 “influences”. The proper references have been added in this section of the revision, including ref. 36 and ref. 38. (Page 12, the second paragraph)

7. Conclusions:

They would require thorough rephrasing, as the presented data do not allow such conclusions. „Great“ contribution to colorectal carcinogenesis is based on the
heterozygous variants, occurring in about 4% of (sporadic?) colorectal cases. What about the 96% of cases? By the way, the study on cell lines showed the effect of homozygous variant configuration, but these carriers have not been identified either in cases or in controls.

The conclusions in the revision have been greatly modified on the base of the results in the revision. The hMYH variation affecting protein transportation is likely to be associated with cancer susceptibility. A portion of hMYH protein arising from the haplotype allele was not able be transported into mitochondria, while the variation could be responsible for the increased risk for the development of CRC.

The potential limitations have also been pointed out: 1) these data from a small-sized patient population are still preliminary; and 2) it has not been directly identified the activation of adenine glycosylase in mitochondria and mitochondrial DNA stability. Thus, validation of these results on larger cohorts and further functional studies are needed.

8. Tables section:

1) It is missing a table describing the population included in the study (number of cases and controls, distribution for sex, age average etc).

According to this suggestion, Table 1 has been added in the revision. A total of 138 colorectal cancer patients (85 men and 53 women, mean age 59.6±13.6 years) and 343 healthy controls (207 men and 136 women, mean age 57.9±14.5 years) were screened in the present study. We did not observe any characteristic difference in association between the cases and controls. (Page 19)

2) Table 2 presents some editing errors such as: „wild-type genotype“, „wild-type allele“, „variant allele“.

We are very sorry for the mistakes. All the errors have been corrected. We thank the review very much for helping us preparing the revision. (Page 21)

Response to reviewer Haruhiko Sugimura:
1. Although the numbers of the cases are modest, this functional study provides important information on Asian colorectal cancer genetic predisposition. The readers would expect the author address any perspective on the possibility of somatic G > T transversion in tumors occurring in these particular genotype people.

   Actually, somatic G > T transversion in tumors is the important evidence of impact of the variation on MYH gene. In this study, we tried to illustrate a variant allele related to oxidative mitochondrial DNA damage. As mutant and wild DNA coexist in mitochondria, it is difficult to detect the significant G > T transversion in these particular genotype people. Further model is processing to directly investigate the excision repair function of the variation.

2. Minor comments: 1) In the abstract, result section line 2 ration > ratio; page 7, immunofluorescence analysis, line 3,6; The author list of Ref11 is wrong. Correctly, Shinmura K, Yamaguchi S, Saitoh T, Takenoshita S, Kuwano H. Yokota J

   We are very sorry about it. We have corrected all the errors and checked the revision carefully. (Page 2, line 19; Page 15, Ref10) I hope the revision will be correctly in your system.