**Comments and responses (Manuscript ID: 70252)**

**Reviewer #1:**

**Specific Comments to Authors:**

1. It is hard to understand the data why NOCs increases MGMT expression while inducing malignant transformation of epithelium cells and why inhibition/knock down of MGMT slows down this transformation. Therefore, the significance of MGMT upregulation by NOCs needs to be discussed and the conclusion that MGMT prevents this transformation needs reconsideration.

Author answer: In this study, we established a malignant transformation model of gastric epithelial cells by MNNG or MNU repeated exposure. MNNG and MNU are DNA alkylating agents, which can induce the $O^6$-methylguannine to cause DNA damage. In the early phase of malignant transformation, cells were rapidly damaged after MNNG/MNU treatment. And the $O^6$-methylguannine damage was accumulated in the cells. MGMT was activated in feedback to precipitate the DNA damage repair following MNNG/MNU treatment. However, high expressed MGMT is not conducive to the accumulation of gene mutations and the induction of genomic instability. We speculate that in the early stage of cell malignant transformation, the up regulation of MGMT is conducive to cell survival, but in the late stage of malignant transformation, the inhibition of MGMT is conducive to promoting cell carcinogenesis. According to the reviewer's comments, we have discussed the significance of dynamic changes of MGMT in cell malignant transformation in the part of discussion.

2. In result section, it should be indicated that MGMT expression in Fig. 1A is from IHC. How many samples were examined and relevant data should be presented. Fig. 1B is not clear whether they are from qRT-PCR result in which normalized and relative level of gene of interest is presented generally. Fig 1C is very confuse. In the left panel, samples of qRT-PCR results should be explained and whether these are normalized to a house keeping gene, here likely from the GAPDH, should be explained and expressed. I cannot see how Fig. 1B shows MGMT expression comparison between cancer and adjacent normal tissues. Rational to examine MGMT expression in 6 cell lines should be provided. On the right panel, just examples are presented. Quantitative analysis must be analyzed. Protein size should be marked along with protein ladders on the example blots. In addition, as stated in the method, both primary antibodies against MGMT and GAPDH are mouse monoclonal. Authors must explain how they differentiated MGMT from GAPDH using the 2ndary antibody which must be one against mouse IgG. How CCLE database was analyzed for correlation of MGMT expression and promoter methylation should be provided. In addition, Fig. 1D is not appropriate in this figure since all other data have no correlation with DNA methylation yet. This subfigure belongs to Fig. 4 for rationale of methylation analysis.

Author answer: According to the reviewer's comments, in the part of Results and Figure Legends, we have illustrated the number of clinic samples used to analyzed MGMT expression by IHC, and GAPDH was used to normalize relative level of MGMT gene expression by qPCR.

For the Fig 1C, we also provided the rational examination of MGMT in the part of Figure legends. GAPDH was used to normalize relative level of MGMT gene expression. We also analyzed the MGMT expression by quantitative analysis and presented in Figure 1C. The information of primary antibodies against MGMT and GAPDH are presented in Materials and Methods. We used the primary

antibodies against MGMT and GAPDH diluted to 1:1000 and 1:2000, respectively. The same IRDye 800- or IRDye 680-conjugated 2ndary antibody was used to against mouse IgG of primary antibodies against MGMT and GAPDH.
According to the reviewer's suggestion, we added the Figure 1D to Figure 4, and made the corresponding changes in results.

3. Regarding results presented in Fig.2, the stage of malignant transformation should be analyzed and explained so that the statement of MGMT upregulation at the early stage of carcinogenesis can stand.
What is the difference between Fig. 2B and 2C? It should be described in method and in result analysis.
Description of the method for Fig. 2F is missed.

Author answer: The MNNG or MNU induced cell malignant transformation was analyzed by the malignant phenotype analysis, such as cell proliferative activity and cell migration activity. We used colony formation assay, soft agar assay and cell migration assay showed that the NOCs-transformed cells have an increase of cell proliferative activity and cell migration activity. We also showed that the NOCs-transformed cells have a tumor formation activity by xenograft assay. It is widely regarded that cell malignant transformation was the early molecular events of carcinogenesis. Therefore we focused on the gene expression of MGMT in cell malignant transformation process.
Fig. 2B and 2C showed the anchorage-independent growth capability and colony formation ability by soft agar assay and colony formation assay, respectively. We have described these two methods in Material and Methods and Results.
According to the reviewer's comments, we have described the xenograft assay (Fig. 2F) in Material and Methods.

4. In Fig. 3, "W" needs to be explained even though it seems to represent week. In addition, explanation of these numbers is missed even though they may be the time following NOCs treatment. Combining data in Fig. 2 and 3, how can authors explain that MGMT constantly stays at a high level from 1 week to 8 weeks? What does the time relevant to different malignant transformation stages?

Author answer: In Fig. 3, "W" means week. We have described in corresponding figure legend.
The malignant phenotypes were analyzed after removal of MNNG or MNU exposure for 4 weeks to verify the cell transformation model. MGMT was rapidly upregualted in the beginning upon NOCs treatment, indicating that MGMT upregulation was induced by the DNA damage response. But MGMT expression gradually decreased after removal of MNNG/MNU exposure for 8w compared with 1w. In fact, MGMT expression returned as the normal after removal of MNNG/MNU exposure for 12w. We use the different times of MNNG/MNU exposure removal to represent different stages of cell malignant transformation.

5. More detailed information is needed for Fig. 3C. What are Cs? What is MGMT (+) or (-)? Authors mentioned overexpressing MGMT, but never described how it was done. These questions are applied to Fig. 3 D, too.

Author answer: According to the reviewer's comments, we described the significance of C1-28,

and MGMT (+) or (-) in corresponding figure legends. C1-28 means the different subcolones of MNNG/MNU-transformed cells. MGMT (+) or (-) represents the MGMT expression in these subcolones. MGMT (+) represents the upregulation of MGMT expression. MGMT (-) represents the downregulation or no-change of MGMT expression.

6. Regarding data in Fig. 4A, how p53 and JunD served as positive control should be explained. No relevant information can be found from method or results.

Author answer: It is reported that p53 and JunD were the transcriptional activator of MGMT promoter. Therefore, we used them as positive control to verify the constructed promoter activity of MGMT. According to the reviewer's comments, we have described this in results.

7. For Fig. 4B, more information should be provided, such as what M and U represent, labeling/samples for each lane, PCR location on the genome and relevant possible methylated cytosine(s). Critically, this method can only detect one or very few possible methylated cytosine(s). Rationale for primer design or sequence selection should be provided. In addition, quantitative analysis with reasonable repeats of experiments should be provided. The source of anti-H3 antibody and control IgG for ChIP experiments are missed from method section. The rationale to examine H3K9me3 and H3K4me2 is missed. Logically, methylated DNA binding proteins such as Mecp2 should be examined using ChIP to find out which transcription factors related to DNA methylation status could be involved in regulation of MGMT transcription.

Author answer: M and U represent methylated and unmethylated. According to the reviewer's comments, we have described the specific primers for MSP PCR in Material and Methods, and added the quantitative analysis of MSP PCR in figure 4B.
For the ChIP assay, we have described the primers information in the material and methods, and illustrated the use of H3K9me3 and H3K4me2 of ChIP assay in results.
The suggestion of finding out which transcription factors related to DNA methylation status could be involved in regulation of MGMT transcription from reviewer is very interesting, and analyzing effect of Mecp2 MGMT methylation by ChIP assay is also very significant. But in this study it is difficult to conclude it, we will explore these in the future studies.

8. Data in Fig. 4C look good, but the genomic location of these sequence and experimental repeats much be provided. Even though generally circles in figure represent cytosines in CpGs, open ones for unmethylated, and solid ones for methylated, authors still need to state these in figure legend. Most critically, the time following MG-C or MU-C treatment is missed.

Author answer: According to the reviewer's comments, we have described the analyzed sequence in material and methods. And we also described the significance of open ones for unmethylated, and solid ones for methylated in the figure legends. MG-C and MU-C mean the subcolone of MNNG and MNU transformed cells. The exposure mode is consistent with the treatment method of malignant transformation model.

9. For Fig. 4D, primers and PCR amplication position should be provided in the same way as to Fig. 4C.

Repeats of experiments should be provided.
Author answer: According to the reviewer's comments, we have added the primers sequence in material and methods

10. In the method, miRNA analysis was mentioned, but no such data is presented in the whole manuscript. Authors should explain it.

Author answer: I so sorry. This is a mistake of typewriting.

11. Sample preparation for immunohistochemistry (IHC), RNA analysis, western blot analysis, and genomic DNA isolation should be provided.

Author answer: According to the reviewer's comments, we have added the sample preparation for different analysis.

12. What did authors mean "the full-length of MGMT promoter was synthesized"? First, promoter size or length for a give gene needs experimental data to define. Therefore, the term of "promoter in full-length" must be followed by genomic location and size in bp. Second, generally, "synthesized" is used for chemical synthesis. So-called full-length promoter is normally a number of kilo base pairs. DNA in such length can be chemically synthesized, but is very costly. Authors should clearly provide the means to obtain this promoter and the basis or reference of promoter identification in term of sequence function analysis. Regarding reporter assay, a second reporter driven by a universal promoter such as the CMV promoter should be cotransfected with promoter-reporter of interest to correct differences of transfection efficiency among samples/plates tested. The time by which the reporter assay was measured after transfection should be provided.

Author answer: "The full-length of MGMT promoter was synthesized" means we cloned the MGMT promoter sequence (-954/+24) from the genomic DNA by PCR and constructed into pGL3-promoter vector. For luciferase reporter assay, the cells were co-transfected with 0.5μg of pGL3-MGMT-promoter, 0.02μg of pRL-SV40 renilla luciferase reporter plasmids into MNNG/MNU-transformed cells by X-treme GENE HP. After transfection for 24 hours, Dual-Luciferase Reporter Assay System was used for testing relative luciferase activity. According to the reviewer's comments, we have corrected "Dual-luciferase reporter assay" in material and methods.

13. Anywhere once antibody is used, its dilution and visualization means of result should be provided.

Author answer: According to the reviewer's comments, we have added the dilution ratio of the antibodies used in this study.

14. In qRT-PCR, reverse transcriptase is missed for miRNA. SYBR premix Ex Taq II is for PCR. RNA samples must be treated by DNase to remove contaminated genomic DNA and this contamination is commonly seen in RNA prepared with Trizol reagent.

Author answer: in this study, we don't use the qPCR of miRNA. This is a mistake of typewriting.

15. BSP was done by outside lab, and then its mechanism, reference, manufacturer source, and data analysis should be provided so that readers can understand the data.

Author answer: According to the reviewer's comments, we have provided the details of BSP analysis in material and methods.

16. Regarding ChIP, no cross-link process was described, suggesting that native ChIP was used. Normally, enzymes will be used to fragment genomic DNA in such a ChIP. Authors should provide information and reference(s) regarding how chromatin and genomic DNA were fragmented. Normalization approach should be provided to correct enrichment change of chromatin of interest, too. Method of ChIP data analysis should be provided.

Author answer: According to the reviewer's comments, we have provided the details of ChIP assay in material and methods.

17. In immunoblot analysis, missed information includes 1) cell lysate in protein mass per lane on SDS-PAGE gel; 2) the nature of solution for 5% skim milk.

Author answer: According to the reviewer's comments, we have provided the cell lysate (50μg) in protein mass per lane on SDS-PAGE gel in material and methods. The solution for 5% skim milk was used for blocking the membrane to reduce the non-specificity.

18. siRNAs were mentioned in Fig. 5. However, relevant information is missed from the method section.

Author answer: According to the reviewer's comments, we have added the RNA interference in material and methods.

19. Sequences of primers, their location relative to the genomic sequence or cDNA sequence should be provided.

Author answer: According to the reviewer's comments, we have added the sequences of primers used for the amplification of genomic sequence or cDNA sequence in material and methods.

20. Authors showed in Fig. S1 that MGMT expression at protein level is downregulated by O6-BG at 2uM to a level lower than that in non-treated cells. This inhibition also increased cell proliferation and anchorage-independent growth in NOCs treated cells. Did authors examine whether O6-BG alone could produce such effects on naïve cells? Authors further applied siRNAs against MGMT to GES-1 cells (name of this cell line should be provided for Fig. 5) and obtained similar results. However, NC treated cells also largely and significantly promoted such changes, but upregulated, instead of downregulated, MGMT protein. This discrepancy should be explained and discussed. Information of NC and control in Fig. 5C and 5D should be provided. How overexpression of MGMT was conducted

should be described in the method section. Labelings of columns in Fig. 5E and 5F are unclear. What is EVMGMT? In Fig. 5G, MGMT mRNA level should be labeled as level relative to GAPDH. Figure legend should provide explanation of all labelings.

Author answer: We examined the effect of $O^6$-BG alone on cell proliferative activity, which showed that it had no effect on cell proliferation.
NC is a synthetic unrelated sequence that does not affect MGMT expression. According to the reviewer's comments, we have repeated the experiment with the new synthesized NC. The result showed that NC has no effect on anchorage-independent cell proliferation. Control means the blank cells without MNNG/MNU treatment.
For figure 5E and F, we added the illustration for EV and MGMT in figure legend. According to the reviewer's comments, we corrected the Y-axis in figure 5G.

21. Authors claimed that promoter methylation is the mechanism underlying the upregulation of the MGMT gene. Whether inhibition of DNA methylation with DNMT inhibitor will interfere with this upregulation in cells treated with NOCs should be examined to support authors' hypothesis. This hypothesis also needs support from human samples from which authors observed high level of MGMT vs normal tissue. This means that DNA methylation status of the MGMT gene/promoter should be examined in these tissue samples, too.

Author answer: According to the reviewer's comments, we treated the gastric epithelial cells with DNMT specific inhibitor 5-aza for 48 and 72 hours. The result also showed that DNA methylation level dependent DNMT1was involved in the upregulation of MGMT.

23. Writing should be largely improved in scientific logical, English grammar, and wording. Examples are listed below. 1) Title is awkward in English. Do authors mean that Carcinogen NOCs-induced hypomethylation of the O6-Methylguanine-DNA methyltransferase gene is involved in malignant transformation of gastric epithelium cells? 2) In the 2nd sentence of the abstract, what does which represent? Statement following the which is too strong and too general. Authors may want to say that "which is the cause of cancer initiation by NOCs. 3) In the next sentence, it should be read as " …, the regulatory mechanism underlying the MGMT involvement in NOCs-induced tumorigenesis, … " 4) The 4th sentence in abstract is better read in " … malignant transformation of gastric epithelium cells induced by NOCs." 5) Function of MGMT is to correct O6-methylguanine and prevent mutation. Therefore, inhibition of MGMT activity will promote, instead of induce, mutation and carcinogenesis because MGMT does not produce O6-methylguanine. 6) Examples, but not limited to, of incorrect wording and grammar are listed below with correct one vs incorrect one. O6 vs O6; carcinogenesis vs tumorigenesis; MGMT DNA methylated tissues vs MGMT methylated tissues; a protype of epigenetic mechanism of gene expression vs common significant; CG rich sequence vs GC sequence; specially increasing risk vs specially increased risk; Abbreviation should be provided when it appears first time in the manuscript and only for these that are used more than once, e.g., MSP and BSP; incomplete sentences: ChIP assay section of method, the first sentence in the last section of result.

Author answer: According to the reviewer's comments, we applied for professional revision of native English.

Reviewer #2:

**Specific Comments to Authors:**

1. The title refers to NoCs induce methylation changes but the manuscript has not detailed why MNNG and MNU compounds were specifically selected and how only these two compounds suffice as representative of all NOCs. This should be elaborated in the manuscript to make the title more suitable for the work described. Also, the study has only investigated the DNA methylation changes in the promoter region of the gene and title refers to the whole gene of MGMT. This also needs to be clarified.

Author answer: According to the reviewer's comments, we clarified the manuscript title with "Upregulation of $O^6$-methylguanine DNA methyltransferase is mediated by DNA hypomethylation of its gene promoter in gastric epithelial cells malignant transformation induced by NOCs".
We elucidate that MNNG and MNU are widely accepted model chemical carcinogen for studying the mechanisms of mutagenesis and carcinogenesis induced by *N*-nitroso compounds in the part of introduction.

2. The abstract of the manuscript summarizes the key findings though the opening sentence/first sentence is elusive and 'suicide enzyme' should be elaborated for better clarity.

Author answer: MGMT is a "suicide enzyme", because to remove alkylating lesions at the $O^6$ position of guanine, like $O^6$-methylguanine adducts, MGMT needs to transfer the alkyl group to itself. Then MGMT becomes inactive and it is ubiquitinated and targeted for proteasomal degradation. In this DNA repair process, an MGMT molecule can remove an $O^6$-methylguanine adduct, it can't be recycled for new reaction.

3. Introduction has provided a comprehensive background of the previous work. In paragraph 2, line 2, 'epigenetic silencing of MGMT geen...' should be explained in bit more detail as to what epigenetic mechanisms in particular are participating in regulation. Also, O-6-methylguanine needs explanation upon first referral in the manuscript to highligh its importance. On page 4, last line of first paragrapg 'critical molecular mechanism may interplay with epigenetic regulation...' would benefit from elaboration of those 'critical molecular mechansims' that authors are referring to.

Author answer: According to the reviewer's comments, we corrected the above sentence in manuscript.

4. Methods sections has detailed the experiments performed. Primer sequecnes for the MS-PCR, RT-PCR and ChIP analysis are not provided. They should be included either in methods or in appendix. There are some typos like 'QRT-PCT' should be 'qRT-PCR' and 'miRNA' by convention refers to microRNA and not messenger RNA that the manuscript is detecting.

Author answer: According to the reviewer's comments, we added the details of the experiments performed in material and methods. The Primer sequences for the MS-PCR, RT-PCR and ChIP analysis. And we corrected the typing errors based on the reviewer's comments.

5. Results are detailed in sections to summarize the findings of the experiments undertaken. DNA hypomethylation needs to be detailed in the section decrbing the results of DNA methylation experiments. Also, the rationale for using the H3K9Met3 and H3K4Met2 for chromatin immunopreciptation and the region targeted for investigation should be clearly explained. At present, it does not clarify as to why this analysis was carried out.

Author answer: According to the reviewer's comments, we described in detail the results of DNA hypomethylation and the principle of chromatin immunoprecipitation using h3k9met3 and h3k4met2 in results.

6. Discussion has elaborated the experimental findings in context of previously described work. Elaboration of 'molecualr mecanisms of gene regualtion' and 'epigenetic mechanisms of gene regualiton' is needed for better clarity and focus of the work described.

Author answer: According to the reviewer's comments, we further discussed the molecular and epigenetic mechanisms of gene regulation in the part of discussion.

7. Minor note, manuscript will benefit from editing for languauge.

Author answer: According to the reviewer's comments, we applied for professional revision of native English.