Prognostic value of matrix Gla protein in breast cancer

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Abstract. To assess the prognostic value of matrix Gla protein (MGP) expression in cases of breast cancer, 9 samples from patients diagnosed with breast cancer who were followed up for more than 10 years were microdissected and then analyzed using Affymetrix U133 Plus 2.0 Arrays. Genes that exhibited significant differences in expression between patients with a good prognosis and those with a poor prognosis were identified. The MGP gene was among the genes up-regulated in cases where the prognosis was poor, indicating that the mRNA levels of MGP are a potential prognostic indicator of breast cancer. However, immunohistostaining of breast tissue microarrays (n=207) did not reveal a correlation between the protein expression of MGP and overall survival, neither was there a correlation between the protein expression of MGP and ER status or bone metastasis. In breast cancer cases, the mRNA level of MGP may be a marker indicating poor prognosis; however, protein expression determined by immunohistostaining is not.

Introduction

Matrix Gla protein (MGP) is a calcium-binding protein isolated from bone matrix and cartilage that requires vitamin K-dependent γ-carboxylation (1-3). In addition to its presence in bone or cartilage, MGP is distributed in organs such as the lung, heart, kidney and spleen (4). Mutations in the MGP gene are noted in Keutel Syndrome (5), a rare condition characterized by ectopic calcification and ossification, peripheral stenosis of the pulmonary artery and midfacial hypoplasia. It has been suggested that the function of MGP in bone and cartilage is to inhibit the formation of hydroxyapatite.

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The role of MGP in oncogenesis is unclear, and the correlation between MGP expression and cancer differs according to tumor type. MGP mRNA expression that was increased in comparison to normal tissue levels was found in ovarian cancer (6) and renal cell carcinoma (7). In renal cell carcinoma, a significant inverse correlation was observed between the level of MGP expression and tumor size, lymph node metastasis and tumor grade. In contrast, the expression of MGP mRNA was down-regulated in colorectal cancer cells compared to adjacent normal tissue, though this down-regulation was not correlated with histopathologic features, such as tumor progression, size and cell differentiation (8). MGP mRNA levels were significantly increased in high- compared to low-grade astrocytic gliomas (9).

In breast cancer, a study using cDNA hybridization revealed the expression of MGP to be 20-fold higher in the human metastatic breast cancer cell line 600PEI than in normal breast epithelium (10). In this study, we found MGP to be overexpressed in breast cancer patients with a poor prognosis compared to those with a good prognosis, on the basis of gene expression analysis using microarrays. We then used the tissue microarrays to clarify whether MGP protein levels determined by immunohistostaining could serve as a prognostic marker of breast cancer, and analyzed the correlation between MGP and ER status or bone metastasis.

Materials and methods

Breast cancer samples. Gene expression microarray samples were obtained from patients who underwent surgical resection between May 1995 and October 1997 at the Department of Breast Oncology of the Cancer Institute Hospital of the Japanese Foundation for Cancer Research (JFCR). Patients had tumors >3 cm in diameter, and sufficient follow-up data spanning more than 10 years were available (n=9). Tissue microarray (TMA) samples were obtained from patients who underwent surgical resection between October 1994 and December 1995 at the Department of Breast Oncology of the Cancer Institute Hospital of JFCR. Patients were selected for the study if the available paraffin block was large enough for the fabrication of TMA samples, and if sufficient follow-up data were available (n=207; median follow-up time, 8.2 years).

Table I. Clinical and histopathological characteristics of breast cancer patients involved in the microarray analysis.

Patient no.	Tumor size (cm)	LN	ER	PgR	Adjuvant treatment	Prognosis
1	6.0	1/23	-	-	CMF	Deceased
2	5.3	5/30	-	-	CMF	Deceased
3	5.2	16/28	+	-	CMF TAM	Deceased
4	5.2	4/19	-	-	Radiation	Deceased
5	5.0	0/20	-	-	CMF	Surviving
6	3.7	0/34	-	-	-	Surviving
7	6.0	0/50	-	-	-	Surviving
8	4.8	0/23	-	-	5'-DFUR,EXE	Surviving
9	5.5	0/33	+	+	CMF	Surviving

LN, lymph node metastasis; ER, estrogen receptor; PgR, progesteron receptor; CMF, cyclophosphamide, doxorubicin and fluorouracil; 5'-DFUR, 5'-deoxy-5-fluorouridine; EXE, exemestane; TAM, tamoxifen.

Gene expression microarray analysis. Laser microbeam microdissection was used to collect pure populations of tumor cells in all samples, and total RNA extraction was carried out (RNeasy Micro Kit, Qiagen). With the total RNA (100 ng) of each sample, two-cycle cDNA synthesis and labeling of cRNA were carried out according to Affymetrix (Santa Clara, CA, USA) protocol (11). We used 20 μg of biotin-labeled cRNA and broke down the full length to 35-200 base fragments. Then, 15 μg of the broken cRNA was used to make a cocktail solution, which was placed in Gene Chip HG U133 plus 2.0 and hybridized for 16 h at 45˚C. The arrays were washed and stained using Fluidic station 450 (Affymetrix) and scanned using an Affymetrix GeneChip Scanner 3000. Expression values for each gene were calculated using Affymetrix GeneChip analysis software MAS 5.0.

The microarray data were analyzed using R software (http://www.r-project.org/). Expression data were normalized by the Robust Multichip Average (RMA) method (12) using Bioconductor and associated packages (Bioconductor 2.6.1, http://www.bioconductor.org). The data were then $\log_2$-transformed. Genes expressed differently between the poor and good prognosis groups were identified using the Significance Analysis of Microarrays (SAM) method with a fold change cutoff >3.5 (up-regulated in the poor prognosis group) or <0.4 (down-regulated); p-value <0.01 (13).

Real-time RT-PCR analysis. Expression of MGP was quantified by real-time RT-PCR. The templates and primer set were mixed with 2X QuantiTect SYBR-Green PCR Master Mix (Qiagen). ß-actin was used as a control. Reactions were performed in triplicate in 96-well microtiter plates in an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA). Primer sequences for MGP were 5'-GCTCAATAGGGAAG CCTGTGAT-3' (forward primer) and 5'-TTTCTTCCCTCA GTCTCATTTGG-3' (reverse primer); primer sequences for ß-actin were 5'-TCACCCACACTGTGCCCATCTACGA-3' and 5'-CAGCGGACCGCTCATTGCCAATGG-3'.

Tissue microarray. TMAs of 207 breast cancer cases with at least two cores of 2 mm in diameter were made by punching out of the donor block and transferring to a recipient block according to the manufacturer's instructions using a dedicated TMA instrument (KIN-1, Azumayaikakikai, Tokyo, Japan).

Immunohistochemistry. Expression of MGP in breast cancer cells was validated by immunohistochemistry. Immunohistochemical analysis was performed according to the dextran-polymer method (EnVision+; Dako, Glostup, Denmark) using monoclonal antibodies against MGP (Proteintech Group Inc., Chicago, IL, USA; 1:100). Heat-induced antigen retrieval pretreatments were performed with Target Retrieval Solution (Dako). Antibody binding was scored in a blinded fashion by two pathologists. A score of 1 was assigned when ≥5% of the neoplastic cells were definitely positive and a score of 0 when <5% of tumor cells were stained (Fig. 1).

Statistical analysis. Survival curves were plotted according to the Kaplan-Meier method and compared using log-rank tests by Statistica 5.5. Univariate and multivariate analyses of prognostic indicators of overall survival were performed using the Cox proportional hazards regression model with R software. Variables that were statistically significant at $p<0.05$ were retained in the model.

Results

Gene expression analysis. The characteristics of the patients included in the gene expression microarray are summarized in Table I. We selected genes that were differentially expressed between the good prognosis group (overall survival >10 years) and the poor prognosis group (overall survival <10 years), and obtained 18 up-regulated (FC>3.5; $p<0.01$) and 23 down-regulated (FC<0.4; $p<0.01$) genes in the poor prognosis group (Table II). MGP was among the genes overexpressed in the poor prognosis group (FC=9.25, p=0.0012), and had the lowest p-value for discriminating between the two groups. Moreoever, the differential expression of MGP was determined by two probe sets (ID 238481_at and 202291_s_at).

Confirmation of differential expression. To confirm the results of gene expression microarray analysis, we carried out

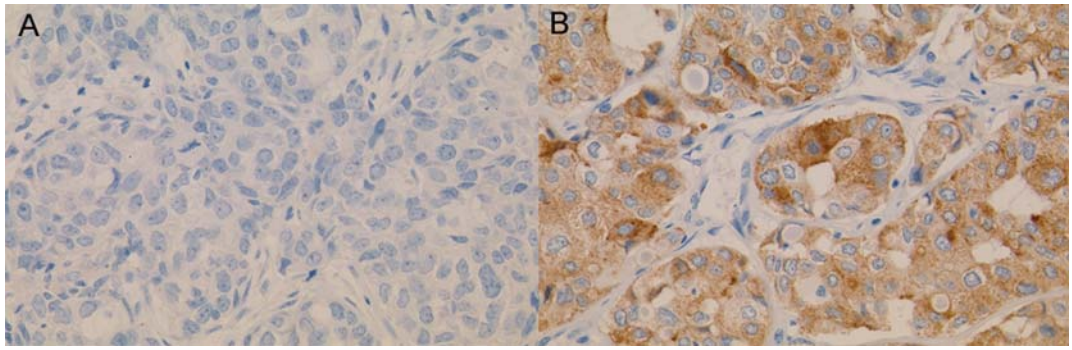

Figure 1. Representative images of the immunohistochemical staining of MGP in breast cancer tissue on a tissue microarray. (A) A score of 0 was assigned when <5% of tumor cells were stained. (B) A score of 1 was assigned when at least 5% of the neoplastic cells were definitely positive. Original magnification x40 (objective).

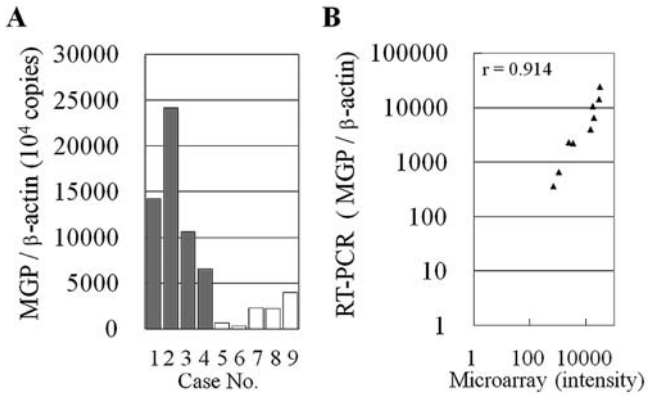

Figure 2. Gene expression microarray data were validated by quantitative RT-PCR. (A) Quantitative RT-PCR data showing upregulated expression of MGP in the poor prognosis group (gray bar). (B) Pearson correlation for MGP gene expression between microarray data and RT-PCR data, 0.914.

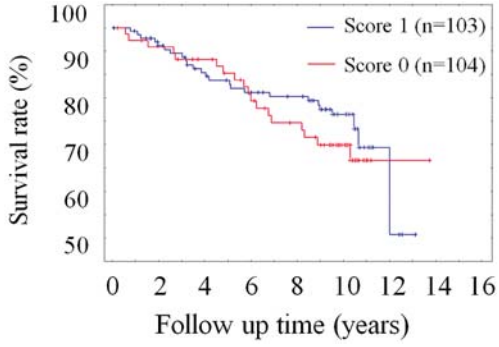

Figure 3. Kaplan-Meier curves of overall survival for MGP-negative (score 0) and MGP-positive (score 1) breast cancer cases. MGP protein expression was not significantly associated with overall survival (log-rank, p=0.79).

real-time quantitative RT-PCR for the MGP gene and the quantitative control gene, ß-actin. Expression levels quantified by real-time RT-PCR were highly correlated with those determined by gene expression microarray analysis (Pearson rank correlation; r=0.914), confirming the reliability of our microarray experiments (Fig. 2). ß-actin was used as a reference gene, because it was the gene with the least fluctuation in our samples according to the microarray data. Expression of MGP was significantly higher in the poor than in the good prognosis group according to RT-PCR data ($p<0.01$). MGP mRNA levels were increased >3-fold in all the patients in the poor prognosis group, as compared to the mean expression level of the good prognosis group. The highest up-regulation of MGP observed was a 12-fold increase, as shown in Fig. 2. Based on the above findings, MGP was selected for further analysis.

Immunohistochemical analysis. MGP expression was examined in 207 breast cancer specimens by immunohistochemistry. The clinical and histopathological characteristics are listed in Table III. In the immunohistochemical evaluation, 104 patients had a score of 0 and 103 a score of 1. We tested for associations between the stainings and overall survival. Kaplan-Meier overall survival curves are shown in Fig. 3. A score of 1 was not associated with a poor prognosis compared to a score of 0 (log rank, p=0.79).

Regarding the correlation between MGP expression and ER status, the ER-positive rate was 60.6% for a score of 0 and 60.2% for a score of 1. MGP protein expression was not related to ER status (χ^2 test, p=0.34). The bone metastasis rate was 17.3% for a score of 0 and 12.6% for a score of 1. MGP protein expression was not related to bone metastasis (χ^2 test, p=0.96).

Discussion

Gene expression analysis revealed the mRNA level of MGP to be much higher in breast cancer patients with a poor prognosis than in those with a good prognosis. To investigate the utility of MGP protein expression as a prognostic marker of breast cancer, we examined the correlation between MGP protein expression and overall survival using tissue microarrays. First, the percentage of stained tumor cells, -5, -25, -50, -75, or -100%, was determined, then the correlation between each of these groups and overall survival was analyzed. No difference between the groups was observed, and the correlation between MGP and overall survival was not confirmed.

A correlation between MGP expression and tumor development or tumor progression has been reported in ovarian cancer, renal cell carcinoma, colorectal cancer and glioma (6-9). There have also been several reports of the up-regulation of MGP mRNA expression in human breast cancer cell lines (10,14).

Table II. Differentially expressed genes in the good and poor prognosis groups.

Probe set	Gene symbol	Accession no.	P-value	Fold change
Up-regulated genes in the poor prognosis group (FC>3.5, p<0.01).				
227850_x_at	CDC42EP5	AW084544	0.0076	9.78
238481_at	MGP	AW512787	0.0012	9.26
202291_s_at		NM_000900	0.0072	3.81
202859_x_at	IL8	NM_000584	0.0062	8.73
205476_at	CCL20	NM_004591	0.0020	7.38
37892_at	COL11A1	J04177	0.0065	7.26
203875_at	SMARCA1	NM_003069	0.0047	5.57
219359_at	ATHL1	NM_025092	0.0039	4.76
210306_at	L3MBTL	U89358	0.0087	4.75
1561042_at	ITGB1	AF086249	0.0076	4.64
228877_at	RGL3	AI379517	0.0028	4.31
224646_x_at	H19	BF569051	0.0048	4.07
241937_s_at	WDR4	AA577678	0.0065	3.94
223380_s_at	LATS2	AF207547	0.0017	3.79
206595_at	CST6	NM_001323	0.0014	3.64
223251_s_at	ANKRD10	BC001727	0.0061	3.64
230136_at	LOC400099	AI573252	0.0033	3.60
219543_at	PBLD	NM_022129	0.0028	3.54
238785_at	C3orf63	AI632091	0.0053	3.53
Down-regulated genes in the poor prognosis group (FC<0.4, p<0.01).				
223604_at	GARNL3	AL136573	0.0014	0.39
204560_at	FKBP5	NM_004117	0.0014	0.38
223824_at	C10orf59	BC005364	0.0088	0.37
209823_x_at	HLA-DQB1	M17955	0.0099	0.36
205073_at	CYP2J2	NM_000775	0.0097	0.35
229332_at	HPDL	AI653050	0.0072	0.35
205818_at	DBC1	NM_014618	0.0073	0.31
219389_at	SUSD4	NM_017982	0.0063	0.31
228160_at	LOC400642	AI433706	0.0093	0.29
209728_at	HLA-DRB4	BC005312	0.0023	0.29
211685_s_at	NCALD	AF251061	0.0020	0.28
222774_s_at	NETO2	AI335263	0.0025	0.28, 0.19
218888_s_at		NM_018092	0.0048	
202741_at	PRKACB	AA130247	0.0096	0.26
203290_at	HLA-DQA1	NM_002122	0.0094	0.26
209539_at	ARHGEF6	D25304	0.0022	0.26
223467_at	RASD1	AF069506	0.0057	0.23
206254_at	EGF	NM_001963	0.0017	0.23
239911_at	ONECUT2	H49805	0.0020	0.22, 0.15
233446_at		AU145336	0.0037	
209315_at	HBS1L	AW297143	0.0058	0.18
201943_s_at	CPD	NM_001304	0.0088	0.15
205267_at	POU2AF1	NM_006235	0.0088	0.14
205029_s_at	FABP7	NM_001446	0.0096	0.14, 0.035
205030_at		NM_001446	0.0057	
225491_at	SLC1A2	AL157452	0.0063	0.12

Table III. Clinical and histopathological characteristics of the breast cancer patients.

	No. of patients (n=207)
MGP	
Score 0	104
Score 1	103
Age, years	
<40	19
40-49	74
50-59	67
60-69	35
≥70	12
Lymph node metastasis	
Negative	96
Positive	111
Tumor size (mm)	
≤19	51
20-29	61
30-39	47
40-49	30
50-59	12
≥60	6
Estrogen receptor	
Negative	82
Positive	125
Progesterone receptor	
Negative	114
Positive	93
Her2	
Negative	161
Positive	46
Adjuvant therapy	
None	43
Done	164
Bone metastasis	
Negative	176
Positive	31

In this study, using both microarray analysis and quantitative RT-PCR, we found that the mRNA of MGP was overexpressed in patients with a poor prognosis. This indicated that the mRNA levels of MGP could be a prognostic factor for breast cancer. However, we could not establish a correlation between the protein levels of MGP and overall survival. The reason for the difference in results regarding the mRNA and protein levels of MGP is unclear, though further study of the function of MGP may elucidate these findings. Estrogen has been found to strongly induce MGP gene expression in ER-positive breast cancer cells (14). However, in the present study, MGP expression was not significantly affected by ER status.

In summary, MGP mRNA expression was up-regulated in breast cancer patients with a poor prognosis, and may have the potential to serve as a prognostic indicator of the disease; however, no correlation was established between the protein levels of MGP as determined by immunohistostaining and overall survival.

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