Author’s response to reviews

Title: NBS1 rs2735383 polymorphism is associated with an increased risk of laryngeal carcinoma

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Author’s response to reviews:

Dear Editors and Reviewers:

Thank you for your letter and for the reviewers’ comments concerning our manuscript entitled “NBS1 rs2735383 polymorphism is associated with an increased risk of laryngeal carcinoma” (ID: BCAN-D-17-00095).

The comments are all valuable and helpful for us to revise and improve our manuscript. We have studied these comments carefully and have made modifications and corrections accordingly. Here we provide our responses, point by point, to the comments as listed below and highlighted the changes we have made in the revised manuscript.

We hope that the manuscript has been revised satisfactorily and will be acceptable for publication.

With best wishes,

Yours sincerely,
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Responds to the reviewer’s comments:
Response to Reviewer #1 (Ilhan Yaylim)

Comment 1. Dear Authors, in the present study, they studied a case-control study including 342 cases and 345 controls to detect the associations between two polymorphisms of NBS1 and the risk of laryngeal carcinoma. This study is a good sample for the risk for laryngeal carcinoma. They used the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to determine the genotypes of the functional SNPs in NBS1 gene. According to their results, rs2735383CC and variant genotypes (GC+CC) were significantly associated with an increased risk of laryngeal carcinoma. They used proper methods and statistical evaluation. For this study, although sample size is well, there is a weak side for obtaining some pathological or clinical data of patients. This might have been very valuable data for the study. This study is an original article and may be published in your valuable journal.

Response: Thanks for the reviewer’s encouraging and positive comments. Indeed, there was a considerable portion of patients with unknown differentiation level at diagnosis, of which the data were failure to obtain. Meanwhile, other clinical feathers were also lacked. These limited our analysis on associations between NBS1 variants and clinical feathers of laryngeal carcinoma. However, because the current study mainly focused on the relationship between NBS1 polymorphisms and laryngeal carcinoma susceptibility, and these patients were all histopathologically confirmed, this weak would not affect the validity of above association. We have described this weakness in the revision as a limitation of current study as follows: “Moreover, a considerable portion of patients was short of differentiation level at diagnosis, and other data such as clinical stages were lacked, these limited our analysis on association between NBS1 variants and clinical feather.” (Discussion section, lines 5-7, page 14)
2. Response to comment (Reviewer 2): Although Hayriye Arzu ERGEN has not given us any suggestion, we still expressed our sincere thanks to him.

3. Response to comments (Reviewer 3):

1) The manuscript should be generally improved in terms of bad English. There are lots of language errors throughout the whole paper.

Response: Thank you for your suggestions. We have asked some professional to help with scientific editing. The language errors throughout the whole paper has been corrected and marked in red in the paper.

2) There are ambiguities concerning studied population: in the Study subjects section the authors wrote: "we only recruited people whose ethnicity is unrelated Han or Zhuang", while from Table 1 we learn that only Han and Zhuang patients were included to the study group. Another term is used at page no. 4 (Chinese population of Guangxi of China). It should be unified.

Response: Sorry for this omission. Only Han and Zhuang Chinese were recruited in the current study. We have unified the term and marked in red in the paper. (Background section, line 4, page 5 and Study subjects section, line 19, page 5)

3) One system concerning describing PCR mixture components should be accepted (page 6, line 15-17) - i.e. 5 mM MgCl2, 25 µl and so on... - there should be a space between the quantity and its measure.

Response: Thank you for your suggestions. We have revised and marked in red in the paper. (Genotyping analysis section, line 22, page 6 and lines 1-12, page 7)

4) There are numerous discrepancies concerning cited literature references, that has to be clarified. In this layout it does not generally support the selection of both polymorphisms to this study.

   a) In the Background the authors maintain that "in recent years the incidence of laryngeal carcinoma around the world has been rising dramatically". The available reports, f.e. the
newest “Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015” (JAMA Oncol. 2017;3(4):524-548) indicate that the global incidence of larynx cancer decreases.

Response: Thank you for your suggestions. We feel very sorry about that we did not check the whole scientific reports about laryngeal cancer and thus made a huge mistake about this sentence. We have changed the sentence of “in recent years the incidence of laryngeal carcinoma around the world has been rising dramatically” to “Although the overall incidence is declining [2], laryngeal cancer is still a big problem throughout the world” and marked in red in the paper. (Background section, lines 9-10, page 3)

b) The NBS1 polymorphic variants were previously analyzed in laryngeal carcinoma, therefore I suggest to change the cited paper in the Background, [14] from Lu et al 2009 to Ziolkowska et al 2007 [34].

Response: Thank you for your suggestions. We have changed the cited paper [14] to cited paper [34] and marked in red in the paper, which became cited paper [15] in revision paper. (Background section, line 8, page 4)

c) In the paper cited on page 4, lines 30-31 [18] by Zheng et al 2011, the authors confirmed only the association between rs1805794 and NPC but not between rs2735383 and NPC. The same situation is for paper [19] (Jiang et al 2011) - no difference in the susceptibility to ALL was found concerning rs2735383 genotypes. Therefore, I suggest to exclude these citations from the Background.

Response: Thank you for your suggestions. We have removed the sentence “In 2011, a group of Chinese researchers found that the association between rs2735383 and the risk of nasopharyngeal carcinoma and acute lymphoblastic leukemia” and the two cited paper by Zheng et al 2011 and Jiang et al 2011 in the Background section. (Background section, line 17, page 4).

d) Also during Discussion some cited papers are selected improperly, the authors cite Yao et al [24] (Tumor Biol 2013; page 11, line 17) after they wrote: "NBS1 rs1805794 polymorphism which has been frequently investigated showed that an association between the variant genotype and breast cancer risk". However, the cited authors outright suggests lack of NBS1 Glu185Gln polymorphism association with breast cancer risk in any populations. The same concerns Broberg et al [30] (Carcinogenesis 2005 (page 11, line 20))
Response: Thank you for your comments. We are very sorry for our inadvertence on citing those improper references. In the revision, we have carefully revised these references and legitimately cited them to support described comment. For example, the references Yao et al [24] and Broberg et al [30] were instead of Smith TR et al ([27] in the revision) and Zhang Y et al ([32] in the revision). The Smith TR et al showed that there was a significant association between NBS1 rs1805794 and breast cancer risk, while Zhang Y et al conducted a meta-analysis to demonstrate that the NBS1 Glu185Gln polymorphism was significantly associated with urinary system cancer susceptibility, including bladder cancer. (Discussion section, page 12, lines 8-9)

e) Discussion, page 11, lines 39-40 - authors claim, that in the Reference paper [32] (by Han J et al 2009) authors indicate association of rs2735383G>C in NBS1 with breast cancer. Indeed, they found a significant trend of increased risk with increasing numbers of risk alleles in the DSB-NHEJ pathway but the NBS1 gene (named NBN there) was included into the pathway of genes participating in DSB-HR pathway, which brought opposite results. Thus, the authors should carefully verify the available reports.

Response: Sorry for misuse of the reference here. Indeed, the data did not find a significant association between rs2735383G>C and breast cancer risk. In the revision, we cited novel reference and modified the description on this association as follows: “In addition, although studies revealed an insignificant association between the SNP and breast cancer risk [34-36], rs2735383G>C was significantly associated with progesterone receptor positivity of breast cancer [36]”. (Discussion section, page 13, line 1-2)

f) The same concerns "association rs2735383 G>C in NBS1 with lymphoid malignancies" - the cited authors (Rollinson et al 2006 [25]) indicated a lack of: "significant differences in allele or genotype frequency, global haplotype distribution between the cases and control, nor effect for individual haplotypes when analyzed by unconditional logistic regression for either RAD50 or NBS1".

Response: Thanks for the reviewer’s comments. As no study showed significant association between rs2735383 G>C and lymphoid malignancies risk, we removed the reference in the revision. (Discussion section, line 19, page 12)

5) The authors should explain the origin of "major" and "minor" allele naming, reffering to adequate database. In the manuscript, the authors describe allele G for rs1805794 as major one and allele G for rs2735383 as minor one. According to dbSNP G alleles for both polymorphisms are the minor variants. On the contrary, the results collected in Table 2
indicate that G allele is the major allele for rs1805794 and rs2735383. This part of Genotyping analysis should be clarified. (zheng2011)

Response: Thanks for the reviewer’s comment. According to the dbSNP database, the G allele of the two SNP is defined as minor allele, because their frequency was lower than C allele. We also used this norm here. Actually, the “C” allele in the current study is same as “G” allele in the dbSNP database, because the G and C is base complemental. In dbSNP database, the sense strand carried “G” allele, while the antisense strand carried “C” allele, and we detected the “C” allele here is actually “G” allele in the dbSNP database. This is why in the dbSNP database, the SNPs are defined as rs1805794C>G and rs2735383C>G. We still used rs1805794G>C and rs2735383G>C here followed the description of Zheng J et al [20] and Yang L et al. [22]. We added the description in the revision as follows: “According to the dbSNP database, the current study defined the C allele of both SNPs in the antisense strand (i.e., the corresponding allele is G in the sense strand in the database) was defined as minor allele followed with previously published studies [20, 22].” (Genotyping analysis section, lines 11-14, page 6)

6) In the Discussion, page 11, lines 25-26 - I suggest to replace the formulation "risk of CRC" with "risk of different tumors, including those of head and neck" - as this conclusion results from the cited paper.

Response: Thank you for your suggestions. We have replaced the formulation "risk of CRC" with "risk of different tumors, including those of head and neck" and marked in red in the paper. (Discussion section, line 11-12, page 12).

4. Response to comment(Reviewer 4):

1) The authors should describe the type of cell (e.g. squamous cells) cancer started in.

Response: Thank you for your suggestions. According to your suggestion, we have added description of the type of cell cancer in the “Study subjects” and marked in red in the paper. (Materials and methods section, lines 13-16, page 5)

2) The abbreviation words should be described in the manuscript, independent of presented in the "Abstract" section.

Response: Thank you for your suggestions. We had listed the abbreviation words in the paper, but we presented after “Discussion” section according to the rules of this journal. (Abbreviation section, lines 11-16, page 15)
3) The nomenclature of SNPs should follow recommendations for the description of sequence variants of Human Genome Variation Society.

Response: Thanks for the reviewer’s suggestion. We thus replaced rs1805794G>C and rs2735383G>C with c.553G>C and g.90947269G>C in the revision following recommendations for the description of sequence variants of Human Genome Variation Society. (Background, lines 13-17, page 4)

4) The roles of SNPs in gene and protein expression should be presented in "Introduction" section and discussed individually in the manuscript with the observed results.

Response: Thanks for the reviewer’s suggestion. We have added some descriptions in the revision as follows: “The transition of G to C resulted in reduced DNA repair capacity of NBS1 and promoted tumor migration [19-20]” (Background section, lines 15-16, page 4), “This SNP was also functional by decreasing NBS1 expression [22]”. (Background section, lines 19-20, page 4). “Moreover, functional studies have demonstrated that the rs1805794G>C is functional, which can recede the DNA repair capacity of NBS1 (Zheng J et al [20]). This transition also impairs NBS1's capacity of inhibit tumor invasion. (Fang W et al 2014 [19])” (Discussion section, lines 14-16, page 12)

5) All previous results described in scientific literature about referred SNPs and laryngeal carcinoma should be presented in "Introduction" section and better discussed.

Response: Thanks for the reviewer’s suggestion. We have added some descriptions in the revision as follows: “Ziólkowska I et al. demonstrated that heterozygous carriers of the c.I171V variant are prone to the development of larynx cancer [15]. Nowak J et al. reported that the NBS1 g.657del5 contributed to significantly higher risk of laryngeal carcinoma (Nowak J, et al [16]). However, the frequencies of these loci are rare in Chinese. Anyway, these data suggested the NBS1 gene to be susceptible gene of laryngeal carcinoma.(Background section, lines 6-11, page 4)” and “In addition, although c.I171V and g.657del5 have been reported to be risk loci of laryngeal carcinoma risk in other ethnics, we did not test these two loci because they are rare in Chinese. All these data suggest that the role of NBS1 polymorphisms in laryngeal carcinoma risk may have been affected by some ethnic difference, which warrants further investigations.”(Discussion section, lines 12-17, page 13).
6) In "Materials and Methods", diagnosis, clinical and pathological information methods; and tobacco and alcohol classification should be included and referenced.

Response: Thank you for your suggestions. We have added the diagnosis, clinical and pathological information methods marked in red (Study subject section, lines 11-13, page 5). We also added two cited papers in our study to explain the tobacco and alcohol classification. They were “Environmental tobacco smoke and lung cancer risk in nonsmoking women (Stockwell HG, et al [23])” and “Patterns of alcohol consumption and ischaemic heart disease in culturally divergent countries: the Prospective Epidemiological study of myocardial infarction(PRIME)” (Ruidavets JB, et al [24]), respectively. We have marked in red in the paper. (Study subject section, line 2-4, page 6)

7) The tumors were classified by TNM stages?

Response: Thank you for your suggestions. The tumors were not classified by TNM stages. They were classified by histopathological diagnosis instead. We classified them in poorly differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma, well-differentiated squamous cell carcinoma and unknown.

8) The positive and negative controls were used in the experiments?

Response: Thank you for your suggestions. As for this question, we feel very sorry about this question, because we did not include positive and negative controls in the paper, we only used the empty vector plasmid as control. Thanks for your suggestions again, it would be very useful for our future studies.

9) The method of quantitative PCR should be better described, including dye description and threshold method.

Response: Thank you for your suggestions. We have revised the method of description including dye description and threshold method marked in red. (Detection of gene’s expression by real-time PCR section, lines 21-22, page 7 and lines 4-5, page 8)

10) The authors didn't present in the "Statistical analysis" section the gene expression and study power statistical tests. It's not necessary include the variables that were not significant between groups in the regression models.
Response: Thank you for your suggestions. We have added the gene expression and study power statistical tests in the paper and marked in red. (Statistical analysis section, lines 16-20, page 8).

11) The combined SNPs analysis could be performed and predicted microRNAs could be presented, including in silico analysis.

Response: Thank you for your suggestions. We have done the combined SNPs (rs2735383 and rs1805794) analysis, as shown in Supplementary Table, but no statistically significant differences were observed. The result indicated that the combined variant genotypes were associated with a boardline increased cancer risk. It showed that the main effect was from the role of rs2735383, but not from rs1805794.

On the other hand, bioinformatics analysis with the 1000 Genomes Project (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) showed that rs2735383 G>C was in complete linkage disequilibrium with rs1063053 G>A and rs1063054 A>C. The prediction by using SNPinfo Web Server (http://snpinfo.niehs.nih.gov/) revealed that these two SNPs are all potent functional. The transition from common allele G of rs1063053 to variant allele A may lose binding site of hsa-miR-517b, and rs1063054A>C could result in new binding site of hsa-miR-654-3p but loss binding site of hsa-miR-513a-3p. These findings indicate that the functional polymorphism rs2735383G>C in the 3’-UTR of NBS1 gene could predict the risk of laryngeal carcinoma. (Results section, lines 14-22, page 14)

12) It's recommended that the authors should add functional study to enhance the validity of the research, such as luciferase assay. In addition, the role of the studied SNPs in the patient's survival could be important.

Response: Thank you for your suggestions. Being limited by the follow-up data, we cannot perform survival analysis by now.