Maspin Expression Profile in Human Prostate Cancer (CaP) and *in Vitro* Induction of Maspin Expression by Androgen Ablation

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ABSTRACT

Purpose: **Expression of tumor suppressor gene, *MASPIN*, is associated with inhibition of tumor cell invasion and metastasis. Loss of or decreased expression of Maspin is found frequently in breast and prostate cancer cells. The objective of this study is to investigate Maspin expression in prostate tumor specimens and explore the mechanisms of hormonal regulation of Maspin expression in prostate tumors.**

Experimental Design: **Immunohistochemical staining of Maspin expression was performed on surgical whole-mounted prostate specimens. The expression of Maspin was scored on individual tumors. Correlation of Maspin expression with clinicopathological features was analyzed for statistical significance. Androgen ablation-induced Maspin expression was analyzed by Maspin promoter luciferase reporter assay and quantitative reverse transcription-PCR analysis of endogenous Maspin expression in LNCaP cells *in vitro* and in animal model.**

Results: **Comprehensive evaluation of Maspin expression profile in multiple tumor foci from whole mounted prostate specimens of prostate cancer patients revealed absence of Maspin expression in a significant fraction (63%). However, Maspin expression is significantly higher in tumor specimens (92%) of patients treated with neoadjuvant androgen ablation therapy before radical prostatectomy. LNCaP cells cultured in androgen-depleted medium show induction of Maspin promoter activity in a promoter luciferase reporter assay. In addition, Maspin expression is increased after castration in LNCaP prostate cancer cells derived tumors in nude mice.**

Conclusions: **Maspin expression is frequently absent in primary prostate cancers. Up-regulation of *MASPIN* in response to androgen ablation strongly suggests a physiological role of Maspin in growth inhibition and/or apoptosis of prostate cancer cells during androgen ablation.**

INTRODUCTION

Maspin is a serpin with tumor suppressing activity. Decreased expression of Maspin is associated with tumor progression in breast tumor cells. Expression of Maspin in breast tumor cells inhibits tumor cell invasion *in vitro* and tumor cell metastasis *in vivo* (1). Maspin expression is regulated by a variety of factors. We demonstrated recently that Maspin is directly regulated by the *p53* gene. UV irradiation and cytotoxic agents also induce Maspin expression (2). The manganese-containing superoxide dismutase is implicated in the up-regulation of Maspin in human breast cancer cells (3). γ linolenic acid, an essential fatty acid with anticancer properties, is reported to induce Maspin expression and affect motility of cancer cells (4). Zhang *et al.* have reported a comprehensive analysis of the Maspin promoter, which contains multiple *cis* elements that are subject to transcriptional regulation. Transcriptional activity of Maspin expression differs between benign prostatic cells and tumor cells, and the expression of *MASPIN* is subject to androgen regulation (5). Hypermethylation of *MASPIN* promoter is also implicated in the absence of Maspin expression in breast tumors (6).

The functional targets of Maspin remains unclear. Recently, Zhang *et al.* (7) have reported that Maspin may function as an angiogenesis inhibitor. Maspin blocks fibroblast growth factor and vascular endothelial growth factor-mediated endothelial cell migration. Maspin expression also inhibits neovascularization in the rat cornea pocket assay, and Maspin-treated prostate tumor cells are associated with reduced vascularization in a xenograft mouse model. Our observation that p53 directly regulates Maspin expression further emphasizes the biological function of Maspin downstream of the p53. These studies suggest that Maspin could play important roles in tumor cell invasion and metastasis.

The biological effects of androgen on target cells, *e.g.*, prostatic epithelial cell proliferation and differentiation, as well as the androgen ablation-induced cell death, are mediated by transcriptional regulation of ARGs[3] (8). The gain of AR functions is believed to play a role in prostate tumorigenesis. Alter-

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[3] The abbreviations used are: ARG, androgen-regulated gene; PSA, prostate-specific antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum.

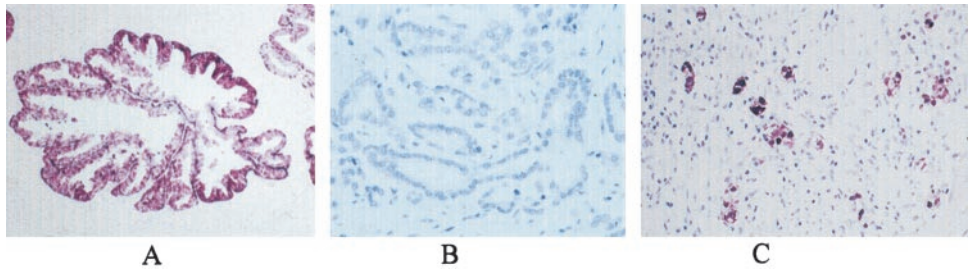

Fig. 1 Immunohistochemical staining of Maspin expression in prostatic tissue. *A*, strong Maspin expression in benign prostatic secretory cells and basal cells; *B*, negative Maspin expression in well-differentiated prostatic carcinoma; *C*, strong Maspin expression in prostatic carcinoma after neoadjuvant therapy.

ations of the androgen receptor gene by mutations in the hormone-binding domain of the AR or by amplification of the ARG have been reported in advanced stages of CaP (8, 9). Amplifications of the ARG in hormone refractory CaP represent yet another scenario where gain of AR function is associated with tumor progression (10, 11).

Several growth factors commonly involved in cell proliferation and tumorigenesis, *e.g.*, insulin-like growth factor-I, epidermal growth factor, etc., have been shown to activate the transcription transactivation functions of the AR (12). Recent studies analyzing expression of the ARGs in hormone-sensitive and refractory CWR22 nude mice xenograft models (13) have also shown sustained expression of several ARGs in AR-positive recurrent tumors after castration. This suggests activation of AR in these tumors. Thus, androgen regulation of critical target genes may play a role in normal prostate growth and prostate tumorigenesis.

In this study, we demonstrate that Maspin expression is absent frequently in primary prostate tumors. Therefore, loss of biological functions linked to Maspin, such as inhibition of angiogenesis and cell invasion, may contribute to the process of tumorigenesis. A higher frequency of Maspin expression in hormonally treated patients may serve as a biomarker for patients to respond to hormonal therapy.

MATERIALS AND METHODS

Specimens and Clinicopathological Features. Ninety-seven patients who underwent radical retropubic prostatectomy at Walter Reed Army Medical Center and 24 patients who had neoadjuvant therapy before the prostatectomy were chosen for this study. The specimens were processed as paraffin embedded, whole mounted sectioned at 2.25-mm intervals at the Armed Forces Institute of Pathology. Clinical information and pathological data were obtained from the Department of Defense Center for Prostate Disease Research Tri-service Multicenter Longitudinal Prostate Cancer Database site at Walter Reed Army Medical Center. The Center for Prostate Disease Research database is an Institutional Review Board-approved retrospective and prospective prostate cancer registry of military health care beneficiaries using standardized forms that physicians and data managers complete during each patient visit.

Histopathological grading was done by a single pathologist (I. A. S) according to the Gleason grading system (14) and the WHO method for nuclear grade and glandular differentiation (15). The specimens were grouped according to the degree of differentiation and nuclear grade. Staging was based on the Tumor-Node-Metastasis system (16).

Immunohistochemistry Analysis. H&E sections were analyzed for the presence of tumors. Sections containing the highest number of tumors and/or the largest tumor were selected for each patient. The corresponding tissue blocks were then recut into 4-μm thin sections and mounted on charged slides. The sections were deparaffined, and endogenous peroxidase was blocked with 0.6% hydrogen peroxide in methanol. Antigen retrieval was accomplished by microwaving for 15 min in 1 mM citrate buffer. Immunological detection was achieved with monoclonal anti-Maspin antibody (PharMingen) at a dilution of 1:320. The avidin-biotin-peroxidase system (Vectastain Elite kit; Vector Labs) was used to visualize the binding of the antibody.

The Maspin Immunohistochemistry slides were reviewed and graded by two pathologists (I. A. S. and W. Z.) in a similar manner as described previously (17). The majority of specimens showed a very focal distribution. The areas of focal positivity with the highest number of immunoreactive tumor cells were used to grade the tumor as: negative; 1+, <25%; 2+, 26–50%; 3+, 51–75%; and 4+, 76–100%.

Statistical Analysis. The Mantel-Haenszel χ^2 test for trend and logistic regression was used to study the relationship of Maspin to age, race, WHO differentiation, WHO nuclear grade, Gleason sum, pretreatment PSA, and pathological stage in 97 radical prostatectomy patients without prior androgen ablation therapy. Kaplan-Meier survival methodology and Cox regression were used to study the relationship of Maspin expression to disease. The Pearson χ^2 test and logistic regression were used to compare Maspin expression in radical prostatectomy specimens obtained from patients with or without androgen ablation therapy before radical prostatectomy.

Plasmid and Constructs. The construction of the *MASPIN* promoter luciferase reporter has been described previously (2). The promoter region of *MASPIN* was amplified by PCR according to the reported DNA sequence. The pM-Luc (−759) was generated by the primers: GAGACTCGAGGCTGAAGTACAGTGGTTAG (with *Xho*I site). The DNA fragment was cloned into the *Xho*I and *Hin*dIII site of the pGL3 basic vector (Promega).

Transfection and Luciferase Assay. The LNCaP cells were plated at 5×10^5 cells/well (six-well plate) 1 day before the transfection. The transfection was performed using the calcium-phosphate method (Clontech). The *MASPIN* promoter reporter plasmid (5 μg), p53 plasmid (2.5 μg), and an internal control plasmid pRL-TK (0.5 μg) were cotransfected into cells for 48 h, and the cells were then harvested for luciferase assay. Luciferase activity was measured by luminometer using the

Table 1 Association of Maspin expression to demographic, clinical, and pathological variables in 97 radical prostatectomy patients with no prior androgen ablation therapy

Variable	Total patients	Maspin expression		% Positive	P^a
		Negative	Positive		
Total	97	58	39	40.2	
Age					0.317
<55	11	7	4	36.4	
55–59	12	4	8	66.7	
60–64	34	21	13	38.2	
65–69	29	20	9	31.0	
≥70	11	6	5	45.5	
Race					0.121
African-American	22	10	12	54.6	
Caucasian	75	48	27	36.0	
WHO differentiation					0.050
Well	31	23	8	25.8	
Moderate	32	18	14	43.8	
Poor	34	17	17	50.0	
WHO nuclear grade					0.108
I	37	25	12	32.4	
II	58	33	25	43.1	
III	2	0	2	100.0	
Gleason sum					0.199
3–5	9	6	3	33.3	
6	54	35	19	35.2	
7	26	13	13	50.0	
8–9	8	4	4	50.0	
Pretreatment PSA					0.765
0–4	11	7	4	36.4	
4.1–10.0	52	32	20	38.5	
10.1–20.0	25	14	11	44.0	
≥20.1	8	5	3	37.5	
Unknown	1	0	1		
Pathological stage					0.199
pT2	42	27	15	35.7	
pT3	49	27	22	44.9	
pT4	2	0	2	100.0	
pTx	6	4	2		

[a] P for the Mantel-Haenszel χ^2 test for trend in the association between Maspin expression and the levels of the variable. Pretreatment PSA = unknown and pathological stage = pTx were not included as levels in the trend tests.

Dual-luciferase reporter assay system (Promega). The results are presented as fold induction of the reporter plasmid alone after normalization with the internal control plasmid pRL-TK.

Quantitative Reverse Transcription-PCR. Total RNA was isolated from LNCaP tumors in nude mice by TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. A portion of total RNA (1 μg) was transcribed reversibly with Superscript reverse transcriptase (Life Technologies, Inc.). The PCR reaction was conducted in 7700 (PE-Biosystems) using the PCR kit from PE-Biosystems. The primers used for *MASPIN* are: TGCTGCCTACTTTGTTGGCAAGT; Forward: TGATACTGTCAATGTTTCCCATACAGA; Reverse, Probe. CCAGTGCAGATGATGAACATGGAGGCCAC.

LNCaP Tumor Growth. Experiments on LNCaP tumor growth in nude mice were performed in the laboratory of Dr. Paul Rennie. LNCaP cells (1×10^6) were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) in the flank region of 6–8-week-old male athymic nude mice (BALB/c strain; Charles River Laboratory, Montreal, Canada). When the tumors reached 200–300 mm^3 in volume, the mice were castrated via scrotal incision under methoxyflurane anesthesia. LNCaP tumors were harvested before and after castration.

Table 2 Association of Maspin expression with tumor differentiation[a]

Differentiation	Total tumors	Maspin expression	
		Positive (%)	Negative (%)
Well	95	26 (27.4)	69 (72.6)
Moderate	58	25 (43.1)	33 (56.9)
Poor	40	21 (52.5)	19 (47.5)

[a] $P = 0.003$ for the Mantel-Haenszel χ^2 test for trend. $P = 0.033$ adjusting for age, race, pretreatment PSA, and pathological stage.

RESULTS

Histological Distribution of Maspin Expression and Correlation with Clinical Parameters. In benign prostatic tissue, the basal cells were uniformly immunoreactive with the anti-Maspin antibody. The secretory cells showed a focally intense reaction product either in isolated glands or in small clusters of glands, particularly in the periurethral region (Fig. 1*A*).

However, there was no specific distribution pattern. In a number of acini, the proteinaceous secretions were strongly positive, often containing needle shaped structures. The transitional epithelium of the prostatic urethra and prostatic ducts was strongly positive. The epithelium of the ejaculatory ducts and seminal vesicles was negative. However, the basally located cells in both structures were positive, which is similar to the basal cells of prostatic acini.

Representative whole mounted prostate sections from 58 of 97 (60%) prostate cancer patients (60%) without neoadjuvant androgen ablation therapy were negative for Maspin expression (Table 1). Association of Maspin expression in whole mounted prostate sections to age, race, differentiation, nuclear grade, Gleason sum, PSA, and pathological stage was analyzed. Intriguingly, Maspin expression was inversely correlated with the degree of tumor differentiation statistically ($P = 0.05$). The Maspin expression was positive in 25.8% of well-differentiated tumors, 43.8% of moderately differentiated tumors, and 50% of poorly differentiated tumors, respectively (Fig. 1*B*). In a multivariable logistic regression analysis of the simultaneous influence of all seven variables on Maspin expression, only tumor differentiation was significant. Because of the multifocal nature of prostate cancers, 193 individual tumors were present in cancerous prostate glands of 97 patients. Only 72 of these 193 tumors (37.3%) showed Maspin expression, and 121 (62.7%) lacked Maspin expression. Maspin expression was significantly correlated with the WHO differentiation grade of prostate tumors with increased Maspin expression associating with fewer differentiated tumors (Table 2). There were 37 individual tumors in prostate glands of 24 patients who had undergone neoadjuvant androgen ablation therapy. All of these cases showed at least focal response to the treatment. Twenty-nine of 37 tumors (78.4%) showed positive Maspin expression, pre-

Table 3 Maspin expression in tumors of patients with and without androgen ablation therapy before prostatectomy[a]

A.	Treatment	Total patients	Maspin expression	
			Positive (%)	Negative (%)
	With treatment	24	22 (91.7)	2 (8.3)
	Without treatment	97	39 (40.2)	58 (59.8)
B.	Treatment	Total tumors	Maspin expression	
			Positive (%)	Negative (%)
	With treatment	36	29 (80.6)	7 (19.4)
	Without treatment	193	72 (37.3)	121 (62.7)

[a] $P < 0.001$ for the unadjusted χ^2 test. $P < 0.001$ adjusting for age race, pretreatment PSA, and pathological stage.

dominantly in the cells with treatment effect (Fig. 1*C*). However, there was no correlation between Maspin expression and the WHO differentiation in residual viable tumors similar to the untreated group. Two of three Maspin-negative cases in the treated group were tumors with only focal treatment effect. Tumor specimens from 22 of 24 patients treated with neoadjuvant androgen ablation treatment were Maspin positive (91.7%), a significantly ($P < 0.001$) higher positivity rate than that of 40.2% Maspin-positive tumors without androgen ablation therapy before surgery (Table 3, *A* and *B*).

During mean follow-up time after radical prostatectomy of 59.2 months for the 97 patients, 27 of 97 patients (27.8%) had biochemical recurrence as determined by levels of serum PSA after surgery. There was no correlation between Maspin expression and progression-free survival (Kaplan-Meier survival methods, Log-rank $P = 0.879$). In multivariable Cox regression analyses simultaneously relating Maspin expression, age, race, differentiation, nuclear grade, Gleason sum, PSA, and pathological stage to progression, Maspin remained insignificant as a predictor of disease progression.

Molecular Mechanisms of Maspin Induction in Response to Androgen Ablation. The strong association of increased Maspin expression in CaP specimens of patients treated with neoadjuvant androgen ablation therapy suggested that Maspin expression could be modulated by the androgen-signaling pathway. To demonstrate that androgen down-regulated *MASPIN* gene expression in prostate tumor cells, *MASPIN* promoter luciferase reporter assay was performed in LNCaP cells. LNCaP cells were cultured in androgen-deprived medium for 4 days, followed by transfection with *MASPIN* promoter luciferase construct. The cells were then cultured in this medium supplemented with 10 nM synthetic androgen R1881 for 48 h. As shown in Fig. 2, *MASPIN* promoter-driven luciferase activity was 3-fold higher in androgen-deprived medium than in regular medium. Furthermore, the addition of synthetic androgen R1881 to androgen-deprived media abolished the increase of luciferase activity. This data suggested that the Maspin promoter was down-regulated by androgens in prostate tumor cells.

To further demonstrate whether or not androgen signaling affected Maspin expression *in vivo*, we analyzed Maspin expression in xenografts of LNCaP cells in nude mice after castration. Total RNA was extracted from tumors harvested at different time intervals after castration. The expression of *MASPIN* was analyzed by real-time PCR. *MASPIN* expression was increased 4-fold in LNCaP cell-derived tumors at 4 and 10 days after castration, as shown in Fig. 3. As an internal control, the expression of GAPDH did not change. The results suggested that androgen ablation induces *MASPIN* expression.

DISCUSSION

Biological function of the tumor suppressor gene *MASPIN*, *e.g.*, inhibition of cell invasion (1), angiogenesis (7), and the direct regulation of *MASPIN* expression by p53 (2), underscores the significance of Maspin in the process of tumorigenesis. Decreased Maspin expression in breast cancer correlates with the disease progression. Although high expression of Maspin in oral squamous carcinoma indicates a good prognosis for the patients, expression of Maspin is detected in high-grade pancreatic tumors (18, 19). More studies are needed to understand how Maspin expression is regulated by microenvironment of different tumors. Although Maspin expression has been evaluated in prostate tumor cell lines, there is a lack of systematic evaluation of Maspin expression and its correlation to clinicopathological features in human tumors. Our study is a comprehensive evaluation of Maspin expression in CaP. With the availability of whole mounted specimens derived from radical prostatectomy of CaP patients, we have been able to map Maspin expression in the context of multifocal lesions of each prostate gland.

Maspin expression was absent in ~60% of tumors. However, an inverse relationship between increased Maspin expression and degree of differentiation is intriguing and warrants additional study. This is the first study of a systematic and comprehensive evaluation of Maspin expression in the context of multifocal prostate cancers. Indeed, one of the unexpected data were the inverse relationship of Maspin expression to the degree of differentiation. This observation is indeed counterintuitive to the Maspin function as an inhibitor of tumor cell invasion and metastasis. However, tumor progression is a complex process involving changes in multiple molecular pathways. Furthermore, Maspin expression detected by immunostaining may not predict functional states of Maspin. At present, we are unable to role out a "p53 scenario" for Maspin, where the presence of p53 protein in tumor cells represents dysfunctional protein. On the other hand, increased Maspin expression in poorly differentiated tumors may indicate that a subset of prostate tumors may have acquired additional changes that bypass the normal regulation of Maspin expression. The regulation of Maspin expression, thus, cannot be attributed exclusively to a positive regulation by tumor suppressor gene *p53* and a negative regulation by androgen signaling. Zhang *et al.* (5) have reported a comprehensive analysis of the Maspin promoter, which con-

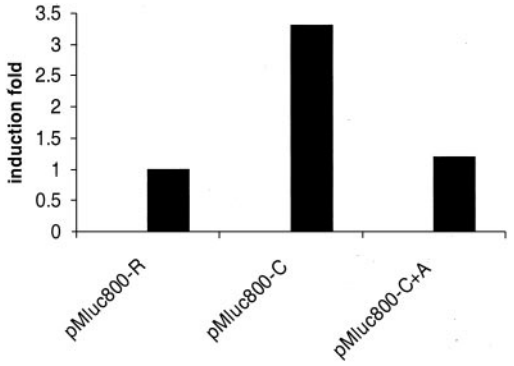

Fig. 2 Induction of Maspin promoter in LNCaP cells. The LNCaP cells were plated at 5×10^5 cells/well (six-well plates) in medium containing charcoal-treated FBS 4 days before the transfection. The transfection was performed using the calcium phosphate method (Clontech). The Maspin promoter reporter plasmid (5 μg), p53 plasmid (2.5 μg), and an internal control plasmid pRL-TK (0.5 μg) were cotransfected into cells for 48 h, and the cells were harvested for luciferase assay. After transfection, the cells were cultured in either regular FBS (*PMluc800-R*), charcoal-treated FBS (*PMluc800-C*), or charcoal-treated FBS supplemented with 10 nM R1881 (*PMLuc800-C+A*). Luciferase activity was measured by luminometer using the dual luciferase reporter assay system (Promega). The results are presented as fold induction of the luciferase activity in regular FBS set as 1. The luciferase activity of the reporter plasmid is normalized with the internal control plasmid pRL-TK.

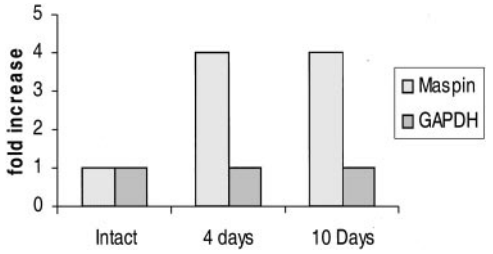

Fig. 3 Real-time PCR analysis of Maspin expression in LNCaP tumors after castration. Total RNA (1 μg) from LNCaP tumors was reversibly transcripted. The cDNA (~100 ng) was used in real-time PCR reaction. Six replica reactions were set for each time point, and the average cycle number for the threshold of detection was calculated. The real-time PCR cycle number comparing the intact and castrated RNA determined the fold induction. The 4-fold increase reflected two cycle difference of PCR. GAPDH was used for internal control. *Intact*, RNA was isolated from LNCaP tumors before castration; *4 days* and *10 Days*, RNA was isolated from LNCaP tumor after castration.

tains multiple *cis* elements that are subject to transcriptional regulation.

The experimental evidence presented here strongly suggests transcriptional induction of *MASPIN* in response to androgen ablation, and perhaps it is one of the important aspects of regulation of *MASPIN* expression in prostate cancer cells. Genes regulated by androgenic hormones are of critical importance for normal physiological function for the human prostate gland, and they contribute to the development of progression of prostate carcinoma.

Although Maspin expression is not detected in a significant portion of prostate cancers, Maspin expression is prevalent in the radical prostatectomy specimens of patients treated with neoadjuvant androgen deprivation therapy before surgery. Tumor cells that exhibit histological response to "hormonal treatment" showed Maspin expression. These data suggest that the androgen withdrawal may unmask Maspin expression in prostate cancer, which frequently lacks Maspin expression. Therefore, Maspin expression may serve as a possible biomarker for prostate cancer cells responding to the androgen ablation therapy.

A previous study reported that the *MASPIN* promoter contains a negatively regulated hormone responsive element (20). Maspin expression on androgen withdrawal indicates either its active role in the androgen ablation-induced apoptosis or just a coincidental expression during the apoptosis. Whether expression of Maspin during androgen ablation is p53 dependent or independent remains to be determined. Regulation of *MASPIN* by p53, as well as androgen, suggests a role of Maspin in prostate cell growth/differentiation. Taken together, alterations of Maspin expression appear to play a role in prostate tumorigenesis, and the induction of Maspin expression in response to androgen ablation suggests for its potential functions in tumor cell growth inhibition and/or apoptosis.

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