Author’s response to reviews

Title: Epigenetic mechanisms involved in differential MDR1 mRNA expression between gastric and colon cancer cell lines and rationales for clinical chemotherapy

Authors:

Tae-Bum Lee (tblee@chosun.ac.kr)
Jung-Hee Park (heeob@hanmail.net)
Young-Don Min (ydmn@chosun.ac.kr)
Kyung-Jong Kim (kjkim@chosun.ac.kr)
Cheol-Hee Choi (chchoi@chosun.ac.kr)

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Author’s response to reviews: see over
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Title: MS:1189197494151297 - Epigenetic mechanisms involved in differential MDR1 mRNA expression between gastric and colon cancer cell lines and rationales for clinical chemotherapy

Authors:

Tae-Bum Lee (tblee@chosun.ac.kr)
Jung-Hee Park (heeeob@hanmail.net)
Young-Don Min (yadmin@chosun.ac.kr)
Kyung-Jong Kim (kjkim@chosun.ac.kr)
Cheol-Hee Choi (chchoi@chosun.ac.kr)

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Authors’s response to reviews: see over
Reviewer #1 (Dr Damian Marlee)

Major Compulsory Revisions

1. Both referees are in agreement that a direct sequencing of the genomic MDR1 promoter region using a bisulfite treatment is needed to provide additional strength to the comparison of the two types of cancer cell lines, let alone the comparison of two distinct cancer types.

   • Various methods have been developed to analyze DNA methylation. Amongst these methods are restriction enzyme- and sodium bisulfite-based approaches which directly detect methylation. Although the latter method is better than the former one, we think that restriction enzyme-based PCR is an appropriate one with many researchers have used. We performed bisulfite DNA sequencing analysis for the definitive evidence of methylation status of MDR1 promoter region as reviewers requested. Methylation status obtained from quantification PCR-based methylation analysis were completely matched with the results obtained by bisulfite DNA sequencing assay. We described abstract (page 1), methods (page 9 and 10), results (page 13) and discussion (page 15) about bisulfite DNA sequencing assay. Also, we revised results of PCR-based methylation analysis on page 2, 12, 15, Table 1 on page 24, Fig. 5 legend on page 26 and Fig. 5. In previous results, MS1 and MS2 sites in the HT-29 cells and only at the MS1 site in the COLO320HSR cells were methylated. Reexperiment showed that only at the MS2 site in the HT-29 cells were methylated but was not methylated in the COLO320HSR cells.

2. The other vulnerable spot of the paper is that the cell lines SNU-668 and SNU-484 almost mirror the expression pattern of COLO320HSR in PCR-based assays. Here, protein-level studies are needed to clarify this issue.

   • We think that a reviewer might be confused with something including the number of PCR cycles. Be aware that our isotopic RT-PCR method is much more sensitive than non-isotopic conventional one that can’t detect MDR1 mRNA in gastric cancer cell lines. In addition, Western blot analysis has
already revealed that p-glycoprotein is not expressed in SNU-668 and SNU-484 cells.

3. The authors are invited to use a library of tumor tissues, obtained in accordance of commonly used protocols and procedures, to solidify the results, obtained from the array of cell lines.

- I totally agree that much samples, the better. But we think that is no doubt for our conclusion. Actually it is not easy obtain a library of tumor tissues.
Reviewer #2 (Dr Jing-Yuan FANG)

Major Compulsory Revisions

1. Why does authors did not assay the methylation of the promoter or/and exon 1 using the bisulfite modification and sequencing (mapping)? Because the result from mapping contains all of data from the MSP and restriction endonuclease digestion as well as direct sequencing for MSP products. The primers design problem? Please explain the reason.

   • Various methods have been developed to analyze DNA methylation. Amongst these methods are restriction enzyme- and sodium bisulfite-based approaches which directly detect methylation. Although the latter method is better than the former one, we think that restriction enzyme-based PCR is an appropriate one with many researchers have used. We performed bisulfite DNA sequencing analysis for the definitive evidence of methylation status of MDR1 promotor region as reviewers requested. Methylation status obtained from quantification PCR-based methylation analysis were completely matched with the results obtained by bisulfite DNA sequencing assay. We described abstract (page 1), methods (page 9 and 10), results (page 13) and discussion (page 15) about bisulfite DNA sequencing assay. Also, we revised results of PCR-based methylation analysis on page 2, 12, 15, Table 1 on page 24, Fig. 5 legand on page 26 and Fig.5. In previous results, MS1 and MS2 sites in the HT-29 cells and only at the MS1 site in the COLO320HSR cells were methylated. Reexperiment showed that only at the MS2 site in the HT-29 cells were methylated but was not methylated in the COLO320HSR cells.


   • We added 5 recent references (1, 2, 3, 7 and 38) on page 19 and 22.
Reviewer #3 (Dr Masakazu Yashiro)

General

1. The data presented in this study is not enough to conclude that their results can provide a better understanding of the efficacy of combined chemotherapy as well as their oral bioavailability.

   - We just have focused on the epigenetic mechanisms involved in MDR1 gene expression between gastric and colon cancer cell lines. The efficacy of combined chemotherapy as well as their oral bioavailability was just implicated in discussion.

2. Their findings are not sufficient for definition of chemotherapeutic regimen. No experimental evidence is provided in the effect on 5-FU, platinum agents and topoisomerase inhibitors in animal models.

   - The choice of animal models for research is one of the most critical aspects of experimental design. Your critical suggestion will be another next big project.

3. More details of the backgrounds of cancer cell lines used in this study should be addressed. Because the chemo-sensitivity of most cancer cell lines depend on p53 status, p53 status in each cancer cell lines should be addressed.

   - Among the ten gastric cancer cell lines, all cell lines except SNU-1 and SNU-620 had p53 mutation. (SNU-1 had wild-type p53, while homozygous deletion of p53 was detected in SNU-620). And p53 mutations were revealed in 5 of the 9 colon cancer cell lines (except SNU-C4, LoVo, HCT-8 and HCT-116). We described p53 status of cell lines used on page 11.

4. Why were both of semi-quantitative RT-PCR and real-time RT-PCR performed? Some of gastric cancer cell lines such as SNU-668,484 showed relative high expression in semi-quantitative PCR, whereas no expression in real-time RT-PCR in despite of more than 35 cycles. In addition, why did these cell lines show almost same expression ratio as COLO320HSR? Data of Pgp protein expression should be necessary.

   - We think that a reviewer might be confused with something, the number of PCR cycles. Be aware that our isotopic RT-PCR method is much more
sensitive than non-isotopic conventional RT-PCR and real-time PCR that can’t detect MDR1 mRNA in gastric cancer cell lines. In addition, Western blot analysis has already revealed that p-glycoprotein is not expressed in SNU-668 and SNU-484 cells.

5. In the chemo-sensitivity assay, expression of Pgp protein is not associated with sensitivity for paclitaxel, because IC50 of SNU-668 was more than 10-fold high compared with SNU-C5 which showed no expression of Pgp protein same as SNU-668. In addition, there might be lack in sufficient explanation about dose of paclitaxel in figure 4B. Growth of SNU-668 which IC50 is 200-fold less than Colo320HSR is not suppressed at all, whereas that of colo320HSR is dramatically reduced only by adding Pgp inhibitor.

- It is well known that paclitaxel is a good substrate for Pgp. The differential sensitivity between SNU-668 and SNU-C5 to paclitaxel is shown to be due to different levels of MDR1 mRNA or protein. We guess that Pgp protein in SNU-668 cells might be too low to be detected by Western blot analysis. Since three cell lines including SNU-668, SNU-C5 and COLO320HSR were treated with approximately IC10 concentration of paclitaxel, they survived in the presence of this concentration of the drug. We added paclitaxel concentrations in Fig 4B legend on page 26.

6. Direct sequencing of promoter region with or without bisulfite treatment might be useful to examine the methylation status at in gastric and colon cancer cell lines.

- Various methods have been developed to analyze DNA methylation. Amongst these methods are restriction enzyme- and sodium bisulfite based approaches which directly detect methylation. Although the latter method is better than the former one, we think that restriction enzyme-based PCR is an appropriate one with many researchers have used. We performed bisulfite DNA sequencing analysis for the definitive evidence of methylation status of MDR1 promotor region as reviewers requested. Methylation status obtained from quantification PCR-based methylation analysis were completely matched with the results obtained by bisulfite DNA sequencing assay. We described abstract (page 1), methods (page 9 and 10), results (page 13) and
discussion (page 15) about bisulfite DNA sequencing assay. Also, we revised results of PCR-based methylation analysis on page 2, 12, 15, Table 1 on page 24, Fig. 5 legend on page 26 and Fig. 5. In previous results, MS1 and MS2 sites in the HT-29 cells and only at the MS1 site in the COLO320HSR cells were methylated. Reexperiment showed that only at the MS2 site in the HT-29 cells were methylated but was not methylated in the COLO320HSR cells.

7. Discussion about treatment with 5AdC and TSA is relative complicated. How is the effect of these treatments on Pgp protein expression or chemo sensitivity? The authors described that MDR1 mRNA expression is differentially regulated in gastric and colon cancer. However, as shown in Table1, SNU-16 (gastric cancer cell line) and SNU-C5 (colon cancer) show similar effect on MDR1 expression as well as SNU-216 and HCT-116.

• Complicated discussion about effects of 5AdC and TSA is due to complicated mechanisms of differential expression of MDR1 gene in gastric and colon cancer cell lines, which can’t be clearly explained with DNA methylation and/or histone deacetylation, suggesting that there might be other mechanisms.