Prognostic value of cytoplasmic expression of S100A4 protein in endometrial carcinoma

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Abstract. The S100A4 protein, a member of the S100 family of calcium-binding proteins, has been considered as a candidate prognostic marker in patients with cancer. The present study was conducted to evaluate the prognostic value of S100A4 and to examine its correlation with the clinicopathological parameters and the overall survival and progression-free survival in patients with endometrial carcinoma (EC). To do this, we performed immunohistochemistry of formalin-fixed tissue sections obtained from 135 cases of EC. In addition, we quantified the level of S100A4 mRNA using the quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). The cytoplasmic expression of S100A4 protein was observed in 35 cases (25.9%). There was a significant association between the expression of S100A4 and clinicopathological parameters such as histologic grade, FIGO stage, lymph node metastasis and loss of progesterone receptor (PR). qRT-PCR demonstrated that the level of S100A4 mRNA was significantly higher in ECs as compared with normal endometrium. The cytoplasmic expression of S100A4 had a significant correlation with shorter overall survival and progression-free survival on the Kaplan-Meyer survival analysis. In multivariate analysis, there was a significant correlation between S100A4 expression and a poorer OS. In conclusion, our results indicate that S100A4 may be a biological marker indicating the recurrence and poor prognosis in patients with EC.

Introduction

Endometrial carcinoma (EC) is one of the most common malignancies that occur in the female reproductive system. In the US, it is estimated that ~49,560 new cases of EC with 8,190 deaths occurred in 2013 (1). In Korea, its incidence has markedly increased, accounting for ~16% of total gynecologic malignancies (2). There are two types of EC depending on the clinicopathological characteristics (3,4). Type I ECs are characterized by the endometrioid histology, accounting for ~80% of total ECs. It is known that type I ECs are hormone dependent, show a predilection in younger patients and are associated with a favorable prognosis. In addition, they are also characterized by a high incidence of loss-of-function alterations in the PTEN tumor suppressor gene as well as defects in DNA mismatch repair genes. By contrast, type II ECs usually have non-endometrioid histology, are not estrogen dependent, are seen in older patients and are associated with a poor prognosis. In addition, they are likely to harbor p53 mutation. The prognosis of EC is dependent on several factors, including the stage, histologic grade, histopathologic subtype and invasion of myometrium (5).

The S100 gene family located on chromosome 1q21, comprises >20 members whose protein sequences encompass at least one EF-hand Ca^{++} binding motif. It has been reported to be involved in a variety of physiological functions, such as cell proliferation, extracellular signal transduction, intercellular adhesion and motility as well as cancer metastasis (6-8). S100A4 belongs to the S100 calcium binding protein family and may be characterized as a cytoplasmic protein that promotes cellular motility via direct interaction with myosin-IIA and also has functions in cell cycle progression. Its overexpression has been documented in breast (9-12), gastric (13), colorectal (14-16), esophageal squamous cell (17) and gallbladder carcinoma (18), ovarian (19) and bladder cancer (20), papillary thyroid carcinoma (21,22) and non-small cell lung cancer (23). In addition, its upregulation has been associated with disease progression, metastasis and decreased patient survival. However, little is known about the exact mechanisms of its action.

In human ECs, however, there have been few findings on the expression and subcellular localization of S100A4. We found only one report on its expression in EC (24). This showed that the level of S100A4 mRNA on quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was significantly higher in the grade 3 EC, uterine papillary serous carcinoma and malignant mixed Müllerian tumor (MMMT)

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compared with grade 1 or 2 EC and normal endometrium. This was also demonstrated in immunohistochemistry. It has also been reported that the positive immunoreactivity for S100A4 was seen in the cytoplasm of cancer cells and overexpressed in the grade 3 ECs, uterine papillary serous carcinoma and uterine MMMT.

Based on the above background, we conducted the present study to evaluate the prognostic value of S100A4 and to examine its correlation with the clinicopathological parameters. To perform this, we carried out immunohistochemistry to determine the expression and localization of S100A4 protein and quantified its mRNA levels using qRT-PCR.

Materials and methods

Patients and tissue samples. Formalin-fixed paraffin-embedded specimens were selected from 135 patients with EC who were diagnosed with EC and underwent surgical resection between January 1998 and December 2009 at Pusan National University Hospital, Busan, Korea. Of these, eleven cases of tumor and normal endometrial tissue were collected immediately after surgery, cut into small pieces, frozen in liquid nitrogen and stored at -70˚C until use for qRT-PCR. The biospecimens for this study were provided by the Pusan National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

Based on the primary pathology reports and the medical records of the patients, we collected clinicopathological data such as age, gender, tumor grading, histologic type, stage, lymphovascular invasion and lymph node metastasis. Surgical staging was performed based on the International Federation of Gynecology and Obstetrics (FIGO) criteria for EC; the stage I and II-IV were considered the early- and advanced-stage EC. Moreover, the histologic types and grades of EC were determined based on the World Health Organization (WHO) criteria. The overall survival (OS) was calculated from the date of surgery to the date of mortality or the last follow-up visit. The progression-free survival (PFS) was calculated from the date of surgery to the date of relapse or progression of EC.

The present study was approved by the Institutional Review Board (IRB) at Pusan National University Hospital after obtaining informed consent.

Immunohistochemistry. Each slide was deparaffinized and rehydrated according to the standard procedure and then treated with 0.01 M sodium citrate buffer (pH 6.0) in a microwave for 5 min. This was followed by a 5-min cooling. This process was performed three times. Then, the slides were incubated for 1 h at room temperature using rabbit polyclonal anti-S100A4 (1:100; Dako, Carpinteria, CA, USA), rabbit monoclonal anti-estrogen receptor (ER) (SP1; 1:200), rabbit monoclonal anti-progesterone receptor (PR) (SP2; 1:200) and rabbit monoclonal anti-p53 (SP5; 1:100) (all from Lab Vision, Fremont, CA, USA). The EnVision Detection System (Dako) was used to detect the antibody responses according to the manufacturer's recommended protocol. The reaction products were visualized with diaminobenzidine (DAB) as a chromogen and then counterstained with Mayer's hematoxylin. For negative controls, we replaced the primary antibody with the phosphate-buffered saline (PBS).

Assessment of immunohistochemical staining. Evaluation of immunohistochemical staining was performed by two independent pathologists. S100A4 immunoreactivity was characterized by cytoplasmic and/or nuclear staining in tumor cells. The results of the immunohistochemical staining for S100A4 were evaluated based on the intensity of immunohistochemistry (none, 0; weak, 1+; moderate, 2+; or strong, 3+) as well as the percentage of positively stained tumor cells (0, none; 1, <10%; 2, 10-49%; and 3, 50-100%). These two values were multiplied and the results served as the immunoreactive score; the negative and positive immunoreactivity were defined as 0 or 1 point and ≥2 points, respectively.

The degree of the expression of ER, PR and p53 was evaluated according to the percentage of tumor cells with nuclei showing positive immunoreactivity. When >10% of tumor cells were positively stained, the tumor was considered positive expression. On the other hand, the tumor was considered negative expression when <10% of tumor cells were positively stained.

Quantitative real-time PCR (qRT-PCR). Total tissue RNA was extracted from frozen tissue samples using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (Qiagen). qRT-PCR analysis was performed according to the manufacturer's instructions (QuantiSpeed SYBR kit; PKT, Seoul, Korea). β-actin was applied as an internal control. The primers for β-actin (205 bp) were: 5'-TGACGTGGACATC CGCAAAG-3' (sense) and 5'-CTGGAAGGTGGACAGCG AGG-3' (antisense). The primers for S100A4 (185 bp) were: 5'-GCCCTGGATGTGATGGTGT-3' (sense) and 5'-TCGTT GTCCCTGTTGCTGTC-3' (antisense). cDNA was amplified with an initial denaturation at 95˚C for 3 min followed by the sequential cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 10 sec, and extension at 72˚C for 10 sec for 45 cycles, with final extension at 72˚C for 5 min. Each assay was carried out in triplicate and results were averaged. For relative quantification, $2^{-\Delta\Delta Ct}$ was calculated and used as an indication of the relative expression levels.

Statistical analysis. We used the Pearson's Chi-square test to analyze the correlation between the expression of S100A4 and various clinicopathological parameters. In addition, we also analyzed the OS and PFS using the Kaplan-Meier method. Furthermore, we performed the Cox regression analysis to determine prognostic factors. Statistical analysis was carried out using SPSS (version 14; SPSS, Inc., Chicago, IL, USA). A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological features are represented in Table I. Based on the FIGO criteria, there were 95 cases of stage I EC, 19 cases of stage II EC, 18 cases of stage III EC and three cases of stage IV EC. Histopathologic grading showed that there were 44 cases of G1, 58 cases of G2 and 33 cases of G3.

Table I. Clinicopathological features of endometrial carcinoma patients (n=135).

Parameters	n (%)
Histologic type	
Endometrioid	123 (91.1)
Serous	8 (5.9)
Clear cell	3 (2.2)
Undifferentiated	1 (0.8)
Histologic grade	
1	44 (32.6)
2	58 (43.0)
3	33 (24.4)
Myometrial invasion	
<1/2 of myometrium	88 (65.2)
≥1/2 of myometrium	47 (34.8)
FIGO stage	
I	95 (70.4)
II	19 (14.1)
III	18 (13.3)
IV	3 (2.2)
LN metastasis	
Absent	117 (86.7)
Present	18 (13.3)
Disease progression	
Progression free	107 (79.3)
Progression	28 (20.7)
Overall survival	
Alive	124 (91.9)
DOD	11 (8.1)
ER expression	
No	58 (43.0)
Yes	77 (57.0)
PR expression	
No	41 (30.4)
Yes	94 (69.6)
p53 expression	
No	100 (74.1)
Yes	35 (25.9)

DOD, died of disease; ER, estrogen receptor; PR, progesterone receptor.

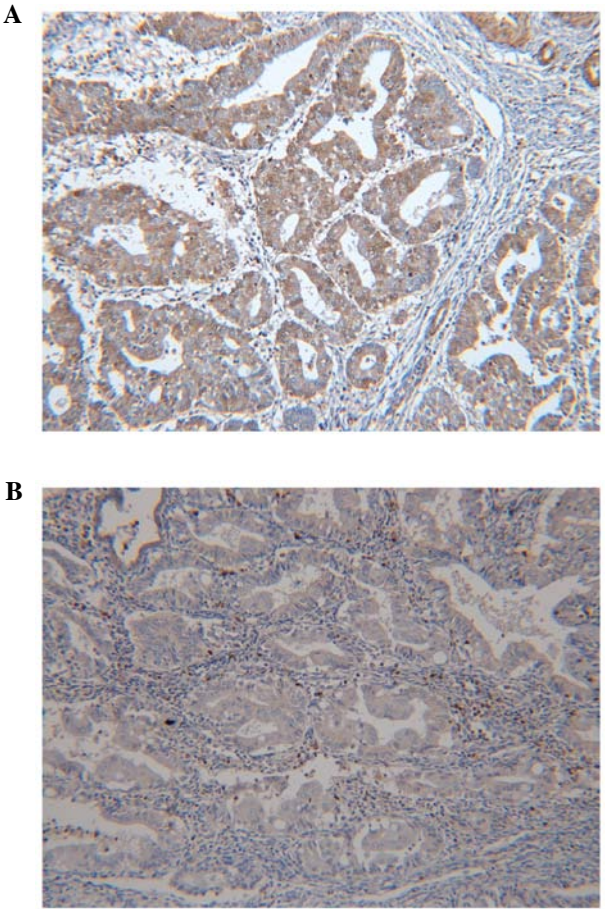

Figure 1. Immunohistochemical study of S100A4 expression. There was (A) positive and (B) negative cytoplasmic staining in cancer cells in EC.

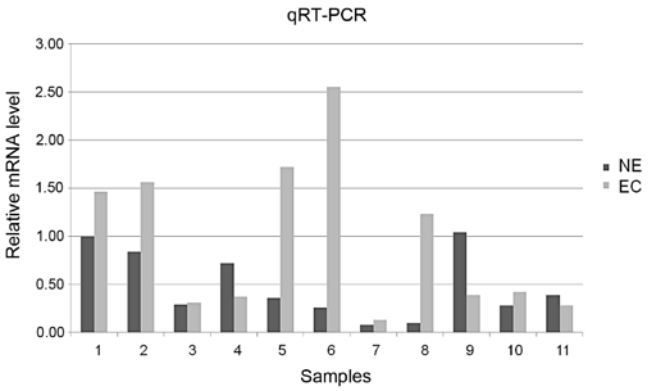

Figure 2. mRNA level of S100A4 via qRT-PCR. S100A4 mRNA was increased in endometrial carcinoma (EC) samples as compared with normal endometrium (NE) in 7 cases.

In our series, the median follow-up period was 43 months (range, 0.25-131 months). Based on the disease progression, we classified the patients into two groups: the progression group (n=28) (20.7%), comprising those who developed either recurrence or metastasis, and the progression-free group (n=107) (79.3%), comprising those who were free of progression. In addition, there were 124 (91.9%) survivors at the last follow-up.

The positive immunoreactivity for S100A4 was commonly seen in the cytoplasm of tumor cells. However, there were some cases in which cytoplasm and nucleus showed a positive immunoreactivity. S100A4 protein was also expressed in histiocytes, lymphocytes, fibroblasts and endothelial cells as well as tumor cells. In the normal endometrium, S100A4 protein immunoreactivity was clearly absent in both cytoplasm and nucleus. Of the total cases (n=135), 35 (25.9%) showed positive immunoreactivity for S100A4 (Fig. 1). The positive immunoreactivity for S100A4 was associated with histologic type and grade, the FIGO stage and lymph node metastasis. Non-endometrioid histologic type exhibited S100A4 expression more frequently than endometrioid type. Reduced

Table II. Association between positive expression of S100A4 and clinicopathological features (n=135).

Parameters	S100A4 expression		P-value
	No (%)	Yes (%)	
Histologic type			0.002
Endometrioid	96 (78.0)	27 (22.0)	
Non-endometrioid	4 (33.3)	8 (66.7)	
Histologic grade			0.000
Low (grade 1)	38 (86.4)	6 (13.6)	
High (grade 2/3)	62 (68.1)	29 (31.9)	
Myometrial invasion			0.149
<1/2	69 (78.4)	19 (21.6)	
≥1/2	31 (66.0)	16 (34.0)	
FIGO stage			0.002
I	78 (82.1)	17 (17.9)	
II-IV	22 (55.0)	18 (45.0)	
LN metastasis			0.007
Absent	92 (78.6)	25 (21.4)	
Present	8 (44.4)	10 (55.6)	
Disease progression			0.040
Progression free	86	24	
Progression	14	11	
Overall survival			0.001
Alive	97	27	
DOD	3	8	
ER expression			0.552
No	41 (70.7)	17 (29.3)	
Yes	59 (71.4)	18 (28.6)	
PR expression			0.003
No	23 (56.1)	18 (43.9)	
Yes	77 (81.9)	17 (18.1)	
p53 expression			0.262
No	77 (77.0)	23 (23.0)	
Yes	23 (65.7)	12 (34.3)	

DOD, died of disease; ER, estrogen receptor; PR, progesterone receptor.

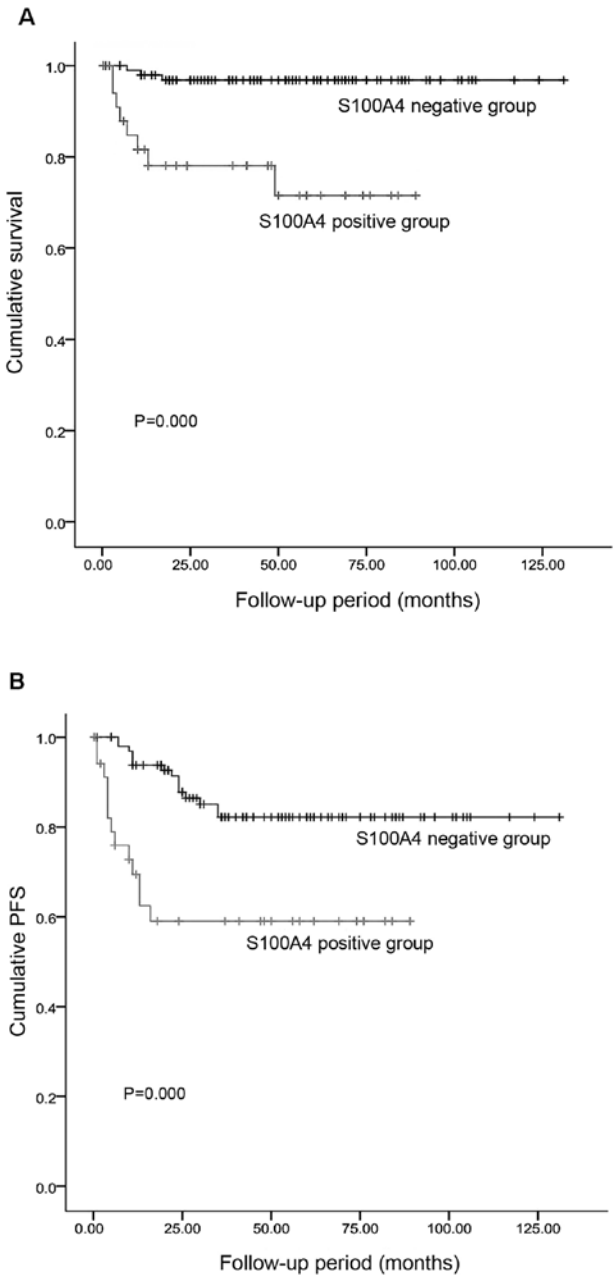

Figure 3. Kaplan-Meier survival analysis shows that the poor (A) OS and (B) PFS are associated with positive cytoplasmic immunoreactivity for S100A4.

expression of PR was significantly correlated with S100A4 protein expression. There was no significant correlation between the expression of S100A4 and that of ER. Moreover, there was no significant correlation between the expression of S100A4 and p53. Correlations between the expression of S100A4 and clinicopathological findings are summarized in Table II.

qRT-PCR revealed that the level of S100A4 mRNA was higher in EC as compared with normal endometrium (7/11, 63.6%), which is consistent with the degree of expression of S100A4 protein (Fig. 2).

The Kaplan-Meier survival analysis showed that poor OS and PFS were associated with histologic grade, the FIGO stage, myometrial invasion, lymph node metastasis, ER and PR expression and positive immunoreactivity for S100A4 (Table III). Disease progression was observed in 37.1% (13/35) of the patients with expression of S100A4 and in 15.0% (15/100) of those with no S100A4 expression. This difference reached a statistical significance (P=0.000). Moreover, the proportion of disease-related deaths was 22.9% (8/35) in the patients with S100A4 expression and 3.0% (3/100) in those with no S100A4 expression (P=0.000) (Fig. 3). There was a negative correlation between the expression of ER and PR and the OS and PFS. In multivariate analysis of the variables defined in Table IV, there was a significant correlation between the cytoplasmic expression of S100A4 and shorter OS after the adjustment for the FIGO stage, lymph node metas-

Table III. Univariate analysis of clinicopathological features, including S100A4 immunoreactivity and OS and PFS (n=135).

	Overall survival			Progression-free survival		
	Alive (n=124)	DOD (n=11)	P-value	Progression-free (n=107)	Progression (n=28)	P-value
Histologic type			0.278			0.287
Endometrioid	114 (92.7)	9 (7.3)		99 (80.5)	24 (19.5)	
Non-endometrioid	10 (83.3)	2 (16.7)		8 (66.7)	4 (33.3)	
Histologic grade			0.078			0.012
Low (grade 1)	43 (97.7)	1 (2.3)		40 (90.9)	4 (9.1)	
High (grade 2/3)	81 (89.0)	10 (11.0)		67 (73.6)	24 (26.4)	
Myometrial invasion			0.004			0.000
<1/2	85 (96.6)	3 (3.4)		78	10	
≥1/2	39 (83.0)	8 (17.0)		29	18	
FIGO stage			0.000			0.000
I	95 (100)	0 (0)		86 (90.5)	9	
II-IV	29 (72.5)	11 (27.5)		21	19	
LN metastasis			0.000			0.000
Absent	113 (96.6)	4 (3.4)		100	17	
Present	11 (61.1)	7 (38.9)		7	11	
S100A4 expression			0.000			0.000
No	97 (97.0)	3 (3.0)		85 (85.0)	15 (15.0)	
Yes	27 (77.1)	8 (22.9)		22 (62.9)	13 (37.1)	
ER expression			0.006			0.002
No	49 (84.5)	9 (15.5)		39	19	
Yes	75 (97.4)	2 (2.6)		68	9	
PR expression			0.001			0.000
No	33 (80.5)	8 (19.5)		25	16	
Yes	91 (96.8)	3 (3.2)		82	12	
p53 expression			0.120			0.066
No	94 (94.0)	6 (6.0)		83	17	
Yes	30 (85.7)	5 (14.3)		24	11	

Table IV. Multivariate analysis of prognostic factors for OS in EC.

Variables	Grouping	P-value	Ratio of risk	95% of CI
FIGO stage	I vs. II-IV	0.016	0.898	0.000-1.136
Myometrial invasion	<1/2 vs. ≥1/2	0.250	0.435	0.105-1.800
LN metastasis	Absent vs. present	0.032	0.180	0.038-0.859
ER expression	No vs. yes	0.051	25.676	0.980-67.286
PR expression	No vs. yes	0.083	0.095	0.007-1.354
S100A4 expression	No vs. yes	0.037	0.162	0.029-0.893

ER, estrogen receptor; PR, progesterone receptor.

tasis, myometrial invasion and the expression of ER and PR, all of which were significant variables on univariate analysis. The cytoplasmic expression of S100A4 was not significantly correlated to PFS in multivariate analysis (Table V).

Discussion

In the present study, we immunohistochemically examined whether S100A4 has a prognostic value in cases of EC. It has

Table V. Multivariate analysis of prognostic factors for PFS in EC.

Variables	Grouping	P-value	Ratio of risk	95% of CI
Histologic grade	Low vs. high	0.244	0.502	0.157-1.602
FIGO stage	I vs. II-IV	0.040	3.374	1.059-10.744
Myometrial invasion	<1/2 vs. ≥1/2	0.023	0.357	0.147-0.869
LN metastasis	Absent vs. present	0.034	0.366	0.144-0.925
ER expression	No vs. yes	0.076	0.392	0.139-1.102
PR expression	No vs. yes	0.207	0.423	0.111-1.611
S100A4 expression	No vs. yes	0.056	0.424	0.176-1.021

ER, estrogen receptor; PR, progesterone receptor.

been reported that the high degree of S100A4 expression is correlated with a shorter prognosis in cases of cancer. In breast cancer, the expression of S100A4 was increased in the metastatic lesion (9,11,12). Moreover, it has also been reported that the expression of S100A4 is an indicator of lymph node metastasis and tumor recurrence in colorectal cancer. Thus, its prognostic value has been suggested (14-16). There are several recent published studies on the correlation between the survival rate and the increased expression of S100A4 in other types of human malignancies (17-23). These reports suggest that S100A4 is associated with the aggressiveness of cancer and it may play a role in the pathogenesis of advanced-stage cancer. However, there are also some contradictory reports in this series. According to some studies, there is no significant correlation between the expression of S100A4 and OS in colon cancer, non-small cell lung carcinoma (NSCLC) and melanoma (25-27).

There is a limited amount of data available on the prognostic value and clinical implication of the expression of S100A4 in patients with EC. Xie *et al* reported that the level of S100A4 mRNA and the positive cytoplasmic immunoreactivity of S100A4 were increased in patients with high grade EC (24). Consistent with the previous report, we observed that the cytoplasmic expression of S100A4 and the level of S100A4 mRNA were increased in EC compared to the normal endometrium. We also analyzed the correlation between the expression of S100A4 and the clinicopathological parameters. The present study is of significance in that we first analyzed the correlation between the immunohistochemical properties of S100A4 and survival in patients with EC. Our results showed that 25.9% of the patients with EC had positive immunoreactivity for S100A4. In our series, the positive immunoreactivity for S100A4 had a significant correlation with higher histologic grade, FIGO stage and lymph node metastasis. This suggests that it plays a role in progression in EC. The OS and PFS were significantly shorter in the patients with positive immunoreactivity for S100A4 as compared with their negative counterparts. In multivariate analysis, the positive immunoreactivity for S100A4 had a significant correlation with shorter OS after the adjustment of the FIGO stage, the depth of myometrial invasion, lymph node metastasis, and ER and PR expression, all of which were significant variables in univariate analysis. These results suggest that the expression of S100A4 may be an indicator of tumor progression as well as poor prognosis.

The loss of steroid receptors has been considered an indicator of the aggressiveness of 'hormone-dependent cancers' such as breast cancer and EC. It has been previously shown that the expression of S100A4 was associated with the loss of ER in breast cancer (11,12). On the other hand, Xie *et al* failed to demonstrate an inverse correlation between the expression of S100A4 and that of the steroid hormone receptors, thus suggesting that the expression of S100A4 was not subject to the hormonal status (24). Our results showed that the expression of S100A4 had a significant correlation with loss of PR rather than that of ER. Further studies are therefore warranted to clarify the correlation between the expression of S100A4 and that of steroid hormone receptors in patients with EC.

There have been attempts to clarify the mechanisms of action of S100A4. To explain this, it has been hypothesized that the hypomethylation of the S100A4 gene leads to the increased expression of S100A4. It has been reported that hypomethylation of the S100A4 gene is associated with gene activation and overexpression of S100A4 in human malignancies (28,29). Moreover, it has also been reported that the methylation of the S100A4 gene was detected in normal endometrium and grade 1 EC with the decreased expression of S100A4. In grade 3 EC with increased level of S100A4 mRNA and the increased expression of S100A4, however, there was no methylation of the gene. These findings indicated that hypomethylation may play a role in the progression and aggressive behavior of EC (24). S100A4 binds to actin, non-muscle myosin and the p53 tumor suppressor protein (7,30). These interactions may increase the cell motility and modulate the function of p53. Of note, S100A4 binds to p53 protein and thereby inhibits its phosphorylation (16,30). Presumably, S100A4 may be involved in the expression of p53 and the inhibition of its activity to regulate the G1/S checkpoint pathway. It has therefore been speculated that S100A4 may be involved in the inhibition of the function of p53. In the present study, we also examined the possible correlation between the expression of S100A4 and that of p53, considered a possible target of S100A4, in patients with EC. As shown in the present study, however, we failed to demonstrate such correlation using immunohistochemistry.

It has been reported that the positive cytoplasmic expression of S100A irrespective of its nuclear expression has a prognostic value in many human malignancies (9,11,12,14,16,21,22). Kicuchi *et al* reported that S100A4 could translocate between the cytoplasm and nucleus in ovarian cancer cells (19). Although little is known about the exact mechanisms by which S100A4 translocates between the cytoplasm and nucleus and its nuclear expression has a prognostic value in carcinomas

only in a limited scope, its nuclear localization has been considered an indicator of poor prognosis in patients with ovarian or colorectal cancer (19,31).

In conclusion, the positive cytoplasmic immunoreactivity for S100A4 is closely associated with the progression of EC. However, there is no correlation between the expression of S100A4 and that of p53. The OS and PFS were significantly shorter in the patients with positive immunoreactivity for S100A4 as compared with their negative counterparts. Based on the above results, it can be concluded that the expression of S100A4 may be an indicator of poor prognosis in patients with EC. Therefore, these patients should be given more intensive care and meticulous monitoring of the clinical course. Finally, our results indicate that S100A4 may be a biological marker indicating the recurrence and poor prognosis in patients with EC. However, further large-scale studies are warranted to establish its prognostic value and clinical implications in patients with EC.

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