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ORIGINAL ARTICLE

Clinicopathological significance of WT1 expression in ovarian cancer: a possible accelerator of tumor progression in serous adenocarcinoma

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Abstract Recently, oncogenic potential of the WT1 gene has been proposed in some human solid tumors and leukemias. Although previous studies have shown the frequent expression of the WT1 protein in ovarian serous adenocarcinomas (OSAs), its clinicopathologic significance is still unclear. We immunohistochemically examined the expression status of WT1 in 119 OSAs and analyzed the correlation of the intensity of WT1 immunoreactivity with the level of WT1 mRNA expression by quantitative real-time polymerase chain reaction, clinicopathologic variables, expression of p53, Bcl-2, and Ki-67 labeling index (LI). Of 119 OSAs, nuclear WT1 immunoreactivity was positive in 99 (83%), of which 44 (44%) and 55 (56%) exhibited high and low WT1 immunoreactivities, respectively. The quantitative WT1 mRNA levels were significantly correlated with the intensity of WT1 immunoreactivity ($P<0.05$). In comparison with WT1-negative OSAs, the WT1-positive OSAs showed a higher grade ($P=0.007$), advanced stage ($P=0.018$), and higher Ki-67 LI ($P<0.001$). Additionally, high WT1 immunoreactivity was correlated with a higher grade ($P=0.003$), Ki-67 LI ($P=0.012$), Bcl-2 expression ($P=0.003$), and poorer patient outcome (5-year survival, 36.5 vs 63.8%, $P=0.008$ by log-rank test). The WT1 protein may be an accelerator of the progression of OSA.

Keywords Ovarian carcinoma · Ovarian serous adenocarcinoma · Prognostic factor · Tumor progression · WT1

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Introduction

The WT1, located on chromosome 11p13, was at first identified as a gene responsible for the development of Wilms' tumor [10]. Although the role of the product of the WT1 gene was initially described as transcriptional regulation, recent researchers have demonstrated its multifunctional roles sometimes with contradictory features, including being an activator vs a repressor of transcription, transcription in the nucleus vs RNA metabolism and

translational regulation in the cytoplasm, and even being an oncogene vs a tumor supressor gene [12].

Although the *WT1* gene was primarily considered to be a tumor-suppressor gene, recent studies have demonstrated an overexpression of the wild-type *WT1* gene in leukemias [15] and various solid neoplasms such as those of the breast [16, 19], lung, digestive tract [25, 26], pancreas [23], bone, and soft tissues [33, 37]. Moreover, several reports indicating an oncogenic, rather than a tumor-supressor, role of the wild-type *WT1* gene have been accumulated. For example, high expression levels of WT1 messenger ribonucleic acid (mRNA) are correlated with poor prognosis in leukemias [15], breast cancer [19], and soft tissue sarcomas [33]. Treatment with WT1 antisense oligomers specifically causes growth inhibition in leukemic blast cells [2, 39] and in cell lines derived from cancers of the lung, stomach, colon, and breast [24, 41]. These findings indicate that the *WT1* gene product is a potent target of immunotherapy because *WT1* gene expression is restricted to a limited set of normal tissues including the gonad, uterus, kidney, and mesothelium [4, 27].

Ovarian carcinoma behaves more aggressively and has a worse prognosis than any other cancer involving the female genital tract [28]. Because of the paucity of specific early symptoms, up to 70% of patients present with advanced disease [28]. Of the various histological subtypes of ovarian carcinoma, ovarian serous adenocarcinoma (OSA) is the most common and usually shows aggressive behavior and secondary resistance to current adjuvant chemotherapies.

It is a generally accepted idea that the expression of a single or multiple specific oncogenes and subsequent activation of cell proliferation contribute to neoplastic transformation of cells, tumorigenicity, and tumor progression. Such oncogenes and oncogene products may be of a clinical utility as predictive or prognostic factors and as therapeutic targets. So far, it has been shown that the incidence of WT1-positive tumors was highest in OSA among the various histological types of ovarian carcinoma [1, 13]. However, there is limited evidence for the clinicopathologic significance of WT1 in ovarian carcinomas [13, 21].

In the present study, to evaluate the clinicopathologic significance of the WT1 expression in OSA, we analyzed WT1 protein expression status by immunohistochemistry (IHC) applied to a tissue microarray (TMA) containing 119 specimens of OSA. Tumors showing a distinctly intense immunoreaction were further tested for their expression levels of WT1 mRNA by real-time, quantitative, reverse transcriptase polymerase chain reaction (RT-PCR) analysis. We demonstrated that these features of OSA were correlated with clinicopathologic parameters, expression of other molecular markers, and patient outcome.

Materials and methods

Patients and tissue samples

This study was performed with the approval of the Internal Review Board on ethical issues. All patients involved gave their informed consent to participate. We reviewed the clinicopathologic records of 119 patients who underwent initial surgery followed by platinum-based chemotherapies for primary ovarian OSA at the Department of Obstetrics and Gynecology, National Defense Medical College Hospital, Tokorozawa, Japan, between 1987 and 2004. All 119 patients underwent surgical staging according to the International Federation of Gynecology and Obstetrics (FIGO) system. Radical cytoreductive surgery was performed for patients with advanced disease. The chemotherapeutic regimens comprised cyclophosphamide, doxorubicin, and cisplatin for 74 patients, paclitaxel and carboplatin for 32, docetaxel and carboplatin for 10, cyclophosphamide and cisplatin for one, cyclophosphamide and carboplatin for one, and irinotecan and cisplatin for one. All pathology specimens were reviewed at our institution, and the tumors were classified histologically as OSAs according to the World Health Organization criteria [34]. The clinicopathologic details, such as patient age, FIGO clinical stage, tumor histologic grade, residual tumor after initial surgery, and overall patient survival were assessed for all 119 patients.

Histologic grading was performed by reference to the system proposed by Shimizu et al. [31] and Silverberg [32] (Table 1). Clinical response to chemotherapy was evaluated by ultrasonography or computed tomography and classified as complete response (CR), partial response (PR), stable disease, or progressive disease in accordance with the new Response Evaluation Criteria for Solid Tumours [35].

Table 1 Scoring system used for grading

Score	Criteria
A. Predominant architectural pattern	
1	Tubular and/or cystic
2	Papillary
3	Solid
B. Cytologic atypia	
1	Slight
2	Moderate
3	Marked
C. Mitotic Figures/10 high-power fields[a]	
1	0–9
2	10–24
3	≥25

Sum of the scores for A, B and C: 3–5=Grade 1; 6, 7=Grade 2; 8, 9=Grade 3

[a] Counted in the most active region at 10× 40× using an Olympus Optiphot microscope (field area 0.345 mm^2)

Tumor size was determined as the product of the maximum diameter and the length perpendicular to the maximum diameter. Clinical response to chemotherapies was assessed for the 63 patients who had residual tumors 2 cm in size or larger, after initial surgery.Tumors that showed CR or PR to first-line chemotherapies were considered to be chemosensitive.

Follow-up was calculated from the date of initial definitive surgery to the date of either last follow-up or death. The average follow-up period after initial surgery was 46.4 months, ranging between 2 and 227 months. Forty-two of the 119 patients died because of OSA spread, and two patients died of other causes.

Tissue microarray and immunohistochemistry

To construct TMA blocks, we selected formalin-fixed paraffin-embedded tissue blocks containing the areas where histologic grading had been performed, from the 119 OSA cases. Two core specimens 2.0 mm in diameter were taken from a tissue block of each case and transferred to recipient blocks using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD).

These TMA blocks were cut into 4-μm-thick sections, mounted on silane-coated glass slides, and subjected to IHC using the following primary monoclonal antibodies and dilutions: anti-WT1 (clone 6F-H2, 1:50, Dako, Grostrup, Denmark), anti-p53 (clone DO7, 1:100, Dako), anti-Bcl-2 (clone 124, 1:50, Dako), and anti-Ki-67 (clone MIB-1, 1:50, Dako).

These tissue sections were deparaffinized, subjected to antigen retrieval by digestion with proteinase K (ready to use, Dako) for 10 min for WT1, or by autoclaving in sodium citrate buffer (pH 6.0) for 15 min at 121°C for p53, Bcl-2, and Ki-67, then being allowed to cool at room temperature. Endogenous peroxidase was blocked using 5% hydrogen peroxide. Nonspecific staining was blocked in 2% normal swine serum. The slides were incubated with primary antibodies at 4°C overnight and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision Plus; Dako) for 1 h at room temperature. Specific antigen–antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. Counterstaining was performed using Mayer's hematoxylin. As positive controls, we used leukemic cell line K562 for WT1, a breast cancer with nuclear p53 immunoreactivity, and a lymph node showing reactive hyperplasia positive for Bcl-2 and Ki-67. As negative controls, the primary antibodies were omitted from each reaction process.

A case showing any degree of nuclear immunoreactivity for WT1 in more than 10% of the core tissue was considered to be the WT1-positive case. The intensity of WT1 expression was classified as 0 (nonstaining), 1+ (weak), 2+ (moderate), or 3+ (intensive; Fig. 1). Cases showing heterogeneous intensity of WT1 immunoreactivity were classified according to the highest degree of immunoreactivity of the WT1-positive cells in each core when the area with such immunoreactivity occupied more than 10% of the core tissues. If the part with the highest degree of immunoreactivity occupied less than 10% of area, the intensity of the largest area was adopted. Tumors scored as 1+ were defined as a low-level immunoreactivity group and those with a score of 2+ or 3+ as a high-level immunoreactivity group. Based on the literature, p53 and Bcl-2 were regarded as positive if more than 10% of tumor cells showed nuclear immunoreactivity for p53 and cytoplasmic immunoreactivity for Bcl-2 [3, 29, 36]. The Ki-67 labeling index (LI) was determined using the formula: Ki-67 LI (%)=100× number of tumor cells with nuclear Ki-67 immunoreactivity/ number of tumor cells with and without nuclear Ki-67 immunoreactivity. In total, 400 cell nuclei were counted twice independently, and both sets of data were combined.

RNA extraction and reverse transcription

To compare the intensity of WT1 immunoreactivity with its level of messenger ribonucleic acid (mRNA) expression, we selected a total of 21 OSA samples based on the pattern of their WT1 immunoreactivity—seven different samples each with negative, low-level, and high-level immunoreactivity—and prepared them for mRNA expression analysis. As controls for WT1 mRNA expression, the K562 leukemic cell line and five samples of non-neoplastic ovarian tissue obtained from separate patients who had undergone radical surgery for uterine cancers confined to the uterus were used [15]. All these non-neoplastic ovarian

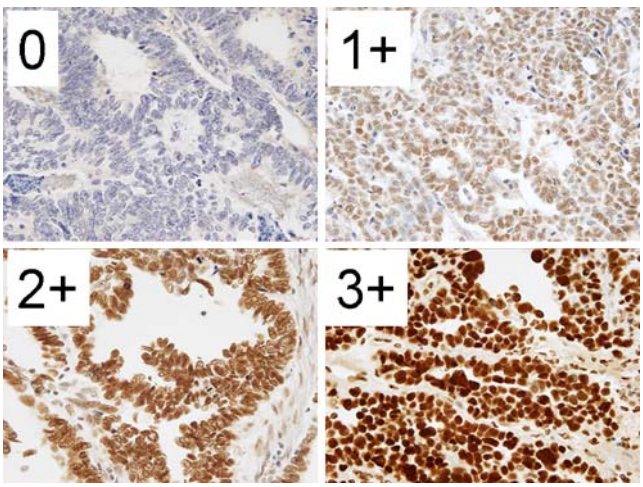

Fig. 1 Nuclear immunoreactivity of WT1 in ovarian serous adenocarcinomas (OSAs). OSA with negative WT1 (*0*), OSA with low-level WT1 immunoreactivity (*1+*), OSA with high-level WT1 immunoreactivity (*2+*), and OSA with high-level WT1 immunoreactivity (*3+*). Original magnification, ×400

tissues contained the ovarian surface epithelia, which are reportedly immunoreactive for the WT1 [30], on their peritoneal surface. All tissue samples were snap-frozen immediately after resection and stored until use in liquid nitrogen.

Total RNA was isolated from the tissues and cell line using RNeasy Mini (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Real-time PCR amplification of synthesized complementary deoxyribonucleic acid (cDNA) was performed in a total volume of 50 μl of reaction mixture containing 2 μl cDNA, corresponding to 100 ng of total RNA, 0.4 pmol/μl of each primer, and 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). We used TaqMan, a set of primers, and a probe (assay ID: Hs01103751_m1) for WT1 cDNA amplification. Cycling conditions were 95°C for 10 min, followed by 40 repeats of 95°C for 0.5 min and 63°C for 1 min. Standard curves for quantification of WT1 and β-actin (TaqMan β-actin Control Reagents: no. 401846) were constructed from the results of simultaneous amplification of serial dilutions of the cDNA from K562 cells, in which the expression level of WT1 mRNA was defined as 1.0, as described previously [15]. WT1 mRNA expression levels in the 21 samples of OSA and five non-neoplastic ovaries were determined according to the standard curves. To normalize the difference in RNA degeneration and in RNA loading for RT-PCR in individual samples, the level of *WT1* gene expression divided by the level of β-actin gene expression were defined as the relative WT1 mRNA expression level in each sample.

Statistical analysis

Statistical analyses were performed using the StatMate III software (ATMS, Tokyo, Japan). Comparisons between parameters were computed by the Chi-squared test or by the Student's *t* test or the Mann–Whitney test for unpaired data. For survival analysis, Kaplan–Meier curves were drawn, and differences between the curves were calculated by the log-rank test. Independent prognostic significance was computed by the Cox proportional hazards general linear model for disease-specific survival. Differences at $P<0.05$ were considered to be statistically significant.

Results

Correlation of nuclear WT1 immunoreaction with the corresponding mRNA expression level

Of the 119 OSAs, 99 (83.2%) were found to be WT1 positive and the remaining 20 (16.8%) were considered to be WT1 negative. Of the 99 WT1-positive cases, 55 (55.6%) were classified as having low-level immunoreactivity (1+) and 44 (44.4%) as having high-level immunoreactivity (2+, 24 cases; 3+, 20 cases).

The relative WT1 mRNA expression levels in the groups showing different intensities of immunoreactivity were quantified using real-time PCR assay and represented relative to the level in the human leukemia cell line K562 (defined as 1.0; Fig. 2). The median levels of mRNA expression in the WT1-negative, low-level, and high-level immunoreactivity groups were 0.66, 3.15, and 8.17, respectively. The WT1 mRNA expression level in the WT1-immunonegative group was statistically different both from that in the group with low-level WT1 immunoreactivity ($P=0.009$; Mann–Whitney *U* test) and from the group with high-level WT1 immunoreactivity ($P=0.0027$; Mann–Whitney *U* test). The mRNA expression levels were also significantly different between the low-level and high-level immunoreactivity groups ($P=0.017$; Welch's *t* test) and between the non-neoplastic ovarian tissues and the WT1 high-level immunoreactivity group ($P=0.028$; Mann–Whitney *U* test). WT1 mRNA expression in the immunohistochemically WT1-negative group was significantly lower than that in the non-neoplastic ovarian tissues (median, 0.66 vs 3.21, $P=0.028$; Mann–Whitney *U* test). There was no significant difference between non-neoplastic

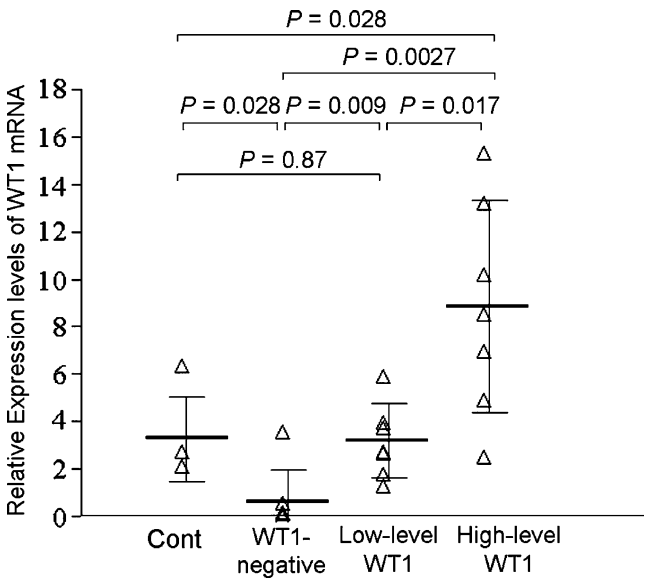

Fig. 2 Relative levels of WT1 mRNA expression in ovarian serous adenocarcinomas (OSAs) with distinct levels of immunoreactivity and in normal ovarian tissues. WT1 mRNA expression levels were calculated by comparison with the WT1 expression level in the K562 cell line. There was a significant difference in the mean relative expression level of WT1 mRNA between control ovarian tissues and WT1-negative OSAs ($P=0.028$), between the WT1-immunonegative group and the group with low-level WT1 immunoreactivity ($P=0.0088$), between the WT1-immunonegative group and the group with high-level WT1 immunoreactivity ($P=0.0027$), between the low-level and the high-level immunoreactivity groups ($P=0.017$), and between control ovarian tissues and the high-level immunoreactivity group ($P=0.028$). *Cont* Normal ovarian tissue samples

ovarian tissues and the low-level WT1 immunoreactivity group (median, 3.21 vs 3.15, P=0.871; Mann–Whitney U test).

Correlation of nuclear WT1 immunoreactivity with clinicopathologic factors

Eight (40%) of the 20 cases in the WT1-negative group were in FIGO stage I to II, compared with only 13 (13.1%) of the 99 WT1-positive cases (P=0.019; Table 2). Moreover, the WT1-positive group showed a higher proportion of histologically high-grade tumors (P=0.0072) and a higher Ki-67 LI (mean, 51.0 vs 29.3, P<0.0001) than the WT1-negative group. With regard to patient age, residual tumors (2 cm in size or more), the proportion of chemosensitive tumors, and expression of Bcl-2 and p53, there were no significant differences between the WT1-negative and WT1-positive groups. Although not statistically significant, patient outcome in the WT1-negative group tended to be better than that in the WT1-positive group (5-year survival, 73.6 vs 52.0%, P=0.387; log-rank test; Fig. 3).

As well as the comparison between the WT1-negative and WT1-positive groups, the group showing a high level of WT1 immunoreactivity had a higher histological grade (P=0.0032) and a higher Ki-67 LI (mean, 57.3 vs 46.0, P=0.0121) than the low-level immunoreactivity group (Table 3). Moreover, the high-level immunoreactivity group showed a higher frequency of Bcl-2 expression than the low-level immunoreactivity group (52.3 vs 23.6%, P=0.0032). There was no significant difference in age, FIGO stage distribution, residual tumors (2 cm in size or more), proportion of chemosensitive tumors, or p53 expression status between these two groups. Patient outcome was significantly poorer in the high-level WT1 immunoreactivity group than in the low-level WT1 immunoreactivity group (5-year survival, 36.5 vs 63.8%, P=0.0082; log-rank test; Fig. 3).

Table 2 Comparison of clinicopathologic factors and WT1 immunoreactivity in ovarian serous adenocarcinomas

Factor	Total	Number of cases (%)		P value
		WT1 negative (n=20)	WT1 positive (n=99)	
Age (year, median)		53.4	56.7	0.242
FIGO[a] stage				0.019
I	14	6 (43)	8 (57)	
II	7	2 (29)	5 (71)	
III	71	7 (10)	64 (90)	
IV	27	5 (19)	22 (81)	
Residual tumor				0.298
0 cm	21	6 (29)	15 (71)	
<2 cm	30	5 (17)	25 (83)	
≥2 cm	65	9 (14)	56 (86)	
Details unknown	3	0 (0)	3 (100)	
Response to chemotherapies				0.517
CR/PR[b]	41	5 (12)	36 (88)	
SD/PD[b]	22	4 (18)	18 (82)	
NE[c]	2	0 (0)	2 (100)	
Histologic grade				0.0072
1	17	7 (41)	10 (59)	
2	53	9 (17)	44 (83)	
3	49	4 (8)	45 (92)	
Ki-67 LI[d] (median)		29.3	51.0	<0.0001
p53				0.150
Positive	66	8 (12)	57 (88)	
Negative	54	12 (22)	42 (78)	
Bcl-2				0.249
Positive	40	4 (10)	36 (90)	
Negative	79	16 (20)	63 (80)	
5-year survival (%)		73.6	52.0	0.387[e]

[a] International Federation of Gynecology and Obstetrics
[b] *CR* Complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease
[c] Not evaluated
[d] Labeling Index
[e] Calculated by log-rank test

Univariate and multivariate analyses of disease-specific survival

The results of univariate analysis for disease-specific survival according to various clinicopathologic factors, including the intensity of WT1 immunoreactivity, are summarized in Table 4, a. Among the clinicopathologic factors, residual tumor (2 cm in size or more; P=0.007) was

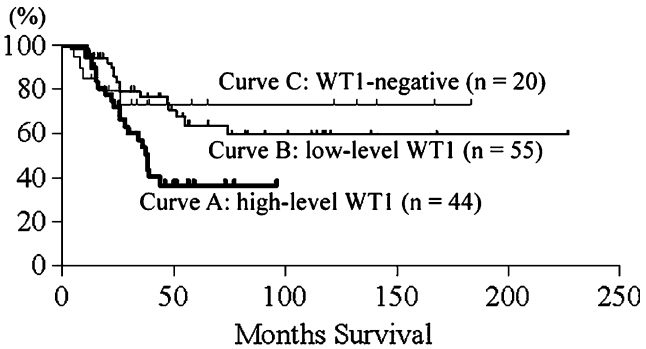

Fig. 3 Overall survival curves for 119 patients with ovarian serous adenocarcinoma (OSA), stratified by WT1 immunoreactivity status. *Curve A* for 44 OSA patients with high-level WT1 immunoreactivity, *Curve B* for 55 OSA patients with low-level WT1 immunoreactivity, and *Curve C* for 20 OSA patients with negative WT1 immunoreactivity. Significant differences were evident between the high-level WT1 immunoreactivity group (*Curve A*) and the low-level immunoreactivity group (*Curve B*; P=0.0082, log-rank test)

Table 3 Comparison of clinicopathologic factors and intensity of WT1 immunoreactivity in WT1-positive ovarian serous adenocarcinomas

Factor	Total	Number of cases (%)		*P* value
		WT1 low-level (*n*=55)	WT1 high-level (*n*=44)	
Age (year, median)		55.9	57.8	0.397
FIGO[a] stage				0.595
I	8	5 (63)	3 (37)	
II	5	4 (80)	1 (20)	
III	64	33 (52)	31 (48)	
IV	22	13 (59)	9 (41)	
Residual tumor				0.464
0 cm	15	9 (60)	6 (40)	
2 cm	25	16 (64)	9 (36)	
≥2 cm	56	28 (50)	28 (50)	
Details unknown	3	2 (67)	1 (33)	
Response to chemotherapies				0.563
CR/PR[b]	36	19 (53)	17 (47)	
SD/PD[b]	18	8 (44)	10 (56)	
NE[c]	2	1 (50)	1 (50)	
Histologic grade				0.003
1	10	10 (100)	0 (0)	
2	44	26 (59)	18 (41)	
3	45	19 (42)	26 (58)	
Ki-67 LI[d] (median)		46.0	57.3	0.012
p53				0.133
Positive	57	28 (49)	29 (51)	
Negative	42	27 (64)	15 (36)	
Bcl-2				0.003
Positive	36	13 (36)	23 (64)	
Negative	63	42 (67)	21 (33)	
5-year survival (%)		63.8	36.5	0.008[e]

[a] International Federation of Gynecology and Obstetrics
[b] *CR* Complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease
[c] Not evaluated
[d] Labeling Index
[e] Calculated by log-rank test

significantly correlated with worse patient outcome. Higher histological grade (grade 3/2/1) and the intensity of WT1 immunoreactivity (high-level immunoreactivity or others) showed only marginal significance (P=0.052 and 0.083, respectively). Patient age greater than or equal to 57 years (median; P=0.801), advanced FIGO clinical stage (stage III or IV; P=0.113), Ki-67 LI greater than or equal to 47.4 (median; P=0.563), p53 positivity (P=0.244), and Bcl-2 positivity (P=0.649) were not correlated with disease-specific survival in the present analysis.

We also performed a multivariate analysis using the Cox proportional hazards model including residual tumor size (≥2 vs <2 cm), histologic grade (3/2/1), and the intensity of WT1 immunoreactivity (high-level immunoreactivity vs others) as parameters (Table 4, b). The residual tumor size was identified as an independent prognostic value for overall survival (P=0.0052). The high-level WT1 immunoreactivity also showed nearly significant prognostic value (P=0.054).

Discussion

In the present, real-time PCR analysis clearly demonstrated that the intensity of WT1 immunoreactivity in OSAs was associated with the mRNA expression level, indicating that the intensity of WT1 immunoreactivity is a good indicator of the level of true WT1 protein expression. Taken together with clinicopathologic features, it appears that WT1 could assist the progression of OSAs in proportion to the level of expression of both its mRNA and protein.

The frequency of immunohistochemical detection of WT1 protein expression in OSA was 83.2% (99 of 119

Table 4 Cox model estimates of the significance of prognostic factors

Variables	*P* value	RR (95% CI)
a. Univariate Cox regression model		
Age (≥57[a] vs <57 years)	0.801	0.92 (0.49–1.72)
FIGO stage (IV or III vs II or I)	0.113	2.13 (0.84–5.45)
Residual tumor (≥2 vs <2 cm)	0.007	2.40 (1.26–4.55)
Histologic grade (3/2/1)	0.052	1.58 (1.00–2.50)
Ki-67 (LI≥47.4[a] vs <47.4)	0.563	1.20 (0.65–2.20)
p53 (positive vs negative)	0.244	0.68 (0.36–1.30)
Bcl-2 (positive vs negative)	0.649	0.86 (0.44–1.67)
WT1 immunoreaction (high level vs other)	0.083	1.73 (0.93–3.20)
b. Multivariate Cox regression model		
Residual tumor (≥2 vs <2 cm)	0.005	2.64 (1.34–5.21)
Histologic grade (3/2/1)	0.874	1.04 (0.63–1.73)
WT1 immunoreaction (high level vs no)	0.054	1.92 (0.99–3.73)

Other abbreviations as in Tables 2 to 3
RR Relative risk, *CI* confidence interval
[a] Mean values

cases), which was compatible with the range reported in previous studies (50.5–97%) [1, 13]. The introduction of TMA technology allowed a large number of specimens to be processed and was especially useful for comparative scoring of WT1 protein expression with specimens subjected to IHC under identical conditions.

Principally, the clinicopathologic significance of the *WT1* gene and its gene product in tumor tissues has been discussed from the viewpoints of (1) diagnostic utility, (2) functional roles in tumor progression, and (3) potential as a target for antigen-specific immunotherapy. From the viewpoint of diagnostic utility, a high level of WT1 protein expression could be a marker of the clinical aggressiveness of OSA. In relation to clinicopathologic parameters, the WT1-positive group showed a more advanced stage and higher histological grade than the WT1-negative group. Moreover, compared with the low-level WT1 immunoreactivity group, the group with a high immunoreactivity level showed a higher histological grade and a poorer patient outcome. Hylander et al. [13] demonstrated that WT1-positive OSAs tended to have a higher grade and more advanced stage than WT1-negative tumors. Netinatsunthorn et al. [21] divided WT1-positive OSAs into subgroups by the intensity of their immunostaining, as we did in the present study, and demonstrated that a high intensity of WT1 immunoreactivity had an independent prognostic value. In our multivariate analysis, the prognostic value of the high-level WT1 immunoreactivity was also nearly significant (P=0.054).

Some reports have advocated the practical utility of IHC for detecting the WT1 protein in differentiating OSA from other histological subtypes of ovarian carcinoma or from adenocarcinomas of other primary sites such as the endometrium or the pancreatobiliary system [8, 9]. However, its application to differential diagnosis in surgical pathology is currently limited and controversial, as a recent study by Nakatsuka et al. [20] demonstrated WT1 immunoreactivity in carcinomas of various primary sites such as the stomach, prostate, biliary tract, and urinary tract, and even in malignant melanomas.

The functional role of the *WT1* gene and its product in ovarian carcinomas is largely unknown, and few studies have investigated the issue. Shimizu et al. [30] hypothesized that the *WT1* gene is related to cell differentiation in OSAs, based on the detection of WT1 expression in normal female genital tissues such as the ovarian surface epithelium, the lining of inclusion cysts, and the tubal epithelium, but not in the cervical or endometrial epithelium.

The *WT1* gene encodes a zinc-finger transcription factor that represses the transcription of growth factor genes such as the platelet-derived growth factor A chain [7], colony-stimulating factor 1 [11], insulin-like growth factor (IGF)-II [5], and growth factor receptor genes such as the IGF-I receptor (IGF-IR) [38] and epidermal growth factor receptor [6]. Because the transcription of these growth factors and growth factor receptors can be regulated by WT1 via its interaction with wild-type p53, it has been recognized that WT1 functions as a transactivator in the absence of wild-type p53 [14, 17]. In OSAs, especially those with a histologically high grade, p53 mutation, which commonly contributes to nuclear p53 immunoreactivity, is associated with tumor progression [3, 29, 36]. Genetic or molecular interaction between WT1 and mutant p53 may be related to tumor progression and aggressive biological activity of OSA cells.

The different subcellular distribution of the WT1 protein among tumor types is of interest. In the reports by Nakatsuka et al. [20], a majority of WT1-positive tumors showed diffuse or granular immunoreactivity in the cytoplasm, whereas ovarian tumors and desmoplastic small round cell tumors frequently showed nuclear immunoreactivity. To date, the pathological significance of such contrasting distribution (nucleus vs cytoplasm) is unclear. Although the WT1 protein is principally a DNA-binding transcription factor distributed in the nucleus, recent reports have demonstrated that phospholylation in the DNA-binding domain alters the affinity for DNA and subcellular distribution of the WT1 [40]. Moreover, nucleus-cytoplasmic shuttling of WT1 and the association between WT1 and actively translating polysomes were reported [22]. Therefore, distinct subcellular localization of WT1 might alter the properties of tumor cells through the regulation of variable genes and/or proteins.

The WT1-positive group showed a higher Ki-67 LI than the WT1-negative group. Moreover, compared with the low-level WT1 immunoreactivity group, the group showing high immunoreactivity had a higher Ki-67 LI. Interestingly, expression of Bcl-2, an apoptosis regulator, was more frequent in the high-level than in the low-level WT1 immunoreaction group. WT1 can also directly activate the transcription of several apoptosis-related genes including Bcl-2 [18]. The present study demonstrated that a high level of WT1 immunoreactivity was significantly associated with both Ki-67 LI and Bcl-2 expression. These findings suggest transcriptional interaction of WT1 with cell cycle accelerators and apoptosis inhibitors.

It is intriguing that WT1-negative OSAs showed lower mRNA expression than non-neoplastic ovarian tissues. Moreover, the mRNA expression level of OSAs with low WT1 immunoreactivity was similar with that of non-neoplastic ovarian tissues. We additionally studied the WT1 immunoreactivity in the ovarian surface epithelium and/or the lining of inclusion cysts in seven unremarkable ovaries and the tumor cells of seven cases of serous tumor of borderline malignancy, which is thought to be a precursor of OSAs. Consequently, all the ovarian surface

epithelia, linings of inclusion cysts, and tumor cells of serous borderline tumors studied showed a weak WT1 immunoreactivity (data not shown), corresponding to the low-level immunoreactivity demonstrated in the present study. These findings suggest that low-level expression of WT1 may be associated with cell differentiation rather than an accelerator of tumor progression, and moreover, the low-level WT1 positivity might be a native characteristic of ovarian serous neoplasms. However, at present, it is unknown what kind of molecular mechanisms give rise to the different WT1 expression pattern, i.e., high-level immunoreactivity and the negativity, in OSAs.

Comprehension of WT1 expression in ovarian carcinomas should aid its utility for diagnosis or prognostication and might also help to clarify whether the *WT1* gene product could be an attractive tumor antigen for immunotherapy. Recent evidence that the presence of intratumoral T cells in advanced ovarian carcinomas is correlated with better clinical outcome has prompted a search for a well-characterized tumor antigen [42]. Generally, the ideal tumor antigen for immunotherapy should demonstrate a high level of expression in tumors, limited expression in normal tissues, and a functional role in tumor progression. Therefore, WT1-positive OSAs, especially those with a high level of WT1 immunoreactivity, could be a prime target for antigen-specific immunotherapy.

In conclusion, the results of the present study suggest that WT1 can be an accelerator of tumor progression in OSAs, possibly with a dose-dependent manner. A high level of WT1 immunoreactivity in high-grade and advanced-stage OSA may have not only a diagnostic and prognostic value but also a potential for application of antigen-specific immunotherapy against this highly lethal malignancy.

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