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The mitogen-activated protein kinase phosphatase-1 (*MKP-1*) gene is a potential methylation biomarker for malignancy of breast cancer

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Abbreviations: ATCC, American Type Culture Collection; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LN meta, lymph node metastasis; MKP-1, mitogen-activated protein kinase phosphatase-1; MSP, methylation-specific PCR; PR, progesterone receptor; ROC curve, receiver-operating characteristic curve

Abstract

The mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) belongs to the MAPK cascades which are central to cell proliferation and apoptosis. The carcinogenic role of MKP-1 has been reported in many types of cancer but it has rarely been investigated in breast cancer. The present study was designed to evaluate the *MKP-1* mRNA expression and its possible regulation by methylation of *MKP-1* promoter in the model of several breast cancer cell lines and tissues as well as controls. Our data demonstrate *MKP-1* mRNA expression significantly decreased in five breast cancer cell lines compared to breast controls ($P < 0.01$). Using the methylation-specific PCR (MSP) analysis, the unmethylated reaction (U) is dominant in both normal cell lines and benign breast tumors (100% vs. 86.2%), whereas the methylated reaction (M) is dominant in both breast cancer cell lines and invasive breast tumors (100% vs. 57.2%). In terms of methylation ratio (M/M+U), methylation level in *MKP-1* promoter is significantly higher in the invasive breast tumor tissues ($n = 152$) than in benign breast tumor tissues ($n = 29$) ($P < 0.0001$). Assessing the methylation ratio of the promoter of *MKP-1* gene to diagnose the breast malignancy (invasive vs. benign), the area under the receiver-operating characteristic (ROC) curve was 0.809 (95% CI: 0.711-0.906, $P < 0.001$). The best performance for this prediction has a sensitivity of 76.32% and a specificity of 82.76% at the cutoff value of 0.38. Taken together, we firstly demonstrated that the promoter methylation of *MKP-1* gene is a potential breast cancer

biomarker for breast malignancy.

Keywords: biological markers; breast neoplasms; DNA methylation; DUSP1 protein, human; epigenesis, genetic

Introduction

Breast cancer remains a common malignancy among women. The high incidence and prevalence of breast cancer represents a major public health problem and understanding the molecular features of breast cancer has been a central target in biomedical research. Among the many signaling pathways central to cell proliferation and apoptosis are the mitogen-activated protein kinase (MAPK) cascades (Whitmarsh and Davis, 1999). The mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), also known as the dual-specificity phosphatase 1 (DUSP1), mediates the dephosphorylation and inactivation of MAPKs in insulin-responsive tissues. The actions of MKP-1 may play an important role in the maintenance of metabolic homeostasis (Roth Flach and Bennett, 2010a, 2010b).

Recently, accumulating evidence has shown that the MKP-1 is also involved in carcinogenesis. Some different types of cancers including prostate (Rauhala *et al*., 2005), liver (Loda *et al*., 1996), renal (Mizuno *et al*., 2004), and urothelial (Shimada *et al*., 2007) cancers showed decreased *MKP-1* mRNA or protein expression. For example, the *MKP-1* mRNA in hormone-refractory prostate carcinomas was expressed at a lower level than in benign prostate hyperplasia or untreated prostate carcinomas (Rauhala *et al*., 2005). This down-regulation of MKP-1 is regarded as an early event in prostate carcinogenesis. In liver cancer, most (80%) hepatocellular carcinomas expressed low levels of mRNA based on in situ hybridization analysis (Loda *et al*., 1996). In renal cancer, both Caki-1 and KU 20–01 cell lines showed decreased MKP-1 protein expression (Mizuno *et al*., 2004). In urothelial cancer, MKP-1 protein showed lower expression in high-grade/invasive phenotype than in low-grade/noninvasive in the urothelial cancer cell lines (Shimada *et al*., 2007). In contrast, primary gastric adenocarcinomas were found to express higher MKP-1 protein than normal gastric tissues (Bang *et al*., 1998). Hence, the MKP-1 expression in tumors relative to normal tissue is either still controversial or shows a cancer type-dependent expression manner (Boutros *et al*., 2008), i.e., increase or decrease. However, the relative MKP-1 expression in breast cancer compared to normal tissues has not been adequately studied.

Alterations in DNA methylation are a hallmark of human cancer, especially occurring at CpG islands within the promoter regions of genes (Issa, 2004; Cairns, 2007). There is accumulating evidence to show that abnormal DNA methylation occurs in early carcinogenesis (Esteller *et al*., 2001; Shih *et al*., 2010) and may have an important role in the early detection and prognosis monitoring of cancer (Baylin and Ohm, 2006). For breast cancer, altered methylation in malignant is higher than that of benign breast tissue (Zhu *et al*., 2010), especially on the promoters of genes (Brooks *et al*., 2009). The CpG methylation signatures have been reported to be the potential diagnostic, prognostic, and predictive tools for breast cancer (Jovanovic *et al*., 2010; Parrella, 2010). The methylation analysis also improves early breast cancer detection (Caldeira *et al*., 2006; Noetzel *et al*., 2008; Henneges *et al*., 2009; Seniski *et al*., 2009; Veeck *et al*., 2009; Wu *et al*., 2010; Zurita *et al*., 2010; Suijkerbuijk *et al*., 2011). However, the relationship between *MKP-1* gene expression and methylation in breast cancer remains unclear.

The aim of this study was to investigate the mRNA expression and methylation status for *MKP-1* gene of breast cancer tissues and cell lines compared to those of the normal tissues and cell lines. The potential use of methylation status for *MKP-1* gene as the breast cancer biomarker was also evaluated.

Results

MKP-1 mRNA levels in breast normal and cancer cell lines

Using the real time RT-PCR analysis, *actin* and

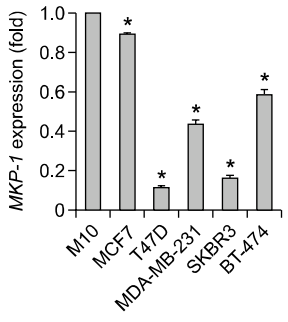


Figure 1. The mRNA expression of *MKP-1* gene in several breast cancer cell lines. Normal breast cell lines (M10) and breast cancer cell lines (MCF7, T47D, MDA-MB-231, SKBR3, and BT474) were included. The quantity of cDNA in each preparation was estimated by real time RT-PCR in the reference of the internal control *actin* gene. Experiments were repeated in triplicate. *$P < 0.01$.

MKP-1 mRNA expressions were examined in all 6 cell lines. After adjustment, the *MKP-1* fold activation in normal breast cell line M10 is regarded as control. In Figure 1, the tested breast cancer cell lines (MCF7, T47D, MDA-MB-231, SKBR3, and BT474) show the fold activation of 0.894 ± 0.003, 0.118 ± 0.008, 0.437 ± 0.020, 0.164 ± 0.014, 0.586 ± 0.026 (mean ± SD; $n = 3$), respectively. It shows significantly reduction for *MKP-1* mRNA expressions in these five breast cancer cell lines compared to normal control ($P < 0.01$).

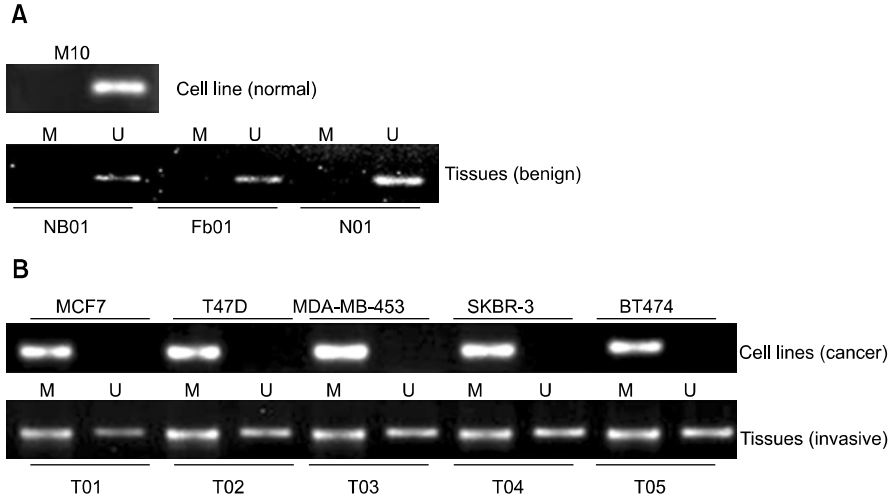
MSP of the promoter of MKP-1 gene in breast normal and cancer cell lines

The possible reasons for the breast cancer cell lines expressing lower *MKP-1* mRNA level than that of breast normal cell line (Figure 1) were examined in terms of the methylation status for *MKP-1*. Using the MSP assay, the breast normal cell line (M10) shows the unmethylated (U) band only (the upper part in Figure 2A and the left side in Figure 2C) without the detectable methylated (M) band. In contrast, the breast cancer cell lines (MCF7, T47D, MDA-MB-231, SKBR3, and BT474) show the methylated (M) bands only (the upper part in Figure 2B and the left side in Figure 2C). Regarding the methylation ratio (for band intensity) as described in Materials and methods, the methylation results in breast normal and cancer cell lines are 0 ($n = 1$) and 1 ($n = 7$), respectively.

MSP of the promoter of MKP-1 gene in breast normal and cancer tissues

Using the MSP, we also examined the methylation status of *MKP-1* gene in several breast normal and cancer tissues. Several representative MSP results are demonstrated in Figures 2A and 2B. In Figure 2A (bottom), three breast benign tissues (NB01, Fb01, and N01) show unmethylated reaction (U) only. In contrast, the breast cancer (invasive) tissues such as T01, T02, T03, T04, and T05 show both methylated (M) and unmethylated (U) reaction (bottom in Figure 2B).

Actually, MSP analysis of the *MKP-1* gene was carried out with DNA extracted from 29 benign and 152 invasive breast tumor tissues (left side, Figure 2C). The presence of U vs. M of MSP in the benign and the invasive breast tumor tissues is 25 vs. 4 and 65 vs. 87, respectively. Of these, the U is dominant (86.2%) in benign breast tumors whereas the M is dominant (57.2%) in invasive breast tumors. Similarly, the methylation ratios for the benign ($n = 29$) and invasive ($n = 152$) breast tumor tissues show 0.24 ± 0.24 and 0.52 ± 0.26, respectively.



C

MKP-1	Cell lines		Tumor tissues	
	Normal % (n)	Cancer % (n)	Benign % (n)	Invasive % (n)
Un-methylated (U)	100.0 (1)	0.0 (0)	86.2 (25)	42.8 (65)
Methylated (M)	0.0 (0)	100.0 (7)	13.8 (4)	57.2 (87)
M/(U+M) ratio	0.0 (1)	1.0 ± 0.0 (7)	0.24 ± 0.24 (29)	0.52 ± 0.26 (152)

Figure 2. MSP of the promoter of *MKP-1* gene in several breast cell lines (normal vs. cancer) and breast tissues (benign vs. invasive tumor). (A) Demonstration of MSP in a normal breast cell lines and several benign breast tissues. (B) Demonstration of MSP in several breast cancer cell lines and invasive breast tumor tissues. (C) Methylation status in all breast cell lines (normal and cancer) and tissues (benign and invasive) tested. M, methylated (155-bp); U, unmethylated (158-bp).

Table 1. Correlation of methylation ratio for *MKP-1* gene with clinicopathological characteristic in breast cancer

Characteristic	Patient no.	%	Mean* ± SD	ANOVA (*P value*)
LN meta				0.515
Negative	85	55.9	0.51 ± 0.29	
Positive	67	44.1	0.54 ± 0.22	
Grade				0.893
I	9	6.2	0.55 ± 0.28	
II	93	63.7	0.52 ± 0.27	
III	44	30.1	0.52 ± 0.23	
ER				0.615
Negative	50	33.9	0.54 ± 0.26	
Positive	102	67.1	0.52 ± 0.26	
PR				0.751
Negative	67	44.1	0.53 ± 0.27	
Positive	85	55.9	0.52 ± 0.25	
HER2				0.283
Negative	101	66.9	0.51 ± 0.25	
Positive	50	33.1	0.56 ± 0.28	
Stage				0.280
I	68	44.7	0.49 ± 0.28	
II	52	34.2	0.54 ± 0.24	
III + IV	32	21.1	0.57 ± 0.24	
Recurrence				0.310
Absent	137	90.1	0.52 ± 0.26	
Present	15	9.9	0.59 ± 0.25	

*Methylation ratio, intensity of M/intensity of U + M. LN meta, lymph node metastasis; SD, standard deviation; ER, estrogen receptor; PR, progesterone receptor.

The methylation ratio of invasive breast tumor tissues is significantly higher than that of benign breast tumor tissues ($P < 0.0001$).

Methylation of the promoter of MKP-1 gene and clinicopathological characteristic in breast cancer

As shown in Table 1, the differences of methylation ratios in lymph node metastasis (LN meta), grades, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), stages, and recurrence to breast cancer are not significant.

Diagnostic performance of MKP-1 promoter methylation for malignancy in breast cancer

ROC curves were used to assess the feasibility of using methylation ratio of the promoter of *MKP-1* gene as a diagnostic tool to detect the malignancy of breast cancer. The area under the ROC curve assessing methylation ratio of the promoter of *MKP-1* gene serving as the diagnostic tool to detect the malignancy (invasive vs. benign) of breast cancer was 0.809 (95% CI: 0.711-0.906, $P < 0.001$) (Figure 3).

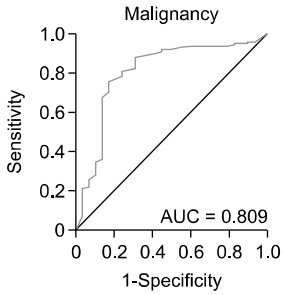


Figure 3. Receiver-operating characteristic (ROC) analysis for malignancy prediction of breast cancer based on the mathylation ratio for *MKP-1* gene. AUC, area under curve.

Table 2. Different cutoffs and their relative sensitivities and specificities of methylation ratios of the promoter of *MKP-1* gene for assessing the malignancy of breast cancer

Sensitivity (%)	Specificity (%)	Cutoff (methylation ratio)
67.76	86.21	0.43
76.32	82.76	0.38
78.95	75.86	0.35
88.16	68.97	0.24

In Table 2, the sensitivities and specificities of the methylation ratio of *MKP-1* promoter assessing the malignancy of breast cancer for different cutoff values are presented, i.e., 67.76 vs. 86.21, 76.32 vs. 82.21, 78.95 vs. 75.86, and 88.16 vs. 68.97 for the cutoff values of 0.43, 0.38, 0.35, and 0.24, respectively. The methylation ratio of *MKP-1* promoter assessing the malignancy of breast cancer showed the best performance with a sensitivity of 76.32% and a specificity of 82.76% at the cutoff value 0.38.

Discussion

The identification of genes that change the promoter methylation status might help to discover new biomarkers for tumor diagnosis and prognosis. For example, hypermethylation of RASSF1A, HIN-1, RAR-β, Cyclin D2, and Twist genes has been reported in early diagnosis for breast cancer (Fackler *et al*., 2003). In this study, we focused on breast cancer and examined the mRNA expression of *MKP-1* gene as well as its promoter methylation status.

Since breast cancer is a heterogeneous disease with several subtypes (Perou *et al*., 2000), many subtypes of breast cancer cell lines were included to measure its mRNA expression in our study. The

mRNA expression of *MKP-1* gene was down-regulated in five subtypes of breast cancer cell lines compared to controls (Figure 1), which is consistent with many other types of cancer (Loda *et al*., 1996; Mizuno *et al*., 2004; Rauhala *et al*., 2005; Shimada *et al*., 2007).

Among these studies, the mRNA expression level of *MKP-1* gene in prostate cancer was reported to be down-regulated, and its regulation involved the DNA methylation (Rauhala *et al*., 2005). Similarly, we found that the methylation status of *MKP-1* gene in terms of methylation ratio was significantly higher in the normal breast cell line and 29 benign breast tumors than in five breast cancer cell lines and 152 invasive breast tumors (Figure 2). The ROC curve (AUC = 0.809) shows that the methyation ratio of the promoter of *MKP-1* gene has high sensitivity and specificity for discriminating between malignant breast tumor and benign breast tumor. With the increase of the methylation ratio the promoter of *MKP-1* gene, the specificity for predicting the malignancy of breast cancer increases (Table 2).

However, the methyation ratio of the promoter of *MKP-1* gene may not be an effective prediction indicator for discrimination of several clinicopathological characteristics of breast cancer as listed in Table 1. For example, the area under the ROC curve assessing methylation ratio of the promoter of *MKP-1* gene as the diagnostic tool to detect the recurrence (absent vs. present) of breast cancer was 0.634 (95% confidence interval (CI): 0.502-0.765; $P = 0.087$).

In breast cancer cell lines (Figure 2B), unmethylated portion was not detected. However, the *MPK-1* gene expressed differently (10-90% of control) without complete inhibition of gene expression. These results suggested that the methylation of the promoter of *MPK-1* gene in terms of our MSP result cannot fully explain the downregulation of *MPK-1* gene. This finding may partly be due to the MSP (Lee *et al*., 2004; Park *et al*., 2005) only focusing on part of the possible methylation sites within the designed primers. Therefore, the methylation status of this gene needs further assay by bisulfite sequencing (Park *et al*., 2007) or pyrosequencing (Marsh, 2007; Shames *et al*., 2007) of the cell lines to provide the detailed methylation profile for the promoter of *MKP-1* gene. Moreover, the role of methylation in modulating the *MKP-1* gene expression is not clearly addressed in this study. Methylation as a possible mechanism of gene silencing of *MKP-1* gene would be answered when it is investigated by treatment of breast cancer cell lines with the methyltransferase inhibitor, such as demethylating agent, 5-azadeoxycytidine (Park *et al*., 2005, 2007). We cannot exclude the possibility that other non-methylation factors may be involved in the regulation of *MKP-1* gene expression. In our study, we demonstrated that methylation level of *MKP-1* is a potential diagnostic tool for assessing the malignancy. However, the relationship between gene expression of *MKP-1* gene and the methylation status have not been addressed, and further investigation is required.

In conclusion, the methylation level is significantly higher in malignant breast cancer than benign breast cancer, and it is a potential diagnostic tool for assessing malignancy in breast cancer.

Methods

DNA, breast tumor tissues, and cell lines

Genomic DNA was obtained from breast cell lines, primary tumors, and normal tissue. The benign ($n = 29$) and invasive ($n = 152$) breast tumor tissues were kindly provided by the Cancer Center of Kaohsiung Medical University Hospital approved by the IRB in Kaohsiung Medical University. Human breast cancer cell lines MCF7, SKBR3, MDA-MB-453, T47D, BT474 and MDA-MB-231 were obtained from the National Centre for Cell Science, Pune, India and the American Type Culture Collection (ATCC), respectively. T47D cell line was maintained in RPMI 1640, others cells were grown as monolayer cultures in Dulbecco's modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) (Sigma Aldrich, Bangalore, India) supplemented with 10% fetal bovine serum. Normal mammary epithelial cell H184B5F5/M10 (M10) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). These cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37^{o}C in a humidified atmosphere of 95% air and 5% carbon dioxide. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

RNA extraction and real time RT-PCR

Total RNA was extracted by Trizol reagents (Invitrogen, corp.) in accordance with the manufacturer's manual. Total RNA (2 μg) was reverse-transcribed using oligo-dT primer and Superscript III reverse transcriptase (Invitrogen, USA). Quantitative real time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) (Yen *et al*., 2009) in an iCycler MyiQ single color real-time PCR detection system (Bio- Rad Laboratories, Hercules, CA). The mRNA levels of *MKP-1* and controls were further validated using real time-PCR. *MKP-1* primers were based on NCBI/UniSTS:156846/GDB:371394 (found by e-PCR in sequences from *Homo sapiens*) and human *actin* primers for the internal control were described previously (Hall *et al*., 2008). Primer sequences are included in Supplemental Data Table S1.

The fold activation for *MKP-1* mRNA expression was analyzed using the -$\Delta\Delta$Ct method (Livak and Schmittgen 2001), where the Ct (threshold cycle) value of an *MKP-1*

gene was subtracted from the Ct value of a reference housekeeping gene, *actin*. After real time PCR, melting curve analyses and gel electrophoreses were performed to ensure the specificity of the quantitative RT-PCR reactions (Chang *et al*., 2008; Yen *et al*., 2009).

Methylation-specific PCR (MSP) (Herman *et al*., 1996)

DNA was modified with 1 μg of genomic DNA using a CpGenomet DNA modification kit (Chemicon, Temecula, CA) (Shieh *et al*., 2005) according to the manufacturer's protocol. Modified DNA was resuspended in TE buffer stored at -80°C. Modified DNA was amplified in a total volume of 10 μL solution containing 1x PCR buffer, 1.5 mM $MgCl_2$, 200 ng of each primer, 0.2 mM of each dNTP and 1 U Platinum *Taq* Polymerase (Invitrogen). Primer sequences for MSP are included in Supplemental Data Table S1. The PCR conditions used were same for both PCRs and are as follows: 5 min of initial denaturation at 94°C, 35 amplification cycles (denaturation for 30 s at 94°C, annealing for 30 s at 54°C, and extension for 30 s at 72°C), and final extension for 10 min at 72°C. PCR products were then loaded and electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized under UV illumination.

Methylation ratio

The methylation status was detected by MSP and the intensities of methylated (M) and unmethylated (U) bands were individually quantified with the aid of the Gel Pro Analyzer ver. 4. The relative amount of methylation (Yan *et al*., 2006) in the sample was slightly modified and calculated using the following formula: methylation ratio = M/(M + U), where M and U indicated their band intensities measured by the gel scanning.

Statistics

Data analyses were performed using the SPSS 13.0 package. Comparisons of the means of each case group to that of the control group were performed by *t* test. Receiver-operating characteristic (ROC) curves (Chang *et al*., 2002, 2007) were used to assess the methylation ratio as diagnostic tools for detecting breast cancer. The area under the ROC curve was a measure of the overall ability of a diagnostic test with multiple cutoffs of methylation ratio to distinguish between breast cancer patients and controls.

Supplemental data

Supplemental Data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-5-06.pdf.

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