Author's response to reviews

Title: The NFkB1 polymorphism (rs4648068) is associated with the cell proliferation and motility in gastric cancer

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Reviewer 1:

Major compulsory revisions

1. Lines 210-222, the section on C/EBP-beta, are unclear. I am unsure from reading them whether C/EBP-beta is binding to the NF-kappaB promoter and regulating it, or if NF-kappaB1 is binding to the C/EBP-beta and regulating it. **Some clarity or perhaps a diagram would be useful here.** I am having trouble believing that the presence of a promoter can bind up enough C/EBP-beta to “regulate” protein activity on other genes, which is what the authors seem to be saying. Alternately, they may be saying that C/EBP-beta binds better to the GG version of the NF-kappaB promoter and enhances transcription of NF-kappaB. This is a reasonable finding, but does not explain the effects seen in figure 5, as explained below in point 2.

   Answer: Thank you so much for your comments and suggestions. We think there might be the interaction between NFKB1 promoter and C/EBP-beta as the followings:

   In this study, we aim to investigate NFKB1 transcriptional activity and biological function influenced by rs4648068 (A>G) polymorphism. Since the complete coding region for NFKB1 contains 100,000bp, it is difficult to construct a plasmid containing this entire gene. Thus, the recombinant plasmid pGL3-AA, pGL3-GG, pGL3-AA-NFKB and pGL3-GG-NFKB were constructed and transfected into cells, which wasn’t clarified in the previous manuscript. The recombinant plasmid pGL3-AA and pGL3-GG containing the promoter region were constructed for luciferase assay, while the recombinant plasmid pGL3-GG-NFKB and pGL3-AA-NFKB containing the promoter region of NF-kappaB1 and adjacent three consecutive exons (Figure 2) were constructed for cell biological experiment. Basically, as is considered by the reviewer, it seems that C/EBP-beta binds better to the GG version of NFKB1 promoter (Figure 4A) and enhances the transcription of NFKB1, giving rise to the upregulated p50 expression, consisted with western blot result in Figure 4B.

   On the other hand, NF-kappaB1 p50 could bind C/EBP-beta and reciprocally induce the partner’s expression, forming a transcriptional positive feedback loop [1]. Upregulated C/EBP-beta expression was accompanied by a higher level of NF-kappaB1, which was confirmed by western blot analysis (Figure 4B). A diagram was shown to clarify the interaction between NFKB1 promoter and C/EBP-beta (Figure 4C). The mechanism for regulating C/EBP-beta expression remains unclear, and will be performed in our future work. C/EBP-beta:p50 interaction might induce target genes to influence the cell biological activity. Thus, the SNP site rs4648068 might have effects on cell proliferation and motility by regulating NF-kappaB1 and C/EBP-beta expression. To elucidate the relationship between NFKB1 promoter and C/EBP-beta, more details of our experiments have been added and described in revised manuscript text (from line 248 to 267).
2. I am unsure what the recombinant plasmid contains. Is it just the promoter region of NF-kappaB with the polymorphisms, or is it the entire gene, complete with NF-kappaB coding sequence? This needs to be better specified. If it is just the promoter region, how does this explain the effects seen, since endogenous NF-kappaB would still be regulated by its own promoter?

Answer: Thank you so much for your considerations. Since the complete coding region for NFKB1 contains 100,000bp, it is difficult to construct an expression plasmid containing this entire gene. Correspondingly, the recombinant plasmid pGL3-AA, pGL3-GG, pGL3-AA-NFKB and pGL3-GG-NFKB were established and transfected into cells, which wasn’t clarified in our previous manuscript. Actually, the recombinant plasmid pGL3-AA and pGL3-GG containing the promoter region were made for luciferase assay, while the recombinant plasmid pGL3-GG-NFKB and pGL3-AA-NFKB containing the promoter region of NF-kappaB1 and adjacent three consecutive exons (Figure 2) were used for cell biological experiment. Description on the recombinant plasmids was elaborated in Method Section of revised manuscript (from line 83 to 108).

Minor essential revisions

1. There are a few missing words and minor mistakes throughout the manuscript.

Answer: Thank you so much for your suggestions. We tried our best to correct the manuscript. Definitely, we’re also going to seek the assistance of a professional editing service to polish them, as soon as the revised manuscript is accepted.

Reviewer 2:

Major Compulsory Revisions

1. Figure 1 shows NF-kappaB immunohistochemistry of tissues from gastric cancer patients. However, the actual origin and tissue type is not described in either methods or results. Quantification is presented but it is not clear what the denominator is. Is it all nuclei in the tissue? Were multiple sections from multiple patients evaluated? Also, the presented tissue sections are not comparable between genotypes. There seem to be different tissue types in each panel. Panel c) shows mostly connective tissue and fat, b) epithelial tissue in one panel, connective tissue in the other, and a) a mix of tissues with what appears to be mostly lymphocytes in the right hand panel. The method section provides a formula but does not describe how positive cells were counted. If subjective measures were used, the intensity of staining may be variable between slides and not necessarily indicative of quantity. How was the statistical analysis done? A better approach to quantify the expression of NF-kappaB in gastric cancer tissue is using qPCR and/or western blot analysis.

Answer: Thank you so much for your comments and suggestions. We replaced with more representative results of immunohistochemical staining in revised figure 1. Detail information about tissues were added in the revised manuscript and described as follows:
Paraffin-embedded gastric cancer tissues were collected from 20 patients with gastric cancer who were admitted to Fudan University Cancer Hospital (Shanghai, China) July 2014. All cases were gastric adenocarcinoma confirmed by pathologist (clarified in the method from line 40 to 44). Only those patients whose clinical data (include diagnosis, age, sex, address, disease history, etc) were intact and the blocks were enough to be cut into 5 slides were selected. NF-κB1 (brown) is mainly expressed in cytoplasm of gastric adenocarcinoma tissue. The tissue sections for immunohistochemical staining were from different patients. (a) The patients’ genotype was homozygote GG, and the dark brown staining for NF-κB1 was shown in cytoplasm of adenocarcinoma cells. (b) The patients’ genotype was AA. NF-κB1 immunohistochemical staining of AA group exhibited faint. (c) The patients’ genotype was GA. NF-κB1 immunohistochemical staining of adenocarcinoma cells was also not intensive. Left panels magnification, ×200; Right panels magnification, ×400.

The method and formula for immunohistochemical staining calculating were described as follows: The NF-kappaB1 positive tissues were quantified based on the percentage of positive cells which were serially counted in one microscopic field. The cell counting was repeated in five random microscopic fields at ×400 magnification. Two pathologists who were blinded to patient group independently interpreted the IHC staining results using positive index (PI). The positive index (PI) was calculated using the following formulation: PI= i × p, where i is intensity of staining (0 for negative, blue; 1 for weakly-positive, light yellow; 2 for medium positive, yellow; 3 for strong positive, brown), and p is positive percentage of staining (1 for ≤10%; 2 for 11%-50%; 3 for 51%-75%; 4 for >75%) [2]. Then, the positive index (PI) was calculated for each case. If there were divergences in the PI determined by the two pathologists, slides were rescored until a consensus was reached. Details were described in the method of manuscript text (from line 69 to 81). Besides, differences in NF-kappaB1 expression between different groups were investigated using Kruskal-Wallis non-parametric test in the section of Statistical Method.

2. Figure 3 includes a nice approach to show the role of NFKB polymorphisms in transcriptional activity. However, the result section should be expanded to provide a clear description of all panels and a summary of the results of these experiments in both non-gastric and gastric cancer cell lines. For data presented in table 1, it is not clear which luciferase plasmid was used. If the description in the legend means that the data presented are the difference between luciferase activities of GG and AA reporters, it should be clearly described in the results section.

Answer: The result of luciferase assay in both non-gastric and gastric cancer cell lines has been analyzed and included in revised manuscript with a summary in the end of paragraph (line 213 to 229). Both of recombinant plasmid pGL3-AA and pGL3-GG containing the promoter region were transduced to SGC7901 cells for data presented in table 2. The difference between luciferase activities of GG and AA reporters was estimated under the stimulation of LPS, which was clarified in the results section (from line 236 to 238).
3. Figure 5 provides data supposed to demonstrate the physiological importance of the described effect. However, there are several problems with the presentation. It is not clear whether the plasmids are stably transfected into cells. A transient transfection will usually become ineffective within 3 days, so it is not clear whether the cells still contained the constructs after 6 days. More importantly, it is not clear to me how transfected promoter regions regulating a luciferase gene can change the transcription of endogenous NFKB target genes. The authors should explain how that works and provide evidence from the literature that this is a valid approach.

**Answer:** To guarantee the effective transduction, all the experiments were performed within six days after the recombinant plasmids were transfected to cells using lipofectamine 2000. We thought that the effects might exist within 6 days according to the literature [3], and our results are satisfied for the cell proliferation evaluation. Definitely, it is better for all experiments completed within 3 days.

In this study, we aim to investigate NFKB1 transcriptional activity and biological function influenced by rs4648068 (A>G) polymorphism. Since the complete coding region for NFKB1 contains 100,000bp, it is difficult to construct a plasmid containing this entire gene. Therefore, the recombinant plasmid pGL3-AA, pGL3-GG, pGL3-AA-NFKB and pGL3-GG-NFKB were constructed and transduced into cells, which wasn’t clarified in the previous manuscript. The recombinant plasmid pGL3-AA and pGL3-GG containing the promoter region were constructed for luciferase assay, while the recombinant plasmid pGL3-GG-NFKB and pGL3-AA-NFKB containing the promoter region of NF-kappaB1 and adjacent three consecutive exons (Figure 2) were constructed for cell biological experiment (clarified from line 83 to 108). C/EBP-beta binds better to the GG version of NFKB1 promoter (Figure 4A) and enhances the transcription of NFKB1, giving rise to the upregulated p50 expression, consisted with western blot result in Figure 4B.

On the other hand, NF-kappaB1 p50 could bind C/EBP-beta and reciprocally induce the partner’s expression, forming a transcriptional positive feedback loop [1]. A diagram was shown to clarify the interaction between NFKB1 promoter and C/EBP-beta (Figure 4C). The mechanism for regulating C/EBP-beta expression remains unclear, and will be perfomed in our future work. C/EBP-beta:p50 interaction might induce target genes to influence the cell biological activity. Thus, the SNP site rs4648068 might have effects on cell proliferation and motility by regulating NF-kappaB1 and C/EBP-beta expression. To elucidate the relationship between NFKB1 promoter and C/EBP-beta, more details of our experiments have been added and described in revised manuscript text (from line 248 to 267).

**Reference:**
