Abstract. - OBJECTIVE: In this study, we aimed to investigate the underlying mechanisms of MGP downregulation in chemoresistant ER+ breast cancer cells and its association with survival outcomes in breast cancer patients.

MATERIALS AND METHODS: Microarray data of dysregulated genes in chemoresistant ER+ breast cancer cells were searched in GEO datasets. MGP expression in breast cancer patients and its DNA methylation status were analyzed in TCGA database. MGP promoter methylation was assessed using Methylation-Specific PCR (MSP) assay. The association between MGP expression and survival outcomes in different subtypes of breast cancer patients after systemic therapy was analyzed by data mining in Kaplan Meier plotter and in Breast Cancer Gene-Expression Miner Version 4.0 (bc-GenExMiner 4.0).

RESULTS: MGP is significantly downregulated in MCF-7/ADR cells compared to the parental MCF-7 cells. MCF-7/ADR cells had a significantly higher level of methylation in MGP promoter than MCF-7 cells. Demethylation treatment significantly restored MGP expression at both mRNA and protein levels. High MGP expression is associated with better relapse-free survival (RFS) in luminal A and luminal B breast cancer patients, but the association was not observed in HER2+ and basal-like subtype breast cancer patients. High MGP expression was associated with significantly lower risk of any event (AE) and also lower risk of metastatic relapse (MR). Survival curve showed that high MGP expression was associated to both better AE-free survival and MR-free survival.

CONCLUSIONS: MGP is downregulated due to promoter hypermethylation in chemoresistant ER+ breast cancer and high MGP expression predicts better survival outcomes among ER+ breast cancer patients.

Key Words: MGP, Hypermethylation, ER+, Breast cancer, Survival.

Introduction

Currently, chemotherapy is still the major therapy for patients with advanced breast cancer1,2. Since sensitivity to chemotherapy is one of the most important prognostic factors, it is quite valuable and important to identify the biomarkers and potential mediators of chemotherapy responses in breast cancer patients. Some previous researches reported that dysregulated epigenetic regulations, such as DNA hyper- and hypo-methylation may confer induced adaptive responses of cancer cells to chemotherapeutic agents3,4. One recent paper found that in human breast cancer cells, ERα can enhance aberrant global DNA hypermethylation via activating the DNMT1 gene, leading to enhanced anticancer drug resistance5. Matrix Gla protein (MGP), is a vitamin K2-dependent, Gla-containing protein. Previous studies found that MGP is a multi-functional inhibitor of normal and abnormal angiogenesis, thereby acting as an endogenous inhibitor of tumor angiogenesis6. MGP downregulation was observed in some cancers, including lung cancer7 and colorectal adenocarcinoma8. In breast cancer, the role of MGP is still controversial. One recent work observed that oncomiR-155 can decrease MGP expression in breast cancer MCF-7 cells, leading to enhanced cell proliferation and cell invasiveness9. However, another previous paper showed that MGP upregulation at mRNA level may predict poor prognosis in breast cancer10. This finding is not verified at the protein level and also did not consider breast cancer subtypes10. In the current study, by re-analysis of one publically available gene microarray data, we found that MGP expression was downregulated in chemoresistant ER+ breast cancer cells compared to the
sensitive parental cells. Therefore, we investigated the underlying mechanisms of its downregulation and its association with survival outcomes in breast cancer patients.

Materials and Methods

Bioinformatic Data Mining
Microarray data that compared the gene transcriptional profile of adriamycin-selected MCF-7/ADR cells and weakly tumorigenic parental MCF-7 cells was searched in and downloaded from NCBI GEO Datasets (accession No. GDS4084). The raw SOFT data file was re-analyzed to identify the most downregulated genes. The expression of MGP in breast cancer patients and the DNA methylation status was analyzed based on the data of the breast cancer cohort in TCGA database, using the UCSC Xena (http://xena.ucsc.edu/). MGP expression at protein level in breast cancer tissues and in the normal control tissues were reviewed in Human Tissue Atlas (http://www.proteinatlas.org/). The promoter sequence of MGP was obtained by the MGP promoter clone information in Genecopoeia (HPRM23032, NM_000900). Methylation sites in the promoter region of MGP were predicted using MethPrimer (http://www.urogene.org/methprimer/). The association between MGP expression and relapse-free survival (RFS) in different sub-types of breast cancer patients after systemic therapy was analyzed by data mining in Kaplan Meier plotter (http://kmplot.com/analysis/), which is an online public database with assessment of different gene expression on survival among breast cancer, ovarian cancer, lung cancer and gastric cancer patients, including 22,277 genes in 5,143 breast cancer patients. To pool previous studies that assessed the association between MGP expression and survival outcomes in ER+ breast cancer patients, data mining was performed in Breast Cancer Gene-Expression Miner Version 4.0 (bc-GenExMiner 4.0), a statistical mining tool of published annotated genomic data. In this database, meta-analysis could be performed to assess the association between gene expression and any event (AE, defined as any relapse or death)-free survival and metastatic relapse (MR)-free survival among different subtypes of breast cancer patients. Survival curves were generated by Kaplan-Meier method and differences between the curves were assessed using the Log-rank test.

Cell Culture
Human ER+ breast cancer cell line MCF-7 was obtained from ATCC (Rockville, MD, USA). To generate adriamycin (ADR)-resistant MCF-7/ADR cells, the parental MCF-7/ADR cells were treated with stepwise increased concentrations of ADR for over 8 months. The cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml at 37°C and 5% CO2.

Demethylation Treatment
MCF-7/ADR cells were seeded onto 6-well plates at 1.0×10⁶ cells/well and were treated with 1 or 2.5 μM 5-AZA-dC for 24 h.

Methylation-Specific PCR (MSP)
Genomic DNA of MCF-7/ADR and MCF-7 cells were extracted using a DNeasy tissue kit (Qiagen, Hilden, Germany). Methylation-specific PCR (MSP) analysis was performed as previously described using methylated-specific primers (M): forward, 5’-GGAAATAGAATTTATGGTGTATCGG-3’ and reverse, 5’-ACCTCTTCTTCTAAAAAACCTTCGC-3’; and unmethylated-specific primers (U): forward, 5’-TTAAGGAAATAAGTTATGTTGATCGG-3’ and reverse, 5’-ACCTCTTCTTCTAAAAAACCTTCAGT-3’. PCR products were verified by 2% agarose gel electrophoresis.

Western Blot Analysis
Cell samples were lysed using a lysis buffer (Beyotime, Shanghai, China) and the protein concentration was quantified using a BCA protein assay kit (Beyotime). Then, the denatured protein samples were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were further transferred to polyvinylidene fluoride (PVDF) membranes. After blocking and washing, the membranes were subjected to incubation with anti-MGP (ab70193, Abcam, Cambridge, MA, USA) and anti-β-actin (Ab1801, Abcam). Next, the membranes were incubated with corresponding horse radish peroxidase (HRP) conjugated secondary antibodies. The protein band signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA). The relative expression of the proteins was quantified using ImageJ software.
Statistical Analysis

Statistical analyses were performed using SPSS 18.0. software (SPSS Inc., Chicago, IL, USA). Data were presented as the mean ± SD. Data were analyzed for statistical significance by two-tailed Student’s t-test or ANOVA with Student-Newman-Keuls test as a post hoc test. p< 0.05 was considered as statistically significant.

Results

MGP is Downregulated in Chemoresistant ER+ Breast Cancer

To identify the dysregulated genes in chemoresistant ER+ breast cancer cells, we re-analyzed the raw data one available microarray data (GDS4084) that assessed the transcriptional profiles between MCF-7/ADR cells and the parental MCF-7 cells. The results showed that MGP is significantly downregulated in MCF-7/ADR cells compared to the parental MCF-7 cells (Figure 1A). Then, we examined MGP expression profiles in different subtypes of breast cancer and the DNA methylation status by using the breast cancer cohort (N=1218) in TCGA database. The heap map showed that MGP downregulation is closely related to DNA hypermethylation in both luminal A and luminal B subtypes of breast cancer (Figure 1B, green frames).

Demethylation Restores MGP Expression in ER+ Breast Cancer Cells

Next, we examined MGP expression at protein level in normal breast tissues and in breast cancer tissues in Human Protein Atlas. MGP is strongly expressed in glandular cells and myoepithelial cells in normal breast tissues (Figure 2A, orange arrows). MGP is also widely expressed among 11 cases of breast cancer tissues in the database, in which 7 cases have strong MGP staining (Figure 2B, red frames), while 4 cases have moderate

Figure 1. MGP is downregulated in chemoresistant ER+ breast cancer. A, The heap map the 30 most downregulated genes in MCF-7/ADR cells compared to the parental MCF-7 cells. Red: up-regulation; Blue: down-regulation. Raw microarray data was obtained from GEO dataset (accession: GDS4084). B, The heap map of MGP in different subtypes of breast cancer and the corresponding DNA methylation status by using the breast cancer cohort (N=1218) in TCGA database. Green frames: hypermethylation cases in luminal A and luminal B groups.
MGP expression (Figure 2B, blue frames). These results suggest that MGP downregulation might occur in some breast cancer cases. Since we observed a possible role of hypermethylation in MGP downregulation in breast cancer patients, we further investigated the association between DNA hypermethylation and MGP downregulation in chemoresistant ER+ breast cancer cells. MCF-7/ADR cells were treated with 5-AZA-dC, a demethylation reagent. QRT-PCR and following Western blot analysis showed that 5-AZA-dC treatment significantly increased MGP mRNA (Figure 2C) and MGP protein (Figure 2D-E) expression in a dose dependent manner. To further verify the involvement of DNA methylation in MGP expression, DNA methylation status of the MGP promoter region was detected by MSP analysis. Two pairs of methylation-specific (M) or unmethylation-specific primer amplification. **p<0.01.
unmethylation-specific (U) primer sets were designed to detect methylation status of two CpG sites (Figure 2F). MSP analysis showed that MCF-7/ADR cells, but not MCF-7 cells, had hypermethylation in this region (Figure 2G). However, 5-AZA-dC treatment significantly decreased hypermethylation in this region (Figure 2G).

**High MGP Expression is Associated with Better RFS in ER+ Breast Cancer Patients**

Resistance to chemotherapy is an important mechanism of therapeutic failure. Since we confirmed MGP downregulation in chemoresistant ER+ breast cancer cells, we then examined the association between MGP expression and RFS in patients with breast cancer and received systemic therapy. By data mining in Kaplan Meier plotter, we found that high MGP expression is associated with better RFS in patients with luminal A breast cancer (HR=0.71, 95% CI: 0.60-0.84, p<0.01, N=1933) (Figure 3A) and in patients with luminal B breast cancer (HR=0.68, 95% CI: 0.56-0.82, p<0.01, N=1149) (Figure 3B). However, the association was not observed in HER2+ and basal-like subtype breast cancer patients (Figure 3C-D).

![Figure 3](http://kmplot.com/analysis/). High MGP expression is associated with better RFS in ER+ breast cancer patients. A-D. RFS curves of different breast cancer subtype patients (A: luminal A; B: luminal B; C: HER2+; D: Basal-like) with high or low MGP expression after systemic therapy. Data was retrieved from the Kaplan Meier plotter (http://kmplot.com/analysis/).
High MGP Expression is Associated with better AE-free and MR-free Survival in ER+ Breast Cancer Patients

To further examine whether the association between high MGP expression and better survival outcome is a generalized finding in ER+ breast cancer, we performed data mining in bc-GenExMiner 4.0. Data searching in the database found 33 available microarray data including 3941 patients assessed the association
between MGP expression and AE-free survival in ER+ breast cancer patients (Figure 4A). Pooled results showed that high MGP expression was associated with significantly lower risk of AE (HR=0.86, 95% CI: 0.81-0.91, p<0.0001) (Figure 4A). Also, 23 available microarray data including 2820 patients assessed the association between MGP expression and MR-free survival (Figure 4B). The results showed that high MGP expression was also associated with significantly lower risk of MR (HR=0.84, 95% CI: 0.77-0.90, p<0.0001) (Figure 4B). Survival curve showed that high MGP expression was associated to both better AE-free survival (HR=0.79, 95% CI: 0.70-0.88, p<0.0001) (Figure 4C) and MR-free survival (HR=0.70, 95% CI: 0.60-0.81, p<0.0001) (Figure 4D).

Discussion

There is emerging evidence showed that DNA methylation is an important epigenetic modulation in the development of chemoresistance in breast cancer. The chemoresistant breast cancer cells usually have aberrant global DNA hypermethylation. This global DNA hypermethylation causes a series of dysregulated signaling pathways associated with chemoresistance. For example, the tumor-suppressor gene Notch3 was inactivated due to DNA hypermethylation. MiR-320a is downregulated in chemoresistant breast cancer cells due to promoter hypermethylation. MiR-320a can directly target transient receptor potential channel C5 (TRPC5) and nuclear factor of activated T-cells isoform c3 (NFATC3), two essential genes for cancer chemoresistance. MiR-149 is also downregulated by promoter methylation, which acts as an inhibitor of breast cancer chemoresistance by targeting GlcNAc N-deacetylase/N-sulfotransferase-1 (NDST1). In this study, by re-analysis of the raw data of GDS4084, a microarray that compared the transcriptional profiles between MCF-7/ADR cells and the parental MCF-7 cells, we observed that MGP is significantly downregulated in MCF-7/ADR cells. Previous studies found that MGP may have an anti-tumorigenic effect in breast cancer cells, which might be related to its putative protein-protein interactions with BMP-2 and vitronectin. Another recent work found that MGP had anti-proliferative in ER+ MCF-7 cells and it is targeted and downregulated by oncomiR-155. However, whether other mechanisms are involved in its downregulation is not clear. By reviewing MGP expression and the corresponding DNA methylation microarray data in breast cancer cohort in TCGA database, we found that MGP downregulation might be closely related to DNA hypermethylation. The following MSP assay showed that MCF-7/ADR cells had significantly higher level of methylation in MGP promoter than MCF-7 cells. Demethylation treatment using 5-AZA-dC significantly decreased methylation and restored MGP expression at both mRNA and protein levels. Based on these findings, we infer that promoter hypermethylation is an important mechanism of MGP downregulation in chemoresistant MCF-7 cells. Since MGP is considered as a tumor suppressive gene in ER+ breast cancer, we decided to further investigate whether its expression is associated to survival outcomes in this group of patients. Although one previous paper reported that MGP upregulation at mRNA level may predict poor prognosis in breast cancer. However, this study did not consider breast cancer subtypes and the statistical power is low due to small patient number (N=9). Our data mining in Kaplan Meier plotter based on 3081 breast cancer patients showed that high MGP expression is associated with better RFS in luminal A and luminal B breast cancer patients. However, the association was not observed in HER2+ and basal-like subtype breast cancer patients. To generalize this finding, we further performed meta-analysis in bc-GenExMiner 4.0. The pooled results showed that high MGP expression was associated with significantly lower risk of AE and MR. Survival curve showed that high MGP expression was associated better AE-free survival and also better MR-free survival.

Conclusions

MGP is downregulated due to promoter hypermethylation in chemoresistant ER+ breast cancer cells. High MGP expression may predict better survival outcomes among ER+ breast cancer patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.
References


