

# Clinical significance of *Maspin* promoter methylation and loss of its protein expression in invasive ductal breast carcinoma: correlation with VEGF-A and MTA1 expression

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**Abstract** *Maspin* is a serine protease inhibitor with tumor-suppressor activity. *Maspin* can suppress tumor growth and metastasis in vivo and tumor cell motility and invasion in vitro. Previous studies indicate that the loss of *Maspin* expression is closely linked to aberrant methylation of the *Maspin* promoter. We examined the promoter methylation status of *Maspin* in tumor and corresponding serum of breast cancer patients. In addition, protein expression of this gene was also assessed to determine possible correlation between promoter hypermethylation and gene silencing. Further, we investigated the correlation of *Maspin* expression with vascular endothelial growth factor (VEGF-A) and MTA1 expression. *Maspin* methylation was analyzed by methylation-specific PCR in 100 invasive ductal breast carcinoma patients' tumors and circulating DNA in a prospective study. Promoter hypermethylation was correlated with

expression of the encoded protein in tumors by immunohistochemistry. Significant correlation was observed between promoter hypermethylation of *Maspin* ( $r=+0.88$ ;  $p\leq 0.0001$ ) in tumors and paired sera. Significant association was found between *Maspin* promoter hypermethylation and loss of its protein expression ( $p=0.01$ , OR=3.1, 95% CI=1.3–7.4). The expression of VEGF-A and MTA1 was lower in tumors with high *Maspin* expression compared to tumors with loss of *Maspin* expression. Our results indicate that aberrant promoter methylation is associated with loss of *Maspin* immunoreactivity in breast cancer tissues. Further, loss of *Maspin* expression is significantly correlated with increased expression of VEGF-A and MTA1.

**Keywords** Breast cancer · DNA methylation · *Maspin* · MTA1 · Gene silencing

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## Introduction

Maspin, a serine proteinase inhibitor (serpin) family, plays a role in development of the mammary gland [1] and is expressed in myoepithelial cells and normal secretory epithelial cells [2]. In vitro studies and animal models have shown that Maspin inhibits tumorigenesis by modulating tumor cell growth, invasion, and metastasis [2, 3]. Maspin also inhibits endothelial cell motility and angiogenesis [4]. Earlier studies have demonstrated a tight link between the loss of Maspin expression in breast cancer cells and the aberrant cytosine methylation and histone deacetylation of its promoter [5–7]. Maspin interacts with diverse group of intercellular and extracellular proteins, regulating cell adhesion, motility, apoptosis, and angiogenesis and is critically involved in mammary gland development [8]. The tissue-specific expression of Maspin is epigenetically controlled, and aberrant methylation of *Maspin* promoter is closely associated with *Maspin* gene silencing [5, 9–15]. The promoter methylation of the *Maspin* gene leads to gene silencing in cancers, such as breast, thyroid, skin, and colon [5, 11, 13]. In contrast, overexpression of Maspin in gastric, pancreatic, and ovarian cancers results from promoter CpG demethylation [10, 12, 14]. This clearly indicates that both methylation and demethylation of *Maspin* promoter could regulate *Maspin* gene expression.

Recent studies suggest that Maspin overexpression correlates with increased expression of vascular endothelial growth factors A, C, and D in human ovarian carcinoma and melanoma [16, 17]. The most potent of these cytokines is vascular endothelial growth factor (VEGF-A), a heparin binding glycoprotein with potent angiogenic, mitogenic, and vascular permeability-enhancing activities specific for endothelial cells.

The metastasis tumor antigen (MTA) family of proteins, a group of 70 to 80 kD polypeptides, have been shown to be functional components of the Mi-2/NuDR complex, a major macromolecular form of histone deacetylases [18], which has been linked to transcriptional repression, cellular proliferation, and cancer [19–21]. In vertebrates, the MTA family is comprised of three members: MTA1, MTA2, and MTA3. MTA1 is overexpressed in a variety of human malignancies, and its overexpression is also associated with tumor progression and metastasis in multiple cancer types [19, 22, 23]. The expression level of MTA1s, a natural variant of MTA1, is increased in estrogen receptor (ER)-negative human breast cancers [21].

In a parallel study in an independent cohort of Invasive ductal carcinomas of breast (IDCs), we recently demonstrated loss or reduced cytoplasmic expression of Maspin in 36 of 59 (61%) tumors by immunohistochemical analysis and upregulation of Maspin expression by curcumin in breast cancer cells (MCF7 and MDA-MB-231) that correlated with

the upregulation of p53 protein and downregulation of Bcl-2, suggesting Maspin-mediated apoptosis in MCF7 cells. The upregulation of Maspin expression by curcumin in breast cancer cells taken together with the clinical data suggested a potential therapeutic role for curcumin in inducing Maspin-mediated inhibition of invasion of breast carcinoma cells.

In the present report, we hypothesized that methylation-induced gene silencing of *Maspin* may account for the loss of its protein expression in IDCs of breast. In an independent cohort of 100 IDCs, we determined the promoter methylation status of *Maspin*, its relationship with its protein expression in these tumors. Further, we also examined the relationship of Maspin with expression of the other key components involved in angiogenesis and metastasis such as VEGF-A and MTA1. The association between promoter hypermethylation of *Maspin* in primary breast tumors and paired serum DNA was also investigated to explore the possibility of using serum DNA methylation analysis of this gene as surrogate non-invasive marker for their tumor status.

## Materials and methods

### Tissue specimens

Surgically resected specimens or trucut biopsies from untreated primary breast carcinoma patients, matched normal breast tissues (5–10 cm away from the site of the tumor), and peripheral blood samples were collected from 100 breast cancer patients enrolled in the Out Patients Department of Surgical Disciplines, All India Institute of Medical Sciences, New Delhi, India, after approval of the study by Institutional Human Ethics Committee. Written consent was taken from all the patients enrolled in the study. The age of the patients ranged from 30 to 81 years (median age is 50 years). All patients were diagnosed with Invasive Ductal Carcinoma.

Ten milliliters of blood was collected from breast cancer patients at the time of surgery. Blood was also collected from 30 healthy females (age range 22–70 years); ten of these women were premenopausal and 20 were postmenopausal; four were nulliparous. All these 30 healthy females did not have history of any chronic disease and had no evidence of disease at the time of enrollment in the study. Serum was isolated from clotted blood by centrifugation at 1,000×g for 10 min. A part of each tumor and representative adjacent normal tissue was kept in formalin for histopathological characterization to confirm the diagnosis and immunohistochemistry and the other part was snap frozen and stored at –70°C.

### DNA extraction

DNA was extracted from breast tumor and normal tissues, breast cancer cell lines MDA-MB-231, and normal lym-

phocytes using standard technique of digestion with proteinase K in the presence of sodium dodecyl sulfate (SDS) at 37°C overnight, followed by phenol/chloroform extraction. Serum DNA was extracted using Qiamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturers' instructions. The quality and integrity of DNA from tissues was checked by electrophoresis on 0.8% agarose gel, quantitated spectrophotometrically, and stored at -20°C till further use.

#### Methylation-specific PCR

The sensitive methylation-specific PCR (MSP) was used to detect promoter methylation, as it can detect 0.1% cancer cell DNA from a heterogeneous cell population [24]. Bisulfite modification of the DNA (up to 2 µg) from various sources was carried out as described [25]. Primers and PCR conditions used for MSP of *Maspin* are from published study [26] and have been standardized in our laboratory. For positive and negative controls of the MSP, breast cancer cell line (MCF7) was used as a positive control, maternal blood DNA collected after 3rd trimester was used as positive control for unmethylated DNA, and water with no DNA template as a control for contamination were included in each experiment. After amplification, each PCR product was electrophoresed using a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

#### Immunohistochemistry

Immunohistochemical analysis was carried out using paraffin-embedded breast carcinoma tissue sections as described by us previously [27]. Briefly, after antigen retrieval by microwave heating in Tris–Cl<sub>2</sub> Buffer (pH 9.0) for 15 min, tissue sections were rinsed with 0.01 M phosphate buffer saline, pH 7.4 (PBS). Sections were incubated with primary antibody (1:100 dilutions) at 4°C overnight in a humidified chamber. Monoclonal antibodies were purchased against *Maspin* (550839) from BD Pharmingen (San Diego, CA), VEGF-A (sc-7269), and MTA1 (sc-9446) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). After extensive washing with PBS, sections were incubated with biotin-linked universal antibodies and subsequently with horse radish peroxidase-streptavidin conjugate (LSABTM Kit, Dako Cytomation, Glostrup, Denmark) and color was developed using 3, 3'-diaminobenzidine hydrochloride as chromogen, counterstained with Mayer's hematoxylin, and mounted for evaluation using microscope (OLYMPUS BX-51, Japan). In the negative control, primary antibody was replaced by isotype-specific IgG.

The results of immunohistochemistry were assessed by the pathologist (S.D.G), who independently examined the immunostaining scoring of the tissue sections. The slides were scored for the percentage of cells showing immunoreactivity: -, no detectable staining and +, ≥10% positive tumor cells showed reactivity. The nuclear/cytoplasmic immunoreactivity of the *Maspin* [28] and MTA1 [29] was considered as positive. Tumors were regarded as ERα, PR, and p53 positive, if the staining was localized in nuclei [30]. While for VEGF-A tumors were regarded as positive, if the staining was localized in cytoplasm [31].

For HER2/*neu*, the scoring system was as follows: no staining or membrane staining in fewer than 10% of tumor cells, 0; faint, barely perceptible membrane staining in more than 10% of tumor cells, the cells are stained only in part of the membrane, 1+; weak to moderate complete membrane staining observed in more than 10% of tumor cells, 2+; and strong, complete membrane staining in more than 10% of tumor cells, 3+ [32].

#### Cell lysis and Western blot

Cells and tumor tissues were lysed on ice using radio-immunoprecipitation buffer (0.05 mol/l Tris–HCl, pH 7.4, 0.15 mol/l NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, and 10 mg/ml aprotinin). Lysates were then centrifuged at 13,000 rpm. at 4°C for 10 min. Protein extracts were solubilized in SDS gel loading buffer (60 mmol/l Tris base, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol). Samples containing equal amounts of protein (50 µg) were separated on a 10% SDS–polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA) in a transfer buffer. Immunoblotting was performed using antibodies against *Maspin* (1: 1,000), VEGF-A (1: 1,000), MTA1 (1: 1,000), and anti-β actin antibodies (1: 1,500), as an internal control. The signal was developed with enhanced chemiluminescence (Santa Cruz, CA) after incubation with appropriate secondary antibodies.

#### Statistical analysis

Statistical analysis of correlation of methylation of genes and protein expression with known clinicopathological characteristics was performed with Fisher exact test (two-sided). Two-sided *p* values were calculated, and *p* ≤ 0.05 was considered to be significant. Sensitivity and specificity was calculated using receiver-operating characteristic analyses. All of the statistical analyses were performed using SPSS software (version 10.0; SAS Institute, Cary, NC).

## Results

Promoter methylation analysis of *Maspin* was carried out in 100 IDCs of breast and paired serum DNA. Figure 1 shows representative methylation status of *Maspin* in invasive breast carcinomas and paired serum DNA. Immunohistochemical analysis of Maspin, VEGF-A, and MTA1 proteins was carried out in 89 of 100 IDCs; 11 cases could not be analyzed by immunohistochemistry because limited amount of tissue available could be used for MSP analysis only.

### Analysis of *Maspin* methylation and protein expression in IDCs

Hypermethylation of *Maspin* promoter was detected in 54 (54%) IDCs (Table 1). For determining the clinical utility of the study, it is important that the target genetic alteration is cancer specific and is not present in normal cells. Therefore, as a control, we checked methylation status of this gene in 15 paired normal breast tissues and 30 sera collected from healthy females. *Maspin* promoter was unmethylated in majority of adjacent normal tissues (12/15) and of the 30 normal sera analyzed, 24 were unmethylated, while six normal sera showed methylated *Maspin* promoter. *Maspin* promoter hypermethylation in tumor and serum was significantly associated with lymph node metastasis ( $p=0.001$ , OR=5.4, 95% CI=2.2–13.7, and  $p=0.004$ , OR=3.7, 95% CI=1.5–9.2, respectively). No other significant association was found between *Maspin* hypermethylation and any of the clinicopathological parameters analyzed (Table 1).

There was a significant correlation between *Maspin* methylation status in tumor and serum DNA ( $r=+0.88$ ;  $p\leq 0.0001$ ). No aberrant methylation was found in serum DNA, if this alteration was not present in the primary tumor.

Immunohistochemical analysis showed loss or markedly reduced expression of Maspin protein in 48 of 89 (54%) of IDCs analyzed (Fig. 2, Table 2). Of the 50/89 tumors

showing *Maspin* promoter hypermethylation, 33/50 (66%) cases showed loss or reduced expression of Maspin protein, while of the 39 unmethylated tumors, 24 (62%) showed moderate to high level of nuclear and cytoplasmic expression, 15 (39%) showed reduced or loss of expression ( $p=0.01$ , OR=3.1, 95% CI=1.3–7.4). The paired normal breast tissues harboring the unmethylated *Maspin* promoter showed high levels of Maspin protein in myoepithelial cells (Fig. 2a–d).

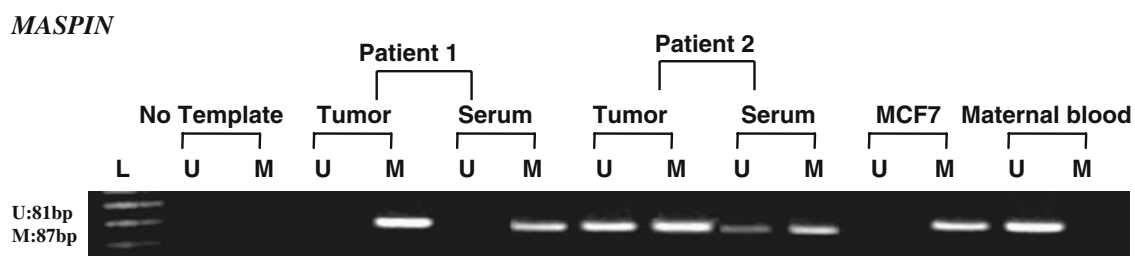
The expression of Maspin was significantly associated with p53 positivity in breast tumors ( $p\leq 0.001$ , OR=10.08, 95% CI=3.3–30.2). No significant association was found between Maspin protein expression and any of the clinicopathological parameters analyzed (Table 3).

### Association of VEGF-A protein expression with clinicopathological parameters

VEGF-A expression was localized in the cytoplasm of the IDCs (Fig. 2e). Of the 89 IDCs examined, 50 (48%) displayed intense cytoplasmic staining in tumor cells. Intense VEGF-A expression was significantly associated with late tumor stage ( $p=0.01$ , OR=3.1, 95% CI=1.3–7.5). However, VEGF-A expression did not correlate with tumor grade, status of ER, and PR or nodal metastasis (Table 3).

### Association of MTA1 protein expression with clinicopathological parameters

In normal duct, MTA1 was expressed in 10% of the epithelial cells with a weak immunoreactivity in the nuclear compartment where as of the 89 tumor tissues examined, 50 (48%) displayed intense nuclear and cytoplasmic staining (Fig. 2g). A marked increase in nuclear MTA1 immunoreactivity was observed in late carcinoma stage ( $p=0.03$ , OR=2.5, 95% CI=1.1–6.1) and lymph node metastasis ( $p=0.05$ , OR=2.4, 95% CI=1.0–6.0; Table 4).



**Fig. 1** MSP analysis of *Maspin* gene in breast tumors and serum DNA. Panel viewed from left to right shows a 50-bp ladder as molecular weight marker, a water control for contamination in the PCR reaction. Patient 1 shows presence of methylated DNA in tumor and corresponding sera. Patient 2 shows presence of both unmethylated

and methylated DNA detected in both tumor and corresponding sera. Breast cancer cell line MCF7 DNA was used as a positive control for methylated DNA, maternal blood collected during 3rd trimester is used as a positive control for unmethylated DNA

**Table 1** Association of *Maspin* methylation with clinicopathological parameters of breast cancer patients

Variables	<i>Maspin</i> methylation					
	Tumor			Serum		
	U	M	<i>p</i>	U	M	<i>p</i>
Total no.=100	46	54		52	48	
Age (years) <sup>a</sup>						
>50 (59)	26	33	0.64	31	28	0.89
≤50 (41)	20	21		21	20	
Menopausal status						
Pre (38)	17	21	0.84	21	17	0.61
Post (62)	29	33		31	31	
Tumor size						
T <sub>1</sub> +T <sub>2</sub> (56)	28	28	0.36	30	26	0.72
T <sub>3</sub> +T <sub>4</sub> (44)	18	26		22	22	
Node involvement						
Negative (33)	24	9	0.001	24	9	0.004
Positive (67)	22	45		28	39	
Stage						
I & II (51)	28	23	0.68	29	22	0.32
III (49)	18	31		23	26	
Grade						
Well Diff. (50)	21	29	0.70	24	26	0.63
Undiff. (28)	13	15		15	13	
p53 Status						
Positive (33)	13	20	0.50	14	19	0.23
Negative (56)	26	30		31	25	
Unknown <sup>b</sup> (11)	7	4		7	4	
ERα status						
Positive (46)	20	26	0.64	18	22	0.80
Negative (54)	26	28		23	26	
PR status						
Positive (48)	24	24	0.44	28	20	0.22
Negative (52)	22	30		24	28	
Her2/ <i>neu</i>						
Positive (23)	10	13	0.86	12	11	0.91
Negative (57)	26	31		29	28	
Unknown <sup>b</sup> (20)	10	10		10	9	
HR status <sup>c</sup>						
Positive (53)	25	28	0.80	30	23	0.4
Negative (47)	21	26		22	25	
ERα/PR/Her2/ <i>neu</i> status						
Positive (55)	24	31	0.72	28	27	0.90
Negative (25)	12	13		13	12	
Unknown <sup>b</sup> (20)	10	10		10	9	

TNM refers to UICC classification

ERα estrogen receptor, PR progesterone receptor

<sup>a</sup> Mean and median age is 50 years

<sup>b</sup> Immunohistochemical analysis was not carried out in these breast cancer patients due to non-availability of tissue

<sup>c</sup> HR (hormone receptor) status: ERα(-), PR(+)/ERα(+), PR(-)/ERα(+), PR(+), ERα(-), PR(-)

## Relationship of *Maspin* expression with VEGF-A and MTA1

The expression of VEGF-A and MTA1 was significantly lower in tumors with high *Maspin* expression compared to tumors with loss of *Maspin* expression. Forty-four percent of the cases with intense *Maspin* expression had high VEGF-A expression compared to 67% of cases with negative *Maspin* expression ( $p=0.03$ , OR=2.6, 95% CI=1.1–6.1, Table 4).

Similarly, only 11% of the cases with intense *Maspin* expression had high MTA1 expression as compared to 82% of those with negative *Maspin* ( $p=0.001$ , OR=12.0, 95% CI=4.3–32.2, Table 4).

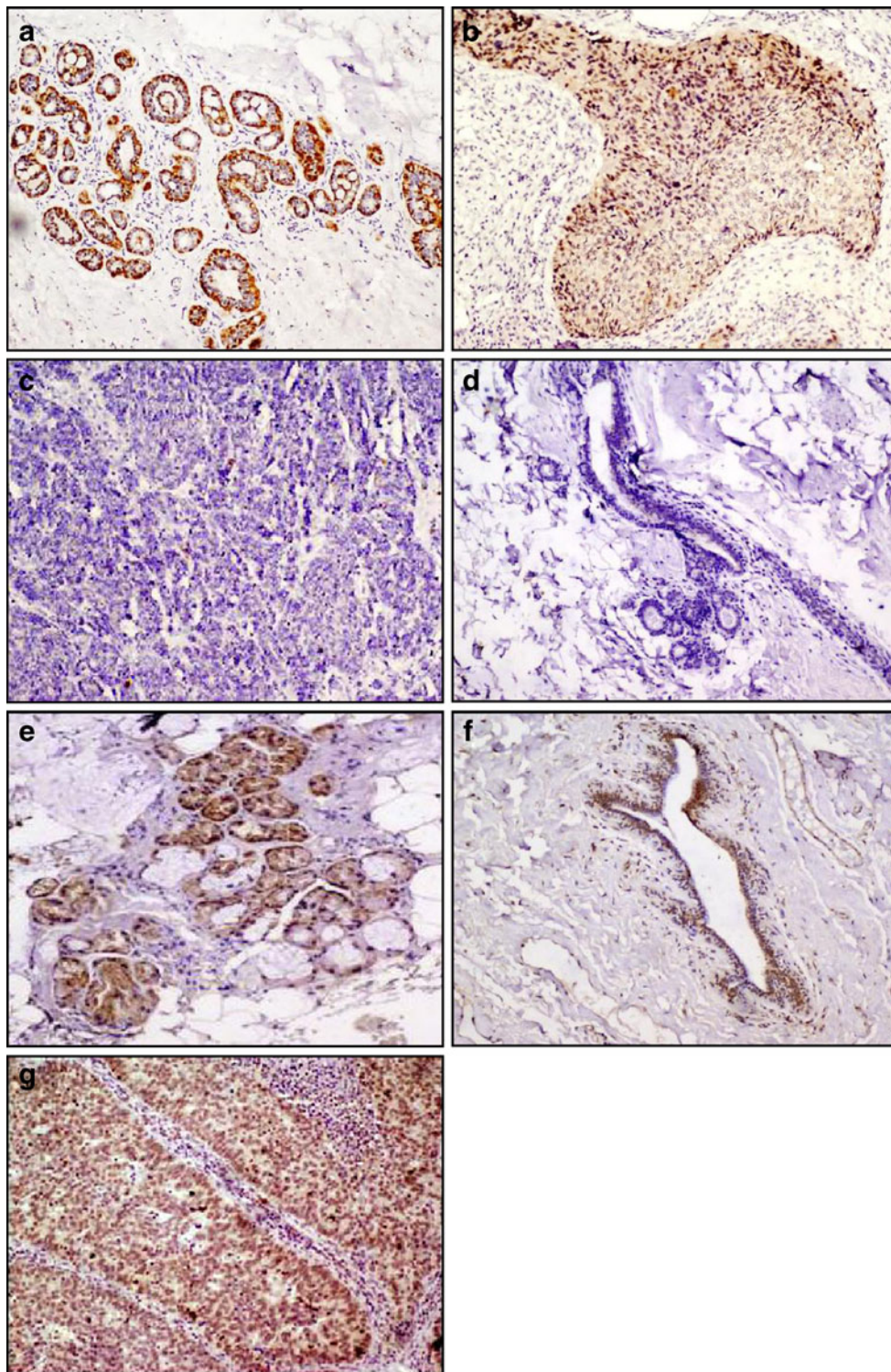
The immunohistochemistry results for *Maspin*, VEGF-A, and MTA1 were further confirmed by Western blot analysis using MCF7, MDA-MB-231, and breast tumor tissue. Immunoblot analysis showed clear increased expression of VEGF-A and MTA1 and decreased expression of *Maspin* in both the breast cancer cell lines and tumor Tissue. (Fig. 3).

## Discussion

The salient findings of our study are: (a) correlation of *Maspin* promoter methylation with loss of its protein expression in IDCs; (b) association of loss of *Maspin* expression and cytoplasmic accumulation of VEGF-A and nuclear accumulation of MTA1 suggesting role of *Maspin* in angiogenesis and metastasis; (c) association of loss of *Maspin* expression with nuclear accumulation of p53; (d) correlation between *Maspin* hypermethylation in tumor and paired serum DNA, suggesting the utility of serum DNA as a surrogate non-invasive tool for methylation analysis.

In this study, we analyzed 100 IDC breast cancer tissues as well as 15 adjacent normal breast tissues for *Maspin* promoter methylation by MSP. The data revealed that *Maspin* promoter was methylated in over 54% of the breast tissues, suggesting that promoter methylation of *Maspin* can be a frequent event in human breast carcinogenesis, which is in general agreement with earlier studies [9, 33, 34]. With respect to the epigenetic status of the *Maspin* promoter in normal breast tissues, we found that majority of adjacent normal breast tissues as well as normal serum taken from healthy females had unmethylated *Maspin* promoters.

In general, aberrant methylation of the *Maspin* promoter was associated with loss of *Maspin* immunoreactivity; however, some cases (17 breast tumor tissues) that were scored as *Maspin*-positive also showed an aberrant methylation of the *Maspin* promoter. In most of these cases, only some cells were *Maspin*-positive and other cells were



**Fig. 2** Immunohistochemical analysis of Maspin, MTA1, and VEGF-A in breast tissues. Paraffin-embedded tissue sections from invasive ductal carcinoma of breast and normal breast tissues were used for immunohistochemical analysis of Maspin, MTA1, and VEGF-A proteins using monoclonal antibodies against Maspin, MTA1, and VEGF-A as described in the “Materials and methods” section were counterstained with hematoxylin. The photomicrographs show **a** normal breast tissue section showing expression in myoepithelial cells, **b** invasive carcinoma depicting cytoplasmic and nuclear immunolocalization of Maspin, **c** invasive ductal carcinoma from patient showing no detectable Maspin immunopositivity, **d** normal breast tissue section showing no detectable VEGF-A immunopositivity, **e** invasive ductal carcinoma from patient showing intense cytoplasmic expression of VEGF-A, **f** normal breast tissue section showing faint nuclear expression of MTA1, and **g** invasive ductal carcinoma from patient showing intense nuclear and cytoplasmic expression of MTA1 (**a–g**, original magnification  $\times 200$ )

alization of Maspin, **c** invasive ductal carcinoma from patient showing no detectable Maspin immunopositivity, **d** normal breast tissue section showing no detectable VEGF-A immunopositivity, **e** invasive ductal carcinoma from patient showing intense cytoplasmic expression of VEGF-A, **f** normal breast tissue section showing faint nuclear expression of MTA1, and **g** invasive ductal carcinoma from patient showing intense nuclear and cytoplasmic expression of MTA1 (**a–g**, original magnification  $\times 200$ )

**Table 2** Relationship between Maspin gene methylation and protein expression

Gene	Genes Methylated (N=89)		Protein Expression (N=89)		
	Methylation Status	Tumor (N)	Protein Expression (N)		Loss of Protein Expression (N)
			Nu	Nu + Cyto	
<i>Maspin</i>	U	39	7	17	15
	M	50	3	14	33

M methylated DNA, U unmethylated DNA, N number of patients analyzed is 89, Nu nuclear, Nu + Cyto nuclear and cytoplasmic

**Table 3** Association of Maspin, VEGF-A, and MTA1 protein expression with clinicopathological parameters

Variables	Maspin			VEGF-A			MTA1		
	+	-	p	+	-	p	+	-	p
(Total no.=89)	41	48		50	39		50	39	
Age (years) <sup>a</sup>									
>50 (52)	25	27	0.65	34	18	0.04	29	23	0.92
≤50 (37)	16	21		16	21		21	16	
Menopausal status									
Pre (34)	14	20	0.46	25	9	0.009	22	12	0.20
Post (55)	27	28		25	30		28	27	
Tumor size									
T <sub>1</sub> +T <sub>2</sub> (54)	24	30	0.70	27	27	0.14	27	27	0.14
T <sub>3</sub> +T <sub>4</sub> (35)	17	18		23	12		23	12	
Node involvement									
Negative (29)	14	15	0.70	14	15	0.29	12	17	0.05
Positive (60)	27	33		36	24		38	22	
Stage									
I & II (53)	23	25	0.70	21	27	0.01	22	26	0.03
III & IV (47)	18	23		29	12		28	13	
Grade									
Well Diff (48)	19	31	0.10	32	18	0.30	30	20	0.71
Undifferentiated (28)	15	12		14	13		15	12	
p53 status									
Positive (33)	5	28	0.001	19	14	0.84	19	14	0.84
Negative (56)	36	20		31	25		31	25	
ERα status									
Positive (38)	16	22	0.50	22	16	0.77	24	14	0.25
Negative (51)	25	26		28	23		26	25	
PR status									
Positive (40)	18	22	0.85	23	17	0.82	25	15	0.27
Negative (49)	23	26		27	22		25	24	
Her2/neu									
Positive (22)	10	12	0.88	13	9	0.70	12	10	0.88
Negative (57)	27	30		31	26		30	27	
Unknown <sup>b</sup> (20)									
HR status <sup>c</sup>									
Positive (44)	20	24	0.90	25	19	0.90	27	17	0.33
Negative (45)	21	24		25	20		23	22	
ERα/PR/Her2/neu									
Positive (54)			0.53			0.90			0.53
Negative (25)	24	30		30	24		30	24	
Unknown <sup>b</sup> (20)	13	12		14	11		12	13	

TNM refers to UICC classification

ERα estrogen receptor, PR progesterone receptor

<sup>a</sup> Mean and Median age is 50 years

<sup>b</sup> Immunohistochemical analysis was not carried out in these breast cancer patients due to non-availability of tissue

<sup>c</sup> HR (hormone receptor) status: ERα(-), PR(+)/ERα(+), PR(-)/ERα(+), PR(+). vs. ERα(-), PR(-)

**Table 4** Relationship between expression of Maspin and VEGF-A and MTA1 proteins

Variables	Maspin Positive	Maspin Negative	<i>p</i>
VEGF-A Positive	18	32	0.001
VEGF-A Negative	23	16	
MTA1 Positive	11	39	0.03
MTA1 Negative	30	9	

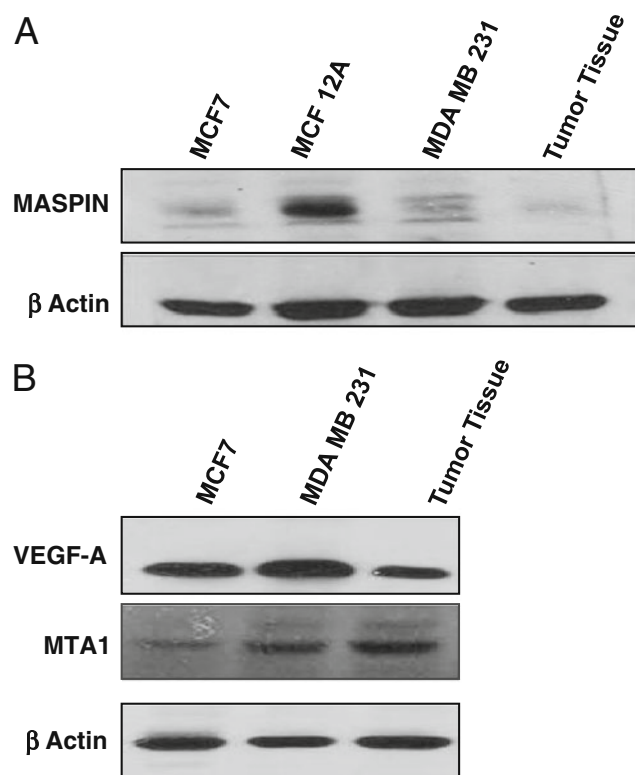
Maspin-negative. Moreover, in these few cells also Maspin staining was localized in cytoplasm. We speculate that it may be possible that aberrantly methylated *Maspin* promoter sequences were derived from the Maspin-negative cells in the population.

Maspin is a serine protease that interacts with cell surface plasminogen activators. It has been shown to be involved in numerous biological processes including (but not limited to) apoptosis, cell motility, matrix remodeling, angiogenesis, regulation of cell phenotype, and redox system [8, 35]. VEGF-A has been shown in breast cancer to constitute a marker of angiogenesis, correlating with vessel density, predict a worse prognosis, and constitute an effective target of molecular therapy [31]. Correlation of Maspin and VEGF-A expression have been reported in other tumor types [36–38], but to the best of our knowledge this is the first study to find this link in breast cancer. We observed a significant association between loss of Maspin expression and cytoplasmic accumulation of VEGF-A, suggesting the role of Maspin in angiogenesis. These results are consistent with previous findings in gastric, colon, and ovarian carcinomas that showed inverse correlation between Maspin expression and microvessel density [39, 40]. Overexpression of Maspin in breast cancer cell lines is known to reduce their metastatic potential [41, 42], and re-expression of Maspin in metastatic mammary cell line reduces cell migration [43]. Maspin has been reported to lower the expression of cell-associated uPA/uPA receptors and uPA activity. Maspin may induce apoptosis by reducing the cell surface-associated pro-survival uPA–uPA receptor complex [1, 44].

The transition mutations of p53 in human cancer are common and occur in all malignant cell types, and the relationship observed between Maspin expression and p53 is biologically relevant. Several studies have reported that p53 activates the maspin promoter by binding directly to the p53 consensus-binding site present in the maspin promoter [4, 45, 46]. In our study, p53 immunopositivity, which suggests loss of functional p53 protein, also seems to contribute to the loss of Maspin expression in primary breast tissues.

Contradictory results have been reported in the literature concerning the role of Maspin in breast cancer and its

prognostic impact. Some earlier studies on Maspin demonstrate its tumor-suppressive properties [47–49]. Overall, our study supports previous clinical studies which have shown that Maspin plays an important tumor-suppressor role in breast tumorigenesis. Ferrucci et al. [50] has confirmed that elevated Maspin expression, quantified by RT-PCR in bone marrow samples of patients with breast cancer, correlates with extended relapse-free survival. However, the biological relevance of Maspin expression and subcellular localization remains controversial. Some data support the hypothesis that the tumor-suppressor properties of Maspin are linked to its nuclear localization [28]. Recent study, demonstrated a high cytoplasmic expression of Maspin in early-relapsing breast cancer and a significantly lower expression in late-relapsing cancer, both in the primary tumors and in the metastatic lesions [51]. In our study, overall nuclear Maspin staining was quite low in the present tumor material and mainly expression was cytoplasmic. The most compelling data regarding the prognostic significance of Maspin in cancer emerges from cancer patient survival studies. Our study was limited by the fact that we had a follow-up of patients only of limited period and in this period prognostic significance of Maspin expression could not be determined.



**Fig. 3** Immunoblot blot analysis of Maspin, VEGF-A, and MTA1 in MDA-MB-231, MCF7, and breast tumor tissue. **a** Decreased expression of Maspin in breast cancer lines: MCF7, MDA-MB-231, and tumor tissue; MCF12A is used as positive control. **b** Increased expression of VEGF-A and MTA1 in breast cancer lines: MCF7, MDA-MB-231, and tumor tissue



In conclusion, to our knowledge, this is the first report demonstrating the relationships between *Maspin* promoter methylation, and loss of expression of *Maspin*. Our data also suggest that expression of *Maspin* is significantly correlated with *MTA1* and *VEGF-A* expression. Furthermore, the correlation between *Maspin* methylation in tumor and paired serum DNA underscores the utility of serum DNA as a surrogate non-invasive tool for methylation analysis.

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