

The Wilms' Tumor Gene *WT1* – 17AA/–KTS Splice Variant Increases Tumorigenic Activity Through Up-Regulation of Vascular Endothelial Growth Factor in an *In Vivo* Ovarian Cancer Model

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Abstract

The Wilms' tumor 1 gene *WT1* encodes a zinc transcription factor involved in a variety of cancer-related processes. In this study, we sought to investigate the effects of *WT1* splice variants on tumorigenic activity and survival in an *in vivo* ovarian cancer model. To this end, we established stable ovarian cancer cell lines transduced with lentiviral constructs containing each of the four *WT1* splice variants (– 17AA/– KTS, + 17AA/– KTS, – 17AA/+ KTS, and + 17AA/+ KTS). In mice inoculated intraperitoneally with SKOV3ip1 cells expressing *WT1* – 17AA/– KTS, disseminated tumor weights and production of ascites were significantly increased compared with those in mice inoculated with cells expressing the control vector. The overall survival in mice inoculated with *WT1* – 17AA/– KTS-expressing cells was significantly shorter than that in mice inoculated with control cells ($P = .0115$). Immunoblot analysis revealed that *WT1* – 17AA/– KTS significantly increased the expression of vascular endothelial growth factor (VEGF) compared with the control. Greater numbers of CD31-immunopositive vessels were observed in tumors from mice injected with cells expressing *WT1* – 17AA/– KTS than in tumors from control mice. Finally, *WT1* – 17AA/– KTS significantly increased tumor microvessel density compared with that in the control ($P < .05$). Treatment with anti-VEGF antibody (bevacizumab) inhibited tumor growth, dissemination, and ascites production in mice injected with cells expressing *WT1* – 17AA/– KTS. The overexpression of *WT1* – 17AA/– KTS induced a more aggressive phenotype in ovarian cancer cells through VEGF up-regulation in an *in vivo* ovarian cancer model. Our findings indicated that *WT1* – 17AA/– KTS enhanced tumorigenic activity and could decrease patient survival through up-regulation of VEGF expression in ovarian cancers.

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Introduction

Wilms' tumor gene *WT1* is located on chromosome 11q13 and it encodes a zinc finger transcription factor [1]. The *WT1* protein activates or represses the transcription of many target genes involved in the cell cycle, proliferation, differentiation, and apoptosis [2–4]. *WT1* was initially identified as a tumor suppressor gene due to its inactivation in Wilms' tumor (nephroblastoma), the most common pediatric kidney tumor [5]. However, recent findings have shown that *WT1* acts as an oncogene in some cancers, including ovarian cancer [6–11]. Previous studies have demonstrated that high expression levels of *WT1* correlate with poor prognosis in leukemia

[12] and breast cancer [13] and with more advanced tumor stages in testicular germ cell tumors [14] and head and neck squamous cell carcinoma [15].

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In ovarian cancer, WT1 is highly expressed in high-grade serous carcinoma, a more aggressive subtype [16]. Moreover, our unpublished data demonstrated that high levels of WT1 expression yielded tumors with more aggressive International Federation of Gynecology and Obstetrics (FIGO) stages, lymph node metastasis status, omentum metastasis status, and ascites production in ovarian cancers. Several studies examining the correlation between WT1 expression and survival have found WT1 to be indicative of unfavorable prognoses in patients with ovarian cancers [16,17]; however, other studies have reported that WT1 expression may be of limited prognostic value in ovarian cancers in the clinical setting [18,19]. This raises important questions about the lack of a significant correlation between WT1 expression levels and survival, despite the observation that WT1 acts as an oncogene and is highly expressed in more aggressive histological subtypes.

WT1 is spliced alternatively at two sites: exon 5 with 17AA and the KTS site, which exists between exons 9 and 10. Splicing at these sites yields four variants (-17AA/-KTS, +17AA/-KTS, -17AA/+KTS, and +17AA/+KTS) [20–23]. Several studies have reported that the four WT1 splice variants have different functions in various cancers. WT1 +17AA/-KTS induces programmed cell death through transcriptional repression of the *EGFR* gene in osteosarcoma cells [24]. WT1 +17AA/+KTS can cause a morphological transition from an epithelial phenotype to a more mesenchymal phenotype in mammary epithelial cells [25]. In ovarian cancers, WT1 -17AA/-KTS induces morphological changes and promotes cell migration and invasion *in vitro* [20]. Moreover, a recent study investigated the expression of WT1 splice variants using real-time PCR and reported that the ratio of WT1 variants, particularly +17AA variants, is probably crucial for the process of malignant transformation in acute myeloid leukemia [26]. Therefore, it is possible that the ratio of expressed WT1 splice variants is associated with the lack of a significant correlation between total WT1 expression and survival in patients with ovarian cancers.

Therefore, in this study, we examined four WT1 splice variants having distinct functions in ovarian tumorigenicity using stable ovarian cancer cell lines overexpressing each splice variants. We also examined the effects of WT1 variants on tumor growth, dissemination, and ascites production using an ovarian cancer mouse model.

Materials and Methods

Cell Cultures

The SKOV3ip1 cell line was generated from ascites developed in *nu/nu* mice by administering an intraperitoneal injection of human ovarian carcinoma cell line SKOV3 [27]. The SKOV3ip1 cell line was cultured at 37°C in M199:105 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO₂.

WT1 Plasmids

Four pcDNA 3.1(+) vectors (Invitrogen, Carlsbad, CA, USA) were engineered to contain one of the four human WT1 splice variants (-17AA/-KTS, +17AA/-KTS, -17AA/+KTS, or +17AA/+KTS) [20]. The sequences of each of these four WT1 variants were amplified from the corresponding vector by PCR using primers containing *Bgl*II and *Not*I restriction sites (sense primer sequence, 5'-AGA TCT GAC TTC CTC TTG CTG CA-3'; antisense primer sequence, 5'-GCG GCC GCT TGA AAG CAG TTC ACA CAC T-3'), digested, and ligated into the lentiviral vector plasmid, pHR-SIN-CSGW dlNotI [28] (a gift from Y. Ikeda, Mayo Clinic, Rochester,

MN, USA). We introduced each of the four WT1 splice variants in place of eGFP using the *Bam*HI and *Not*I sites in pHR-SIN-CSGW dlNotI. The resulting plasmids were designated as WT1 -17AA/-KTS-, +17AA/-KTS-, -17AA/+KTS-, or +17AA/+KTS-pHR-SIN-CSGW dlNotI. We deleted the eGFP sequence in pHR-SIN-CSGW dlNotI and used this modified vector as a control vector. Preparation of infectious particles was performed according to established protocols [29].

Intraperitoneal Xenograft Model and Treatment *in Vivo*

All of the procedures involving animals in this study were approved by the animal care committee of Yamagata University in accordance with institutional and Japanese government guidelines for animal experiments. WT1 splice variants (-17AA/-KTS, +17AA/-KTS, -17AA/+KTS, and +17AA/+KTS) or control lentivirus vectors were co-transfected with packaging plasmids (pVSV-G and pGag/pol) into 293 T cells to generate lentiviral particles. These particles were then used to transduce SKOV3ip1 cells, and cells stably expressing the vectors were selected with 1 µg/mL puromycin. SKOV3ip1 cells (2×10^6) expressing WT1 variants (-17AA/-KTS [n = 13], +17AA/-KTS [n = 13], -17AA/+KTS [n = 8], or +17AA/+KTS [n = 10]) or control vector (n = 8) were suspended in 250 µL PBS and inoculated by intraperitoneal injection (i.p.) into 5- to 7-week-old female BALB/CA *nu/nu* mice. Abdominal circumference and body weight were measured twice weekly. Mice injected with WT1 -17AA/-KTS-expressing cells were euthanized with CO₂ after 36 days and mice injected with cells expressing control vector or the other variants were euthanized after 40 days to assess tumorigenicity by measuring volume of ascites, extent of dissemination, and weight of tumors. We used data from mice that were euthanized precisely after 36 or 40 days.

In a second experiment, 2×10^6 SKOV3ip1 cells expressing the control vector or each WT1 variant were injected i.p. into 5- to 7-week-old female BALB/CA *nu/nu* mice (n = 30, with 6 mice per group of cells), and survival was evaluated from the first day of inoculation until death.

In a third experiment, SKOV3ip1 cells (2×10^6) expressing WT1 -17AA/-KTS were implanted by i.p. into 5- to 7-week-old *nu/nu* nude mice (n = 10). After two week after inoculation, one group of mice (n = 5) was treated i.p. with PBS twice weekly for 3 weeks. A second group of mice (n = 5) was treated ip with bevacizumab (5 mg/kg) twice weekly for 3 weeks. Bevacizumab was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Bevacizumab were diluted in 200 µL of PBS. Abdominal circumference and body weight were measured once a week. At 3 weeks after initiating treatment, mice were euthanized with CO₂ to assess antitumor efficacy of bevacizumab by measuring volume of ascites and weight of tumors.

Reverse Transcription (RT)-PCR

Briefly, tumors were homogenized in RLT buffer. Total RNA was isolated and purified with an RNeasy[™] Mini kit (Qiagen, Valencia, CA, USA). cDNA was generated from 0.4 µg RNA using a QuantiTect Reverse Transcription kit (Invitrogen).

cDNA (1.0 µL) was used for PCR amplification in total volume of 25 µL. The PCR primer sets used for identifying WT1 splice variants [9] were as follows: forward primer (F2), 5'-GAC CTG GAA TCA GAT GAA CTT AG-3'; reverse primer (R2), 5'-GAG AAC TTT CGC TGA CAA GTT-3'; forward primer (F3), 5'-GTG TGA AAC CAT TCCAGT GTA-3'; and reverse primer (R3), 5'-TCC TGA CAA CTT GGC CAC CG-3'. WT1 forward (F2) and reverse primers (R2)

spanned the 17AA coding sequences, and forward (F3) and reverse primers (R3) spanned the KTS coding sequence. The thermal cycle profile used for amplification of WT1 splice variants was 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s. PCR products were electrophoresed on 2% agarose gels containing ethidium bromide and photographed.

Western Blotting

Tumors were homogenized in 400 μ L lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1 nmol/L Na₂EDTA,

1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium PPI, 1 mmol/L β -glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride). Homogenates were centrifuged at 10,000 rpm at 4 °C for 10 min, and the protein concentrations of the supernatants were determined using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of protein isolated from tumors expressing each WT1 splice variant was separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was carried out in 5% skim milk. Protein spots were immunoblotted with anti-WT1 (c-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti- β -actin (AC74, Sigma),

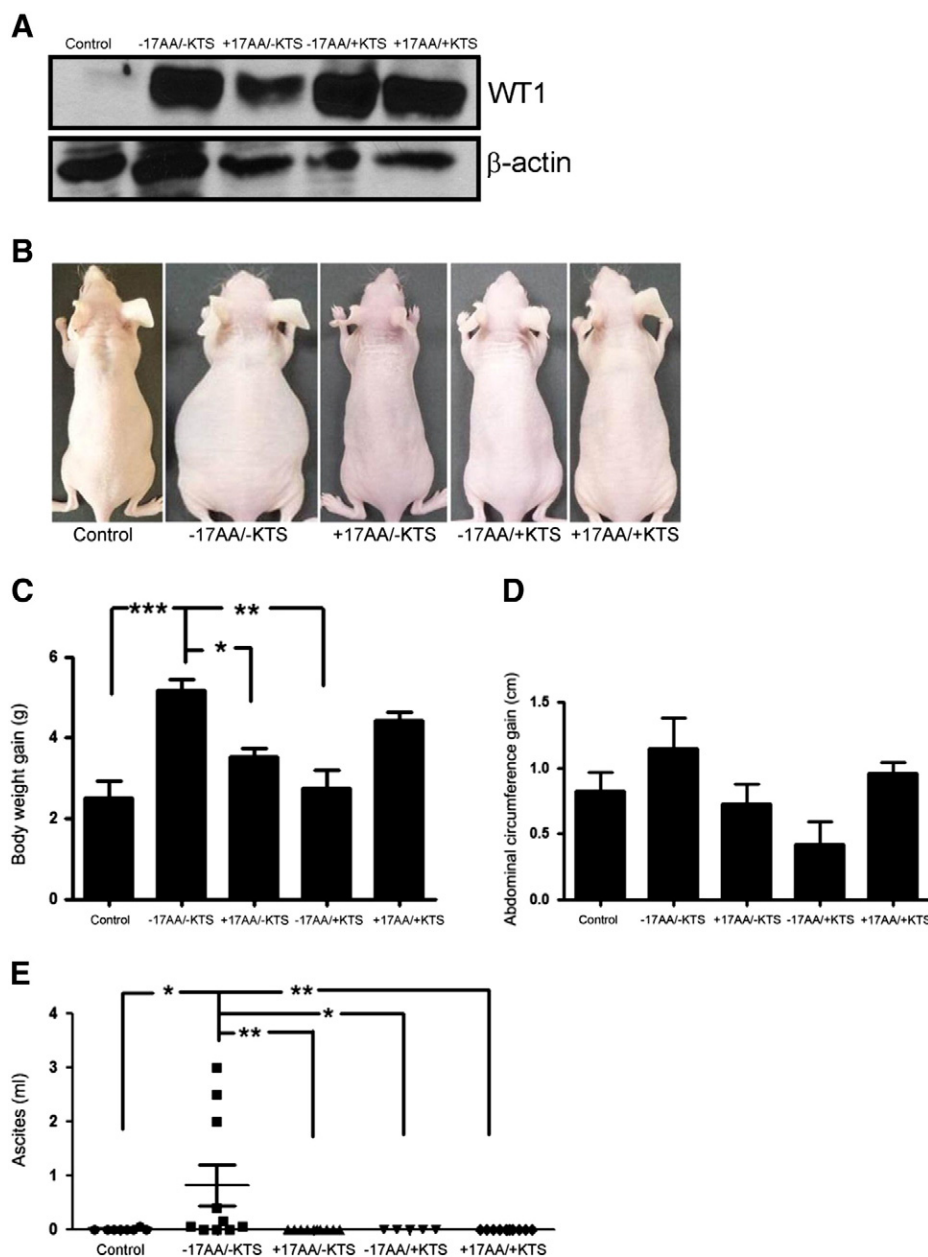


Figure 1. Confirmation of each WT1 splice variant and the appearance, weight gain, abdominal circumference gain, and ascites of mice. (A) SKOV3ip1 cells expressing control vector, WT1 -17AA/-KTS, +17AA/-KTS, -17AA/+KTS, or +17AA/+KTS were lysed and subjected to western blotting using anti-WT1 and anti- β -actin antibodies. (B) Representative images of the mice at the end of the experiment. (C) Body weight gain was calculated by subtracting the body weight at the end of the experiment from that at the start in mice from each of the five groups. (D) Abdominal circumference gain was calculated by subtracting the abdominal circumference at the end of the experiment from that at the start in mice from each of the five groups. (E) The volume of ascites was measured at autopsy. Values are shown as means \pm SEs. Significant differences are indicated by asterisks. ****** $P < .01$; ***** $P < .05$.

anti-VEGF (A-20, Santa Cruz Biotechnology), and anti-CD31/PECAM-1 antibodies (M-20, Santa Cruz Biotechnology).

Immunohistochemistry

Tumor tissues that had disseminated into the abdomen were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded tissue sections were incubated with anti-CD31/PECAM-1 antibodies (Santa Cruz Biotechnology; 1:50 dilution) followed by peroxidase-conjugated secondary antibodies. The tissue sections were viewed at 100× magnification, and images were captured. Four fields per section were

randomly analyzed. The microvessel density (MVD, number/mm²) in each field was calculated (number of CD31-positive objects/0.644 mm²). Mean values of MVD in each group were calculated from the intra-abdominally disseminated tumors developed in mice injected with cells expressing control vector or WT1 -17AA/-KTS.

Statistics

Statistical analysis was performed using one-way ANOVA in Graph-Pad Prism 5 software, and *P* values of less than .05 indicated significant differences. Data are expressed as the mean ± SE.

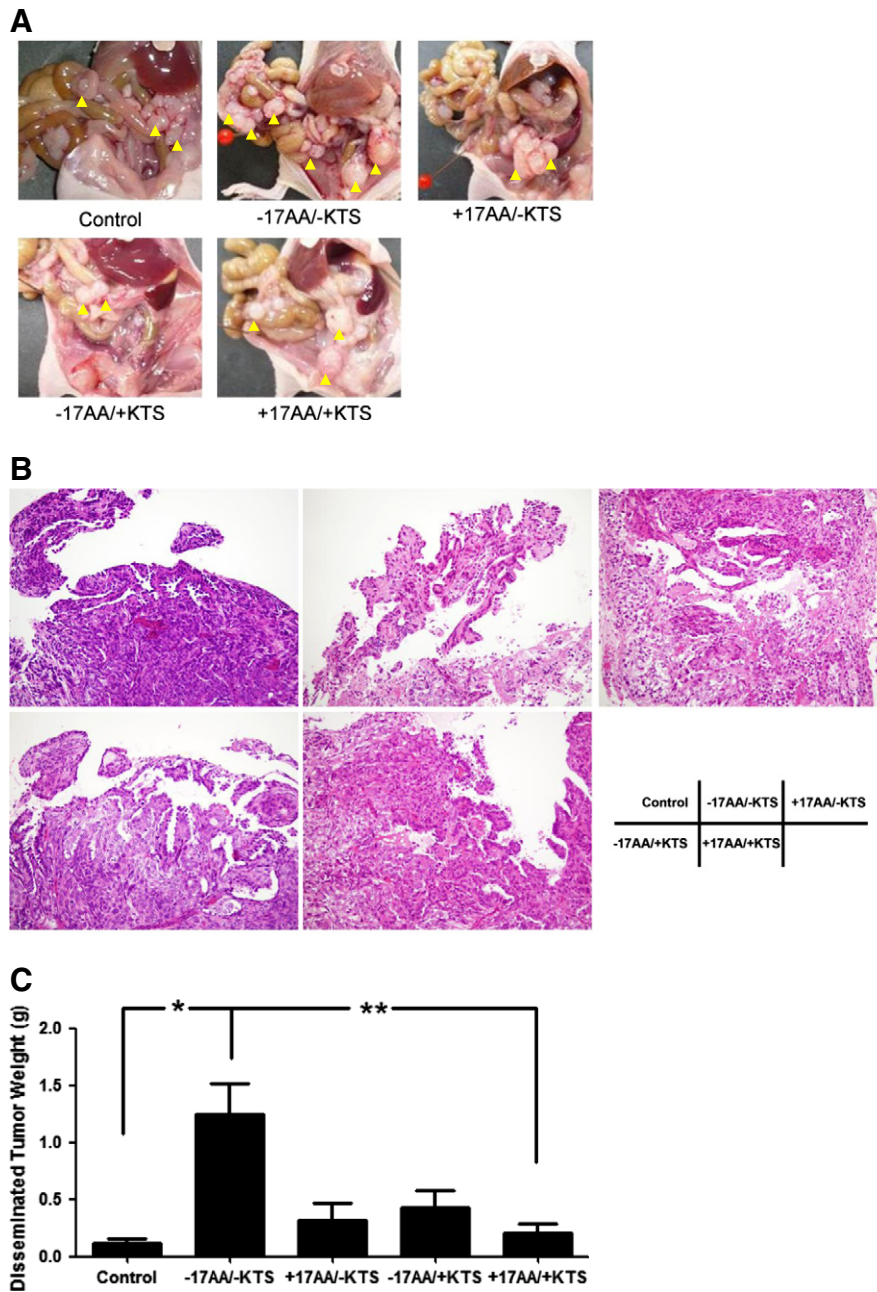


Figure 2. The effects of each WT1 splice variant on tumorigenesis *in vivo*. (A) Representative image of mouse appearance on laparotomy. The yellow arrowhead indicates the intra-abdominal dissemination of the tumor. (B) The disseminated tumor was removed, and the weight was measured. (C) The disseminated tumor was fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded tissue sections were stained with hematoxylin and eosin. The disseminated tumor showed histological findings of serous cystadenocarcinoma. Values of disseminated tumor weights are shown as the mean ± SE. Significant differences are indicated by asterisks. ****P* < .05, ***P* < .01; **P* < .05.

Results

Effects of WT1 Splice Variants on Tumorigenicity in Ovarian Cancer

SKOV3ip1 cells were stably transduced with lentiviral constructs containing control vector, WT1 -17AA/-KTS, WT1 +17AA/-KTS, WT1 -17AA/+KTS, or WT1 +17AA/+KTS, and immunoblot analysis showed high levels of WT1 expression in SKOV3ip1 cells transduced with each WT1 variant (Figure 1A). To test whether SKOV3ip1 cells transduced with each WT1 variant or control vector could be tumorigenic *in vivo* and to observe the pattern of tumor growth, we implanted these cells into nude mice (n = 3 for each of the five different cell type). SKOV3ip1 cells expressing WT1 -17AA/-KTS rapidly produced tumors (3/3), and mice injected with the cells were usually dead within 40 days, while mice injected with SKOV3ip1 cells expressing control vector (3/3), WT1 +17AA/-KTS (3/3), WT1 -17AA/+KTS (3/3), or WT1 +17AA/+KTS (3/3) developed only small tumors, even after 40 days. Based on these preliminary data, we euthanized mice injected with WT1 -17AA/-KTS-expressing cells on day 36 and mice injected with cells expressing control vector or the other variants on day 40.

The appearances of the mice are shown in Figure 1B. Mice injected with cells expressing -17AA/-KTS showed a significant increase in body weight gain compared to mice injected with cells expressing

control vector, +17AA/-KTS, or -17AA/+KTS (Figure 1C). However, there were no significant differences in abdominal circumference gains among the five groups of mice (Figure 1D). Interestingly, overexpression of the -17AA/-KTS splice variant resulted in a significant increase in the volume of ascites, compared with that in mice infected with cells expressing the control vector or other WT1 variants (Figure 1E).

The extent of intra-abdominal dissemination is visually shown in Figure 2A. Massive intra-abdominal dissemination was detected in mice injected with cells expressing the WT1 -17AA/-KTS variant. Mice injected with cells expressing the control vector, WT1 +17AA/-KTS, WT1 -17AA/+KTS, or WT1 +17AA/+KTS showed a little intra-abdominal dissemination. Histological analysis of intra-abdominal lesions developed in mice injected with cells expressing the control vector or each variant confirmed the findings of serous adenocarcinoma (Figure 2B), which was consistent with SKOV3ip1 cells, as described previously [30]. There was no difference in histological findings in cells expressing each of the four WT1 variants (Figure 2B). Tumors that had disseminated within the abdomen were measured by resected tumor weight (Figure 2C). Overexpression of -17AA/-KTS resulted in a significant increase in the disseminated tumor weight, as compared with that in tumors expressing the control vector or +17AA/+KTS variant. There were no significant differences in disseminated tumor weights in mice injected with cells expressing the control vector or other variants.

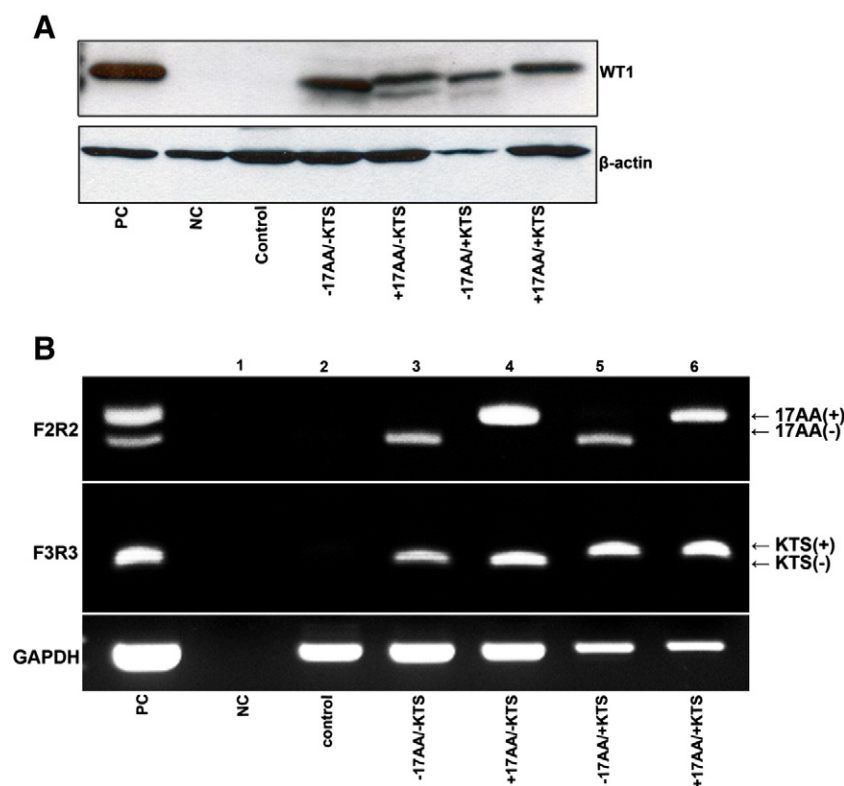


Figure 3. Confirmation of WT1 expression in tumors. (A) Intra-abdominally disseminated tumors were harvested from mice injected with cells expressing the control vector, -17AA/-KTS, +17AA/-KTS, -17AA/+KTS, or +17AA/+KTS, and WT1 expression was assessed by immunoblot analysis with anti-WT1 antibodies, using β -actin as a loading control. Whole cell lysates of SKOV3ip1 cells transduced with WT1 -17AA/-KTS were used as positive controls (PC) and those of SKOV3 ip1 cells were used as negative controls (NC). (B) PCR analysis using F2 and R2 primers showed two bands; the upper band indicated the presence of 17AA, while the lower band indicated the absence of 17AA. PCR analysis using F3 and R3 primers showed two bands; the upper band indicated the presence of KTS, while the lower band indicated the absence of KTS. cDNA from OV202 cells, which expressed WT1, was used as a positive control (PC), while distilled water was used as a negative control (NC).

Confirmation of WT1 Variant Expression *In Vivo*

Immunoblot analysis showed that WT1 was abundantly expressed in tumors obtained from mice inoculated with cells expressing the four variants (Figure 3A). Absence of WT1 expression was confirmed in tumors from mice inoculated with cells expressing the control vector. Additionally, PCR analysis of RNA extracted from the tumors confirmed the expression of each WT1 variant, including the specific 17AA/KTS insertion/deletion, and the absence of WT1 from the control (Figure 3B).

The Effects of WT1 Splice Variants on Survival

We also examined the effects of the WT1 variant on the survival of mice with ovarian cancer. The survival curve of all mice injected with cells expressing the control vector or each WT1 variant are shown in Supplementary Data 1. The median survival times of mice inoculated with cells expressing control vector, WT1 -17AA/-KTS, +17AA/-KTS, -17AA/+KTS, and +17AA/+KTS were 54.5 (range, 52-107), 45 (range, 43-53), 56.5 (range, 44-177), 78 (range, 60-94), and 60.5 (range, 54-178) days, respectively. Moreover, WT1 -17AA/-KTS alone significantly shortened survival compared with the control ($P = .0115$; Figure 4).

WT1 -17AA/-KTS Increased the Expression of VEGF and Promoted Angiogenesis *In Vivo*

Our data showed that overexpression of WT1 -17AA/-KTS enhanced tumorigenic activity and resulted in a poor outcome in our ovarian cancer model. However, it was unclear how WT1 -17AA/-KTS contributed to tumorigenicity and influenced survival in ovarian cancers. Previous study have shown that WT1 splice variants regulate various genes, such as *CCND2*, *PCNA*, *IGFBP5*, *EGR-1*, and *VEGF* [31,32]. Therefore, we next examined the mRNA expression levels of these genes in tumors from mice inoculated with cells expressing the control vector or WT1 -17AA/-KTS by RT-PCR. We confirmed that WT1 -17AA/-KTS increased the mRNA expression of *VEGF* compared with the control vector; however, WT1 -17AA/-KTS did not affect the expression of other genes, such as *CCND2*, *PCNA*, *IGFBP5*, or *EGR-1* (Supplementary Data 2). Moreover, immunoblot analysis revealed that WT1 -17AA/-KTS significantly increased the expression of VEGF at the protein level, as compared with the control (Figure 5A). We next examined whether WT1 -17AA/-KTS promoted angiogenesis *in vivo*. As shown in Figure 5B, larger numbers of

CD31-immunopositive vessels were observed in tumors from mice injected with cells expressing WT1 -17AA/-KTS than in tumors from control mice. WT1 -17AA/-KTS significantly increased tumor MVD compared with the control ($P < .05$; Figure 5C).

The Effects of Anti-VEGF Antibody on WT1 -17AA/-KTS-Induced Tumor Growth and Ascites Production

To investigate whether anti-VEGF antibody inhibited tumor growth and ascites formation enhanced by WT1 -17AA/-KTS overexpression, we administered bevacizumab to athymic mice inoculated with SKOV3ip1 cells (2×10^6) expressing WT1 -17AA/-KTS. Two weeks after inoculation, the mice were randomized into two groups; the first group received PBS ($n = 5$) twice weekly for 3 weeks, while the second group received 5 mg/kg bevacizumab ($n = 5$) twice weekly. One of the mice treated with PBS was dead before the end of the experiment. The appearances of the mice are shown in Figure 6A. Body weight and abdominal circumference were measured at the end of the experiment. Mice treated with bevacizumab showed a significant decrease in body weight and abdominal circumference compared to mice treated with PBS (Figure 6, B and C). Treatment with bevacizumab completely inhibited ascites production (Figure 6D). Moreover, mice treated with bevacizumab showed a significant decrease in the disseminated tumor weight, as compared to mice treated with PBS (Figure 6E). These results suggested that inhibition of VEGF attenuated the enhancing effect of WT1 -17AA/-KTS on tumorigenic activity and ascites formation.

Discussion

This is the first report to examine the effects of WT1 splice variants on tumorigenic activity using an ovarian cancer mouse model. We established stable SKOV3ip1 cell lines overexpressing each of the four WT1 variants (-17AA/-KTS, +17AA/-KTS, -17AA/+KTS, or +17AA/+KTS) using lentiviral constructs and found that WT1 -17AA/-KTS increased tumor growth, dissemination, and ascite production and shortened survival. We also found that WT1 -17AA/-KTS induced the expression of VEGF and that anti-VEGF antibody inhibited the tumor growth and ascites formation enhanced by WT1 -17AA/-KTS overexpression. Collectively, these data indicated that WT1 -17AA/-KTS enhanced tumorigenicity through up-regulation of VEGF and induced cellular transform into a more aggressive phenotype in ovarian cancers.

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 [33]. However, recent findings have shown that WT1 acts as an oncogene in some tumors, including ovarian cancers [6-11]. Several studies have reported that the four WT1 splice variants have different functions in various cancers. For example, WT1 -17AA/-KTS has been shown to induce morphological changes and promote cell migration and invasion in ovarian cancer (TYK) cells [20]. In mammary cells, WT1 +17AA/+KTS causes a morphological transition from an epithelial to a more mesenchymal phenotype [25]. Our *in vivo* data showed no difference in histological findings in cells expressing each of the four WT1 variants (Figure 2B). We also examined the function of WT1 splice variants on cell invasion *in vitro* using SKOV3ip1 cells transduced with lentiviral constructs containing an empty (control) vector or each WT1 variant. All isoforms enhanced cell invasion compared with the control, and there was no significant difference among each of the four WT1 splice variants (data not shown). Our *in vivo* data showed that WT1 -17AA/-KTS increased tumor growth, dissemination, and ascite production in ovarian cancers. This result was consistent

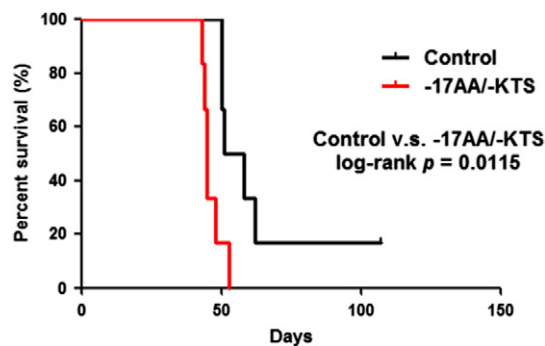


Figure 4. The effects of WT1 -17AA/-KTS on overall survival *in vivo*. Mice were inoculated with cells expressing control vector or WT1 -17AA/-KTS. The mice were observed until death. Overall survival was estimated using the Kaplan-Meier method.

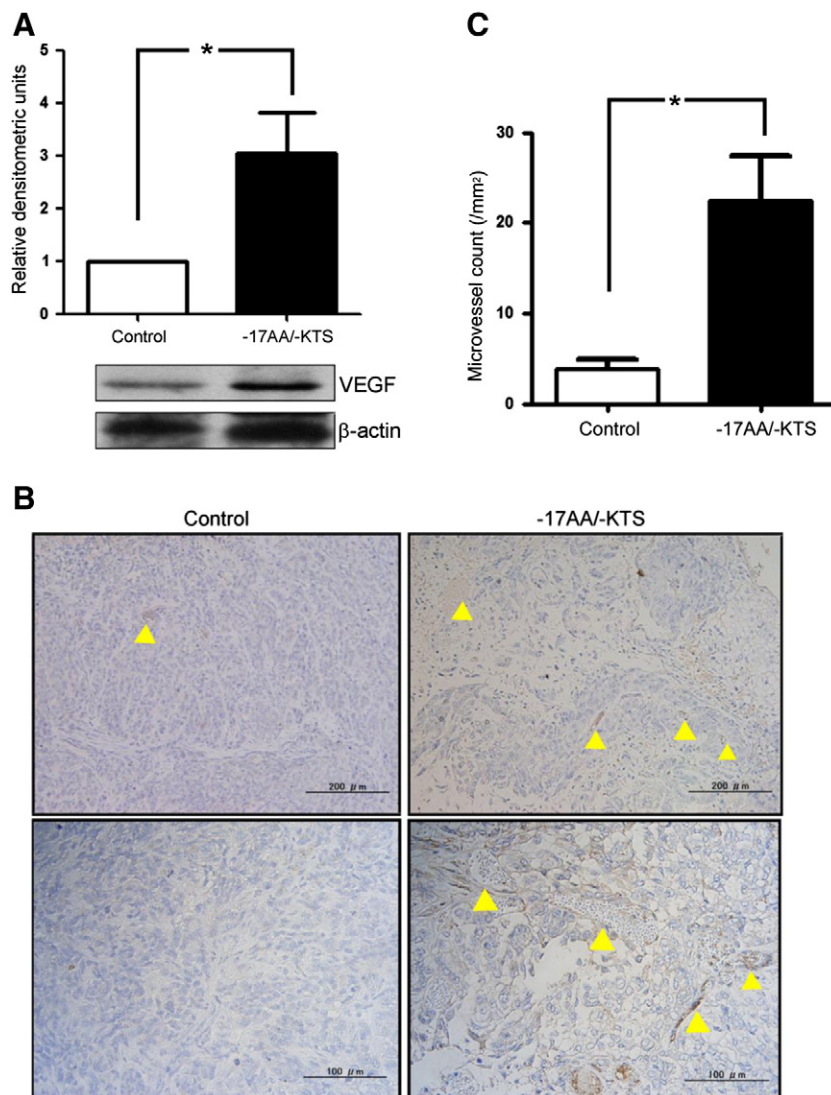


Figure 5. The effects of the WT1 -17AA/-KTS splice variant on the expression of VEGF protein and angiogenesis. (A) Intra-abdominally disseminated tumors were harvested from mice injected with cells expressing control vector or -17AA/-KTS and assessed for VEGF expression by immunoblot analysis with anti-VEGF and anti- β -actin antibodies (bottom panel). Relative densitometric units of VEGF bands are shown, with the density of control bands set arbitrarily to 1.0 (top panel). The values shown represent the mean \pm SE from at least three separate experiments. (B) Tumor tissues that had disseminated in the abdomen were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded tissue sections were incubated with anti-CD31/PECAM-1 antibodies and imaged at magnification of 200 \times (top panel) and 400 \times (bottom panel). (C) The number of CD31-immunopositive microvessels was measured in tissue sections harvested from mice injected with cells expressing control vector or WT1 -17AA/-KTS. The yellow arrow-head indicates CD31-immunopositive microvessels. The values shown represent the mean \pm SE. Significant differences are indicated by asterisks. * $P < .05$.

with a previous study demonstrating that WT1 -17AA/-KTS increases tumor growth through EGR-1 up-regulation in adenovirus-transformed baby rat kidney (AdBRK) cells *in vivo* [32]. In contrast, several studies have shown that WT1 variants act as tumor suppressors. WT1 -17AA/-KTS and +17AA/-KTS suppress the invasive ability of lung cancer cells by regulating p21 expression [34]. Moreover, WT1 -17AA/-KTS suppresses proliferation and induces a G₂-phase cell cycle arrest in mammary epithelial cells [25]. Thus, each of the four WT1 variants has distinct functions depending on the cancer type. Our data suggested that WT1 -17AA/-KTS increased tumorigenic activity and acted as an oncogene in ovarian cancers.

Several reports have demonstrated that high expression of WT1 correlates with aggressiveness and poor outcomes in various cancers

[12–15]. Additionally, while WT1 is indicative of unfavorable prognoses in patients with ovarian cancers [16], WT1 expression may be of limited prognostic value in ovarian cancers in the clinical setting [18,35]. This may be attributed to inconsistent results in analysis of the association of prognosis with the ratio of WT1 variant expression in patients with ovarian cancers. Our results showed that WT1 -17AA/-KTS shortened survival in mice in our ovarian cancer model (Figure 4). These findings indicated that the presence of WT1 -17AA/-KTS could affect the survival of mice with ovarian cancer. Therefore, the expression of WT1 -17AA/-KTS may be more important in the prognoses of patients with ovarian cancers than that of total WT1.

In addition, our data showed that WT1 -17AA/-KTS increased the expression of VEGF protein and promoted angiogenesis

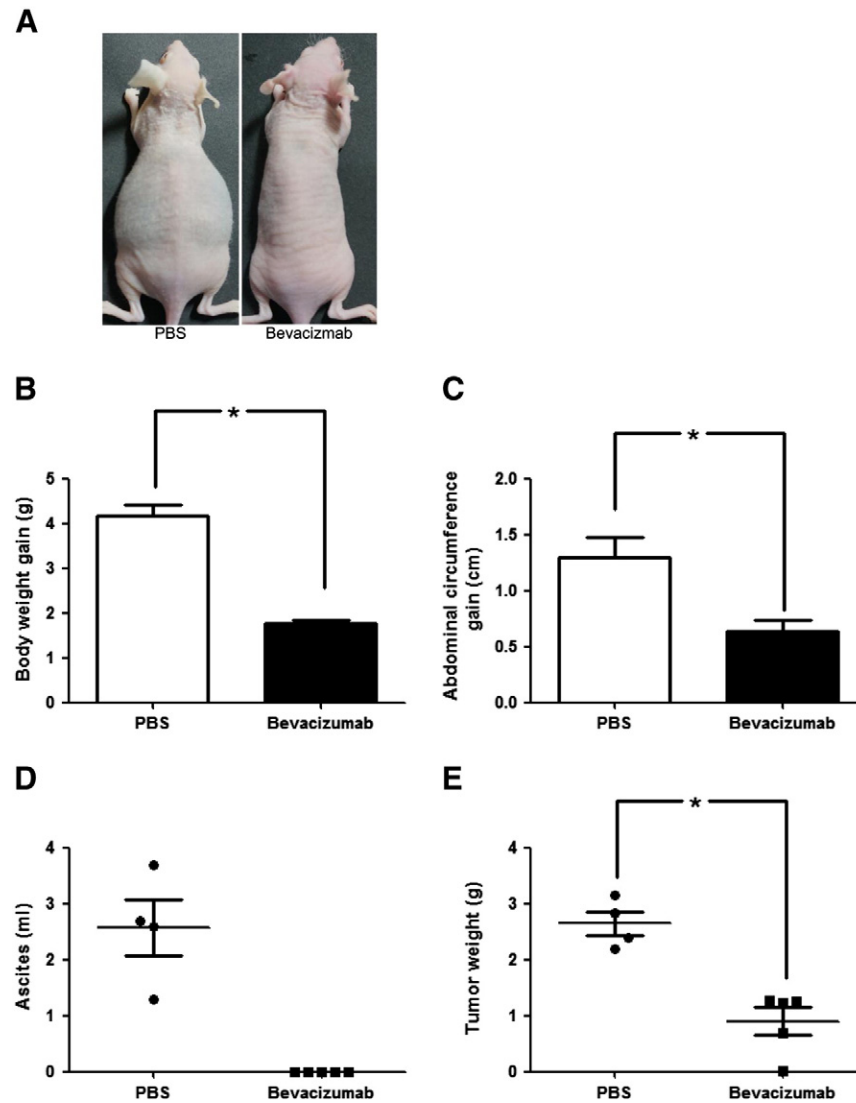


Figure 6. The effect of bevacizumab on tumor growth *in vivo*. Athymic nude mice inoculated with SKOV3ip1 cells expressing WT1 -17AA/-KTS (n = 10) were randomly placed into two groups. One group of mice (n = 5) was treated with PBS and two group of mice (n = 5) were treated with bevacizumab twice weekly for 3 weeks, as described in Materials and Methods. (A) The representative appearance of the mice at the end of the experiment. (B) Body weight gain was calculated by subtracting the body weight at the end of the experiment from that at the start in mice from the two groups. (C) Abdominal circumference gain was calculated by subtracting the abdominal circumference at the end of the experiment from that at the start in mice from two groups. (D) The volume of ascites was measured at autopsy. (E) Disseminated tumors were removed, and tumor weights were measured. The values shown represent the means \pm SEs. Significant differences are indicated by asterisks. * $P < .05$.

compared with the control vector. Inhibition of VEGF using bevacizumab attenuated the enhancing effect of WT1 -17AA/-KTS on tumorigenic activity. VEGF regulates cell proliferation and angiogenesis through the activation of PI3K/Akt and MEK/ERK signaling [36], and is associated with poor prognoses in many human cancers, including ovarian cancers [37–40]. Moreover, VEGF-targeting therapy using bevacizumab prolongs the median progression-free survival [41,42] and has an important role in current therapies for patients with advanced ovarian cancer. Our data indicated that overexpression of WT1 -17AA/-KTS could increase tumorigenic activity and shorten survival through up-regulation of VEGF expression in ovarian cancers. Therefore, WT1 -17AA/-KTS expression may be biomarker of VEGF-targeting therapy, including bevacizumab, in patients with ovarian cancer.

In summary, we concluded that the overexpression of WT1 -17AA/-KTS could enhanced tumorigenicity and could decrease survival through up-regulation of VEGF in an *in vivo* ovarian cancer model. WT1 -17AA/-KTS expression may be correlated with the poor survival of patients with ovarian cancer and may be a promising therapeutic target for ovarian cancer. Furthermore, since the present study was performed using a mouse ovarian cancer model without a true immune system, additional work is required to identify the role of WT1 splice variants in the patients with ovarian cancer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.tranon.2014.07.008>.

References

- Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, and Lewiss WH, et al (1990). Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* **60**, 509–520.
- Sugiyama H (2001). Wilms' tumor gene WT1: its oncogenic function and clinical application. *Int J Hematol* **73**, 177–187.
- Maheswaran S, Englert C, Bennett P, Heinrich G, and Haber DA (1995). The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. *Genes Dev* **9**, 2143–2156.
- Englert C, Hou X, Maheswaran S, Bennett P, Ngwu C, Re GG, Garvin AJ, Rosner MR, and Haber DA (1995). WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. *EMBO J* **14**, 4662–4675.
- Coppes MJ, Campbell CE, and Williams BR (1993). The role of WT1 in Wilms tumorigenesis. *FASEB J* **7**, 886–895.
- Shimizu M, Toki T, Takagi Y, Konishi I, and Fujii S (2000). Immunohistochemical detection of the Wilms' tumor gene (WT1) in epithelial ovarian tumors. *Int J Gynecol Pathol* **19**, 158–163.
- Bruening W, Gros P, Sato T, Stanimir J, Nakamura Y, Housman D, and Pelletier J (1993). Analysis of the 11p13 Wilms' tumor suppressor gene (WT1) in ovarian tumors. *Cancer Invest* **11**, 393–399.
- Hwang H, Quenneville L, Yaziji H, and Gown AM (2004). Wilms tumor gene product: sensitive and contextually specific marker of serous carcinomas of ovarian surface epithelial origin. *Appl Immunohistochem Mol Morphol* **12**, 122–126.
- Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka S, Yao M, Takahashi E, Nakao Y, and Hirabayashi H, et al (2002). Overexpression of the Wilms' tumor gene WT1 in de novo lung cancers. *Int J Cancer* **100**, 297–303.
- Oji Y, Yamamoto H, Nomura M, Nakano Y, Ikeba A, Nakatsuka S, Abeno S, Kiyotoh E, Jomgeow T, and Sekimoto M, et al (2003). Overexpression of the Wilms' tumor gene WT1 in colorectal adenocarcinoma. *Cancer Sci* **94**, 712–717.
- Ueda T, Oji Y, Naka N, Nakano Y, Takahashi E, Koga S, Asada M, Ikeba A, Nakatsuka S, and Abeno S, et al (2003). Overexpression of the Wilms' tumor gene WT1 in human bone and soft-tissue sarcomas. *Cancer Sci* **94**, 271–276.
- Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T, and Nasu K (1994). WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood* **84**, 3071–3079.
- Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, Sugiyama H, and Noguchi S (2002). High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. *Clin Cancer Res* **8**, 1167–1171.
- Harada Y, Nonomura N, Nishimura K, Tamaki H, Takahara S, Miki T, Sugiyama H, and Okuyama A (1999). WT1 gene expression in human testicular germ-cell tumors. *Mol Urol* **3**, 357–364.
- Oji Y, Inohara H, Nakazawa M, Nakano Y, Akahani S, Nakatsuka S, Koga S, Ikeba A, Abeno S, and Honjo Y, et al (2003). Overexpression of the Wilms' tumor gene WT1 in head and neck squamous cell carcinoma. *Cancer Sci* **94**, 523–529.
- Kobel M, Kalloger SE, Boyd N, McKinney S, Mehl E, Palmer C, Leung S, Bowen NJ, Ionescu DN, and Raiput A, et al (2008). Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. *PLoS Med* **5**, e232.
- Netinatsunthorn W, Hanprasertpong J, Dechsukhum C, Leetanaporn R, and Geater A (2006). WT1 gene expression as a prognostic marker in advanced serous epithelial ovarian carcinoma: an immunohistochemical study. *BMC Cancer* **6**, 90.
- Hylander B, Repasky E, Shrikant P, Intengan M, Beck A, Driscoll D, Singhal P, Lele S, and Odunsi K (2006). Expression of Wilms tumor gene (WT1) in epithelial ovarian cancer. *Gynecol Oncol* **101**, 12–17.
- Hogdall EV, Christensen L, Kjaer SK, Blaakaer J, Kjaerbye-Thygesen A, Gayther S, Jacobs IJ, and Hogdall CK (2007). CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study. *Gynecol Oncol* **104**, 508–515.
- Jomgeow T, Oji Y, Tsuji N, Ikeda Y, Ito K, Tsuda A, Nakazawa T, Tatsumi N, Sakaguchi N, and Takashima S, et al (2006). Wilms' tumor gene WT1 17AA(-)/KTS(-) isoform induces morphological changes and promotes cell migration and invasion in vitro. *Cancer Sci* **97**, 259–270.
- Davies RC, Calvio C, Bratt E, Larsson SH, Lamond AI, and Hastie ND (1998). WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. *Genes Dev* **12**, 3217–3225.
- Bickmore WA, Oghene K, Little MH, Seawright A, van Heyningen V, and Hastie ND (1992). Modulation of DNA binding specificity by alternative splicing of the Wilms tumor wt1 gene transcript. *Science* **257**, 235–237.
- Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC, Weissman BE, and Baldwin AS (1999). WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. *EMBO J* **18**, 3990–4003.
- Haber DA, Englert C, and Maheswaran S (1996). Functional properties of WT1. *Med Pediatr Oncol* **27**, 453–455.
- Burwell EA, McCarty GP, Simpson LA, Thompson KA, and Loeb DM (2007). Isoforms of Wilms' tumor suppressor gene (WT1) have distinct effects on mammary epithelial cells. *Oncogene* **26**, 3423–3430.
- Kramarzova K, Stuchly J, Willasch A, Gruhn B, Schwarz J, Cermak J, Machova-Polakova K, Fuchs O, Stary J, and Trka J, et al (2012). Real-time PCR quantification of major Wilms' tumor gene 1 (WT1) isoforms in acute myeloid leukemia, their characteristic expression patterns and possible functional consequences. *Leukemia* **26**, 2086–2095.
- Yu D, Wolf JK, Scanlon M, Price JE, and Hung MC (1993). Enhanced c-erbB-2/neu expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Cancer Res* **53**, 891–898.
- Palmowski MJ, Lopes L, Ikeda Y, Salio M, Cerundolo V, and Collins MK (2004). Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. *J Immunol* **172**, 1582–1587.
- Zufferey R, Nagy D, Mandel RJ, Naldini L, and Trono D (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* **15**, 871–875.
- Yoshioka S, King ML, Ran S, Okuda H, MacLean II JA, McAsey ME, Sugino N, Brard L, Watabe K, and Hayashi K (2012). WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/β-Catenin pathway. *Mol Cancer Res* **10**, 469–482.
- Baudry D, Faussillon M, Cabanis MO, Rigolet M, Zucker JM, Patte C, Sarnacki S, Boccon-Gibod L, Junien C, and Jeanpierre C (2002). Changes in WT1 splicing are associated with a specific gene expression profile in Wilms' tumour. *Oncogene* **21**, 5566–5573.
- Scharnhorst V, Menke AL, Attema J, Haneveld JK, Riteco N, van Steenbrugge GJ, van der Eb AJ, and Jochemsen AG (2000). EGR-1 enhances tumor growth and modulates the effect of the Wilms' tumor 1 gene products on tumorigenicity. *Oncogene* **19**, 791–800.
- Haber DA, Buckler AJ, Glaser T, Call KM, Pelletier J, Sohn RL, Douglass EC, and Housman DE (1990). An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. *Cell* **61**, 1257–1269.
- Moriya S, Takiguchi M, and Seki N (2008). Expression of the WT1 gene-KTS domain isoforms suppresses the invasive ability of human lung squamous cell carcinoma cells. *Int J Oncol* **32**, 349–356.
- Hogdall EV, Christensen L, Kjaer SK, Blaakaer J, Kjaerbye-Thygesen A, Gayther S, Jacobs IJ, and Hogdall CK (2007). Expression level of Wilms tumor 1 (WT1) protein has limited prognostic value in epithelial ovarian cancer: from the Danish "MALOVA" ovarian cancer study. *Gynecol Oncol* **106**, 318–324.
- Jin J, Yuan F, Shen MQ, Feng YF, and He QL (2013). Vascular endothelial growth factor regulates primate choroid-retinal endothelial cell proliferation and tube formation through PI3K/Akt and MEK/ERK dependent signaling. *Mol Cell Biochem* **381**, 267–272.
- Li X, Liu B, Xiao J, Yuan Y, Ma J, and Zhang Y (2011). Roles of VEGF-C and Smad4 in the lymphangiogenesis, lymphatic metastasis, and prognosis in colon cancer. *J Gastrointest Surg* **15**, 2001–2010.
- Nakamura Y, Yasuoka H, Tsujimoto M, Imabun S, Nakahara M, Nakao K, Nakamura M, Mori I, and Kakubo K (2005). Lymph vessel density correlates with nodal status, VEGF-C expression, and prognosis in breast cancer. *Breast Cancer Res Treat* **91**, 125–132.

- [39] Gou HF, Chen XC, Zhu J, Jiang M, Yang Y, Cao D, and Hou M (2011). Expressions of COX-2 and VEGF-C in gastric cancer: correlations with lymphangiogenesis and prognostic implications. *J Exp Clin Cancer Res* **30**, 14.
- [40] Guo X, Chen Y, Xu Z, Xu Z, Qian Y, and Yu X (2009). Prognostic significance of VEGF-C expression in correlation with COX-2, lymphatic microvessel density, and clinicopathologic characteristics in human non-small cell lung cancer. *Acta Biochim Biophys Sin (Shanghai)* **41**, 217–222.
- [41] Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, Mannel RS, Homesley HD, Fowler J, and Greer BE, et al (2011). Incorporation of bevacizumab in the primary treatment of ovarian cancer. *N Engl J Med* **365**, 2473–2483.
- [42] Perren TJ, Swart AM, Pfisterer J, Ledermann JA, Pujade-Lauraine E, Kristensen G, Carey MS, Beale P, Cervantes A, and Kurzeder C, et al (2011). A phase 3 trial of bevacizumab in ovarian cancer. **365**, 2484–2496.