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Hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer

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Abstract

Maspin is a member of the serine protease inhibitor family with tumor suppressing activity in breast cancer. Maspin expression was found in normal breast epithelial cells, but was frequently decreased in breast cancer cells and lost in metastatic cells. In this study, we examined the regulatory mechanism of maspin expression and described the re-activation of maspin expression in a series of maspin-negative breast cancer cell lines. All of the 6 maspin-negative breast cancer cells showed induction of maspin promoter activity in a promoter reporter assay. In addition, the treatment of 5-aza-2′ deoxycytidine, trichostatin A or a combination of both led to the re-expression of maspin in a series of maspin-negative breast cancer cell lines. These findings indicate that DNA methylation and/or histone deacetylation are/is partially responsible for the silencing of maspin gene expression in breast cancer cells. The re-expression of maspin by pharmacological intervention potentially offers a promising new target as a therapeutic option in breast cancer.

Keywords: Maspin; Breast cancer; DNA methylation; Histone deacetylation; 5-AZA-dC; TSA

Maspin is a unique member of the serine protease inhibitor family with tumor suppressive potential in breast and prostate cancer. Initially identified from normal mammary epithelial cells by subtractive hybridization, maspin has been shown to inhibit invasion and motility of breast cancer cell lines [1–4]. It was recently shown that stepwise reduction of maspin expression occurring along with the progression from ductal mammary carcinoma in situ to invasive carcinoma to lymph node metastasis and the lack of maspin expression in breast cancer seem to be associated with the short disease-free survival [5,6].

Maspin is expressed in normal human mammary and prostate epithelial cells but down-regulated during cancer progression. Whereas initial studies indicated that the loss of maspin expression during tumor progression resulted at least in part from the absence of transactivation through the Ets- and AP-1 DNA-binding sites within the maspin promoter region [7], it was recently shown that aberrant cytosine methylation and chromatin condensation of the maspin promoter participate in the silencing of maspin expression during cancer progression [8]. Although the function of maspin has been widely studied, the molecular mechanism underlying its expression still remains unknown. This study examined the regulatory mechanism of maspin expression in a series of breast cancer cell lines. By maspin promoter analysis, we found that maspin-negative breast cancer cells contain factors that were required for maspin transcription. We also demonstrated that maspin expression could be reactivated in maspin-negative cells by treatment with DNA methyltransferase (DNMT) inhibitor, 5-aza-2′ deoxycytidine (5-AZA-dC) or histone deacetylase (HDAC) inhibitor, trichostatin A (TSA). Our results show that epigenetic silencing of the maspin gene expression in breast cancer cells is associated with promoter hypermethylation and/or histone deacetylation.

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Materials and methods

Cell lines. The human breast cancer cell lines, MCF-7, ZR-75-1, SK-BR-3, T-47D, MDA-MB231, MDA-MB468, and BT-20 were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in plastic dishes in RPMI-1640 medium supplemented with 10% fetal calf serum, 1 mM $NaHCO_3$, 2 mM L- glutamine, penicillin–streptomycin at 95% O_2, 5% CO_2 at 37 °C.

Plasmids. To analyze activation of the maspin promoter, the construct of luciferase expression vector was made. The fragment of 1043 bp (−956 to +87) was prepared using restriction enzymes as described previously [7] and was fused with luciferase gene in the pGL2-Basic vector (Promega, Mannheim, Germany) to generate pGL-M956. The construct was confirmed by restriction enzyme mapping and sequence analysis. This region (−956 to +87) contains Ets- and Ap1-binding elements.

Transient transfection and luciferase assay. Cells (2×10^5) were plated into 6-well plates. One microgram pGL-M956 or pGL2 control plasmid was transiently transfected with FuGENE 6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. After 48 h, cells were lysed to determine the luciferase activity. All transfections were standardized by co-transfecting a *Renilla* luciferase control vector (Promega). Luciferase activity was assayed by using the Dual Luciferase Repoter assay system (Promega). Transfections were performed in triplicate.

Treatment of cells with 5-AZA-dC and TSA. Cells were plated at a density of 2×10^5 cells/60-mm dish and treated after 24 h plating with 2 μM 5-AZA-dC (SIGMA, Deisenhofen, Germany) for 96 h or with 1 μM TSA (SIGMA) for 48 h. Reagent and medium were exchanged every 24 h. To assess the effect of a combination of 5-AZA-dC and TSA, cells were exposed sequentially for 48 h to 5-AZA-dC and then to TSA for an additional 48 h.

RNA preparation and RT-PCR. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and treated with DNase I (QIAGEN). The cDNA was generated from DNase-treated total RNA using an oligo(dT)16 primer with Superscript II reverse transcriptase (GIBCO-BRL, Bethesda, MD, USA). The PCR was carried out in a volume of 50 μl consisting of a cDNA (equivalent to the cDNA from 100 ng initial total RNA) using HotStarTaq (QIAGEN) to provide hot-start PCR. The primer sequences designed from the coding region of the human maspin cDNA (GenBank Accession No. U04313) are described previously [5] as follows: 5′-CTTGCCTGTTCCTTTTCCAC-3′ (sense) and 5′-TGGAGAGTTTG ACCTTGGCA-3′ (antisense). The PCR conditions were as follows: initial activation at 15 min, 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, followed by a final incubation at 72 °C for 10 min. The length of the primary PCR product of maspin was 874 bp. Sense and antisense primers for the β-actin gene used as an internal control have been described previously [5]. The amplified product was analyzed by electrophoresis on an agarose gel, stained with ethidium bromide.

Immunohistochemical analysis of maspin. A mouse antihuman maspin monoclonal antibody was purchased from PharMingen (San Diego, CA). Cells were cultured in chamber slides and treated with 5-AZA-dC or TSA. After treatment, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with methanol/3% H_2O_2 before blocking with 10% fetal bovine serum for 30 min. Maspin immunostaining was then performed as previously described [6,9].

Results and discussion

Maspin is highly expressed in normal mammary epithelial cells and myoepithelial cells, but is decreased in breast cancer cells and lost in metastatic cells. It was shown to have tumor suppressive activity attributable to the inhibition of breast cancer cell motility, invasion, and metastasis [1–4,10–12]. Genetic alterations, such as large deletion or maspin gene mutation, have not been reported to play a major role in the silencing of the maspin gene. It was also demonstrated that the maspin gene was regulated at the transcriptional level through elements in the maspin promoter [7]. Although the function of maspin has been widely studied, the molecular mechanism underlying its expression is poorly understood.

To clarify the involvement of epigenetic regulation in maspin expression, we first examine whether loss of expression of maspin results from the absence of transcriptional activation through the maspin promoter, Ets and AP1 sites in breast cancer cell lines. Seven breast cancer cell lines, MCF-7, ZR-75-1, SK-BR-3, T-47D, MDA-MB231, BT-20, and MDA-MB468 were transiently transfected with either maspin reporter construct or empty vector control and their relative luciferase activity was assayed. MDA-MB468 cells were used as a positive control, since we detected maspin expression as described previously [9]. As shown in Fig. 1, high luciferase activity was observed in MCF-7, SK-BR-3, and T-47D by 124-, 73-, and 28-fold of control, respectively. Maspin-positive cell line MDA-MB468 showed increased luciferase activity to 26-fold of control. MDA-MB231, ZR-75-1, and BT-20 showed 11-, 8-, and 4-fold luciferase activity, respectively. It is worth noting that maspin expression is down-regulated in all cell lines with the exception of the MDA-MB468 cells, whereas the activation of maspin promoter is consistently observed not only in MDA-MB468 cell line but also in all of the maspin-negative breast cancer cell lines. These results indicate that maspin-negative cancer cells are able to support transcription from the maspin promoter equally well and contain factors required for transcription.

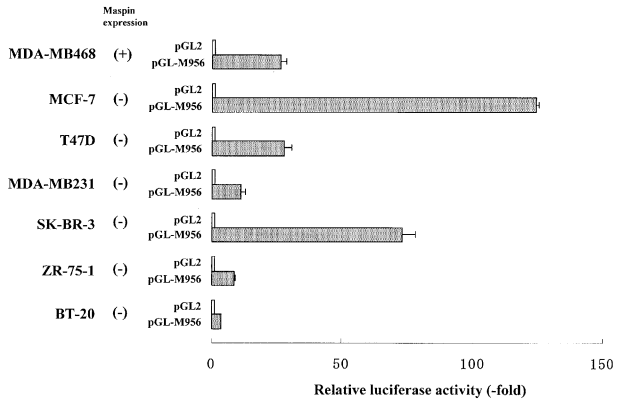

Fig. 1. Maspin promoter activity in breast cancer cell lines. Maspin promoter reporter plasmid, containing the full promoter region, was transiently transfected into 7 breast cell lines. Luciferase activity was measured at 48 h after transfection. The data are presented as—fold induction over the reporter construct alone. Transfection efficiency was normalized by *Renilla*-luciferase activity. Results are means ± SD ($n = 3$).

It has been widely shown that methylation of cytosines within CpG islands is associated with transcriptional silencing during mammalian development and is responsible for the silencing of several cancer-related genes such as Rb, PTEN, CDKN2A, and CDKN2B SKY [13–17]. DNMT inhibitor 5-AZA-dC is widely used to study the re-expression of genes silenced by promoter methylation. Furthermore, methylated DNA is often associated with deacetylated histones, which support the essential roles of both DNMT and HDAC in silencing expression of endogenous methylated genes [18–21]. It has been demonstrated that HDAC inhibitors such as TSA show re-expression of some genes. We next examined the effect of 5-AZA-dC and TSA on the re-activation of maspin in breast cancer cell lines to ascertain whether both DNMT and HDAC activities could play a role in the silencing of maspin expression.

Maspin mRNA expression was evaluated by RT-PCR. The sensitivity of RT-PCR obtained exponential amplification of maspin gene in MDA-MB468 maspin-positive cell line, but no amplification band in the other 6 maspin-negative cell lines. A preliminary experiment showed that optimal gene expression was observed by RT- PCR after treatment of MCF-7 cells with either 2 μM 5-AZA-dC for 96 h or with 1 μM TSA for 48 h as single agents (data not shown). We therefore performed combined treatment in which cells were treated with 5-AZA-dC for 48 h and TSA was added for the last 48 h. As shown in Fig. 2, maspin mRNA was re-expressed in MCF-7, T-47D, MDA-MB231, ZR-75-1, and BT-20 by treatment with 5-AZA-dC alone, but failed to re-express in SK-BR-3. Treatment of the cells with TSA alone also induced re-expression of maspin in MCF-7, T-47D, and ZR-75-1 and weakly re-activated in BT-20. But re-expression was not observed by TSA alone in MDA-MB231 and SK-BR-3. Treatment with TSA showed higher induction of maspin re-expression than 5-AZA-dC treatment in MCF-7 and T-47D cells. These findings indicate that DNA methylation or histone deacetylation is partially responsible for the silencing of maspin gene expression in cancer cells. Whereas re-expression was not observed by treatment with either 5-AZA-dC or TSA alone, the combination of these two agents could activate maspin re-expression in SK-BR-3. Furthermore, re-expression of maspin mRNA by the combination of 5-AZA-dC and TSA was greater than that with either agent alone in ZR-75-1 and BT-20 cells. It appears that maspin gene silencing is due to both DNA methylation and histone deacetylation in some cancer cells. Domann et al. [8] demonstrated that one possible mechanism for loss of maspin expression in breast cancers is cytosine methylation of the maspin CpG island in the promoter region and 5-AZA-dC has been used successfully to re-express maspin gene in MCF-7. But our findings suggest that DNA methylation alone is not sufficient to look for this gene in the silenced state without concomitant histone modification.

The re-expression of maspin by treatment with 5-AZA-dC or TSA in breast cancer cells was confirmed by immunohistochemistry (Fig. 3). Positive staining for maspin was observed by treatment with a combination of 5-AZA-dC and TSA, or TSA alone in SK-BR-3 or MCF-7, respectively (Fig. 3B and D), but no staining was seen in both control cells (Fig. 3A and C). These data are consistent with results of RT-PCR and indicate

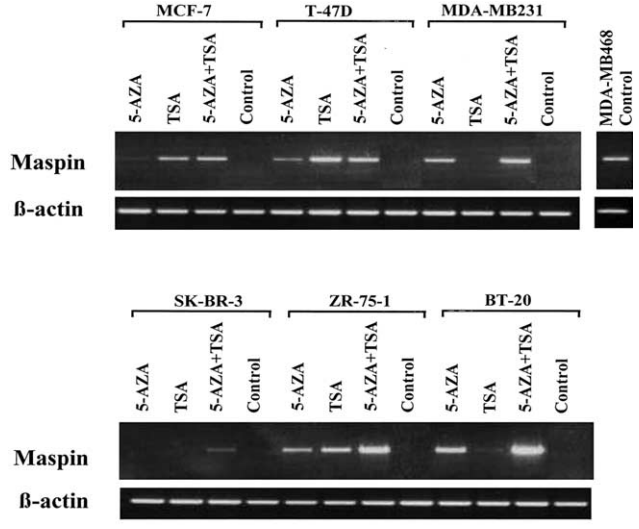

Fig. 2. Re-activation of maspin expression in maspin-negative breast cancer cell lines. Cells were treated with 2 μM 5-AZA-dC for 96 h or with 1 μM TSA for 48 h. To assess the effect of a combination of 5-AZA-dC and TSA, cells were exposed sequentially for 48 h to 5-AZA-dC and then to TSA for an additional 48 h. Total RNA was isolated and maspin expression was determined by RT-PCR. MDA-MB468 cells were used as positive control.

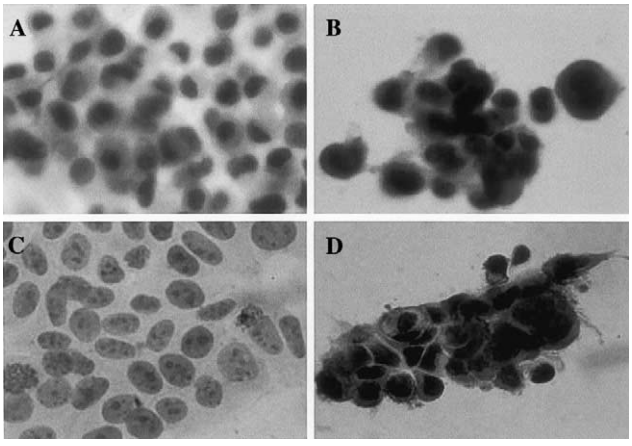

Fig. 3. Maspin staining for maspin re-expression in breast cancer cells. Cells were treated with agents in chamber slides probed with antihuman maspin antibody, and counterstained with hematoxylin. SK-BR-3 (A) and MCF-7 (C) control cells showed no staining. SK-BR-3 treated with a combination of 5-AZA-dC and TSA (B) and MCF-7 treated with TSA alone (D) showed positive staining.

that the re-expression of maspin mRNA by treatment of these agents is translated into protein level.

Recently, in vivo functional studies demonstrated that overexpression of maspin in a bitransgenic and a syngeneic mice model could block mammary tumor progression as well as invasion and metastasis [22,23]. Study of maspin gene expression will not only help us to understand the mechanism of gene regulation but also offers an opportunity for re-expression of tumor suppressor gene maspin in tumors by therapeutic intervention. Here, we demonstrate that maspin-negative cancer cells contain factors that are required for transcription and the treatment of 5-AZA-dC, TSA or the combination lead to the re-expression of maspin. Methylation and HDAC inhibitor are currently considered to be a promising cancer therapy. The re-expression of maspin by pharmacological intervention with such reagents potentially offers a promising approach as a therapeutic option in breast cancer therapy.

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