Manuscript NO: 32067

Title: **Polymorphisms of microRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* in gastric cancer**

Dear Editor,

We would like to thank you and reviewers for valuable comments. We edited the manuscript according to your suggestions and added all obligatory files. We would like to explain that language editing was performed by corresponding author Juozas Kupcinskas whose English language skills were assessed as Grade A (certificate is attached).

Please see the answers to the reviewers' comments below.

Reviewer 1:

In the multi-center study, the authors selected 4 SNPs to evaluate the possible associations between them and the risk of gastric cancer. They observed that INSR rs1051690 SNP was associated with increased risk of GC. What is the basis for selecting these 4 genes?

Response. The basis for selecting four SNPs is outlined in the method section: 'In order to select the candidate SNPs falling within 3'-UTR of genes which are putative targets of frequently deregulated miRNAs in GC, the mirsnpscore database was used (http://www.bigr.medisin.ntnu.no/mirsnpscore). The database contains in silico predictions of SNP effects on miRNA-target gene regulation, which are measured by ΔS score. The higher the ΔS score, the higher the possibility that the miRNA-mRNA interaction is disrupted. The candidate SNPs had to meet the following criteria: a minor allele frequency (MAF) > 0.2, the ΔS value > 0.25 and the target gene had to be previously reported as associated with GC.'

Furthermore, how about the associations between INSR rs1051690 SNP and clinical characteristics of GC? They should be investigated.

Response. We agree that it would be interesting to evaluate the role of this SNP in relation to other clinical characteristics; however, due to the lack of data (e.g. survival) we could not perform analysis, also resulting subgroups were too small for making meaningful analyses. Therefore, these results need to be validated in further larger studies.

Reviewer 2:

In the manuscript entitled "Polymorphisms of microRNA target genes IL12B, INSR, CCND1 and IL10 in gastric cancer" the authors investigated the association of selected

polymorphisms with the risk of developing gastric cancer in European population. Their analyses were performed on relatively large population of patients – 474 individuals. The genotypes were compared in an association study; the control group was composed of 508 controls. Control group was selected from patients with dyspepsia. None of these individuals had at the time of recruitment malignant disease. Analyses showed that the CT genotype and T allele of rs1051690 in INSR gene were significantly more prevalent in the patient-group. The analysis of the dominant model revealed that patients carrying CT and TT genotypes had an increased risk of developing gastric cancer. Overall, the manuscript presents the hypothesis and results well, however, there are few issues concerning this manuscript, which should be corrected.

First, could you rephrase the sentence describing the aim in the Abstract, for example, the authors could write: The aim of the study was to evaluate…..or something appropriate in this context.

Response. The first sentence of the Abstract was written following the requirements of the journal:
Aim. To evaluate associations between miRNA target genes *IL12B*, *INSR*, *CCND1* and *IL10* polymorphisms and gastric cancer (GC) in European population.

Could you explain which software you used for statistical analyses and what was the rationale for selecting the corrected p value?

Response. The analysis was performed using freely available statistical program PLINK v.1.9 available at pngu.mgh.harvard.edu/~purcell/plink. This explanation has been added in the method section of the manuscript.

The Bonferroni method of correcting for multiple testing simply reduces the critical significance level according to the number of independent tests carried out in the study. For M independent tests, the critical significance level can be set at 0.05/M. In our study, the Bonferroni-corrected alpha level was set at 0.013, because four SNPs was analyzed (0.05/4 SNPs).

Also, from the Table 2 it is evident that for some individuals the gender information was missing, were these subjects also evaluated in the study?

Response. The individuals with missing gender information were excluded from logistic regression analysis, because odds ratios of gastric cancer were adjusted by sex.

Specific comments. Abstract. Could you spell out the abbreviation RT-PCR?

Response. Following recommendation of reviewer, the abbreviation was spelled out in the Abstract: 'the real-time polymerase chain reaction'.

Could you also (first sentence of Results in Abstract) use the rs ID for SNP in INSR gene instead of INSR SNPs. Also, you evaluated one SNP in this gene, therefore the abbreviation SNPs is not appropriate here.

Response. As reviewer suggested, SNP was replaced by rs1051690.

Introduction - Second paragraph, is pathogenetic an appropriate term? You could also use "pathogenic".

Response. According to English vocabulary, 'pathogenetic' means related to pathogenesis. Taking this in mind, we think that term 'pathogenetic mechanisms' is correct.

Fourth paragraph, could you rephrase this part, particularly the first two sentences. The second sentence does not explain properly the meaning of or is not associated with the first statement.

Response. We tried to rephrase these sentences in order to provide more clarity as suggested by the reviewers: 'Target gene identification may help to reveal specific functions of individual miRNAs. This process is challenging because miRNAs may bind to multiple target mRNAs. In order to identify potential miRNA targets computational modeling and experimental approaches are applied.'

Methods - First paragraph, last sentence, Could you explain the meaning of this statement. Does it referrs to histopathological data? Thera are no histopathological features associated with examined SNPs. I believe that this sentence does not contribute to the quality and meaning of the article and could be deleted.

Response. With this sentence, we would like to stress that diagnosis of all cases of cancer was verified by histological analysis. So, we decided to leave this sentence. Only the term 'histological verification' was replaced by the term 'histopathological verification'.

DNA genotyping: Was genotyping performed in duplicate? Were any of the samples confirmed by alternative methods?

Response. We mentioned in the Methods section that 'dubious samples had repetitive genotyping analysis.' Duplicate genotyping was performed in 5% of all samples with one hundred percent concordance rates.

Results - Could you explain why you didn't attempt to match control and patient groups regarding the gender? The groups are disproportionate in terms of male and female frequencies.

Response. Gastric cancer was more prevalent in men than in women; therefore, proportion of men was higher in cancer group. We did not have any objective to investigate the gender differences of association between SNPs and gastric cancer; therefore, our case and control groups were not matched by the gender. Furthermore, gender, was included as a covariate in the statistical analysis in order to rule out the potential influence of gender for the results.

Results. Association analysis, last two sentences… Could you also explain in which model the association was found significant? Codominant?

Response. Association was found in dominant and co-dominant models.

Discussion - Second paragraph and other instances in the text, where appropriate: Could you use A recent study also identified… instead of One recent study … -

Response. Thank you for comment. We corrected the sentence.

You mention that the region where rs1051690 is located is putative binding site for miR-146a. Are there any data regarding the expression levels of this miRNA in gastric tissues, both normal and tumour tissues?

Response. A study by Xiao B et al. showed that miR-146a was upregulated in 20 gastric cancer tissues compared with matched non-tumor adjacent tissues by quantitative RT-PCR [Xiao B et al. Oncol Rep 2012]. Increased miR-146a in gastric cancer directly targets *SMAD4* and is involved in modulating cell proliferation and apoptosis. Due to the design of the study we were not able to evaluate whether rs1051690 could mediate the expression of miR-146a and this remains to be evaluated in further studies. This comment has been added in the discussion section of the manuscript.

You mention that SNPs in IL12 and IL10 genes were also evaluated in other studies, did all of these studies evaluated the same polymorphisms?

Response. The other studies evaluated some gene polymorphisms located in IL12 and IL10; however, they were different from the ones selected for our study. This comment has been added in the discussion section of the manuscript.

Last sentence, could you rephrase this, I don't think that this study revealed the nature of the interactions of miRs and polymorphisms.

Response. We completely agree with the comment of the reviewer and tried to change the sentence in the following way: 'Nevertheless, overall our data provide important novel aspects on genetic susceptibility for GC.'

Conclusions - Wouldn't it be more appropriate to use "associations" instead of "link".

Response. Following reviewer's suggestion, we replaced word 'link' with 'association'.

Innovations and breakthroughs - Could you check if it is appropriate to describe this approach as novel? Were there no previous studies that attempted to determine SNPs in putative miRNA binding sites?

Response. We agree that there are other studies in the field have been done using similar approach; however, none of these studies has evaluated above mentioned gene SNPs in relation to gastric cancer and the novelty stems from this aspect.

Tables Table 1 - Could you spell out Chr - Is RsID appropriate? You could also use SNP ID.

Response. We spelled out 'chromosome' and used 'SNP ID.'

Table 2 - last column, Could you explain the test you used in footnotes and mark the appropr

Response. Corrected as reviewer suggested.

Respectfully,

On behalf of all authors
Juozas Kupcinskas