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PEER-REVIEW REPORT

Name of journal: *World Journal of Gastrointestinal Oncology*

Manuscript NO: 70252

Title: O6-methylguanine DNA methyltransferase is upregulated in gastric epithelial cells malignant transformation dependent on its gene promoter DNA hypomethylation

Provenance and peer review: Invited Manuscript; Externally peer reviewed

Peer-review model: Single blind

Reviewer's code: 06079635
Position: Peer Reviewer
Academic degree: BSc, MSc, PhD

Professional title: Assistant Professor

Reviewer's Country/Territory: Pakistan

Author's Country/Territory: China

Manuscript submission date: 2021-07-27

Reviewer chosen by: AI Technique

Reviewer accepted review: 2021-07-28 10:01

Reviewer performed review: 2021-08-05 09:37

Review time: 7 Days and 23 Hours

Scientific quality	[ ] Grade A: Excellent [ ] Grade B: Very good [Y] Grade C: Good [ ] Grade D: Fair [ ] Grade E: Do not publish
Language quality	[ ] Grade A: Priority publishing [Y] Grade B: Minor language polishing [ ] Grade C: A great deal of language polishing [ ] Grade D: Rejection
Conclusion	[ ] Accept (High priority) [Y] Accept (General priority) [ ] Minor revision [ ] Major revision [ ] Rejection

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Re-review	[Y] Yes [ ] No
Peer-reviewer statements	Peer-Review: [Y] Anonymous [ ] Onymous Conflicts-of-Interest: [ ] Yes [Y] No

SPECIFIC COMMENTS TO AUTHORS

The manuscript titled, 'NOCs induce O6-Methylguanine-DNA methyltransferase gene hypomethylation involved in gastric epithelium cells malignant transformation' is an interesting study describing the changes in DNA methylation of MGMT gene promoter in response to MNNG and MNU compounds. The present study has found decrease in DNA methylation level of promoter region of MGMT gene together with changes in H3K9Met3 and H3K4Met2 surrounding that region. This work is important in understanding the role of epigenetic factors during malignant transformation of the normal cells. Epigenetic factors play important roles in cancers and much is still to be learnt about various genes in gastric cancers. Therefore this study provides new insight into DNA methylation changes of a key repair pathway gene. Few comments regarding the mansucript are: 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 sdtudy 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. 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. 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

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

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Academic degree: MD, MSc, PhD
Professional title: Assistant Professor
Reviewer's Country/Territory: United States
Author's Country/Territory: China

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Reviewer accepted review: 2021-07-28 12:52

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Review time: 17 Days and 14 Hours

Scientific quality	[ ] Grade A: Excellent [ ] Grade B: Very good [ ] Grade C: Good [Y] Grade D: Fair [ ] Grade E: Do not publish
Language quality	[ ] Grade A: Priority publishing [Y] Grade B: Minor language polishing [ ] Grade C: A great deal of language polishing [ ] Grade D: Rejection
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Peer-reviewer statements	Peer-Review: [Y] Anonymous [ ] Onymous Conflicts-of-Interest: [ ] Yes [Y] No

SPECIFIC COMMENTS TO AUTHORS

Chen et al in this manuscript entitled "NOCs induce O6-Methylguanine-DNA methyltransferase gene hypomethylation involved in gastric epithelium cells malignant transformation" described their studies of MGMT gene expression and epigenetic regulation in cultured cancer/tumor cell lines and human tissues. They observed that high level of MGMT mRNA and protein in cancer tissues and cell lines at the malignant stage or developing stage after treatment by carcinogenic compound NOCs. During malignant transformation of the human gastric epithelium cell line GES-1 following NOCs treatment, they further observed that significant reduction of DNA methylation of the MGMT gene. This reduction is logically correlated with the upregulation of MGMT in view of the negative role of DNA methylation in transcriptional regulation. However, they also observed that inhibition of MGMT function by pharmacological inhibitor or downregulation of MGMT expression by siRNA resulted in promotion of malignant transformation. Authors, therefore, concluded that MGMT stably up-regulation was induced by its DNA promoter hypomethylation and high expressed MGMT prevented the NOCs-induced cell malignant transformation and tumorigenesis. The observation of DNA methylation reduction of the MGMT gene and upregulation of its expression following NOCs is novel and may provide new insight in epigenetic contribution to our understanding of carcinogenesis. However, the current version of this manuscript suffers from several defects: inappropriate experimental design; lack of sound hypothesis/discussion/explanation why NOCs induces MGMT expression while NOCs produces malignant transformation and why MGMT will prevent this transformation

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instead of promoting this transformation in response to NOCs treatment; inappropriate conclusion; inappropriate presentation and description of results/data; insufficient information of experimental materials, methods and approaches; lack of scientific flow. In sum, the current version of this study is not ready for publication. My specific comments are listed below. Specific comments 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. 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. MGMT expression is too general and unclear. 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.

3. In results for Fig. 1, it should be indicated that MGMT expression in Fig. 1A is from IHC. In addition, details of these data such as types of analyzed tissues and n numbers should be described, and relevant data should be presented in the figure. Fig. 1B is not clear whether they are from qRT-PCR. MGMT expression is too general and unclear. I cannot see how Fig. 1B shows comparison of MGMT expression between cancer and adjacent normal tissues as stated in results. Fig 1C is very confuse. In the left panel, samples of qRT-PCR results should be explained/described/analyzed. Whether these are normalized to a house keeping gene, here likely from the GAPDH, should be

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explained and expressed. Rational to examine MGMT expression in 6 cell lines should be provided and analyzed. 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. 4.

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. 5. 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? 6. 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. 7. 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. The activity of so-called promoter must be confirmed, such as its ability to drive luciferase in comparison to a negative control of promoter-less vector and to a positive control of the CMV, SV40 or RSV promoter. 8. For Fig. 4B, more

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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. 9. 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. Without this information, it becomes difficulty to correlate these data with MGMT expression above. This experiment should be performed for the same time course as examination of MGMT mRNA since this is the major evidence supporting the core story of this manuscript. 10. For Fig. 4D, primers and PCR amplicon position should be provided in the same way as to Fig. 4C. Repeats of experiments should be provided. 11. In the method, miRNA analysis was mentioned, but no such data is presented in the whole manuscript. Authors should explain it. 12. Sample preparation for immunohistochemistry (IHC), RNA analysis, western blot analysis, and genomic DNA isolation should be provided. 13. 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

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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. 14. Anywhere once antibody is used, its dilution and visualization means of result should be provided. 15. 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. 16. 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. 17. 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. 18. 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. 19. siRNAs were mentioned in Fig. 5. However, relevant information is missed from the method section. 20. Sequences of primers, their location relative to the genomic sequence or cDNA sequence should be provided. 21. 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

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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. 22.

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. 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

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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.