COMMENTARY

DNA methylation dependent suppression of GPER1 in colorectal cancer

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Abstract

A paper published by Liu and co-workers in a recent issue of *Molecular Cancer* not only reiterates the epigenetic silencing as a mechanism of GPER1 suppression, but also expands the scope of this emerging paradigm beyond breast cancer to include colorectal adenocarcinoma. This article is a commentary on the relevance and significance of their work. It presents results obtained through an independent analysis of TCGA-COADREAD dataset that supports their claim. It highlights the emerging role of DNA methylation in GPER1 regulation by drawing parallels with other published works, while at the same time draws attention of the readers to the caveats and the points of departures from recent literature.

Keywords: GPER1, GPR30, colorectal carcinoma, methylation, TCGA, CpG island shore

The discovery of the seven-transmembrane G-protein coupled estrogen receptor (GPER1), and its recognition as a transducer of non-genomic effects of estrogen, provided a new mechanistic framework for understanding breast cancer etiology, progression and endocrine resistance. Breast cancer, however, is not the only point of relevance for understanding GPER1 signaling, expression and regulation. GPER1 is now understood to play physiological roles in both reproductive and non-reproductive organs and systems[1]. Given the large body of experimental and clinical data, it is fast

emerging as a potential target for therapeutic intervention.

Amid the significant advancements in our understanding of GPER1 signaling, mechanisms of GPER1 expression and regulation still remain poorly understood. One cannot miss the gradually emerging interest in the epigenetic regulation of GPER1; a direct result of its apparent role as a tumor suppressor[2–5]. A recent article in *Molecular Cancer* authored by Liu et al. is the latest addition to the growing literature, and an independent affirmation of epigenetic silencing as a mechanism of GPER1 suppression[6]. Furthermore, it adds colorectal carcinoma, a non-reproductive tissue cancer, to the growing list of malignancies whose progression appears to be associated with the loss of GPER1 expression. We have read the article with great interest; the subject matter being related to that of our recent publication in *Gene*[2]. The purpose of this communication is two-fold- a) to emphasize the role of DNA methylation in GPER1 suppression on the basis of results obtained from an independent analysis of TCGA data that corroborates with bisulfite sequencing results published by Liu et al., and b) to underscore both the highlights and caveats of their work in the light of the recent literature.

The authors' contribution is set on two basic premises that- a) estrogen plays a role in the pathophysiology of colon and rectum, and b) progression of colorectal cancer (like few other solid tumors) is associated with the loss of GPER1 expression. The outcomes of their clinical, *in vivo* and *in vitro* studies lead the reader to envisage a model in which loss of GPER1 expression is due to DNA methylation- and histone acetylation-dependent epigenetic silencing. GPER1 silencing in turn promotes enhanced rate of proliferation, which leads to tumor initiation and progression. The general framework of the model is consistent with that emerging from other recent studies[2–5]. However, in the following sections, we wish to present our assessment of this interesting piece of work.

Liu et al. have chosen a region (-781 to -461) for bisulfite sequencing. This encompasses 23 CpGs, hereafter referred to as the region of interest (ROI). In cell culture models, the authors claim to have detected an "obvious" difference in methylation within the ROI. However, their claim is not supported by enough sequencing data and statistical analysis. Analysis of bisulfite converted DNA isolated from colorectal tumors and their matched normal counterparts, showed a marked difference in methylation within the ROI. Hyper-methylation was associated with lower GPER1 expression in colorectal tumors. Given the large cohort that the authors had access to, one expects more sequencing data with statistical evaluation.

Human GPER1 has three transcript variants according to the NCBI Nucleotide database. We recently demonstrated the expression of these three variants in MCF-7 cells[2]. Without any reference to one of the two possible transcription start sites, one finds it difficult to map the ROI in the GPER1 locus. We examined the -781 to -461 region with respect to both the transcription start sites, but failed to find the ROI with 23 CpGs. However, we mapped the primer sequences provided by the authors, which demarcate its boundaries. The ROI fell within the upstream CpG island (upCpGi), which we have described recently[2]. So far, to our knowledge, five reports including the one under discussion, have implicated this

region in methylation-dependent silencing of GPER1[2–5]. Thus, there appears to be a consensus as far as the region involved in methylation-dependent GPER1 silencing is concerned. However, we would also like to bring out the points of departure. The upCpGi encompasses a differentially methylated region (DMR) comprising of eight CpGs. Using MCF-7 and MDA-MB-231 breast cancer cell lines as models, we showed that methylation of the DMR inversely correlates with GPER1 expression[2]. The ROI chosen by Liu et al. which is nested within the upCpGi, does not include the DMR (Figure 1). Hence the status of methylation in the DMR remains unaddressed in their study. There also exists a downstream CpG island (dnCpGi) in the GPER1 locus which does not seem to be involved in methylation-dependent modulation of GPER1 expression in breast cancer cells ([2] and supplementary information therein). The analysis of methylation status of CpGs within the DMR and dnCpGi in DNA samples from cell lines, colorectal tumors and their matched controls may yield valuable information.

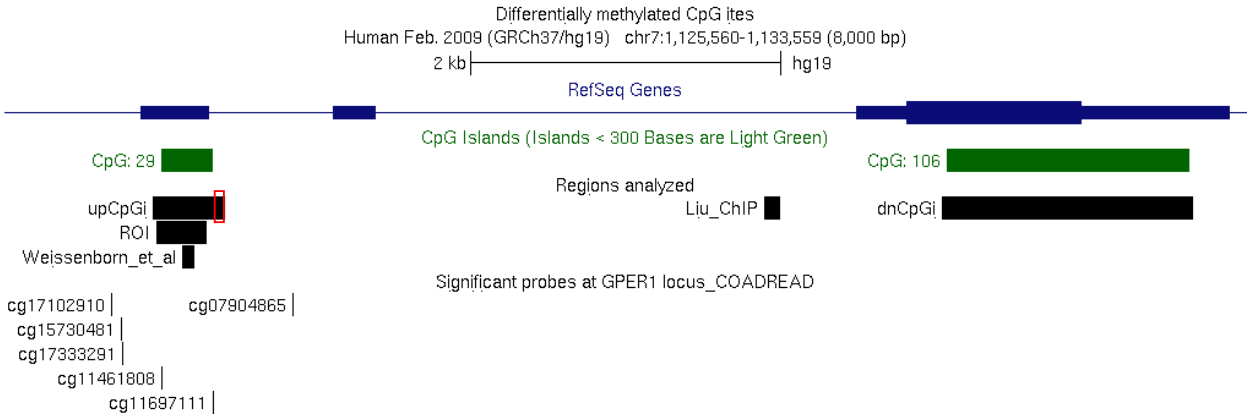

Figure 1: Location of significant probes at the GPER1 locus. A snapshot from the UCSC Genome Browser showing the locations of significant probes (450K Bead chip array) in the GPER1 locus of the human genome (hg19). The significant probes were identified by two-tier selection process (IQR > 0.15 and $\rho < -0.3$) in the EMC analysis. The probe IDs appear in the format cgxxxxxxxx (x is any digit). The vertical line after the probe IDs indicates the location of the probe. The mRNA is shown in blue. The CpG islands in the locus are shown below the mRNA (green rectangles). The locations of upCpGi and dnCpGi, the regions analyzed by Liu et al. for methylation (ROI) and acetylation (Liu_ChIP), and the region studied by Weissenborn et al.[3–5] are shown as black rectangles under the track name "Regions analyzed". The region demarcated within the upCPGi by a red box indicates the DMR[2].

In order to independently verify the claims of Liu et al. we analyzed GPER1 expression in colorectal adenocarcinoma, and their normal counterparts. Towards this end, we analysed the COADREAD dataset from The Cancer Genome Atlas (TCGA)[7]. The details of the data used for analysis is provided in the Supplemental file 1. The

mean GPER1 mRNA expression in tumors was significantly lower than that in normal tissues (Figure 2, $p < 0.00001$), which provides an independent confirmation of the results from Liu et al.

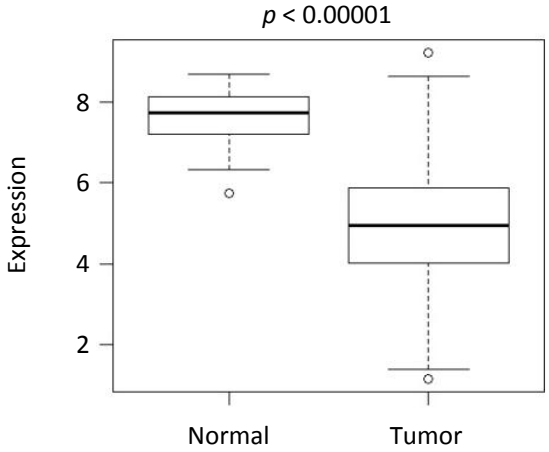

Figure 2. Expression of GPER1 mRNA in colorectal adenocarcinoma. The mRNA expression values, as $\log_2$(RPKM+1), for GPER1 in colorectal adenocarcinoma were retrieved from TCGA-COADREAD dataset. The distribution of GPER1 expression in the normal tissue (21) and the colorectal tumors (373) is shown as boxplot. The difference between the mean GPER1 mRNA expression was tested by Welch two-sample *t*-test and the *p*-value is mentioned above the boxplot.

The relationship between GPER1 expression and CpG methylation in the GPER1 locus was assessed by expression-methylation correlation (EMC) analysis of TCGA-COADREAD dataset as described in Supplemental file 1. Out of the 118 probes in the 450K bead chip array that represent the GPER1 locus, 6 probes (hereafter referred to as significant probes) satisfied the two-tiered selection criteria (IQR > 0.15 and $\rho < -0.3$). The methylation scores of each of these 6 probes significantly correlated inversely with the GPER1 mRNA expression (Supplemental file 2). Two of these 6 probes (cg11461808 and cg11697111), mapped within the upCpGi, whereas the remaining mapped to the upstream (cg17102910, cg15730481 and cg17333291) and downstream (cg07904865) shore regions of the upCpGi (Figure 1). These results partly corroborate with our results, which indicate that the methylation status of the shore region inversely correlates with the GPER1 expression[2]. In case of colorectal adeno-carcinoma, the probes representing upCpGi also seem to significantly correlate inversely with GPER1 expression. Only one probe (cg11461808) mapped to the ROI (Figure 1). With respect to the composite methylation score, computed as an average of beta values of the significant probes, a stronger inverse correlation ($\rho = -0.52502$, $p < 0.00001$) with GPER1 expression was observed (Figure 3A). Further, the classification of tumor samples, based on the composite methylation score threshold of 0.3, into hypo- (≤ 0.3) and hyper-methylated (> 0.3) groups revealed that the mean GPER1 expression was significantly

higher in hypo-methylated group compared to hyper-methylated group (Figure 3B). Taken together, all evidences implicate the ROI in methylation-dependent silencing of GPER1 expression in colorectal carcinoma. It is important to note that the methylation probes of the 450K bead chip array do not represent the DMR. Thus, the importance of DMR in regulating GPER1 expression in colorectal adenocarcinoma remains unaddressed. Liu et al. may consider extending the boundaries of ROI to include the DMR for bisulfite sequencing analysis of DNA samples obtained from their cohort in future studies.

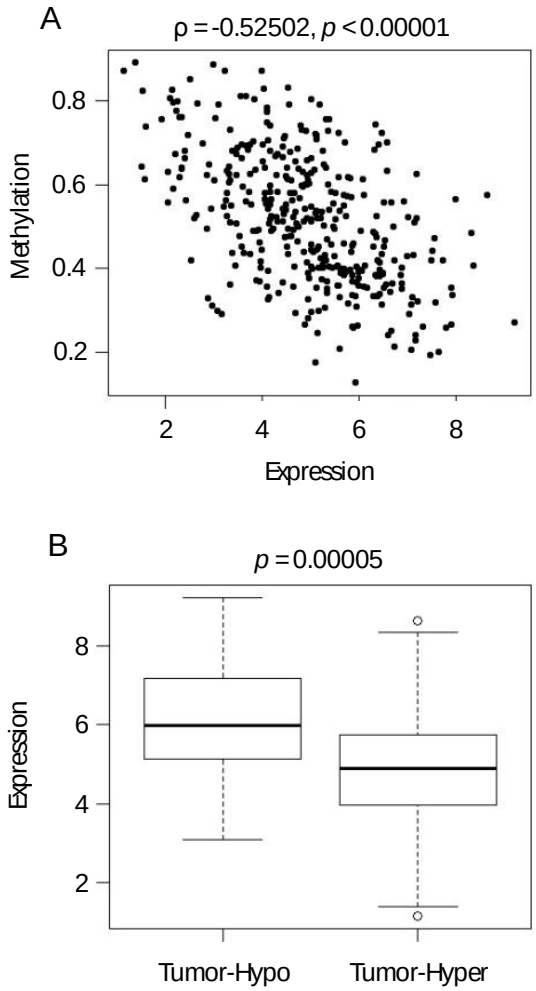

Figure 3: Correlation of DNA-methylation and GPER1 expression. A. Scatterplot of composite methylation score of significant probes versus GPER1 expression. B. Boxplot showing the distribution of GPER1 expression in hypo- and hyper-methylated groups. Tumors were grouped into hypo- and hyper-methylated categories based on the threshold value of 0.3 for the average beta score of the significant probes.

The authors' demonstration of growth inhibition of colorectal carcinoma cells by G1, a GPER1 ligand is consistent with the tumor suppressor role of GPER1. It also underscores the importance of GPER1 in the etiology of colorectal cancers and their

progression. However, we would like to state our concerns about their experiments with G1. The authors reported IC_{50} values of 8.5 μM and 11.7 μM for G1-mediated reduction in viability of HCT-116 and SW480 cells, respectively. On the basis of few studies cited in their article[8–10], they have conducted all their experiments at 1 μM concentration of G1. Recent studies have shown off-target effects of 1 μM G1 in cell culture models[11–13]. Results from experiments conducted in our laboratory show reduced viability of GPER1-negative MDA-MB-453 cells treated with 1 μM G1 (yet unpublished result). Hence, the implications of their results with 1 μM G1 has to be dealt with caution. In the absence of similar experiments done on GPER1 knockout cells, one cannot rule out the contribution of the off-target effects in their observed results.

Liu and co-worker's report is undoubtedly an eye-catcher that provides an insight into the estrogen link in the pathophysiology of the colon, and the contribution of GPER1 therein. With the increasing data on the association of GPER1 expression with the disease, a comprehensive and in-depth understanding of epigenetic mechanisms of its expression will have far-reaching therapeutic implications. Furthermore, it is also important to delineate specific GPER1 mediated effects to understand its therapeutic potential. In this context, we hope that the scientific community, particularly those engaged in GPER1 research will find the issues raised in this communication noteworthy.

List of abbreviations

GPER1: G-protein coupled estrogen receptor 1

ROI: region of interest

DMR: differentially methylated region

TCGA: The Cancer Genome Atlas

COADREAD: colon and rectal adenocarcinoma

EMC: expression-methylation correlation

Acknowledgements

The work was financially supported by a research grant from the Department of Biotechnology, Govt. of India (Sanction order No. BT/506/NE/TBP/2013, dated 11.08.2014). Authors thank the Department of Biosciences and Bioengineering, IIT Guwahati for the infrastructural support. The authors also acknowledge the significant contribution by the entire team behind TCGA project.

References

1. Prossnitz ER, Barton M. Estrogen biology: New insights into GPER function and clinical opportunities. *Mol Cell Endocrinol*. 2014;1421. doi:10.1016/j.mce.2014.02.002.

2. Manjegowda MC, Gupta PS, Limaye AM. Hyper-methylation of the upstream CpG island shore is a likely mechanism of GPER1 silencing in breast cancer cells. *Gene*. 2017;614:65-73. doi:10.1016/j.gene.2017.03.006.

3. Weißenborn C, Ignatov T, Ochel H-J, et al. GPER functions as a tumor suppressor in triple-negative breast cancer cells. *J Cancer Res Clin Oncol*. 2014;140(5):713-23. doi:10.1007/s00432-014-1620-8.

4. Weißenborn C, Ignatov T, Poehlmann A, et al. GPER functions as a tumor suppressor in MCF-7 and SK-BR-3 breast cancer cells. *J Cancer Res Clin Oncol*. 2014;140(4):663-71. doi:10.1007/s00432-014-1598-2.

5. Weissenborn C, Ignatov T, Nass N, et al. GPER Promoter Methylation Controls GPER Expression in Breast Cancer Patients. *Cancer Invest*. 2017;35(2):1-8. doi:10.1080/07357907.2016.1271886.

6. Liu Q, Chen Z, Jiang G, et al. Epigenetic down regulation of G protein-coupled estrogen receptor (GPER) functions as a tumor suppressor in colorectal cancer. *Mol Cancer*. 2017;16(1):87. doi:10.1186/s12943-017-0654-3.

7. Muzny DM, Bainbridge MN, Chang K, et al. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487(7407):330-337. doi:10.1038/nature11252.

8. Wei W, Chen Z-J, Zhang K-S, et al. The activation of G protein-coupled receptor 30 (GPR30) inhibits proliferation of estrogen receptor-negative breast cancer cells in vitro and in vivo. *Cell Death Dis*. 2014;5(October 2016):e1428. doi:10.1038/cddis.2014.398.

9. Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, Picard D. Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J*. 2009;28(5):523-32. doi:10.1038/emboj.2008.304.

10. Pupo M, Pisano A, Abonante S, Maggiolini M, Musti AM. GPER activates Notch signaling in breast cancer cells and cancer-associated fibroblasts (CAFs). *Int J Biochem Cell Biol*. 2014;46:56-67. doi:10.1016/j.biocel.2013.11.011.

11. Lv X, He C, Huang C, et al. G-1 inhibits breast cancer cell growth via targeting colchicine-binding site of tubulin to interfere with microtubule assembly. *Mol Cancer Ther*. 2017:molcanther.0626.2016. doi:10.1158/1535-7163.MCT-16-0626.

12. Wang C, Lv X, Jiang C, Davis JS. The putative G-protein coupled estrogen receptor agonist G-1 suppresses proliferation of ovarian and breast cancer cells in a GPER-independent manner. *Am J Transl Res*. 2012;4(4):390-402. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3493027&tool=pmcentrez&rendertype=abstract.

13. Wang C, Lv X, He C, Hua G, Tsai M-Y, Davis JS. The G-protein-coupled estrogen receptor agonist G-1 suppresses proliferation of ovarian cancer cells by blocking tubulin polymerization. *Cell Death Dis*. 2013;4(10):e869. doi:10.1038/cddis.2013.397.

