Author's response to reviews

Title: Association of Estrogen Receptor beta variants and Serum Levels of Estradiol with Risk of Colorectal Cancer: a Case Control Study

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Version: 2 Date: 7 May 2012

Author's response to reviews: see over
Dear Dr. Bapat,

Thank you and the reviewers for the careful consideration of our manuscript. We have answered the questions/concerns raised by the reviewers, point by point below and have indicated the edits in the text of the manuscript with underlining.

Reviewers: 1

The authors investigated associations of (i) circulating estradiol levels and (ii) ESR2 SNPs rs1256049 and rs4986938 with colorectal cancer risk for Chinese men using a hospital-based case-control study.

Major Materials
- For cases, what were the selection criteria? Age limits? Selected depending on a family history of colorectal cancer?

We set up an age limit of 15 years old and above. We excluded both cases and controls with a family history of colorectal cancer. We edited the manuscript to describe the selection criteria for cases and controls as following.

[Material and methods] The exclusion criteria for both cases and controls included 1) age less than 15 years at the time of diagnosis (cases) or recruitment (control); 2) previous history of malignancies; 3) history of inflammatory bowel disease including familial adenomatous polyposis, ulcerative colitis, and Crohn’s disease; 4) history of chronic hepatic, renal and endocrine disease; 5) family history of CRC; 6) currently taking medication which was known to influence sex hormone level; and 7) diagnosed with hypertension and diabetes mellitus, which could affect sex hormone level.

- For controls, were they frequency- or individual-matched with cases by age or else?

We did not set up match criteria. Controls were selected randomly during the same period.

Methods
- The authors calculated odds ratios for dominant models (CT/TT vs CC).

We performed genotype association analysis under several inheritance models according to the reviewer’s comments. For both SNPs, no significant results were found under codominant model. To make the manuscript more concise, we choose not to present the results in detail but mention the non-significance in the results section, as following. If the reviewer feels it is better to add the results, we would edit the manuscript accordingly.

[Results] No significant association was found for codominant model (data not shown).

- Can you present per allele association (log additive model) for each SNP? i.e. CC=0, CT=1 and TT=2.

The results of risk estimates under log-additive model were added to the results section according to the reviewer’s comments, as follows.
**Results** Similarly, under log-additive model, risk estimates were 0.8 (95% CI, 0.6–1.0) for rs1256049 and 1.5 (95% CI, 1.0–2.0) for rs4986938.

- *Can you present association between total number of risk allele and colorectal cancer risk (per allele association) for combined two SNPs? i.e. risk allele number will range 0-4.*

The result of risk estimate of the association between number of risk alleles and colorectal cancer risk was added to the results section according to the reviewer’s comments.

**Results** Additionally, we evaluated the combined effect of risk alleles of rs1256049 and rs4986938 and found increased risk for CRC associated with cumulative number of risk alleles (OR, 1.3, 95% CI, 1.1–1.6, P, 0.003).

- *Can you present ORs for colon and rectal cancers separately given the authors stated a previous study found that rs1256049 was only associated with rectal cancer?*

In our case populations, majority patients were diagnosed with colon cancer. We did not find significant difference in genotype distribution of the investigated SNPs between patients with colon cancer and rectal cancer. Due to limited sample size and to keep the manuscript concisely, we chose not to present the results of association analysis according to the cancer site. If the reviewer feels it is better to add the results, we would edit the manuscript accordingly.

**Results** - *Page 8: the authors presented the adjusted OR for serum estradiol. Can you please clarify what this OR represented? Per xx pg/ml or High vs Low level (cut-off)?*

The adjusted OR was calculated using logistic regression model in which serum estradiol was taken as a continuous variable. To avoid the confusion, we edited the results section as following.

**Results** Serum estradiol levels were significantly associated with increased risk of CRC in men after adjustment for age; the adjusted OR for serum estradiol levels (continuous) was 1.2 (95% CI, 1.0–1.3, P, 0.009).

- *The authors used the term “adjusted P”. What does it mean? I assumed adjusted OR rather than adjusted P.*

The term was edited according to the reviewer’s comments as follows.

**Results** Serum estradiol levels were significantly associated with increased risk of CRC in men after adjustment for age; the adjusted OR for serum estradiol levels (continuous) was 1.2 (95% CI, 1.0–1.3, P, 0.009).

**Results** The protective effect of rs1256049 CT/TT genotypes on CRC was likely confined to old subjects, ever-smokers and ever-drinkers: rs1256049 CT/TT genotypes were associated with significantly reduced risk of CRC in >48 years old subjects (OR, 0.7, 95% CI, 0.5–1.0, P, 0.047), in ever-smokers (OR, 0.5, 95% CI, 0.4–0.8, P, 0.005), and in ever-drinkers (OR, 0.6, 95% CI, 0.4–1.0, P, 0.038).

**Results** For rs4986938, the ORs for CT/TT genotypes compared to CC genotype were significant for never-smokers only (OR, 2.2, 95% CI, 1.3–4.0, P, 0.005).

**Discussion**
- Page 10: The authors stated that “....the C allele of rs1256049 conferred an increased risk of rectal cancer....” but they stated in introduction (page 4) “....the T allele of rs1256049 confers increased risk of rectal cancer”. What is the correct one for risk allele of that SNP? The authors should use the same reference allele in their analyses for comparison.

We thank the reviewer to point out this typo. We revisit the original article and confirmed that the C allele of rs1256049 conferred an increased risk of rectal cancer in younger population and corrected the manuscript accordingly. To avoid possible typo in other parts, we reviewed the whole manuscript carefully.

- The authors should refer a previous finding of association between rs1256049 and colorectal cancer risk in Japanese population.

We added the finding from the Japanese study to our discussion as following.

[Discussion] A study in Japanese population, however, found an increased risk of CRC linked with rs1256049 TT genotype.

Minor
- Use “multivariable” instead of “multivariate” analysis.

We edited the manuscript according to the reviewer’s suggestion.

- Table 2: For rs1256049+rs4986938, the authors stated 0, 1, 2 risk genotype....Please use “allele” instead of “genotype”.

We grouped the study subjects according to the number of risk genotypes, which were determined on the basis of the risk estimates in Table 2. In this case, the risk genotypes were rs1256049-CC genotype and rs4986938-CT/TT genotypes. To avoid the confusion, we edited the results section as following.

[Results] we categorized all subjects into 3 groups based on the number of risk genotypes which were determined on the basis of the risk estimates in Table 2. The risk genotypes were rs1256049 CC genotype and rs4986938 CT/TT genotypes.

Reviewers: 2
The authors of this study intend to test their hypothesis that endogenous estrogen and ESR2 genetic variation are associated with colorectal cancer (CRC) through a case-control study in a Chinese male population. I read this manuscript with great interest. Overall, this work is of good quality with respect to study design, execution, and manuscript preparation.

Thank you for the complement.

General comments:
While the authors argued their justification for their male-only decision, I feel that had females been included, the overall quality and importance of this study would have been strengthened. For instance, possible differential effects (interaction) of estrogen between two sexes could be investigated. Likewise for endogenous and exogenous estrogen. Hope the authors will be able to address issues in their future research.
As noted by the reviewer, because the correlation between endogenous estrogen and the risk of colorectal cancer in men has not yet been evaluated, we aimed to evaluate the role of estrogen level and ESR2 polymorphisms in colorectal cancer risk. But we agree with the reviewer’s comments that the interaction of estrogen between male and female is worth further investigation and we are considering conducting such a research in future.

Major Compulsory: More detailed descriptions on how cases and controls were recruited are needed. This information will help readers understand how cases and controls represent their corresponding target population (CRC and non-CRC patients in general), for example, inclusion/exclusion criteria and response rates.

We agree with the reviewer’s comments and added the following exclusion criteria to the manuscript and describe the response rate accordingly.

**[Material and methods]** The exclusion criteria for both cases and controls included 1) age less than 15 years at the time of diagnosis (cases) or recruitment (control); 2) previous history of malignancies; 3) history of inflammatory bowel disease including familial adenomatous polyposis, ulcerative colitis, and Crohn’s disease; 4) history of chronic hepatic, renal and endocrine disease; 5) family history of CRC; 6) currently taking medication which was known to influence sex hormone level, and 7) diagnosed with hypertension and diabetes mellitus, which could affect sex hormone level.

**[Material and methods]** Eligible participants were interviewed in person to collect demographic and exposure information including smoking and alcohol drinking status (response rate 98%).

Similarly, why this particular hospital was chosen.

In this study, patients were recruited from two hospitals, Tongji Hospital and Wuhan Eighth Hospital. The former is one of the top-ranking hospitals in China for cancer treatment including CRC with majority of patients from center China region, and the latter specialize in treatment of colorectal disease. These two hospitals were chosen because they are the regional reference center for colorectal tumor. To clarify this point, we edited the manuscript as following.

**[Material and methods]** These two hospitals were chosen because they are the regional reference center for CRC treatment.

The authors stated that controls were volunteers from Tongji Hospital Physical Center during. Were they patients of other health problems? There was very limited information on controls.

All the controls were randomly selected from the health examination clinic of Tongji hospital physical cancer. The controls received a comprehensive health examination and those who had colorectal cancer or other cancers, gastrointestinal pain, inflammatory bowel disease like familial adenomatous polyposis, ulcerative colitis, or Crohn’s disease, detection of blood in stool, or currently diagnosed with tuberculosis, AIDS (acquired immune deficiency syndrome) or other communicable diseases were excluded from this study. We edited the manuscript to describe the recruitment criteria for controls as following.

**[Material and methods]** The exclusion criteria for both cases and controls included 1) age less than 15 years at the time of diagnosis (cases) or recruitment (control); 2) previous history of malignancies; 3) history of inflammatory bowel disease including familial adenomatous polyposis, ulcerative colitis, and Crohn’s disease; 4) history of chronic hepatic, renal and endocrine disease; 5) family history of CRC; 6) currently taking medication which was known to influence sex hormone level, and 7) diagnosed with hypertension and diabetes mellitus, which could affect sex hormone level. In addition, those who had...
gastrointestinal pain, detection of blood in stool, or currently diagnosed with communicable diseases such as tuberculosis and acquired immune deficiency syndrome were excluded from the control group.

Discretionary:
1. More information should be provided in Table 1, for example, BMI, level of education, mean age with s.e., which, I believe, was collected.

We do not have complete information on BMI and level of education in the current study. These two variables, however, may not be significant confounders for the results of association between estradiol level, ESR2 polymorphisms and CRC risk. We added the information of mean age of study subjects to the manuscripts as following.

[Results] The average age (SD) at recruitment was 50.0 (9.4) years (median, 48 years) in the healthy controls.

2. Age 48-years was used as a cut-point, which is very un-conventional. Why was this particular cut-point being used?

Cut-off of the age of 48 years old was based on median age among controls. To avoid the confusion, we added the following description to the statistical analysis part.

[Material and methods] Cut-off of age was based on the median age among controls.

3. Berkson bias is well known when hospital based controls were used and may need to be discussed.

Our control base is healthy individuals who went for preventive health examination and met the exclusion criteria. We agree with the reviewer’s comment that Berkson bias could not be excluded but the possibility is not very big. We edited the discussion to present this potential limitation as following.

[Discussion] Using hospital-based controls could generate Berkson bias which might influence the frequencies of ESR2 genotypes and the susceptibility to CRC risk.

4. I noticed that only 36.4% controls were ever smokers, which seems to be lower than the general male population in China or Wuha. It is likely that the protective effect of smoking on CRC could be explained by bias. I think it is worth discussing.

As shown in table 1, in our study, 63.6% of controls were ever smokers, which is comparable to the smoking rate in the Han Chinese male population in China (59.5%) [WHO Report on the Global Tobacco Epidemic 2008]. But we agree with reviewer’s comments that a possibility of selection bias may be present and we should be in caution in interoperating the results. We edited the manuscript accordingly as following.

[Discussion] Finally, due to the nature of hospital-based case-control study design, a potential selection bias should be taken into consideration when interpreting the results. One of the reasons for the difference between cases and controls in smoking status could be the selection bias.

5. As an epidemiologist (limited knowledge in basic science), I would like to ask the authors the possibility that high Serum estradiol concentrations in cases is result of CRC rather than the cause.
Estradiol is produced as an active metabolic product of testosterone in men. The possibility that high serum estradiol concentration is the consequence of CR instead of etiological factor of CRC is low because: 1) there is no in vitro study indicating that change in estrodiol level is only due to occurrence of CRC; 2) As described in the Discussion section, epidemiological studies found significant association between endogenous estrogen and CRC risk; the association is biologically plausible for several in vitro studies have shown that estrogen may have mitogenic and tumorigenic effects on colorectal cells; 3) although increased in a sequence of normal colonic mucosa-adenoma-carcinoma, strong expression of estrogen receptor was observed in both adenoma and carcinoma, suggest that the estrogens may play a role in colorectal carcinogenesis, not just an late event in colorectal cancer development.

Once again, thank you and the reviewers for the consideration of and careful review of our work. We feel the edits have substantially improved the work, and that it is a significant contribution to the literature and more specifically an interest to your readers. If we can answer any additional questions, please feel free to contact me.

Sincerely,

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