Correlation between WT1 Expression and Cell Proliferation in Endometrial Cancer

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Abstract. *Background: The Wilms' tumor gene WT1 is overexpressed in endometrial cancer. Although recent studies have revealed that WT1 is a new prognostic factor, it remains unclear whether WT1 plays a pathophysiological role including cell proliferation. Patients and Methods: A series of 70 endometrial cancer patients who had undergone a curative resection was studied by immunohistochemistry to determine the correlation between WT1 expression and cell proliferation (proliferating cell nuclear antigen; PCNA). Results: WT1 expression was observed in 64 cases (91%). WT1 expression was associated with advanced FIGO stage (p=0.0228), myometrial invasion (p=0.0114) and high-grade histological differentiation (p=0.0004), indicating up-regulation of WT1 expression with tumor progression. A positive correlation between PCNA labeling index and score of WT1 expression was observed (p=0.0081, ϱ=0.319). Conclusion: These results showed that WT1 might regulate cell proliferation in endometrial cancer.*

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 exists 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 plays an important role in cell growth and differentiation (3, 4). Although *WT1* gene was initially categorized at first as a tumor-suppressor gene, it was recently demonstrated that the wild-type *WT1* gene exhibited an oncogenic rather than a tumor-suppressor function in many kinds of malignancies (5). For example, *WT1* gene is highly expressed in hematological malignancies and solid tumors, including endometrial cancer (6, 7).

Moreover, *in vitro* and *in vivo* studies revealed that WT1 was associated with cell proliferation in malignant melanoma (8, 9), breast cancer (10, 11), myeloid leukemia cells (12), glial tumors (13) and epithelial ovarian tumors (14). However, it remained unclear whether WT1 affected cell proliferation 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 cell proliferation (proliferating cell nuclear antigen; PCNA) in endometrial cancer patients.

Patients and Methods

Patients. This study included 70 primary endometrial cancer patients who had been consecutively admitted, treated and followed-up by 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

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performed in 51 (72.9%) patients. Paraaortic lymph node sampling was performed in two patients because of visible or palpable enlarged lymph nodes. All 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. High-risk patients (*e.g.* those with deep myometrial invasion, cervical involvement, special histology, 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. 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 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 then by microwave heating for 15 minutes using Target Retrieval Solution (Dako Cytomation) for WT1 staining, and using microwave heating for 10 minutes using distilled water for PCNA staining. 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 for WT1, and diluted mouse monoclonal antibodies (clone PC10; DAKO Cytomation) for 2 hours at room temperature for PCNA. 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) for WT1 and diaminobenzidine (DAB) for PCNA. All slides were counterstained with Mayer's hematoxylin.

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.

To quantify expression of PCNA, 3 to 5 representative areas comprising at least 2000 cancer cells were counted from each sample with a light microscope (×200 magnification). Results were expressed as PCNA labeling index (PCNA-LI) representing the number of PCNA-positive cells divided by counted cancer cells and expressed as a percentage. All the histological slides were examined by two observers (S.D. and S.O.) who were unaware of the clinical data or disease outcome.

Statistical analysis. Differences between groups were compared using the Mann-Whitney *U*-test. Relations between continuous variables were investigated by means of the Spearman's rank correlation coefficient test. 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

Patient characteristics. The average patient age at the time of surgery was 57.3 years (range, 26-78 years); 22 patients had premenopausal status, 4 perimenopausal and 44 postmenopausal. The mean patient preoperative body mass index (BMI) was 24.0 (range, 16.9-32.9).

WT1 expression in endometrial cancer. WT1 expression was positive exclusively in cancer cells in 64 cases (91%). The mean score of WT1 expression was 4.114±1.749, and median value was 4. Typical WT1 expression in endometrial cancer cells is shown in Figure 1a. 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 clinicopathological variables is shown in Table I.

WT1 overexpression was associated with advanced FIGO stage (p=0.0228), myometrial invasion (p=0.0114) and high-grade histological differentiation (p=0.0004), indicating up-regulation of WT1 expression with tumor progression in this study. Additionally, WT1 expression was stronger in postmenopausal patients (p=0.0281).

PCNA labeling index (PCNA-LI) in endometrial cancer. The mean score of PCNA-LI was 56.1±30.9 and the median value was 67.6. Typical PCNA expression in endometrial cancer cells is shown in Figure 1b. The association between PCNA-LI and clinicopathological variables is shown in Table II. PCNA-LI was significantly higher in elderly and postmenoposal patients (p=0.0050 and p=0.0314, respectively).

WT1 and PCNA. PCNA-LI was significantly higher in the strong expression WT1 group (final score: 5-7) than the weak expression WT1 group (final score: 0-4) (Mann-Whitney *U*-test: p=0.041). Moreover, considering WT1 expression scores as continuous variables, a strong association was found between WT1 expression and the PCNA-LI (p=0.0081, n=70, ϱ=0.319) using the Spearman rank-correlation coefficient (Figure 2).

Discussion

Recent studies have shown that WT1 influences disease progression and prognosis in various types of cancer. In endometrial cancer, Dupont *et al.* reported that WT1/p53 double positivity were negative prognostic indicators using univariate analysis (15). Chiusa *et al.* showed that elevated

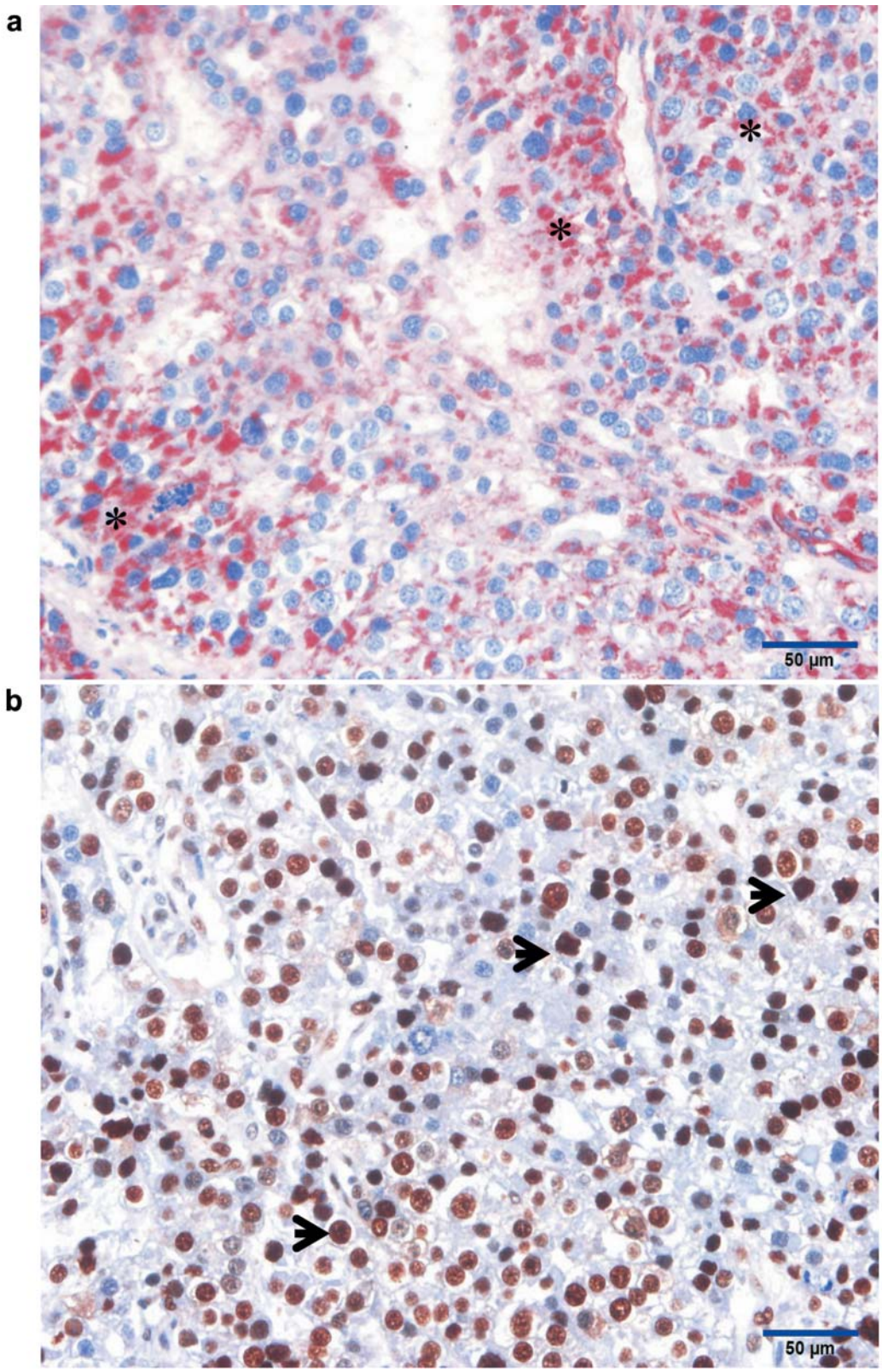

Figure 1. *Immunohistochemical staining of endometrial cancer with WT1 expression and PCNA (original magnification: a, ×200; b, ×200). a, WT1 expression of endometrial cancer as indicated by asterisks showed diffuse or granular staining in the cytoplasm; b, PCNA within endometrial cancer cells as indicated by arrows.*

Table I. *WT1 expression and clinicopathological characteristics.*

Clinical feature	WT1 expression score Median [IQR*] (Mean±SD**)	*p*-Value (Mann-Whitney *U*-test)
Age (years)		
<65 (n=43)	4 [2.00] (3.860±1.684)	0.0933
≥65 (n=27)	5 [1.75] (4.519±1.805)	
FIGO stage		
I (n=52)	4 [2.00] (3.827±1.757)	0.0228
II, III, IV (n=27)	5 [2.00] (4.944±1.474)	
Lymph node metastasis		
Negative (n=65)	4 [2.00] (4.108±1.795)	>0.9999
Positive (n=5)	5 [2.00] (4.200±1.095)	
Myometrial invasion		
a, b (n=53)	4 [2.00] (3.792±1.758)	0.0114
c (n=17)	5 [2.25] (5.118±1.317)	
Histopathology-degree of differentiation		
Grade 1 (n=38)	4 [2.00] (3.447±1.655)	0.0004
Grade 2, 3 (n=32)	5 [2.00] (4.906±1.532)	
Menopause		
Peri, pre (n=26)	4 [3.00] (3.500±2.005)	0.0281
Post (n=44)	5 [2.00] (4.477±1.486)	
Body mass index		
<25 (n=45)	4 [1.00] (4.178±1.800)	0.7012
≥25 (n=25)	4 [2.00] (4.000±1.683)	

IQR*: Interquartile range; SD**: standard deviation.

Table II. *PCNA-LI and clinicopathological characteristics.*

Clinical feature	PCNA-LI Median [IQR*] (Mean±SD**)	*p*-Value (Mann-Whitney *U*-test)
Age (years)		
<65 (n=43)	54.1 [54.3] (48.6±30.9)	0.0050
≥65 (n=27)	78.6 [18.8] (68.0±27.2)	
FIGO stage		
I (n=52)	62.9 [57.7] (52.5±31.6)	0.1503
II, III, IV (n=27)	77.2 [15.0] (66.4±26.6)	
Lymph node metastasis		
Negative (n=65)	67.5 [54.4] (56.1±30.8)	0.9364
Positive (n=5)	67.6 [46.7] (55.5±35.3)	
Myometrial invasion		
a, b (n=53)	64.0 [55.8] (52.4±31.8)	0.0932
c (n=17)	78.6 [19.2] (67.5±25.1)	
Histopathology-degree of differentiation		
Grade 1 (n=38)	53.0 [62.6] (48.8±33.6)	0.1223
Grade 2, 3 (n=32)	72.6 [17.5] (64.7±25.2)	
Menopause		
Peri, pre (n=26)	44.3 [55.8] (45.7±31.2)	0.0314
Post (n=44)	74.3 [28.1] (62.2±29.3)	
Body mass index		
<25 (n=45)	67.6 [55.5] (55.6±31.6)	0.9951
≥25 (n=25)	67.5 [44.8] (56.9±30.1)	

IQR*: Interquartile range; SD**: standard deviation.

levels of WT1 in leukemia were associated with poor prognosis following standard chemotherapy treatment (16). We have also revealed that cytoplasmic expression of WT1 may provide additional prognostic information for endometrial cancer patients (7). Moreover, the present study demonstrated that WT1 overexpression was associated with advanced FIGO stage, myometrial invasion and high-grade histological differentiation.

WT1 protein is vital to cell proliferation and as such serves as a prognostic factor. Wagner *et al*. pointed out that WT1 protein and PCNA were co-localized in malignant melanoma (8). Hashiba *et al*. found a significant correlation between WT1 protein expression score and mindbomb homolog 1 (MIB-1) staining index showing cell proliferation activity (13). We also found that WT1 protein expression was significantly associated with PCNA expression of cell proliferation. Our results are congruent with previous reports of other types of cancer.

Other research may clarify the mechanism by which WT1 can do this. Zepata -Benavides *et al*. showed that WT1 protein might be involved in breast cancer proliferation by regulating cyclinD1 protein levels (11). Rong *et al*. reported that WT1 promoted cell proliferation in the presence of activated signal transducers and activators of transcription (STAT3) (17). Han *et al*. demonstrated that the exogenous expression of WT1 in

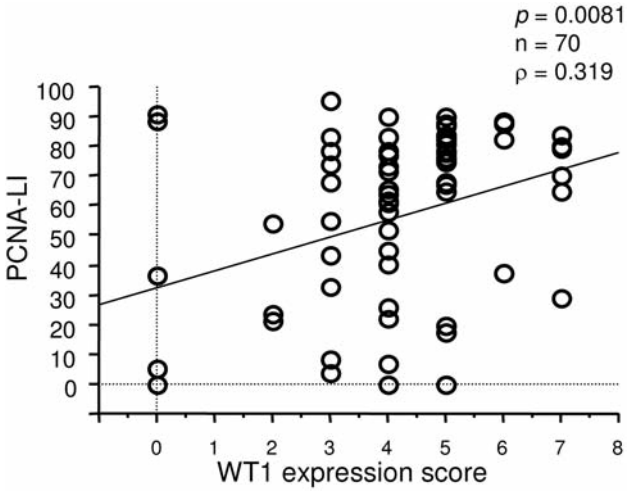

Figure 2. *A positive correlation between PCNA labeling index (PCNA-LI) and score of WT1 expression was observed using the Spearman rank-correlation coefficient.*

the human breast cancer cell lines MDA-MB-468 and MCF-7, and in human leukemic K562 cells can activate the c-Myc promoter and stimulate cellular proliferation (18). Yamazaki *et al*. and Ito *et al*. found that loss of WT1 was associated with

decreased growth of leukemic cells and rapid induction of apoptosis (19, 20). Mayo *et al*. found that stable overexpression of WT1 led to increased endogenous Bcl-2 protein in the rhabdoid tumor cell line G401 (21). These results are worth reflecting on when considering future cancer treatment strategies targeting WT1.

In conclusion, results of the present study showed that WT1 might regulate cell proliferation in endometrial cancer.

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