Immunohistochemical Detection of WT1 Protein in Endometrial Cancer

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Abstract. *Background: The Wilms' tumor gene WT1 is overexpressed in various kinds of solid tumors. However, it remains unclear whether WT1 plays a pathophysiological role in endometrial cancer. Patients and Methods: A series of 70 endometrial cancer patients who had undergone a curative resection was studied to determine the correlation between WT1 expression, clinicopathological characteristics and prognosis. Tissue specimens were evaluated for WT1 expression by immunohistochemistry. Results: The expression of WT1 was strong in 31 patients (44%) and weak in 39 patients (56%). WT1 overexpression was associated with advanced FIGO stage (p=0.0266), myometrial invasion (p=0.0477) and high-grade histological differentiation (p=0.0049). The expression level of WT1 was found to be a significant predictor of disease relapse in univariate analysis (p=0.0233), but not in multivariate analysis (p=0.4757). Conclusion: These results suggested that tumor-produced WT1 provided additional prognostic information in endometrial cancer patients.*

Endometrial cancer is the most common gynecological malignancy in the United States. In Japan, it is the second most common gynecological cancer, but its frequency has dramatically increased in the last decade. Although there are well-established surgical and chemotherapeutic treatments for endometrial cancer, the need for molecular-target therapy has increased, especially for recurrent disease that has acquired radio- or chemoresistance, thus, there is a need for a better understanding of the molecular pathways of endometrial carcinogenesis.

The Wilms' tumor gene *WT1* was isolated as a gene responsible for a childhood renal neoplasm, Wilms' tumor (1, 2). This gene encodes a zinc finger transcription factor and play important roles in cell growth and differentiation (3, 4). Although *WT1* gene was categorized at first as a tumor-suppressor gene, it was recently demonstrated that the wild-type *WT1* gene performed an oncogenic rather than a tumor-suppressor function in many kinds of malignancies (5). *WT1* gene is highly expressed in hematological malignancies and solid tumors, including endometrial cancer (6). However, it remains unclear whether *WT1* plays a pathophysiological role in endometrial cancer.

Therefore, in the present study, we immunohistochemically analyzed the expression of WT1 protein in 70 cases of primary endometrial cancer to study the relationship between WT1 expression and clinicopathological characteristics as well as prognosis to clarify the prognostic significance of WT1 protein expression in endometrial cancer patients.

Patients and Methods

Patients. This study included 70 primary endometrial carcinoma patients who had been consecutively admitted, treated and followed-up at the Department of Obstetrics and Gynecology, Kanazawa University Hospital from January 1995 to December 2002. None of the patients had received any pre-surgical treatment and all had undergone a total abdominal or radical hysterectomy plus bilateral salphingo-oophorectomy. At the time of laparotomy, peritoneal fluid samples were obtained for cytological testing. Systemic pelvic lymphadenectomy was performed in 51 (72.9%) patients. Paraaortic lymph node sampling was performed in two patients because of visible or palpable enlarged lymph nodes. All the patients were classified by the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system (1988). No patient had remaining macroscopic tumors or known distant metastasis immediately after surgery. The high-risk patients (*e.g.* these with deep myometrial invasion, cervical involvement, special histology,

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or peritoneal cytology) underwent external radiotherapy and/or six cycles of chemotherapy (paclitaxel: 180 mg/m^2, carboplatin: according to Chatelut's formula [AUC=5 mg min/ml]) as postoperative adjuvant therapy.

The treatment was followed by a gynecological examination, recording of laboratory data, transvaginal/abdominopelvic ultrasonography and a radiological investigation. The data from regular follow-up visits to the outpatient department were stored in a database specifically designed for endometrial carcinoma patients. A telephone inquiry to update the present status of all surviving patients was made in August 2006. The exact date of disease recurrence was obtained from the referring physicians or from the physicians who attended the patient for the initial diagnosis of the recurrence. All the treatments and clinical research were conducted with written informed consent.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissues from 70 tumors were retrieved with informed consent from archive sources at Kanazawa University Hospital. The histological diagnosis of each tumor was confirmed on the hematoxylin and eosin-stained sections. Representative sections containing both the normal endometrium and the invasive front of the tumor tissue were selected for immunohistochemical staining. The slides were deparaffinized and rehydrated in graded alcohols. Epitope retrieval was performed using enzymatic digestion with Proteinase K for 30 minutes at 37˚C (Dako Cytomation. Carpinteria, CA, USA), and by microwave heating for 15 minutes using Target Retrieval Solution (Dako Cytomation). Endogenous peroxidase activity was quenched by dipping in 3% hydrogen peroxide for 30 minutes. The slides were incubated with mouse monoclonal antibodies (clone 6F-H2; Dako Cytomation) diluted 1:100 at 4˚C overnight. The subsequent steps were carried out according to the manufacturer's instructions by the EnVision+ ® System horseradish peroxidase (HRP)-labelled polymer (Dako Cytomation). Color development was carried out with peroxidase substrate 3-amino-9-ethylcarbazole (AEC). All the slides were counterstained with Mayer's hematoxylin. Formalin-fixed, paraffin-embedded sections of human Wilms' tumor were used as positive controls for WT1.

Evaluation of staining. For evaluation of WT1 expression, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%) according to the percentage of the positive staining area in relation to the whole carcinoma area. The sum of the intensity and extent score was used as the final staining score (0-7) for WT1. Tumors having a final staining score of ≥5 were considered to exhibit strong expression. All the histological slides were examined by two observers (S.O. and Y.O.) who were unaware of the clinical data or the disease outcome.

Statistical analysis. The Chi-square test for 2×2 tables was used to compare the categorical data. Mortality and probability of relapse after surgery were compared by Kaplan-Meier analysis and the log-rank statistic. In the analysis of relapse-free survival rates, those who died of causes unrelated to endometrial cancer and those who had no detected evidence of disease recurrence were considered to be relapse-free. A p-value of <0.05 was considered to indicate statistical significance. All the statistical analyses were performed using the statistical package StatView version 5.0 for Macintosh (Abacus Concepts, Berkeley, CA, USA).

Results

Characteristics of the patients. The patients' average age at the time of surgery was 57.3 years (range, 26-78 years), 22 had premenopausal status, 4 had perimenopausal status and 44 had postmenopausal status. The patients' mean preoperative body mass index (BMI) was 24.0 (range, 16.9-32.9). Among the 70 patients, 12 patients (17.1%) had relapses of endometrial cancer at the time of the last follow-up. The median follow-up time for all the patients was 5.12 years (range, 0.56-11.08 years).

WT1 expression in endometrial cancer. WT1 expression was positive exclusively in cancer cells in 64 cases (91%). The expression of WT1 was strong (final staining score of 5-7) in 31 patients (44%) and weak (final staining score of 0-4) in 39 patients (56%). The typical WT1 expression in endometrial cancer cells is shown in Figure 1. A majority of the positive cases showed diffuse or granular staining in the cytoplasm. The staining of WT1 was heterogeneous in advanced tumors and WT1 was frequently located at the invasion front of the tumor. The association between WT1 expression and clinico-pathological variables is shown in Table I. WT1 overexpression was associated with advanced FIGO stage (p=0.0266), myometrial invasion (p=0.0477) and high-grade histological differentiation (p=0.0049), indicating up-regulation of WT1 expression with tumor progression in this study.

Prognostic impact of WT1 expression in endometrial cancer. Strong expression of WT1 was associated with reduced relapse-free survival in endometrial cancer (Figure 2A). Although there was no clear statistical significance, WT1 expression was a factor negatively influencing the overall survival rate (Figure 2B). Multivariate analysis indicated that WT1 expression had no independent significant effect (data not shown).

Discussion

With the use of anti-WT1 monoclonal (6F-H2) antibody, positive staining in the tumor cells was observed in 91% of the cases. The relatively high rate of positivity for WT1 in the present study contrasts with some previous reports. Acs *et al.* (7) reported that WT1 immunoreactivity was seen in ten of 16 serous, but in none of 35 endometrioid or 18 clear cell carcinomas among endometrial carcinomas. Egan *et al.* (8) also reported that two of 31 serous carcinomas and none of 39 endometrioid carcinomas were reactive for WT1. Meanwhile, Dupont *et al.* (9) confirmed that WT1 expression was found in twenty of 99 endometrioid carcinomas using polyclonal antibody against WT1 (Santa Cruz; clone C-19). The discrepancy between our findings and previous results could be explained by the different criteria employed to judge WT1 positivity: they regarded nuclear but not cytoplasmic

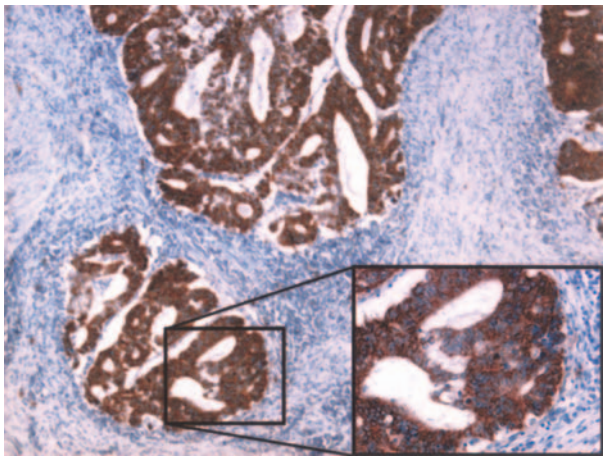

Figure 1. *Representative sections of endometrial cancer with immunohistochemical staining of WT1. Strong cytoplasmic staining is observed in the invasion front of the tumor (×40; inset, ×200).*

staining in the tumor cells as positive, because WT1 is principally a DNA-binding transcription factor mainly distributed in the nucleus. In the present study, granular or diffuse cytoplasmic staining in the tumor cells was judged as positive, for reasons explained below.

Nakatsuka *et al*. reported that Western blot analysis revealed the intracytoplasmic localization of WT1 protein in lung cancer cells (6). Oji *et al*. (10) and Drakos *et al*. (11) showed the cytoplasmic expression of WT1 protein in cell lines derived from glioblastoma and lymphoma. Moreover, Ye *et al*. (12) revealed that phosphorylation in the DNA-binding domain of WT1 alters the affinity for DNA and subcellular distribution of WT1. Post-translational phosphorylation at zinc fingers inhibits the ability to bind DNA, resulting in the cytoplasmic retention of WT1, and also inhibits transcriptional regulatory activity. As established by the interesting study of Niksic *et al*. (13), WT1 shuttles between the nucleus and cytoplasm and might be involved in the regulation of translation through its association with actively translating polysomes. Recent studies found that many types of tumor frequently showed strong cytoplasmic expression of WT1, suggesting that WT1 was involved in the development of tumors (6, 10, 14-16). In the present study, we also found that the majority of endometrial tumors showed strong cytoplasmic WT1 staining, which was associated with advanced FIGO stage, myometrial invasion and high-grade histological differentiation. These results suggest that up-regulation of WT1 expression is linked to tumor progression.

To date, few reports are available on the prognostic impact of WT1 expression in endometrial cancer patients. Miyoshi *et al*. (17) reported that the disease-free survival rate was significantly lower in breast cancer patients with high levels of *WT1* mRNA than those with low levels. Inoue *et al*. (18) showed that leukemia with strong *WT1* mRNA expression

Table I. *WT1 expression and clinicopathological characteristics.*

Variable	WT1 expression		*P*-value (χ^2 test)
	Strong (n=31)	Weak (n=39)	
Age (year)			
<60 (n=43)	16	27	
≥60 (n=27)	15	12	0.1325
FIGO stage			
I (n=52)	19	33	
II, III, IV (n=18)	12	6	0.0266
Lymph node metastasis			
Negative (n=65)	28	37	
Positive (n=5)	3	2	0.4629
Depth (myometrial invasion)			
a (n=17)	4	13	
b, c (b, n=36; c, n=17)	27	26	0.0477
Histopathology-degree of differentiation			
Grade 1 (n=38)	11	27	
Grade 2, 3 (n=32)	20	12	0.0049
Menopause			
Peri, pre (n=26)	8	18	
Post (n=44)	23	21	0.0801
Body mass index			
<25 (n=45)	19	26	
≥25 (n=25)	12	13	0.6410

showed a significantly lower rate of complete remission and significantly worse overall survival than that with weak expression. Moreover, Sera *et al*. (19) reported that overexpressed WT1 protein, which was confirmed by Western blotting and immunohistochemical staining, was an independent prognostic factor for disease-free survival in hepatocellular carcinoma patients. Høgdall *et al*. (20) demonstrated that univariate Kaplan-Meier survival analysis performed on 560 ovarian cancer patients showed a significantly shorter disease-specific survival in patients with positive WT1 protein expression in the tumor tissue. Netinatsunthorn *et al*. (21) also reported that immunohistochemical expression of WT1 was a prognostic predictor in patients with advanced serous epithelial ovarian carcinoma. In the present study, we found that strong expression of WT1 was associated with reduced relapse-free survival in endometrial cancer patients. Our results are congruent with previous reports of other types of cancer.

WT1 could be a novel tumor rejection antigen in immunotherapy for various kinds of WT1-expressing cancer. Clinical trials of WT1 peptide-based cancer immunotherapy showed that WT1 vaccination induced a reduction in tumor size or decrease in tumor marker levels in breast, lung cancer, leukemia and glioblastoma multiforme (22, 23). The results of the present study provide a rationale for immunotherapy targeting WT1 as a new treatment strategy for endometrial cancer.

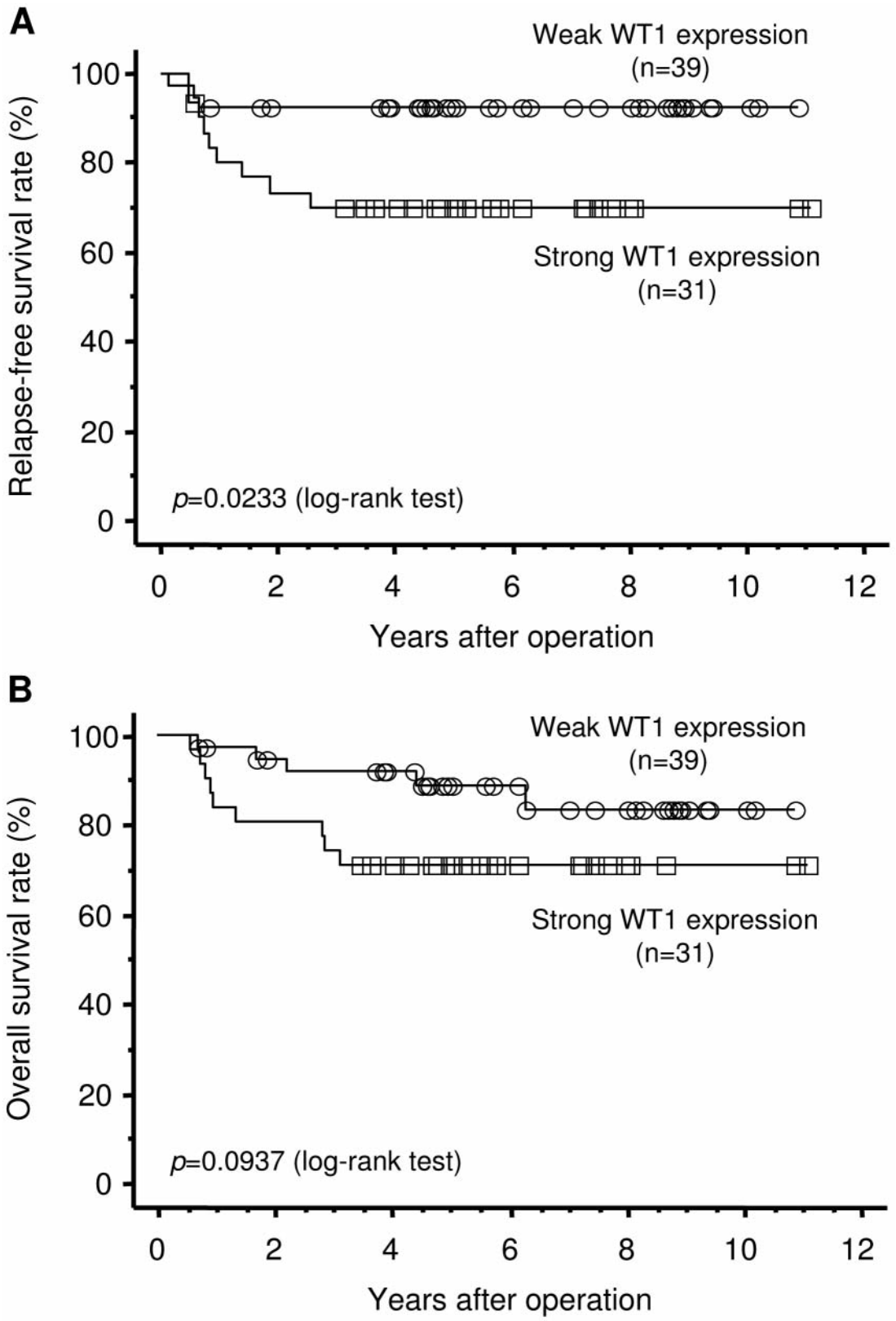

Figure 2. *The Kaplan-Meier survival curves of 70 patients with endometrial carcinoma in relation to WT1 expression are shown. A, Relapse-free survival rate; B, overall survival rate.*

In conclusion, our study now shows the cytoplasmic expression of WT1 might provide additional prognostic information for endometrial cancer patients.

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