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 < 0.0001). 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; Hennekes et al., 2009; Senisi 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
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.

| 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) |
Table 1. Correlation of methylation ratio for \textit{MKP-1} gene with clinicopathological characteristic in breast cancer

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

Table 2. Different cutoffs and their relative sensitivities and specificities of methylation ratios of the promoter of \textit{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                       |

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 \textit{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 \textit{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 \textit{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 \textit{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).

In Table 2, the sensitivities and specificities of the methylation ratio of \textit{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 \textit{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 \textit{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 methylation 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 methylation 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 MKP-1 gene expressed differently (10-90% of control) without complete inhibition of gene expression. These results suggested that the methylation of the promoter of MKP-1 gene in terms of our MSP result cannot fully explain the downregulation of MKP-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 H184BS55/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°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 \( \mu \)g) 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 MgCl₂, 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 status 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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