Maspin expression in endometrial hyperplasia and carcinoma, and its relation with angiogenesis

C. Taskiran[1], O. Erdem[2], A. Onan[1], C. Vural[2], O. Arisoy[1], S. Yildiz[1], H. Guner[1]

[1]Gazi University, Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Ankara
[2]Department of Pathology, Ankara (Turkey)

Summary

Aim: The purpose of this study was to evaluate the maspin expression in endometrial hyperplasia and cancer, and also to investigate its relation with angiogenesis. *Materials and Methods:* A total of 19 women with complex atypical hyperplasia, 44 patients with simple hyperplasia without atypia, and 67 patients with endometrial carcinoma were included. Maspin expression was assessed by immunohistochemistry (IHC), and tested for possible significant relation with age, FIGO stage, histologic type, grade, depth of myometrial invasion (MI), lymphovascular space involvement (LVSI), lymph node metastasis, and overall survival (OS). Angiogenesis was determined by vascular endothelial growth factor (VEGF) staining. *Results:* Maspin expression was detected in only three patients with endometrial hyperplasia (5%). In patients with endometrial cancer, cytoplasmic and nuclear maspin expressions were detected in 36 (53.7%) and 18 (26.9%) patients, respectively. No significant relation was noted between staining localizations and prognostic variables. The five-year OS rate for patients with cytoplasmic staining was 91%, compared to 87% for patients without staining ($p = 0.31$). These values for nuclear expression were 100% and 87%, respectively ($p = 0.16$). The cytoplasmic and nuclear maspin expressions were found to be significantly correlated with VEGF ($r = 0.278$, $p = 0.02$ and $r = 0.295$, $p = 0.01$, respectively). *Discussion:* This is the first study to demonstrate the relation between maspin expression and angiogenesis in endometrial cancer. Although no survival difference was noted for cytoplasmic or nuclear maspin expressions, a tendency was detected for nuclear staining. Further series will clarify the exact prognostic role of maspin expression in gynecological malignancies including endometrial cancer.

Key words: Maspin; Endometrial cancer; Endometrial hyperplasia; Angiogenesis; Survival.

Introduction

Maspin is a member of serpin gene family which inhibits serine protease. It was first isolated in normal mammary epithelium by subtractive hybridization. Reduced tumor formation and metastasis were observed in the presence of maspin suggesting tumor suppressor characteristics of this unique gene [1]. In the subsequent series, it was detected that maspin inhibits breast cancer cell motility, invasion, and metastasis [2, 4]. It is located at 18q21.3 along with other serpin superfamily and encodes 375-amino acid protein with a molecular weight of 42 kDa [5]. Maspin is bound to the active site of the serine protease by the reactive center loop which is situated near the carboxy terminus. At promoter region, several important transcription factor binding sites such as Ets, Ap1, HRE, and p53 were demonstrated [6]. Although the expression of serpin family proteins is limited to the cytoplasmic compartment of the cell, maspin was found to be extracellular, cell-membrane associated, intranuclear, and within the cytoplasmic compartment [7, 8]. The prognostic value of maspin expression has been widely studied in non-gynecological cancers. Although it was found to be protective in breast and prostate cancers; in pancreatic cancer, maspin was reported to be over-expressed in progression from pre-invasive lesions to invasive disease [1, 4 ,9]. Only a few series examined the role of this important gene in gynecological cancers [10-16]. There are only two reports on maspin expression in endometrial cancers [15, 16]. However, hyperplastic tissues were not evaluated in those series, and the effect of maspin expression on overall survival was not investigated. Therefore the authors decided to detect the rate of expression and prognostic importance of maspin in samples of both endometrial hyperplasias and cancers.

Angiogenesis is essential for tumor growth and metastatic spread by supplying metabolic requirements for the growing tumor and providing a vascular pathway for hematogenous spread to distant sites [17, 18]. Although many important promoters of angiogenesis have been reported, the most heavily studied one is vascular endothelial growth factor (VEGF), which induces capillary tube formation, and increases vascular permeability [19].

The purpose of this study was to evaluate the maspin expression in endometrial hyperplasia and cancer, and also to analyze the relation with prognostic variables and survival. In addition, the correlation between maspin expression and angiogenic factor VEGF was also investigated to observe its effect on tumoral angiogenesis.

Materials and Methods

The patients with endometrial hyperplasia and cancer treated at Gazi University Hospital were included in this study. The patients with invasive cancer were subjected to the initial surgical staging procedure including peritoneal cytology, total abdominal

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Table 1. — *Characteristics of the patients and the comparisons of the prognostic variables with respect to cytoplasmic and nuclear maspin stainings.*

		Cytoplasmic maspin staining, n (%)			Nuclear maspin staining, n (%)		
	n (%)	negative	positive	*p*	negative	positive	*p*
Age							
≤55	28 (41.8)	12 (42.9)	16 (57.1)	0.63	22 (78.6)	6 (21.4)	0.39
>55	39 (58.2)	19 (48.7)	20 (51.3)		27 (69.2)	12 (30.8)	
Stage							
I	42 (62.7)	18 (42.9)	24 (57.1)	0.47	30 (71.4)	12 (28.6)	0.68
II-IV	25 (37.3)	13 (52)	12 (48)		19 (76)	6 (24)	
Histology							
endometrioid	59 (88.1)	28 (47.5)	31 (52.5)	0.72	45 (76.3)	14 (23.7)	0.11
others	8 (11.9)	3 (37.5)	5 (62.5)		4 (50)	4 (50)	
Grade							
1	25 (37.3)	11 (44)	14 (56)	0.64	18 (72)	7 (28)	0.96
2	24 (35.8)	10 (41.7)	14 (58.3)		18 (75)	6 (25)	
3	18 (26.9)	10 (55.6)	8 (44.4)		13 (72.2)	5 (27.8)	
Depth of MI[a]							
<1/2	43 (64.2)	21 (48.8)	22 (51.2)	0.57	32 (74.4)	11 (25.6)	0.75
≥1/2	24 (35.8)	10 (41.7)	14 (58.3)		17 (70.8)	7 (29.2)	
LVSI[b]							
negative	54 (80.6)	25 (46.3)	29 (53.7)	0.99	39 (72.2)	15 (27.8)	0.73
positive	13 (19.4)	6 (46.2)	7 (53.8)		10 (76.9)	3 (23.1)	
LN[c] metastasis							
negative	46 (68.7)	21 (45.7)	25 (54.3)	0.97	36 (78.3)	10 (21.7)	0.37
positive	11 (16.4)	5 (45.5)	6 (54.5)		7 (63.6)	4 (36.4)	
NA[d]	10 (14.9)	5 (50)	5 (50)		6 (60)	4 (40)	

[a]Myometrial invasion, [b]Lymphovascular space involvement, [c]Lymph node, [d]Not available.

hysterectomy, bilateral salpingo-oophorectomy, and complete pelvic-paraaortic lymphadenectomy. Ten patients with IA, IB; grade 1-2 tumors did not undergo lymphadenectomy. Staging was performed according to the FIGO 1988 recommendations. Data were obtained from patients' charts, pathology records, special gynecologic oncology files, or from direct contact with the patients and personal physicians. When necessary, personal communication was used to verify patient's status. Maspin expression was assessed by IHC and tested for possible significant relation with age, FIGO stage, histologic type, grade, depth of myometrial invasion (MI), lymphovascular space involvement (LVSI), lymph node metastasis, and overall survival (OS). Angiogenesis was determined by using VEGF and compared with the results of maspin staining to detect any correlation. The co-author pathologist (O.E.) reviewed the paraffin blocks, and the paraffin block with the maximum tumoral tissue was chosen for IHC in each case.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were used for IHC. Four-micrometer-thick sections from tissue blocks were stained with Maspin (Ab-1, EAW24), and VEGF (VEGF Ab-7, Clone VG1) by using the standard streptavidin-biotin indirect method. Primary antibodies were performed for two hours at room temperature after blocking endogenous peroxides and proteins. AEC (3-amino-9-ethylcarbazole) was used as a chromogen. Breast carcinoma (for VEGF), and prostate carcinoma (for Maspin) were used as positive control. Negative controls were incubated with PBS instead of the primary antibody. Nuclear and cytoplasmic stainings were evaluated separately, and percentage of positive cells and staining intensity were recorded. The percentage of cells was rated as follows: 0 point, negative; 1 point, < 10%; 2 points, 10-20%; and 3 points, > 20%. Staining intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The final score was calculated by adding these two scoring systems, and categorized into three groups: mild (score, 1-2), moderate (score, 3-4), and strong (score, 5-6). Cytoplasmic staining was considered positive for VEGF. The staining score was determined according to the intensity of staining (0: no staining, +1: weak staining, +2: moderate staining, + 3: strong staining), and the percentage of cells staining (0: no staining, +1: positive staining in < 25% of glandular epithelial or tumor cells, 2: positive staining in 26%-50% of the glandular epithelial or tumor cells, 3: positive staining in > 50% of the glandular epithelial or tumor cells). The final index score was calculated by addition to results of these two methods. Scores between 0 and 2 were accepted as negative, scores of 3 and 4 were regarded as weakly positive, and scores of 5 and 6 were regarded as strongly positive. Two different scoring systems were used for maspin and VEGF expressions as suggested in the previous publications [10-19].

Statistical analysis

SPSS for windows (Statistical Package for the Social Sciences) was used for statistical analyses. Categorical variables were compared by Chi-square and Fisher's exact tests, and the analyses of continuous variables were performed using Student's *t*, Mann-Whitney *U*, one way ANOVA, and Kruskal-Wallis tests where appropriate. The bivariate Spearman correlation coefficient was used to detect any significant relation. Survival estimates were obtained via the Kaplan-Meier method, and tested for significance by log-rank test. OS rate of the patients was calculated from the date of initial surgery to the date of death or last follow-up. All *p* values were the results of two-sided tests, and they were considered significant if < 0.05.

Results

A total of 19 women with complex atypical hyperplasia, 44 patients with simple hyperplasia without atypia, and 67 patients with endometrial carcinoma were included. Maspin

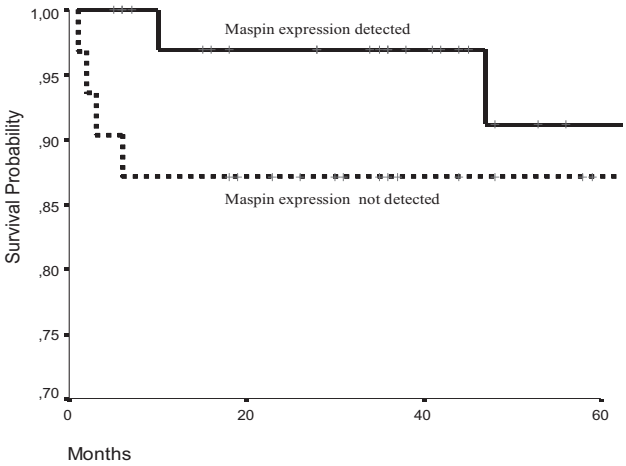

Figure 1. — The comparison of five-year overall survival rates with respect to cytoplasmic maspin expression ($p = 0.31$).

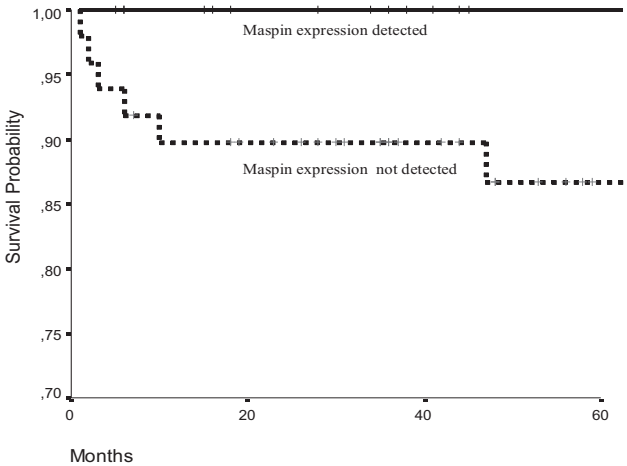

Figure 2. — The comparison of five-year overall survival rates with respect to nuclear maspin expression ($p = 0.16$).

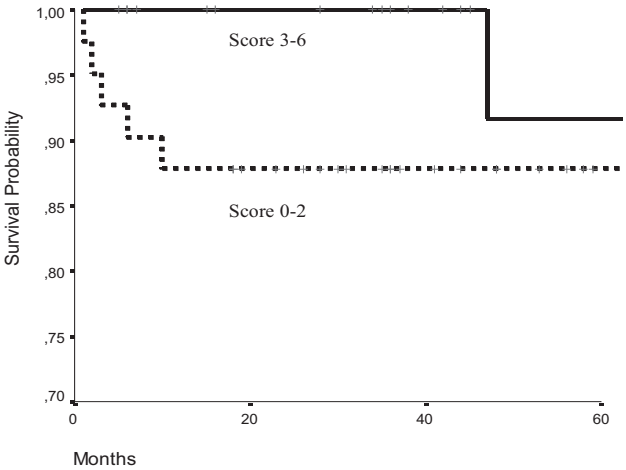

Figure 3. — The comparison of five-year overall survival rates according to the scores of cytoplasmic maspin expression ($p = 0.27$).

expression was detected in one patient with complex atypical hyperplasia, and it was found to be positive in two patients with simple hyperplasia without atypia. Cytoplasmic staining was 2% with a mild intensity for all of these patients. Nuclear staining was not positive for any of women with endometrial hyperplasia. Overall 67 patients with endometrial cancer were subject of this study. The mean age of these patients at the time of diagnosis was 58.2 ± 11.1 years (range, 28-76). The clinical and histopathological characteristics of the patients are documented in Table 1. Of these patients, 42 (62.7%) had Stage I disease, nine (13.4%) had Stage II, 13 (19.4%) had Stage III, and the remaining three (4.5%) patients had Stage IV tumors. The most common histology was endometrioid type endometrial cancer (88.1%). The distribution of patients according to the grades was as follows: grade 1, 25 (37.3%); grade 2, 24 (35.8%); and grade 3, 18 patients (26.9%). Myometrial invasion was negative for seven patients (10.4%), < 1/2 for 36 (53.7%), and ≥ 1/2 for 24 (35.8%) patients. LVSI was detected in 19.4% of the patients (13/67), and the lymph node metastasis was found to be positive in 11 patients (16.4%).

In patients with endometrial cancer, cytoplasmic and nuclear maspin expressions were detected in 36 (53.7%) and 18 (26.9%) patients, respectively. All the patients with nuclear staining had also cytoplasmic maspin expression. Both the cytoplasmic and nuclear staining characteristics were analyzed for possible relation with age, stage, histologic type, grade, depth of MI, LVSI, and lymphatic metastasis, but none of these comparisons revealed significant correlation between staining localizations and prognostic variables (Table 1). The number of patients and the percentages of positive cells according to the cytoplasmic staining were as follows: <10% in 29 patients; 10-20% in five; and > 20% in two patients. These values for nuclear staining were < 10% in 16 patients and 10-20% in two patients. Cytoplasmic staining intensity was +1 for 12 patients, +2 for 17, and +3 for seven patients, and the nuclear staining intensity was +1 for eight patients, +2 for eight, and +3 for two patients (Table 2). The mean score for cytoplasmic staining was 1.67 ± 1.73. Ten patients (14.9%) had mild staining (score 1-2), 22 (32.9%) had moderate (score, 3-4), and four (5.9%) had strong staining characteristics (score, 5-6). When the mean scores were compared with respect to prognostic variables, no significant variance was noted for any of them. The mean nuclear score was 0.7 ± 1.3 (range, 0-5), and it was not significantly different when analyzed with respect to the prognosticators.

The mean follow-up period was 54.2 ± 37.3 months. The five-year OS rate for patients with cytoplasmic staining was 91%, compared to 87% for patients without staining ($p = 0.31$, Figure 1). These values for nuclear expression were 100% and 87%, respectively ($p = 0.16$, Figure 2). When the

Table 2. — *Distribution of the patients according to the staining characteristics.*

Staining localization	Percentage of staining				Intensity of staining			
	0	<10	10-20	>20	0	+1	+2	+3
Cytoplasmic	31	29	5	2	31	12	17	7
Nuclear	49	16	2	0	49	8	8	2

patients stratified into two groups according to the scores of cytoplasmic maspin expression as negative-mild and moderate-strong, the five-year OS rates were 88% and 92%, respectively ($p = 0.27$, Figure 3).

The sections of two patients could not be stained with VEGF, and the mean score for VEGF staining was 4.2 ± 1.6 (range, 0-6). Fourteen patients were negative (22%), 21 (32%) were weakly positive, and 30 (46%) were strongly positive for VEGF staining. The cytoplasmic and nuclear maspin expressions were found to be significantly correlated with VEGF ($r = 0.278$, $p = 0.02$ and $r = 0.295$, $p = 0.01$, respectively). The mean VEGF score for patients with cytoplasmic staining was 3.8 ± 1.7 vs 4.6 ± 1.4 for patients with negative staining ($p = 0.04$). These values for nuclear stainings were 3.5 ± 1.7 and 4.4 ± 1.5, respectively ($p = 0.03$).

Discussion

Targeted therapies will be a part of standard care of cancer patients in near future. Therefore, it is mandatory to identify the critical cellular and molecular pathways. Maspin is one of the most spectacular candidate having tumor suppressive and anti-angiogenic properties [1-4]. In the published literature contradictory findings were reported in the series especially including non-gynecological cancers. Although it was found to be silenced in breast, prostate, and thyroid cancers [1, 20, 21]; in pancreatic, lung, and gastric cancers it was demonstrated that the maspin expression was increased in malignant cells compared to their normal cells of origin [4, 22, 23]. In addition, the elevated levels of maspin expression was found to be related with improved prognosis [24, 25].

Despite the substantial number of studies investigating the value of maspin expression in breast cancers, only a few trials have investigated its' importance in gynecological cancers mainly including the patients with ovarian cancer [10-16]. Sood *et al.* were the first to analyze the role of maspin expression in ovarian cancer tissues and cell lines [10]. They observed that cytoplasmic staining was more predominant in invasive cancers when compared with benign and low-malignant potential tumors, and it was associated with high tumor grade, presence of ascites, and suboptimal cytoreduction. Nuclear expression was related with improved outcome, whereas cytoplasmic localization was related with poor survival. Another striking result of their study was that in vitro invasive potential of the maspin-transfected cell lines was 44-68% lower than the control group. Also, Gynecologic Oncology Group investigated the prognostic value of this important gene by immunoblot analysis including 68 women with advanced stage ovarian cancer, and they showed 72% expression rate with a significant relation with progression free and overall survivals [11]. Solomon *et al*. analyzed 118 patients with high grade advanced stage epithelial serous ovarian carcinoma [12]. Overall, 81.4% of the patients expressed maspin. It was only localized to the nuclear compartment in 21.2% of the cases, and 60.2% of the patients had cytoplasmic staining with or without nuclear expression. The median survival values for negative, cytoplasmic, and nuclear staining groups were 1146, 637, and 1,803 days, respectively, with a significant variance ($p < 0.001$). Maspin localization was also a significant predictor of survival in multivariate analysis. On the contrary, Surowiak *et al*. observed that cytoplasmic expression was related with cisplatin sensitivity in their series including 43 patients with epithelial type ovarian cancers, and these patients had significantly longer progression free and OS rates [13]. Only one study evaluated the maspin expression in the setting of progression from in situ to invasive cervical carcinoma including 18 women with cervical intraepithelial neoplasia-grade 3 (CIN), 7 patients with microinvasive disease, and 11 cases with invasive squamous cell cancers [14]. A significant decrease in maspin scores was reported between CIN 3 vs invasive cancer, and microinvasive vs invasive cancers. Also the maspin scores were lower in tumor emboli, and they speculated that maspin immunopositivity may be related with metastatic potential. No survival analysis was performed in that study.

There are a few reports on maspin expression in endometrial cancer. In the study of Murai *et al*. the samples of 41 patients with endometrioid type adenocarcinoma and 30 women with uterine leiomyoma were stained immunohistochemically [15]. It was completely negative in patients with uterine leiomyoma, whereas 66% of the cases with cancer had maspin expression. No significant relation was noted between the maspin immunoreactivity and the clinicopathological variables including stage, grade, lymph node involvement, distant metastasis, and recurrence. No survival data was given in that study. Interestingly, they showed a significant correlation between aberrant maspin expression and squamous differentiation. Li *et al*. evaluated the expression of maspin gene by reverse transcriptase polymerase chain reaction including 34 endometrial cancer and 28 normal endometrium samples [16]. They reported that maspin expression was significantly higher in Stage I and Stage III patients when compared to normal endometrium ($p < 0.01$ for both comparisons). No significant variance was noted between Stage I and III diseases. In the current study, 19 cases with complex atypical hyperplasia, 44 women with simple hyperplasia without

atypia, and 67 patients with endometrial carcinoma were analyzed for maspin expression. Although only a few cases were positively stained in the group of patients with endometrial hyperplasia, 53.7% of the patients with endometrial cancer had maspin expression. Neither cytoplasmic nor the nuclear staining were found to be significantly related with the clinicopathological prognosticators. The percentages of the cells stained with maspin was not as high as the reported rates for ovarian cancer patients [11, 12]. Similar to the present findings, in the study of Murai *et al*., only 24% of the patients had maspin expression in more than 20% of the cells [15].

In some of the published series on gynecological cancers, the importance of subcellular localization of maspin was reported. Both the Sood *et al*. and Solomon *et al*. showed that nuclear localization was associated with favorable survival in contrast to cytoplasmic staining which was associated with poor outcome [10, 12]. This feature was also supported in the series evaluating non-gynecological cancers [26-31]. Therefore, Hirai *et al*. speculated that nuclear maspin is the active form suppressing tumoral growth, whereas cytoplasmic component has no effect on the carcinogenesis [26]. In the current study, no significant survival difference was found neither for cytoplasmic nor nuclear stainings. However a tendency was noted for patients with nuclear staining with a 13% survival difference between positive (100%) and negative cases (87%).

Maspin was demonstrated to be an inhibitor of angiogenesis [12, 32, 34]. Zhang *et al*. performed in vivo and in vitro tests to explore the relation between maspin and angiogenesis [32]. They observed that maspin blocked the mitogenesis, tube formation, and migration of cultured endothelial cells towards basic fibroblast growth factor and vascular endothelial growth factor in vitro. In a xenograft mouse model it blocked tumor growth and decreased the tumor associated microvessel density. Neovascularization in the rat cornea was also blocked by maspin in vivo. In gastric and colonic cancers, microvessel density was found to be lower in patients with maspin expression [33, 34]. In the gynecological cancer setting, only Solomon *et al*. investigated the relation between maspin expression and angiogenesis in their large series including 118 cases with epithelial serous ovarian carcinoma [12]. They reported that both the VEGF expression and microvessel density were lower in patients with nuclear maspin expression. Although the microvessel density was lower in patients with cytoplasmic maspin expression, VEGF expression was paradoxically higher in these cases. In the present study, the VEGF expression was found to be correlated with both the cytoplasmic and nuclear maspin stainings. The mean scores of VEGF were significantly lower in cases having cytoplasmic or nuclear maspin expression.

In conclusion, this is one of the largest study investigating the existence of maspin expression in patients with endometrial hyperplasia and endometrial cancer. Although it was detected in only 5% of the patients with endometrial hyperplasia (3/63), 53.7% of the patients with endometrial cancer had maspin expression. However, no significant correlation was noted between the expression of maspin and clinicopathological prognosticators as reported by Murai *et al*. In addition, the current study is the first to demonstrate the relation between maspin expression and angiogenesis in endometrial cancer. Although no survival difference was noted for cytoplasmic or nuclear maspin expressions, a tendency was detected for nuclear staining similar to the literature. Further series will clarify the exact prognostic role of maspin expression in gynecological malignancies including endometrial cancer.

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Address reprint requests to:
C. TASKIRAN, M.D.
Israilevleri 33
Sokak (Eski 78.sokak) 8/8
Emek-Cankaya, Ankara (Turkey)
e-mail: cagataytaskiran@yahoo.com