Prostate-Specific Membrane Antigen Expression Is a Potential Prognostic Marker in Endometrial Adenocarcinoma

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Abstract

The aim of this study was to determine the role of prostate-specific membrane antigen (PSMA) as a prognostic marker in endometrial adenocarcinoma (EAC) and to explore whether its down-regulation could be due to epigenetic mechanism. First, we examined the expression and the prognostic value of PSMA by semiquantitative reverse transcription-PCR and immunohistochemistry in EAC tissue samples. Second, to explore the role of CpG methylation in down-regulation PSMA in EAC, we evaluated PSMA CpG island methylation using methylation-specific PCR in cells lines and in a subset of patients' samples. Furthermore, association of the status of tumor methylation to the clinical and histologic variables was also evaluated. Higher PSMA mRNA levels were associated with stage I (P = 0.046) and PSMA protein intensity by immunohistochemistry (P = 0.032). In multivariate analysis, loss of PSMA expression was associated with a worse disease-free survival (P = 0.02). PSMA was methylated in prostate cell lines (DU145 and PC3) and endometrial cell lines. In addition, PSMA was methylated in 5 of 18 samples (all 5 had low PSMA mRNA value). There was a significant association between PSMA methylation and loss of protein expression by immunohistochemistry and PSMA-RNA level with P value of 0.036 and 0.011, respectively. In addition, there was an association between PSMA methylation and tumor size (P = 0.025). In summary, (*a*) PSMA is underexpressed in advanced stage EAC, (*b*) loss of PSMA expression can be considered as a prognostic marker in patients with EAC, and (*c*) loss of PSMA expression in a subset of EAC cases could be due to epigenetic silencing. (Cancer Epidemiol Biomarkers Prev 2008;17(3):571–7)

Introduction

Prostate-specific membrane antigen (PSMA) is a 750–amino acid, class II transmembranous glycoprotein with a short intracellular, NH_2-terminal domain and a large extracellular COOH-terminal domain (1, 2). Low levels of PSMA mRNA and protein were detectable in normal tissues from brain, duodenum, prostate, and kidney (3, 4). Previously, PSMA mRNA and protein expression was seen in the neovasculature of numerous cancer types, and it was exclusively seen in prostate cancer cells, which led us to consider PSMA as prostate specific antigen (5-7). However, due to further detection of PSMA mRNA and protein in renal cell carcinoma and urothelial bladder cancer and the presence of PSMA protein expression in various tumor types, PSMA was no longer considered as prostate specific (3, 8, 9).

PSMA has a folate hydrolase-carboxypeptidase activity that releases glutamate with either α- or γ-linkage (10, 11). PSMA-specific function in prostate cancer is still unclear, but it may function as a tumor metastasis suppressor due to the following lines of evidence. (*a*) The PSMA gene has been mapped to the 11p11.2 locus (12), where a novel inhibitor of tumor progression and metastases, gene KAI1 (CD82), was identified nearby (13). (*b*) Corr et al. showed that when full-length PSMA cDNA was transfected into PC3 cells and orthotopically implanted into nude mice, metastases were fewer and smaller in comparison with those animals implanted with unmodified PC3 cells (14). Furthermore, PSMA monoclonal antibody has also been employed as a part of cancer vaccine therapy and it is widely used as diagnostic method for diagnosing prostate cancer by radioimmunoscintigraphy technique using an indium-labeled monoclonal antibody 7E11-C5 (15-17). All the above data make PSMA peptide an attractive candidate to explore in other malignancies such as endometrial adenocarcinoma (EAC), which constitute our main research interest.

Carcinoma of the endometrium is the most frequently diagnosed malignancy of the female genital tract and is the fifth leading cause of cancer-related deaths in women (18). Despite the recent advances in molecular

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Table 1. Summary of clinical data, histology, and PMSA mRNA protein in patient's population

Variable categories	Frequency (n = 130)	% (of nonmissing)
Diagnosis		
Endometrioid	103	79
Serous and mixed	18	14
Other	9	7
Grade		
1	51	39
2	46	35
3	33	25
Stage		
I	83	64
II	21	16
III	17	13
IV	9	7
mRNA		
≤0.5	15	31
>0.5	34	69
Immunohistochemistry percent		
Negative	52	40
Positive	78	60
Immunohistochemistry intensity		
None/weak	88	68
Moderate/strong	42	32
Immunohistochemistry pattern		
None	45	35
C	47	36
A or A/C	38	29
Recurrence status		
Persistent	6	8
Recurrence	17	23
None	50	68
Status		
AWD	3	4
ANED	44	63
DOD	18	26
DNED	5	7
Age at surgery (y), median (range)	66 (30-87)	
Follow-up time (mo), median (range)	17 (0-90)	

diagnostics, the most important factors in predicting patient prognosis are tumor grade, International Federation of Gynecology and Obstetrics (FIGO) staging, and tumor subtypes: type I, low malignancy neoplasm (endometrioid, secretory, and mucinous), and type II, more virulent type (serous, clear cell carcinoma, and undifferentiated carcinoma; refs. 19-25). In an effort to determine the expression and prognostic value of PSMA in EAC, we have analyzed a large number of human endometrial cancers. A secondary objective was to explore its mechanistic aspect in this tumor. Because PSMA was suggested to have a tumor metastasis suppressor activity and because promoter methylation was able to repress PSMA transcription in prostate cell line (26), we hypothesize that a similar mechanism can occur in EAC. Thus, to validate whether a similar epigenetic mechanism is involved in EAC, we evaluated the relation between down-regulation of PSMA and CpG island methylation in cell lines and in subset of tumors samples using methylation-specific PCR (MSP). Furthermore, association of the status of tumor methylation to the clinical and histologic variables was also evaluated.

Materials and Methods

Human Tissue Samples and Tissue Microarray Construction. Frozen tissue specimens (n = 49) were obtained from patients undergoing surgery for EAC at the Roswell Park Cancer Institute between 2004 and 2005. All tissue specimens were collected under a protocol approved by the institutional review board. All pathology specimens were reviewed in our institution, and tumors were classified according to WHO criteria. From the same tumors that RNA was extracted (n = 49), whole sections from paraffin-embedded block tissues were used for immunohistochemistry study. In addition, tissue from 81 additional patients with EAC was used to construct a tissue microarray as described previously by Kononen et al. (27). Punch biopsies from normal endometrial tissues of patients were also taken for the array as normal controls. Briefly, after carefully choosing the morphologically representative region on the chosen individual paraffin-embedded blocks (donor blocks), a core tissue biopsy of 0.6 mm was punched and transferred to the donor paraffin-embedded block (receiver block). To overcome tumor heterogeneity and the loss of tissue, three core biopsies were done from different areas of each tumor. One section was stained with H&E to evaluate the presence of the tumor by light microscopy. Finally, a panel of normal tissues was also evaluated for PSMA.

Cell Lines. Six urologic and gynecologic cell lines, all from the American Tissue Culture Collection were cultivated according to the supplier's recommendations. The cell lines were as follows: two endometrial cancer cell lines (AN3CA and HEC59), three prostate cancer cell lines (LNCaP, DU145, and PC3), and one ovarian cancer cell line (OVCA429).

RNA Isolation and Reverse Transcription-PCR. Total tissue RNA was isolated from frozen tumor tissues and cancer cell lines as described previously (28) using the TRI Reagent (Molecular Research Center) and according to the manufacturer's protocol. PCR was subsequently done to analyze expression. PCR was done with PSMA-specific primers (sense 5′-CATAGTGCTCCCTTTTGATTGTC-3′ and antisense 5′-CTCTCACTGAACTTGGAAGCAAT-3′). Glyceraldehyde-3-phosphodehydrogenase–specific sense 5′-GCTTCCCGTTCCTCAATTTTGAAG-3′ and antisense 5′-ATGGGAAGGTGAAGGTCGGAG-3′ primers were used to obtain a 195-bp PCR product as control. PCR was done in a PTC-100 thermal cycler (MJ Research) and included a 60-min incubation at 50°C for reverse transcription and 15 min for enzyme inactivation at 95°C followed by 30 cycles of PCR. Each PCR cycle consists of a 1-min denaturation at 95°C followed by a 60-s annealing at 60°C and a 90-s extension at 72°C. After the last cycle, the final extension step was at 72°C for 10 min. The PCR products were visualized by ethidium bromide staining after separation in a 1.5% agarose gel. Semiquantitative PCR was done using AlphaImager software to capture the band density. Relative PSMA mRNA levels were normalized against expression levels of glyceraldehyde-3-phosphodehydrogenase housekeeping gene. Finally, the ratio of PSMA/glyceraldehyde-3-phosphodehydrogenase was given as the final result of PSMA mRNA for each sample. Based on the results obtained from normal endometrium, a cutoff ratio of <0.5

PSMA/glyceraldehyde-3-phosphodehydrogenase mRNA was considered as negative.

Immunohistochemistry. Sections (4 μm) from the formalin-fixed, paraffin-embedded tissue microarray were processed for immunohistochemistry. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase for 5 min. Antigen retrieval was carried out in high pH buffer for 3 min in a steamer-cooker. Then, sections were incubated with the monoclonal antibody PSMA (YPSMA-1, 1:50; Gene Tex) for 30 min at room temperature. This antibody is directed against an immunogen collected from a crude membrane protein preparation from pooled human prostatic adenocarcinoma. It detects prostate cancer but shows little or no cross-reactivity to benign hyperplasia or to normal prostatic tissue. The primary antibody was detected with a biotinylated anti-mouse IgG (DAKO). Diaminobenzidine tetrahydrochloride was then added for development for 10 min followed by counterstaining with hematoxylin solution. Negative control slides omitting the primary antibody were included in all assays. A blind, semiquantitative evaluation of the immunohistochemistry slides was done by two pathologists (P.M. and W.B.) at a double-head microscope. The scores were reviewed, and when a discrepancy was noted between the two readings, the case was discussed and a consensus agreement was used for final scoring.

We observed two different patterns of positive staining including apical and/or apical and cytoplasmic and cytoplasmic. For scoring, the intensity and percentage of positive cells were taken into consideration. The intensity was classified in two categories: cases with negative and weak intensity were considered as negative, and cases with moderate and strong intensity were considered as positive. As for percentage of positive cells, cases with <5% positive cells were considered as negative results and cases with ≥5% positive tumor cells were defined as positive results.

Methylation-Specific PCR. Three prostate cell lines (DU145, LNCAP, and PC3) and two endometrial cell lines (HECLA and RL-95), in addition to 30 samples with low, moderate, and high mRNA value were evaluated by the MSP. A 750 ng genomic DNA from cell lines and formalin-fixed, paraffin-embedded tumor samples was bisulfite treated using the EZ DNA Methylation kit (Zymo Research) as per the manufacturer's instructions and the treated DNA were recovered 75 μL. Bisulfite-treated DNA (1 μL) was used as template for MSP analysis. MSPs were set up as described previously (29). The methyl primers used to amplify the promoter region of the *PSMA* gene are forward 5′-...GGGCGC GTAGTAGAGTAGTAGTATAGGC...-3′ and reverse 5′-...GCGATAACCACAACCGAATCG...-3′. The unmethyl primers used are forward 5′-...AGGGTGT GTAGTAGAGTAGTAGTATAGGT...-3′ and reverse 5′-...CACAATAACCACAACCAAATCA...-3′. Both primer sets were amplified with an annealing temperature of 56°C and the PCRs were run for 35 cycles. The product sizes are 107 and 109 bp for the methyl-specific and unmethyl-specific primers, respectively. The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis. PSMA mRNA level was considered both as a log-transformed quantitative variable and dichotomized. In the former case, comparisons with other explanatory variables were made with *t* tests and ANOVA; in the latter case, comparisons were made with χ tests. Kaplan-Meier estimators and log-rank statistics were used for univariate survival analyses for categorical variables. Stratified models were used to control for stage and grade. Cox regression models and Wald statistics were used for analyses with continuous covariates.

A parsimonious survival model was chosen from the covariates age, stage, and grade. The final model stratified on grade only as the other covariates were not significant.

For disease-free survival (DFS), death without disease was considered independent censoring. Furthermore, an association of the status of tumor methylation to variables, such as age, tumor size, grade, stage, depth of invasion, histology subtypes, and disease outcome, was evaluated by using Fisher's exact test to compare binary variables.

Results

Patient Population. A total of 130 patients with EAC were included in the study. The stage, grade, and tumor subtypes were available for all the patients, and complete clinical and follow-up data were available for 73 patients. The median age was 66 years (range, 30-87). As for tumor subtypes, 103 cases were endometrioid type, 7 serous, 2 clear cell, 4 carcinosarcoma, and 13 mixed tumors. These results are summarized in Table 1.

Expression of PSMA mRNA in Cell Lines and Human Tissues. In tumor cell lines, LNCaP expressed high levels of PSMA mRNA and DU145 and PC3 expressed low levels of PSMA mRNA. In addition, low PSMA mRNA expression was seen in endometrial cancer cell lines (HEC59 and AN3CA) and ovarian cancer cell line (OVCA429; Fig. 1). Very low PSMA mRNA levels were seen in all human normal endometrial tissues. However, EAC cases showed a wide range of PSMA mRNA levels reaching as high as 4.65. Using this dichotomous classification (see Materials and Methods), 15 of 49 (31%) cases were considered negative and 34 of 49 (69%) cases were considered positive (Fig. 2).

Immunohistochemistry. Analyzing the panel of normal tissues, weak PSMA expression was found in prostate glands, duodenum, brain, urinary bladder,

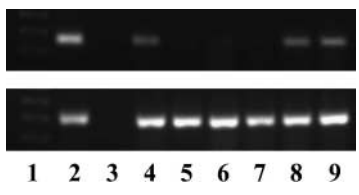

Figure 1. PSMA mRNA by reverse transcription-PCR in cancer cell lines. *Lane 1,* marker; *lane 2,* LNCaP cell line (considered as positive control); *lane 3,* negative control (no template); *lane 4,* OVCA429 (ovarian cancer); *lane 5,* AN3CA (endometrial cancer); *lane 6,* HEC59 (endometrial cancer); *lane 7*, DU145 (prostate cancer); *lane 8,* PC3 (prostate cancer); *lane 9,* U3 (urothelial cancer).

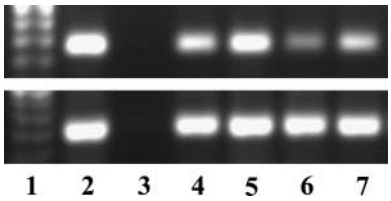

Figure 2. PSMA mRNA by reverse transcription-PCR in human EAC. *Lane 1,* marker; *lane 2,* LNCaP cell line (considered as positive control); *lane 3,* negative control, *lanes 4* to *8,* example of human EAC.

kidney tubules, and endometrial glands. Normal endometrial glands showed a very weak apical and cytoplasmic pattern of PSMA expression. Of all EAC cases, 52 of 130 (40%) cases had negative PSMA expression and 78 of 130 (60%) showed positive PSMA expression. Furthermore, 88 of 130 (67.7%) had no/weak PSMA intensity and 42 of 130 (32.3%) showed moderate/strong intensity. Of the 85 cases with any PSMA intensity, apical and cytoplasmic pattern was found in 38 cases (44.7%) and cytoplasmic pattern in 47 cases (55.2%). There was very sparse or no PSMA expression by endothelial cells of the vasculature seen in these tumors. We also found a significant association between high levels of PSMA mRNA and moderate/strong PSMA protein intensity detected by immunohistochemistry ($P = 0.032$).

Association of PSMA mRNA, PSMA Expression, and Clinical Data. PSMA mRNA expression was significantly associated with early tumor stage. Meaning, high PSMA mRNA levels was significantly associated with FIGO stage I disease ($P = 0.046$). However, PSMA mRNA was not associated with tumor grade ($P = 0.5$), tumor subtype ($P = 0.9$), DFS ($P = 0.5$), or overall survival ($P = 0.6$). As for PSMA protein expression by immunohistochemistry, loss of PSMA expression was not associated with stage ($P = 0.7$), grade ($P = 0.3$), or age ($P = 0.2$). However, in multivariate survival analysis, loss of PSMA expression was significantly associated with worse DFS ($P = 0.02$; Fig. 3).

Association of PSMA CpG Island Methylation, PSMA-mRNA, PSMA Expression, and Clinical Data. Table 2 and Fig. 4 showed the status of PSMA CpG island methylation in cell lines and tumor samples as measured by MSP. In the cell lines, PSMA was methylated in two of three prostate cell lines (DU145 and PC3) and in both endometrial cell lines. In a set of 30 tumor samples, the analysis of DNA methylation was inconclusive in 12 samples (not shown in Table 2) due to poor DNA quality as shown by the inability of any PCR product to be amplified using either the methyl-specific or the unmethyl-specific PSMA primers or nonmethylation specific primers to β-actin (data not shown). However, for the 18 other samples, we did have conclusive data showing three samples that showed moderate levels of methylation (samples 943, 957, and 940) and another two

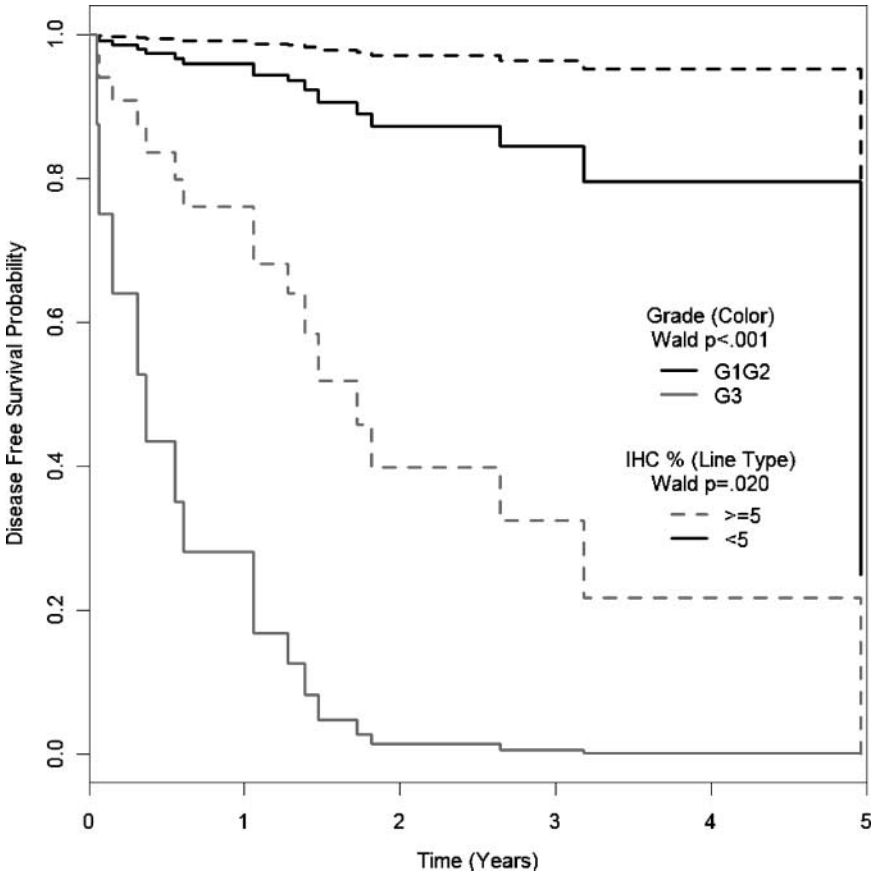

Figure 3. Cox survival curve, indicating an independent prognostic factor of PSMA in predicting DFS after conditioning for disease stage and grade. Patients whose tumors lost PSMA expression have worse DFS than those with positive expression.

Table 2. Summary of the clinical data, histology, PSMA-MS-PCR, PSMA-mRNA, and PSMA expression

Sample	PSMA-MS-PCR methylated	PSMA-RNA	Immuno histochemistry	FIGO grade	FIGO stage	Depth of invasion %	Tumor size in cm	Histology type
941	No	Low	Negative	1	I	82	6	Endometrioid
943	Yes	Low	Negative	3	II	1	7	Endometrioid
957	Yes	Low	Negative	3	IV	93	7	Serous + clear
942	No	Low	Positive	3	II	89	2.2	Endometrioid
940	Yes	Low	Negative	1	II	16	4.5	Endometrioid
945	Yes	Low	Negative	3	I	75	9	Endometrioid + clear
947	No	Low	Negative	1	I	6	5.5	Endometrioid
946	Yes	Low	Negative	2	II	90	6	Endometrioid
932	No	Medium	Negative	1	I	0	1.7	Endometrioid
937	No	Medium	Positive	3	IV	90	7.5	Endometrioid
936	No	Medium	Positive	2	III	87	4.5	Endometrioid
938	No	Medium	Negative	1	I	12	6	Endometrioid
931	No	Medium	Positive	1	I	0	2	Endometrioid
933	No	Medium	Positive	1	I	8	3.2	Endometrioid
948	No	High	Positive	2	I	30	2.5	Endometrioid
954	No	High	Positive	1	I	15	5	Endometrioid
958	No	High	Negative	2	I	10	5.5	Endometrioid
951	No	High	Positive	3	I	75	3	Endometrioid
DU145	Yes							
HECLA	Yes							
LNCAP	No							
PC3	Yes							
RL-95	Yes							

samples that showed very faint methylation (samples 945 and 946) as seen in the Table 2 and Fig 4. There was a significant association between PSMA CpG island methylation and loss of protein expression by immunohistochemistry and low PSMA-RNA level with P value of 0.036 and 0.011, respectively. In addition, there was an association between PSMA methylation and tumor size (P = 0.025) with PSMA hypermethylated in larger tumors. There was no association between PSMA methylation status and other variables such as age, grade, stage, depth of invasion, histology subtypes, and disease outcome.

Discussion

The presence of PSMA mRNA and protein expression by immunohistochemistry in tumors other than prostate cancer along with the possibility that PSMA might be an inhibitor/suppressor of tumor/metastasis and its therapeutic and diagnostic values stimulated our interest to evaluate its expression and prognostic significance in EAC. In normal tissues, previous studies showed low levels of PSMA mRNA expression in small intestine, brain, kidney, and normal prostate, which was confirmed further by immunohistochemistry (3, 4, 8). Similar findings were seen in our panel of normal tissues using immunohistochemistry. In the present study, we detected low levels of PSMA mRNA expression and protein expression in normal endometrial tissue and in EAC at variable levels, thus providing additional data that PSMA is not restricted to prostate adenocarcinoma. Similar to prostate carcinoma and in contrast to other tumors, such as renal cell carcinoma and pancreatic carcinoma, the neovasculature associated with EAC rarely expressed PSMA, which is probably due to the lack of a host desmoplastic response to EAC (6).

In prostate carcinoma, PSMA expression detected by immunohistochemistry by antibody directed against the PSMA internal domain (clone 7E11) showed that the number of PSMA-positive cells was lowest in benign prostate tissue and increased in prostate carcinoma. Furthermore, the intensity of PSMA staining was greatest in the primary tumor and lowest in lymph node

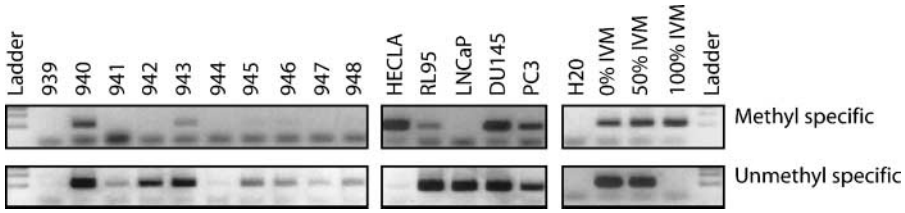

Figure 4. PSMA MSP analysis in selected tumor samples and cell lines. Samples 940 and 943 showed methylated bands. Samples 941, 942, 947, and 948 are conclusively unmethylated. However, samples 939 and 944 were inconclusive. *IVM, in vitro* methylated DNA; *50% IVM,* equal mixture of IVM DNA with nonmethylated DNA.

metastases (30). Other studies on prostate carcinoma revealed that high PSMA expression was correlated with tumor grade and pathologic stage, and it was an independent predictive factor for tumor recurrence (31). However, this was not the case in renal cell carcinoma where one study failed to show either PSMA mRNA or protein expression by immunohistochemistry to be associated with tumor subtype, grade, or stage (8). In urothelial bladder cancer, a correlation between high PSMA mRNA levels and deep tumor invasion (>pT_3) was reported (3). In addition, the study showed that patients with negative serum PSMA mRNA levels had better 2-year survival rate than those with positive PSMA mRNA levels (3). However, the study was limited by number of patients ($n = 27$) and requires confirmation by larger studies.

Herein, we found that high PSMA mRNA level was associated with a very important prognostic factor that is early-stage disease. Because the bulk of EAC seen in clinical practice are low-grade and early-stage EAC, anti-PSMA targeting strategies might represent a potential approach for improving the outcome of a select group of these patients. Most importantly, we found that PSMA expression was an independent prognostic factor for DFS. Patients whose tumors were negative for PSMA staining had shorter DFS than those patients with tumors expressing PSMA. Therefore, PSMA could be of clinical use where patients with tumors negative for PSMA could be managed more aggressively than those with positive PSMA expression. However, these data should be confirmed with other studies before it could be applied.

Our results indicate no association between PSMA-mRNA level and DFS. In contrast, loss of PSMA protein expression by immunohistochemistry was associated with improved DFS. This discordance in results might be related to post-translational modification of PSMA or presence of functional polymorphisms in PSMA protein (32). The drawback of our study is that it might suffer from the limited number of patients with adequate follow-up. Thus, additional studies designed to validate PSMA as a prognostic marker in EAC are warranted.

In addition to distinct genetic mutations found in EAC, epigenetic mechanisms may play an important role in the pathogenesis of this tumor (33, 34). Silencing of gene expression by CpG hypermethylation has been shown to be an early event in cancer development, and it can even precede the neoplastic process (35). By doing PCR analysis for CpG island methylation, we showed that, in a small fraction of EAC cases, loss of PSMA expression is associated with methylation-dependent transcriptional silencing. Additionally, PSMA methylation was associated with tumor size. Strikingly, all five cases where DNA methylation of PSMA was shown showed low mRNA levels and were negative by immunohistochemistry. However, epigenetic silencing of PSMA does not explain entirely the loss of PSMA expression in EAC, as three other negative cases did not exhibit DNA methylation. Thus, other mechanisms responsible of down-regulation of PSMA in endometrial carcinoma are still to be explored.

In summary, we showed that PSMA is highly expressed in early-stage disease, which is the most common presentation and where PSMA monoclonal antibody could be of potential use. In addition, PSMA protein expression was an independent prognostic factor in predicting DFS for EAC. However, additional studies to validate our results are warranted before routine testing of PSMA can be recommended in the clinical setting. Finally, an association between PSMA CpG methylation and low level of expression may explain the mechanism of PSMA in EAC in a fraction of tumors.

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