High Levels of Wilms' Tumor 1 (WT1) Expression Were Associated with Aggressive Clinical Features in Ovarian Cancer

ZHIYANG LIU[1*], KEIKO YAMANOUCHI[1*], TSUYOSHI OHTAO[*], SOHEI MATSUMURA[1], MANABU SEINO[1], VIJI SHRIDHAR[2], TOSHIFUMI TAKAHASHI[1], KAZUHIRO TAKAHASHI[1] and HIROHISA KURACHI[1]

[1]*Department of Obstetrics and Gynecology, School of Medicine, Yamagata University, Yamagata, Japan;*
[2]*Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN, U.S.A.*

Abstact. *Aim: The aim of the present study was to evaluate the correlation between WT1 expression levels and clinical features, to investigate the prognostic value of WT1 expression and to use lentiviral constructs to examine whether overexpression of WT1 affects cell proliferation and invasion in ovarian cancer patients. Materials and Methods: Real-time quantitative PCR (qPCR) methods were employed to analyze WT1 expression levels in clinical samples from 63 patients with ovarian cancer. The correlation between the copy number of WT1 mRNA and clinical variables was analyzed. Results: The median copy number of WT1 mRNA was 53.94 (range=2.135- 32,257) in all subjects and WT1 expression levels were found significantly increased in patients with a higher stage cancer ($p<0.05$), lymphnode ($p<0.001$) and omentum metastasis ($p<0.001$), as well as ascites production ($p<0.05$), compared to patients lacking these clinical variables. No significant difference in WT1 expression levels were observed between patients with and without recurrence. The median disease-free survival time in patients with low WT1 expression levels was significantly longer ($p=0.038$) than that in patients with high WT1 expression. However, overall survival curves showed no statistically significant ($p=0.457$) differences between patients with high- and low-WT1 expression levels. An in vitro study revealed that WT1 over-expression enhanced cell proliferation and invasion in ovarian cancer cells transduced with lentiviral constructs. Conclusion: Using qPCR, we found that high levels of WT1 expression correlated with aggressive clinical features in ovarian cancer. High WT1 expression may impact on median disease-free survival in ovarian cancer.*

Ovarian cancer is one of the most fatal malignancies in women and is the leading cause of gynecological cancer deaths (1). The current standard therapy in patients with advanced ovarian cancer is primary surgical cytoreduction followed by first-line chemotherapy based on platinum and paclitaxel. Although the majority of patients with ovarian cancer respond to initial chemotherapy, most of them eventually relapse during the course of treatment, and once relapse occurs there is no known curative therapy (2). Thus, it is important to develop additional therapeutic approaches for the management of this disease. Immunotherapy is one proposed strategy for reducing the risk of recurrence. Patients with complete response to frontline therapy could be considered for immunotherapy because the majority of these patients may have micrometastases. National Cancer Institute ranks Wilms' tumor 1 (WT1) as the highest prioritization of cancer antigens (3). Clinical trials using peptide vaccines against WT1 in patients with several cancers are showing promising therapeutic effects (4), and WT1 is one of the potential target antigens for a universal vaccine against ovarian cancers (5).

The Wilms' tumor gene *WT1* encodes a protein with four zinc fingers and is considered to be involved in the transcriptional regulation of genes including growth factors, differentiation markers, cell-cycle regulators, and apoptosis regulators (6-9). The *WT1* gene was initially identified as a tumor suppressor gene due to its inactivation in Wilms' tumor (nephroblastoma), the most common pediatric kidney tumor (10). However, recent findings have shown that WT1 acts as an oncogene in ovarian and other tumors (11-14). In addition, several studies have reported that high expression of WT1 correlates with the aggressiveness of cancers and a poor outcome in leukemia (15), breast cancer (16), germ-cell

*These Authors contributed equally to this work.

Correspondence to: Dr. Tsuyoshi Ohta, Department of Obstetrics and Gynecology, Yamagata University, School of Medicine, 2-2-2 Iidanishi, Yamagata 990-9585, Japan. Tel: +81 236285393, Fax: +81 236285396, e-mail: oota-t@med.id.yamagata-u.ac.jp

Key Words: WT1, ovarian cancer, real-time quantitative PCR, clinical features, prognosis, lentivirus, disease-free survival, overall survival.

tumor (17), and head and neck squamous cell carcinoma (18). There are several studies describing WT1 expression in ovarian cancers. A positive expression has been primarily observed in serous adenocarcinoma (19-22), and WT1 is more frequently expressed in high-grade serous carcinoma, which stands-out from other sub-types due to its aggressive nature and because it harbors unique genetic alterations (23). Patients with WT1-positive tumors tend to have a higher grade and stage of tumor (24, 25). Several studies examining the correlation between WT1 expression and survival have found WT1 to be indicative of unfavourable prognosis in ovarian cancer patients (23, 26), whereas other studies report that WT1 expression may be of limited prognostic value for these tumors in the clinical setting (24, 25). In several studies, immunohistochemistry (IHC) was used to measure WT1 expression levels: however, antibody specificity was not always sufficient, and this may have been due to inconsistent results on the association between WT1 expression and prognosis in patients with ovarian cancer.

In the current study we report, for the first time, use of a real-time quantitative PCR (qPCR) method that enables for precise quantification of *WT1* expression levels in clinical samples. To demonstrate clinical relevance, WT1 expression levels were investigated in 63 patients with ovarian cancers, including FIGO stages, lymph node, omentum metastasis status, and ascites production. We examined the expression profile of *WT1* in various histological sub-types of ovarian cancer, and investigated the correlation between *WT1* expression and patients' prognosis. Additionally, we report on the effect of *WT1* over-expression on cell proliferation and invasion using ovarian cancer cell lines transduced with lentiviral constructs containing *WT1*.

Materials and Methods

Patient samples. The present study was conducted using tumor samples obtained from 63 patients with histologically-confirmed ovarian and fallopian tube cancer treated at the Yamagata University Hospital (Yamagata, Japan) from February 2008 through July 2013. Tumor samples were collected after written informed consent and approval of the Ethics Committee of the Yamagata University.

Sample processing. Tissue samples were collected into sterile tubes and stored at –80˚C. Tumors were homogenized in RLT buffer and total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). To determine whether cDNA was successfully extracted from tissue samples, we performed RT-PCR assay for GAPDH.

Real-time PCR analysis. The probe and primer used in the real-time PCR analysis were described in a another study (27) as follows: *WT1* forward primer (5'-TAC ACA CGC ACG GTG TCT TCA-3'), reverse primer (5'-CTC AGA TGC CGA CCA TAC AAG-3'), and *WT1* probe (5'-AGG CAT TCA GGA TGT GCG ACG TGT G-3'). Real-time PCR was performed using ABI Prism 7300 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. All PCR routines were performed in triplicate. Reactions involved an initial incubation for 2 min at 50˚C and then 10 min at 95˚C, followed by cycling at 95˚C for 15 sec and 60˚C for 1 min for 50 cycles. Quantification was performed using the standard-curve method. A standard curve was generated from a dilution series constructed from WT1 (unless otherwise stated, the WT1 isoform used in this study lacked both exon 5 and KTS) plasmid.

Immunohistochemical staining. Paraffin-embedded tissue sections were stained with anti-WT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The scoring system used in the study was previously described elsewhere (20). Briefly, the intensity of immunostaining for WT1 was scored as 0 (staining of single cells or small clusters of cells, approximately <5% cells stained), +1 (5-25%), +2 (>25-50%), +3 (>50-75%), and +4 (>75% of cells stained). Immunohistochemistry was performed in specimens with the ten highest and ten lowest *WT1* mRNA levels.

Cell culture. Human ovarian cancer cell line SKOV3ip1 cells were cultured at 37˚C in M199:105 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptmycin in a water-saturated atmosphere of 95% air and 5% CO_2.

WT1 lentiviral construct. The pcDNA 3.1(+) vector (Invitrogen, Carlsbad, CA, USA) contained the human WT1 (-17AA/-KTS) (28). The *WT1* sequence was amplified from this vector by PCR using primers containing *Bgl*II and *Not*I restriction sites, as follows: forward primer (5'-AGATCTGACTTCCTCTTGCTGCA-3') and reverse primer (5'-GCGGCCGCTTGAAAGCAGTTCACACACT-3'). The amplified sequence of *WT1* was digested, and then ligated into the lentiviral vector plasmid, pHR-SIN-CSGW dlNotI (29) (provided by Y. Ikeda, Mayo Clinic, Rochester, MN). We introduced WT1 genes in place of eGFP using *BamH*I and *Not*I site in pHR-SIN-CSGW dlNotI. The resultant plasmids were designated as WT1-pHR-SIN-CSGW dlNotI. We deleted eGFP region in pHR-SIN-CSGW dlNotI and used it as a control vector. Preparation of infectious particles was performed according to established protocols (30).

Cell proliferation and invasion assays. Both assays were performed as described previously (31, 32). Cells were plated at a density of 1×10^4 cells per well in 12-well plates and grown in M199:105 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin for 7 days. We counted the cell numbers at 1, 3, 5, and 7 days. TC20™ Automated Cell Counter (Bio Rad Laboratories, Berkeley, CA) was used to count the cell number. The cell invasion assay was performed in a modified Boyden chamber. Porous filter (8-μm pores) were coated on the underside by a passive adsorption for Type I collagen (BD Biosciencees, San Jose, CA, USA). Cells (5×10^4 per well) in M199:105 medium were plated in the upper chamber, then transferred to the lower chamber (24-well) containing 10% FBS and allowed to invade for 4 h. Non-invading cells were removed from upper chamber with a cotton swab, and invading cells adherent to the underside of the filter were fixed and stained with Giemsa solution. Filters were mounted onto microscope slides, and stained cells were counted at ×400 magnification in five fields per filter. At least three independent experiments were done.

Statistical analysis. Results were analyzed using the statistical software EZR (http://cran.r-project.org) and GraphPad prism (La

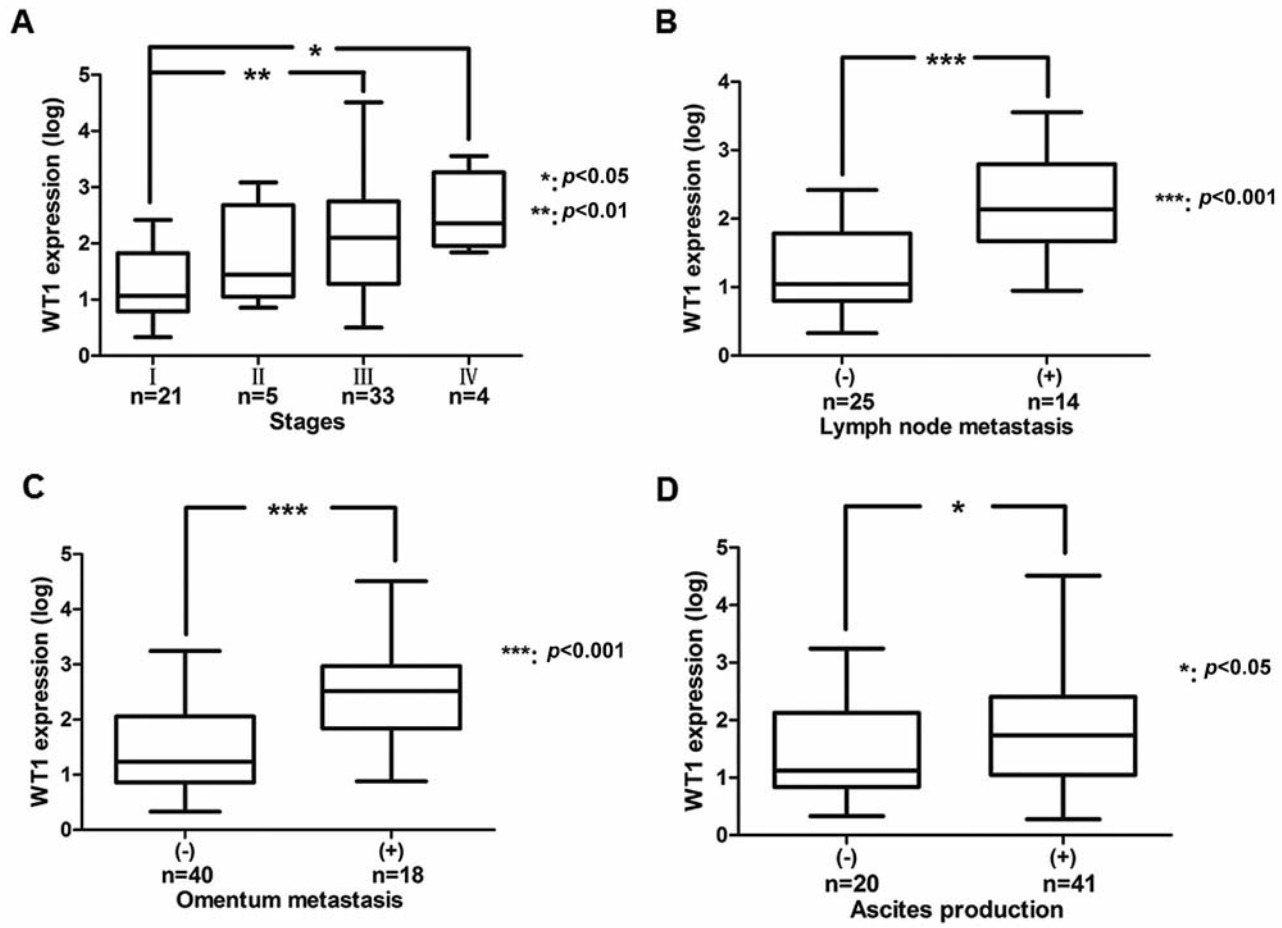

Figure 1. *Correlation between WT1 expression levels and clinical features. We examined WT1 expression levels in groups of different clinical features. (A) FIGO stages, (B) lymph node metastasis status, (C) omentum metastasis status, and (D) ascites production, where "n" refers to the number of patients' samples analyzed for WT1 expression. Boxes indicate the 25th to 75th percentiles. The horizontal lines within the boxes are the median WT1 expression levels and the whiskers indicate the minimum and maximum values.*

Jolla, CA, USA). Group comparisons were performed using the Kruskal-Wallis test. The Mann-Whitney *U*-test was used to determine statistical differences between 2 groups. The prognostic potential of WT1 expression was evaluated by logistic regression. The level of statistical significance was set at a p-value <0.05.

Results

Study population. Patients' characteristics are described in Table I. The mean age of the patients in this study was 57 (range=29-81) years and the medium duration of follow-up was 407 (range=10-1,968) days. At the time of initial diagnosis, the stages were stage I (21 patients), stage II (5 patients), stage III (33 patients), and stage IV (4 patients). Histological examination revealed the following distribution in patients: 58 epithelial ovarian tumors, 4 fallopian tube adenocarcinomas and 1 peritoneal carcinoma; serous in 18 (29%), clear cell in 16 (25%), endometrioid in 12 (19%), mucinous in 2 (3%), carcinosarcoma in 7 (11%), small cell in 2 (3%), mixed 2 (3%) and undifferentiated in 4 (6%). Forty two patients (67%) received the first operation without residual tumors (complete surgery), 4 (6%) had a residual tumor of <1 cm in diameter (optimal surgery), and the remaining 17 (27%) exhibited residual tumors of ≥1 cm in diameter (suboptimal surgery). A complete response to first-line chemotherapy was achieved in 48 (76%) of the 63 patients, one (2%) had partial response, one (2%) had no response, 11 (17%) exhibited disease progression, while the other 2 (3%) were not applicable. The median disease-free survival, excluding patients with persistent/progressive disease after the initial therapy, was 253 (range=42-1,798) days. Forty-five (71%) patients were alive with no evidence of disease, 7 (11%) patients were alive with disease, and 11 (17%) subsequently died from the disease. The median serum CA125 levels were 317.2 (range=10-6,279) U/mL. The copy

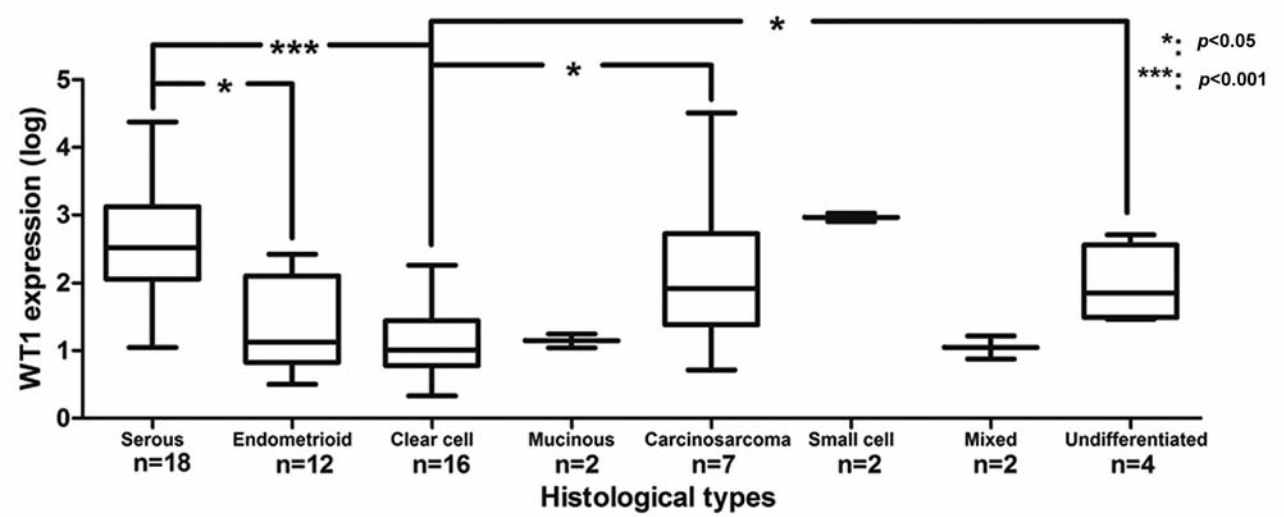

Figure 2. *Changes in WT1 expression levels in different histological sub-types. We examined WT1 expression levels in different histological subtypes where "n" refers to the number of patients' samples analyzed for WT1 expression. Boxes indicate the 25th to 75th percentiles. The horizontal lines within the boxes are the median WT1 expression levels and the whiskers indicate the minimum and maximum values. There were significant differences in WT1 expression levels between clear cell adenocarcinoma or endometrioid and serous, as well as expression levels between carcinosarcoma or undifferentiated and clear cell adenocarcinoma.*

number of *WT1* mRNA was defined as *WT1* expression levels, with median value of 53.94 (ranges=2.135-32,257).

WT1 expression correlated with clinical features. We examined the correlation of WT1 expression levels with FIGO stage, lymph node, omentum metastasis status, and ascites production in all subjects. The median copy number of *WT1* mRNA was 11.67 (range=2.135-262.6) in stage I, 27.69 (range=7.203-1,213) in stage II, 126.7 (rang=3.177-32,257) in stage III, and 227.4 (range=68.85-3,599) in stage IV disease. The levels of *WT1* expression in patients with stage III and stage IV disease increased significantly compared to stage I patients ($p<0.01$ and $p<0.05$, respectively) (Figure 1A). Thirty-nine patients received a staging laparotomy and presented conditions applicable for studying the correlation of *WT1* expression levels with lymph node metastasis status. The median copy numbers of *WT1* mRNA in patients without lymph node metastasis (n=25) was 11.07 (range=2.135-262.6) and that in patients with lymph node metastasis (n=14) was 137.0 (range=8.883-3,599). There was a significant increase in *WT1* expression levels in patients with lymph node metastasis compared to those without (Figure 1B). We were able to assess the omentum metastasis status in 58 patients. The median copy number of *WT1* mRNA in patients without omentum metastasis (n=40) was 17.18 (range=2.135-1,749) and that in patients with metastasis (n=18) was 336.2 (range=7.610-32,257). A significant ($p<0.001$) increase in *WT1* expression levels was found in patients with omentum metastasis compared to patients where metastasis was not observed (Figure 1C). We investigated whether patients exhibited ascites production in 61 cases of ovarian cancers. Ascites production was assessed by computed tomography, where detection of fluid in the Douglas pouch was defined as positive for ascites production. The median copy number of *WT1* mRNA was 13.37 (range=2.135-1,749) in patients without ascites (n=20) and 83.52 (range=4.190-32,257) in patients with ascites (n=41). Patients with ascites exhibited a significant ($p<0.05$) increase in *WT1* expression levels compared to those without (Figure 1D). Collectively, these results suggest that high levels of *WT1* expression were related to aggressive disease conditions in patients with ovarian cancer.

Changes in WT1 expression levels according to various histological sub-types. We next examined WT1 expression levels in different histological subtypes (Figure 2). The median copy number of *WT1* mRNA was 336.2 (range=11.16-23,878) in serous carcinomas, 10.39 (range=2.135-180.8) in clear cell adenocarcinomas, 83.52 (range=5.203-32,257) in carcinosarcomas, 13.57 (range=3.177-262.6) in endometrioid, and 84.92 (range=29.03-506.2) in undifferentiated carcinomas. The *WT1* expression levels were high in aggressive sub-types; serous carcinomas and carcinosarcomas, while the levels of *WT1* expression was low in a slow-growing sub-type; clear cell adenocarcinomas. There was a significant difference in WT1 expression levels between clear cell adenocarcinoma ($p<0.001$) or endometrioid ($p<0.05$) and serous. In addition, there was a

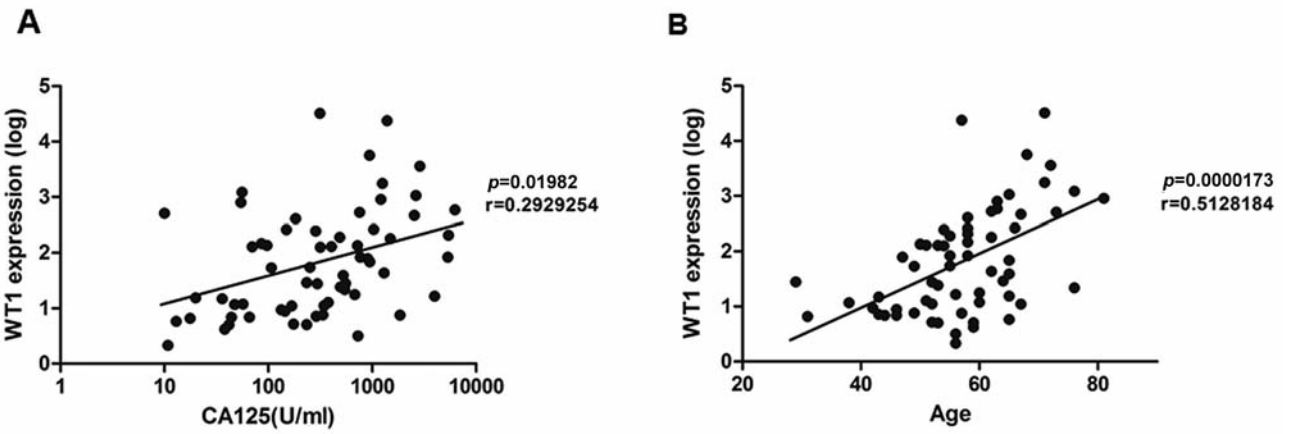

Figure 3. *Correlation between WT1 expression and CA125 or age. (A) Serum CA125 levels increased significantly with WT1 expression levels (r=0.293, p=0.020) in all subjects. (B) Age increased significantly with WT1 expression levels (r=0.513, p=0.00002) in all subjects.*

significant difference in WT1 expression levels between carcinosarcoma ($p<0.05$) or undifferentiated ($p<0.05$) and clear cell adenocarcinoma (Figure 2). Our study is the first to examine changes in *WT1* expression levels among various histological subtypes of ovarian cancer using real-time PCR quantification, and the results were consistent with those reported in previous studies using immunohistochemistry (23-26).

Correlation between WT1 expression and CA125 levels or age. We also examined correlations between WT1 expression levels and other factors, including CA125 levels, age, body-mass index, gravida, blood loss, and operation time. Positive correlations were identified between *WT1* expression levels and serum CA125 levels (r=0.293, $p=0.020$) (Figure 3A) or age (r=0.513, $p=0.00002$) (Figure 3B).

Association between WT1 expression and patient outcome. We investigated the prognostic effect of *WT1* expression in patients with ovarian cancer. Eighteen patients (24.6%) had a recurrence at the time of the last follow-up. The median copy number of *WT1* mRNA in patients without a recurrence was 24.30 (range=2.135-32,257) (n=45) while that with a recurrence was 141.5 (range=7.610-5,668) (n=18), and a significant ($p<0.05$) difference in *WT1* expression levels was observed between the two groups (Figure 4A). The median disease-free survival (DFS) time was 253 days (range=42-1,798 days) (n=48) and the median overall survival (OS) was 407 days (range=22-1,968 days) (n=63). The median copy number of *WT1* mRNA was 53.94, thus we used 53.94 copies as the cut-off level of WT1 expression for dividing the 63 patients into two groups: Group-High comprised of women whose levels of *WT1* mRNA were ≥53.94, and Group-Low comprised of women with *WT1* mRNA levels <53.94. The median DFS was 245 (range=48-1,768) days in Group-High, and 302 (range=42-1,798) days in Group-Low. The median DFS in Group-Low was significantly longer ($p=0.038$) than that in Group-High (Figure 4B). The median OS was 426 (range=117-1,945) days in Group-High, and 383 (range=22-1,968) days in Group-Low. The OS curves showed no statistically significant ($p=0.457$) difference between the two groups (Figure 4C). These results suggested that the levels of WT1 mRNA may affect DFS in patients with ovarian cancers.

Confirmation of correlation between WT1 mRNA and protein expression. Figure 5A shows representative examples of immunohistochemical staining for WT1. Immunohistochemistry was performed in 20 specimens from 10 patients with the highest and 10 patients with the lowest *WT1* mRNA levels. The staining scores of specimens with high *WT1* mRNA expression were significantly higher than those with low *WT1* mRNA expression as a whole (Figure 5B). These results suggested that the amount of *WT1* mRNA expression correlated with the protein expression levels. We also investigated the WT1 staining score and mRNA expression levels in each patient (Table II). There were some dissociations between the levels of WT1 protein and mRNA expression.

Overexpression of WT1 enhanced the ability of cell proliferation and invasion. Since our data indicated that high levels of WT1 expression were related to aggressive clinical features in ovarian cancer patients, we examined whether WT1 overexpression enhanced proliferation and invasion into ovarian cancer cells. We transduced SKOV3ip1 cells with lentiviral constructs, each containing empty vector (control) or WT1, and generated a stably WT1-expressing cell line. Immunoblot analysis revealed WT1 expression at high levels in SKOV3ip1 cells transduced with WT1 (Figure 5A).

Forced expression of WT1 in SKOV3ip1 cells resulted in an increase in cell proliferation compared with the control (Figure 5A). To evaluate the effect of WT1 overexpression on cell invasion, we evaluated random motility using a Boyden chamber assay. Cell invasion was significantly increased by up to 2.5-fold in WT1 overexpressing cells compared to control cells (Figure 5B). These results suggested that overexpression of WT1 transformed the cells into more aggressive cell types in ovarian cancer.

Discussion

To our knowledge, this is the first report to quantify *WT1* expression levels by quantitative PCR and investigate their clinical relevance. We found that high levels of *WT1* expression were associated with advanced FIGO stages, lymph node and omentum metastasis, as well as ascites production. *WT1* was highly expressed in aggressive serous carcinomas and carcinosarcoma. These results suggest that high levels of WT1 expression are associated with aggressive clinical conditions in ovarian cancer.

Several previous studies have used immunohistochemistry to investigate the correlation between WT1 expression levels and clinical features in ovarian cancer patients (19-22, 24, 25). In the present study, we used a real-time quantitative PCR (qPCR) method to examine the precise quantification of WT1 expression levels in clinical samples. WT1 is highly expressed in patients with higher-stage cancers ($p<0.05$), lymph node ($p<0.001$) and omentum ($p<0.001$) metastasis, and ascites production ($p<0.05$) (Figure 1), while serous ($p<0.001$) or carcinosarcoma ($p<0.05$) exhibited high levels of *WT1* expression compared to clear-cell adenocarcinoma (Figure 2). Positive correlations were found between WT1 expression levels and serum CA125 levels (r=0.293, $p=0.020$) (Figure 3A) or age (r=0.513, $p=0.00002$) (Figure 3B). These results were similar to results of previous studies examining the correlation between WT1 expression levels and their clinical relevance using immunohistochemistry (19-26). Moreover, although WT1 was originally defined as a tumor suppressor, subsequent study had reported that WT1 appears to exhibit oncogenic functions in ovarian cancer, as well as in other tumors (11-14), and this was confirmed by our *in vitro* data which indicated that WT1 overexpression enhanced proliferation and invasion in ovarian cancer cells (Figure 6).

In our study, a significant difference in *WT1* mRNA expression levels was observed between subjects with and without recurrence (Figure 4A), and the median disease-free survival in patients with low WT1 expression levels was significantly ($p=0.038$) longer than that with high WT1 expression (Figure 4B). However, overall survival showed no statistically significant ($p=0.457$) difference between the two groups (Figure 4C). Patients with high *WT1* mRNA expression may have early recurrence and receive second-line or third-line chemotherapy. The post-recurrence therapy has a confounding effect on overall survival (33), thus, the levels of *WT1* mRNA expression could not impact on the

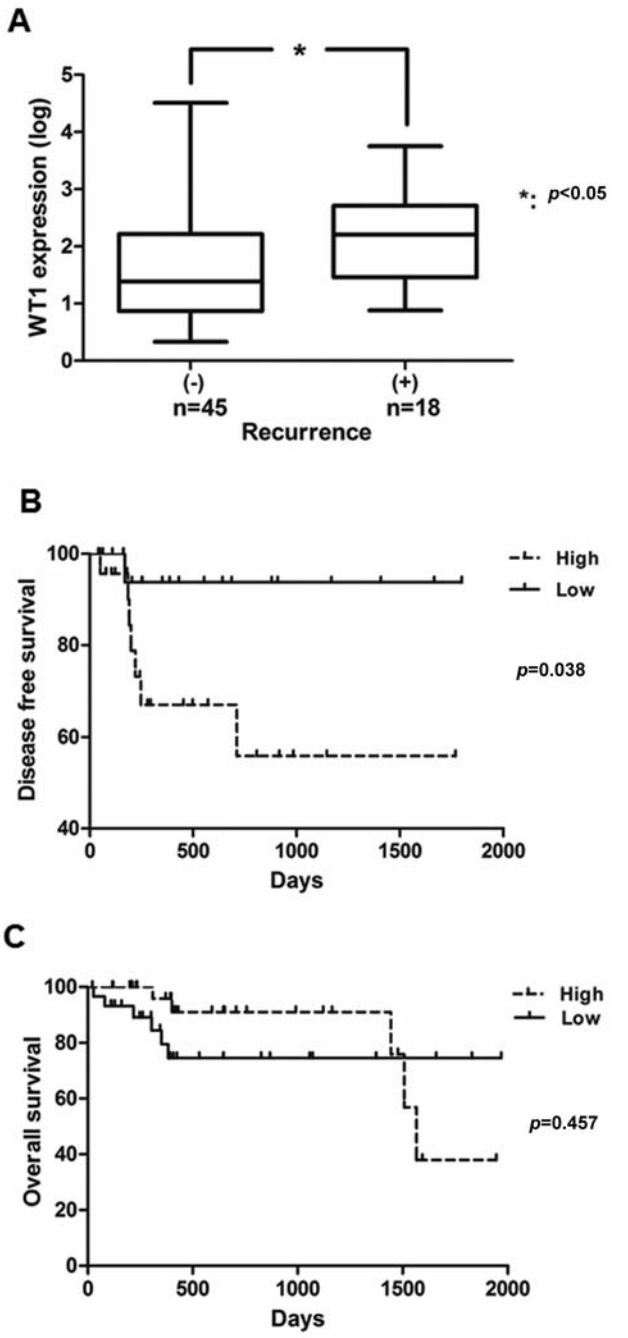

Figure 4. *The prognostic effects of WT1 expression levels in ovarian cancer patients. (A) Kaplan-Meier plots of disease-free survival of groups defined by WT1 expression levels. Group-High comprised of women whose of WT1 mRNA levels were ≥53.94, and Group-Low comprised women whose levels were <53.94. (B) Kaplan-Meier plots of overall survival of groups defined by WT1 expression levels.*

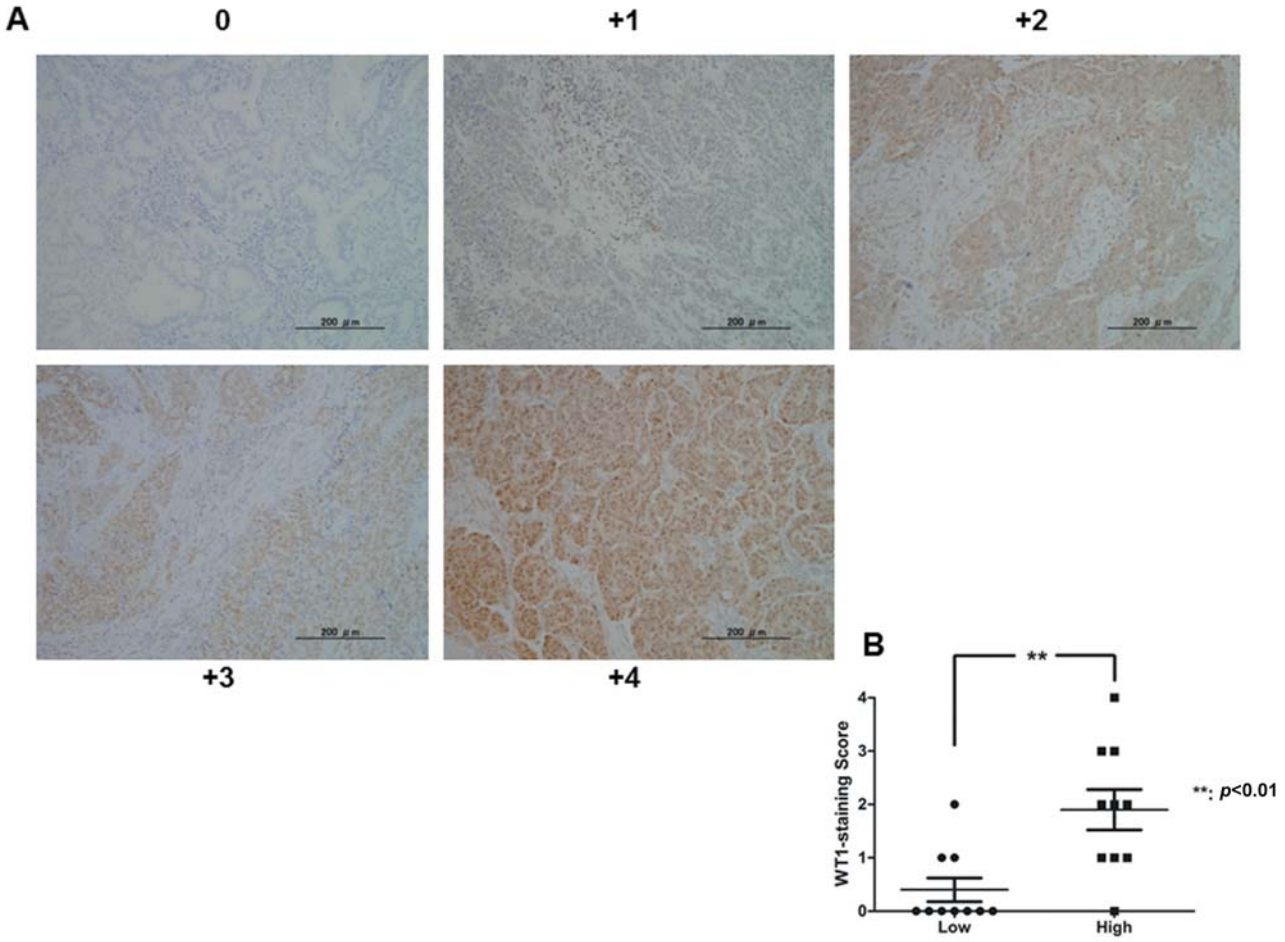

Figure 5. *Correlation between WT1 protein and WT1 mRNA levels. (A) Representative images of staining intensity score (0, +1, +2, +3, and +4) for the WT1 antibody are shown. Magnification, ×200. (B) WT1 staining scores in 20 specimen form 10 patients with the highest (High) and 10 patients with the lowest (Low) WT1 mRNA levels are shown. WT1-staining score in the Low-group was significantly lower than that in the High-group, suggesting that the amount of WT1 mRNA expression correlated with protein expression levels overall.*

overall survival of patients with ovarian cancers. Previous studies examining *WT1* expression levels by use of immunohistochemistry have demonstrated that WT1 expression in the clinical setting may be of limited prognostic value for ovarian cancers (24, 25). We found the prognostic significance of *WT1* mRNA expression levels in the median disease-free survival. We investigated the correlation between *WT1* mRNA and protein expression in 20 specimens from 10 patients with the highest and 10 patients with the lowest WT1 mRNA levels, and found that they correlated as a whole (Figure 5B), but did not correlate in some cases (Table II). These inconsistent results might be explained by the transcript variant.

WT1 is subject to alternative splicing involving exon 5 (17AA) and three amino acids (KTS) at the end of exon 9, producing four major isoforms (-17AA/-KTS, +17AA/-KTS, -17AA/+KTS, and +17AA/+KTS) (34). A recent study investigating WT1 isoform expression using real-time PCR has reported that the ratio of WT1 isoform, particularly +17AA variant, is probably crucial for the process of malignant transformation in acute myeloid leukemia (27). A major limitation of the present study was that we were not able to detect the clinical relevance of WT1 isoforms. A cohort study in a large number of patients with ovarian cancers might show differences in not only disease free but also overall survival by analyzing WT1 isoforms expression.

The National Cancer Institute ranks Wilms' tumor 1 (WT1) as the highest prioritization of cancer antigens (3). Clinical trials using peptide vaccines against WT1 in patients with several cancers are showing promising therapeutic effects (4), and WT1 is one of the potential target antigens for a universal vaccine in ovarian cancers (5). Our data suggest that high levels of WT1 expression could be associated with the aggressive clinical features in ovarian cancers. Taken together,

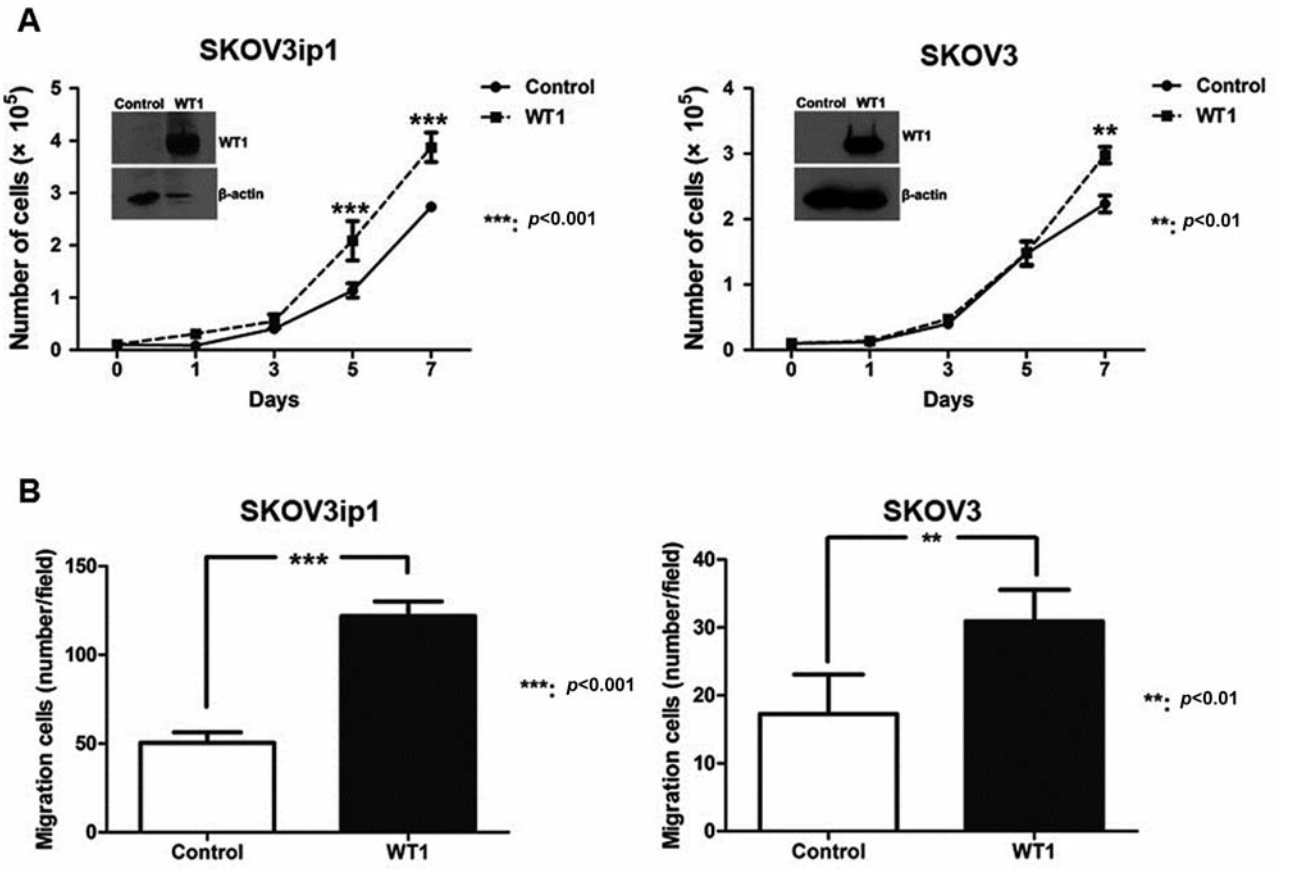

Figure 6. *Enhanced expression of WT1 increases cell proliferation and migration. SKOV3ip1 cells were transduced with lentiviral constructs each containing empty vector (Control) or WT1. The cells were plated at a density of 1×10^4 cells/well in 12-well plates and grown in M199:105 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin for 7 days. We counted cell numbers at 1, 3, 5, and 7 days. All experiments were carried-out in triplicate. The values shown are the mean±SE (n=3) (A). Immunoblot analysis was performed to confirm the expression of WT1 in cells transfected with empty vector or WT1 lentiviral construct (panel). Invasion of both cell lines was analyzed in Boyden chamber (Chemotaxicell). Porous filters (8 μm pores) were coated on the underside by passive adsorption of type I collagen (BD Biosciences, CA, USA). Cells (5×10^4/well) in M199:105 medium were plated into the upper chamber (Chemotaxicell) and transferred to the lower chamber (24-well) containing M199:105 with 10% FBS and allowed to invade for 4 h. Invaded cells adherent to underside of the filter were fixed and stained with Giemsa solution. Filters were mounted onto microscope slides, and stained cells were counted at ×200 magnification in five fields per filter. Three independent experiments were performed with a consistent result. The values shown are the mean±SE (n=3) (B).*

these data suggest that targeting WT1 could have therapeutic implications for ovarian cancers.

In conclusion, this is the first report using a real-time qPCR method that enables for precise quantification of WT1 expression levels in clinical samples. We found that high levels of *WT1* expression were correlated with aggressive clinical features and disease-free survival in patients with ovarian cancer. However, *WT1* expression could not impact on overall survival. Furthermore, since the present study was performed using samples form a small number of patients, additional work is required to identify the role of WT1, including its isoforms in larger numbers of ovarian cancer patients.

Disclosure Summary

The Authors have nothing to disclose.

Acknowledgements

We thank Dr. Yasuhiro Ikeda for providing us with the pHR-SIN-CSGW dINotI plasmid. This work was supported,, in part, by Grants-in-Aid Scientific Research No. 22390308 (to H.K.) and No.24791680 (to T.O.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and in part by Grants-in-Aid for the 21st Century Center of Excellence (COE) Program from the Japan Society for the Promotion of Science.

References

1 Berkenblit A and Cannistra SA: Advances in the management of epithelial ovarian cancer. J Reprod Med *50*: 426-438, 2005.

2 Pfisterer J and Ledermann JA: Management of platinum-sensitive recurrent ovarian cancer. Semin Oncol *33*: S12-16, 2006.

3 Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, prindville SA, Viner JL, Weiner LM and Matrisian LM: The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res *15*: 5323-5337, 2009.

4 Oka Y, Tsuboi A, Oji Y, Kawase I and Sugiyama H: WT1 peptide vaccine for the treatment of cancer. Curr Opin Immunol *20*: 211-220, 2008.

5 Vermeij R, Daemen T, de Bock GH, de Graeff P, Leffers N, Lambeck A, ten Hoor KA, Hollema H, van der Zee AG and Nijman HW: Potential target antigens for a universal vaccine in epithelial ovarian cancer. Clin Dev Immunol pii: 891505, 2010.

6 Rae FK, Martinez G, Gillinder KR, Smith A, Shooter G, Forrest AR, Grimmond SM and Little MH: Anlaysis of complementary expression profiles following WT1 induction versus repression reveals the cholesterol/fatty acid synthetic pathways as a possible major target of WT1. Oncogene *23*: 3067-3079, 2004.

7 Renshaw J, Orr RM, Walton MI, Te Poele R, Williams RD, Wancewicz EV, Monia BP, Workman P and Pritchard-Jones K: Disruption of WT1 gene expression and exon 5 splicing following cytotoxic drug treatment: antisense down-regulation of exon 5 alters target gene expression and inhibits cell survival. Mol Cancer Ther *3*: 1467-1484, 2004.

8 Kim HS, Kim MS, Hancock AL, Harper JC, Park JY, Poy G, Perantoni AO, Cam M, Malik K and Lee SB: Identification of novel Wilms' tumor suppressor gene target genes implicated in kidney development. J Biol Chem *282*: 16278-16287, 2007.

9 Kim MK, McGarry TJ, O Broin P, Flatow JM, Golden AA and Licht JD: An integrated genome screen identifies the Wnt signaling pathway as a major target of WT1. Proc Natl Acad Sci USA *106*: 11154-11159, 2009.

10 Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H and lewis WH *et al*: Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell *60*: 509-520, 1990.

11 Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC, Weissman BE and Baldwin AS: WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. Embo J *18*: 3990-4003, 1999.

12 Richard DJ, Schumacher V, Royer-Pokora B and Roberts SG: Par4 is a coactivator for a splice isoform-specific transcriptional activation domain in WT1. Genes Dev *15*: 328-339, 2001.

13 Ito K, Oji Y, Tatsumi N, Shimizu S, Kanai Y, Nakazawa T, Asada M, Jomgeow T, Aoyagi S, Nakano Y, Tamaki H, Sakaguchi N, Shirakata T, Nishida S, Kawakami M, Tsuboi A, Oka Y, Tsujimoto Y and Sugiyama H: Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway. Oncogene *25*: 4217-4229, 2006.

14 Tatsumi N, Oji Y, Tsuji N, Tsuda A, Higashio M, Aoyagi S, Fukuda I, Ito K, Nakamura J, Takashima S, Kitamura Y, Miyai S, Jomgeow T, Li Z, Shirakata T, Nishida S, Tsuboi A, Oka Y and Sugiyama H: Wilms' tumor gene WT1-shRNA as a potent apoptosis-inducing agent for solid tumors. Int J Oncol *32*: 701-711, 2008.

15 Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T, Nasu K *et al*: WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood *84*: 3071-3079, 1994.

16 Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, Tamaki H, Sugiyama H and Noguchi S: High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res *8*: 1167-1171, 2002.

17 Harada Y, Nonomura N, Nishimura K, Tamaki H, Takahara S, Miki T, Sugiyama H and Okuyama A: WT1 Gene Expression in Human Testicular Germ-Cell Tumors. Mol Urol *3*: 357-364, 1999.

18 Oji Y, Inohara H, Nakazawa M, Nakano Y, Akahani S, Nakatsuka S, Koga S, Ikeba A, Abeno S, Honjo Y, Yamamoto Y, Iwai S, Yoshida K, Oka Y, Ogawa H, Yoshida J, Aozasa K, Kubo T and Sugiyama H: Overexpression of the Wilms' tumor gene WT1 in head and neck squamous cell carcinoma. Cancer Sci *94*: 523-529, 2003.

19 Waldstrom M and Grove A. Immunohistchemical expression of Wilms tumor gene protein in different histologic subtypes of ovarian carcinomas. Arch Pethol Lab Med *129*: 85-88, 2005.

20 Hylander B, Repasky E, Shrikant P, Intengan M, Beck A, Driscoll D, Singhal P, Lele S and Odunsi K: Expression of Wilms tumor gene (WT1) in epithelial ovarian cancer. Gynecol Oncol *101*: 12-17, 2006.

21 Hwang H, Quenneville L, Yaziji H and Gown AM: Wilms tumor gene product: sensitive and contextually specific marker of serous carcinomas of ovarian surface epithelial origin. Appl Immunohistochem Mol Morphol *12*: 122-126, 2004.

22 Cathro HP and Stoler MH: The utility of caretinin, inhibin, and WT1 immunohistochemical staining in the differential diagnosis of ovarian tumors. Hum Pathol *36*: 195-201, 2005.

23 Köbel M, Kalloger SE, Boyd N, McKinney S, Mehl E, Palmer C, Leung S, Bowen NJ, Ionescu DN, Rajput A, Prentice LM, Miller D, Santos J, Swenerton K, Gilks CB and Huntsman D: Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. PLoS Med *5*: e232, 2008.

24 Hylander B, Repasky E, Shrikant P, Intengan M, Beck A, Driscoll D, et al. Expression of Wilms tumor gene (WT1) in epithelial ovarian cancer. Gynecol Oncol *101*: 12-17, 2006.

25 Høgdall EV, Christensen L, Kjaer SK, Blaakaer J, Christensen IJ, Gayther S, Jacobs IJ and Høgdall CK: CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients: From The Danish "MALOVA" ovarian cancer study. Gynecol Oncol *106*: 318-324, 2007.

26 Netinatsunthorn W, Hanprasertpomg J, Dechsukhum C, Leetanporn R and Geater A: WT1 gene expression as a prognostic marker in advanced serous epithelial ovarian carcinoma: immunohistochemical study. BMC Cancer *6*: 90, 2006

27 Kramarzova K, Stuchly J, Willasch A, Gruhn B, Schwarz J, Cermak J, Machova-Polakova K, Fuchs O, Stary J and Boublikova L: Real-time PCR quantification of major Wilms' tumor gene 1 (WT1) iosforms in acute myeloid leukemia, their characteristic expression patterns and possible functional consequences. Leukemia *26*: 2086-2095, 2012.

28 Jomgeow T, Oji Y, Tsuji N, Ikeda Y, Ito K, Tsuda A, Nakazawa T, Tatsumi N, Sakaguchi N, Takashima S, Shirakata T, Nishida S, Hosen N, Kawakami M, Tsuboi A, Oka Y, Itoh K and Sugimaya H: Wilms' tumor gene WT1 17AA(-)/KTS(-) isoform induces morphological changes and promotes cell migration and invasion *in vitro*. Cancer Sci *97*: 259-270, 2006.

29 Palmowski MJ, Lopes L, Ikeda Y, Salio M, Cerundolo V and Collins MK: Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. J. Immunol *172*: 1582-1587, 2004.

30 Zufferey R, Nagy D, Mandel RJ, Naldini L and Trono D: Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biothechnol *15*: 871-875, 1997.

31 Kawagoe J, Ohmichi M, Takahashi T, Ohshima C, Mabuchi S, Takahashi K, Igarashi H, Mori-Abe A, Saitoh M, Du B, Ohta T, Kimura A, Kyo S, Inoue M and Kurachi H: Raloxifene inhibits estrogen-induced up-regulation of telomerase activity in a human breast cancer cell line. J Boil Chem *278*: 43363-43372, 2003.

32 Takata K, Morishige K, Takahashi T, Hashimoto K, Tsutsumi S, Yin L, Ohta T, Kawagoe J, Takahashi K and Kurachi H: Fasudil-induced hypoxia-inducible factor-1α degradation disrupts a hypoxia-driven vascular endothelial growth factor autocrine mechanism in endothelial cells. Mol Cancer Ther *7*: 1551-1561, 2008.

33 Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, Mannel RS, Homesley HD, Fowler J, Greer BE, Boente M, Birrer MJ, Liang SX; Gynecologic Oncology Group: Incorporation of bevacizumab in the primary treatment of ovarian cancer. N Engl J Med *365*: 2473-2483, 2011.

34 Haber DA, Buckler AJ, Glaser T, Call KM, Pelletier J, Sohn RL, Douglass EC and Housman DE: An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. Cell *61*: 1257-1269, 1990.

Received January 21, 2014
Revised February 7, 2014
Accepted February 10, 2014