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FBXW7 is involved in the acquisition of the malignant phenotype in epithelial ovarian tumors

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FBXW7, methylation, mutation, ovarian cancer, p53

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FBXW7 is a ubiquitin ligase that mediates ubiquitylation of oncoproteins, such as c-Myc, cyclin E, Notch and c-Jun. *FBXW7* is a known tumor-suppressor gene, and mutations in *FBXW7* have been reported in various human malignancies. In this study, we examined the sequences of the *FBXW7* and *p53* genes in 57 ovarian cancer clinical samples. Interestingly, we found no *FBXW7* mutations associated with amino acid changes. We also investigated *FBXW7* expression levels in 126 epithelial ovarian tumors. *FBXW7* expression was negatively correlated with the malignant potential of ovarian tumors. That is to say, *FBXW7* expression levels in ovarian cancer samples were significantly lower than those in borderline and benign tumors ($P < 0.01$). *FBXW7* expression levels in serous carcinoma samples were the lowest among four major histological subtypes. In addition, *p53*-mutated ovarian cancer samples showed significantly lower levels of *FBXW7* expression compared with *p53* wild-type cancer samples ($P < 0.001$). DNA methylation arrays and bisulfite PCR sequencing experiments revealed that 5′-upstream regions of *FBXW7* gene in *p53*-mutated samples were significantly higher methylated compared with those in *p53* wild-type samples ($P < 0.01$). This data indicates that *p53* mutations might suppress *FBXW7* expression through DNA hypermethylation of *FBXW7* 5′-upstream regions. Thus, *FBXW7* expression was downregulated in ovarian cancers, and was associated with *p53* mutations and the DNA methylation status of the 5′-upstream regions of *FBXW7*.

In 2008, an estimated 225 500 women were diagnosed with ovarian cancer and 140 200 women died from this disease worldwide.[1] In that year, ovarian cancer was the eighth most common type of cancer and the seventh most common cause of cancer-related death among women. In Japan, the number of deaths due to ovarian cancer has grown from 4006 in 1996, to 4435 in 2006, and to 4705 in 2011, making ovarian cancer the most lethal gynecological cancer.[2]

The F-box protein FBXW7 (also known as Archipelago, hAGO, hCDC4) is a substrate-recognition subunit of an SCF ubiquitin ligase complex. It interacts with substrates undergoing ubiquitylation and mediates the process. Substrates of FBXW7, such as c-Myc, cyclin E, Aurora A, Notch and c-Jun, are positive regulators of the cell cycle. Therefore, the *FBXW7* gene is considered to be a tumor-suppressor gene.[3–6] Mao and colleagues reported that murine *Fbxw7* was a *p53*-dependent haplo-insufficient tumor suppressor gene and that dysfunction of both *p53* and *Fbxw7* contributed to carcinogenesis.[7] Fbxw7 induces proliferating cells to exit from the cell cycle by triggering the degradation of c-Myc. Thus, inactivation of Fbxw7 sustains continuous cell cycling (essential for carcinogenesis). This abnormal cell-cycling is censored by checkpoint activation and eventually restrained by p53 activation. Thus, if both *p53* and *Fbxw7* are dysfunctional, cancer can develop. Indeed, T-cell lymphoma develops in T cell-specific *Fbxw7* knockout mice, and T-cell acute lymphoblastic lymphoma develops in bone marrow-specific *Fbxw7* knockout mice. *p53* inactivation in *Fbxw7* knockout mice promotes the onset of intestinal cancers in addition to lymphomas.[8–10]

Mutations in the *FBXW7* gene have been reported in many human malignancies, and the frequency of *FBXW7* mutations in human cancers has been estimated to be approximately 6%.[11] For example, *FBXW7* mutation rates in cholangiocarcinoma, T-cell acute lymphocytic leukemia and endometrial carcinoma were reported to be 35%, 31% and 16%, respectively.[11–13] However, *FBXW7* mutations are infrequent in ovarian cancer.[14,15]

The *FBXW7* gene encodes three transcripts (*FBXW7*α, −β and −γ) that are produced by alternative splicing. Each mRNA consists of an isoform-specific first exon linked to 10 shared exons, generating three protein isoforms that differ only at their N termini.[3] This genomic organization is highly conserved in mammals. Each isoform occupies a distinct subcellular location. FBXW7α is found in the nucleoplasm, FBXW7β is localized to the cytoplasmic membrane and FBXW7γ is found in the nucleolus.[16,17] *Fbxw7*α is expressed at much higher levels than *Fbxw7*β or *Fbxw7*γ. *Fbxw7*α is ubiquitously

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expressed at high levels, whereas *Fbxw7*β expression is detected at high levels in the brain, and *Fbxw7*γ expression is limited to cardiac muscles and skeletal muscles. Each of the three isoforms is thought to have its own promoter and to be under isoform-specific transcriptional control. Although *Fbxw7*β is reported to be a transcriptional target of p53,[18] the mechanisms regulating *Fbxw7*α/γ expression remain uncharacterized.

Low expression of *FBXW7* is associated with clinicopathological background and prognosis in gastric cancer, colorectal cancer, breast cancer and glioma.[19–22] The mechanisms that regulate *FBXW7* expression in cancers are unclear. However, one study has demonstrated that the methylation status of the *FBXW7*β promoter is inversely correlated with the expression level of *FBXW7* in breast cancer.[23] In addition, some reports have suggested that microRNA regulate *FBXW7* transcript expression in colorectal cancer, esophageal cancer and gastric cancer.[24–26]

In the present study, we examined *FBXW7*(α) mutations in clinical samples of ovarian cancer (n = 57) and gene expression in ovarian tumor clinical samples (n = 126). Mutations of *FBXW7* were rare in ovarian cancers and *FBXW7* expression levels in ovarian cancers were significantly lower than those in borderline and benign tumors. We also investigated the correlation between *p53* mutation status and *FBXW7* expression. *FBXW7* expression was significantly lower in the *p53* mutation group than that in the *p53* wild-type group. In addition, we analyzed the methylation status of the 5′-upstream regions of *FBXW7*. DNA methylation arrays and bisulfite sequencing revealed the hypermethylation of *FBXW7* 5′-upstream regions in *p53*-mutated ovarian cancer samples. Thus, the *FBXW7* expression level would be affected by *p53* mutations through promoter hypermethylation, which might contribute to the acquisition of the malignant phenotype in ovarian tumors.

Materials and Methods

Ovarian cancer tissues. Ovarian tumor specimens from 126 female patients who were treated at Kyushu University Hospital between 2003 and 2010 were included in the present study. Tumors were histologically characterized as serous (benign, 6; borderline malignancy, 9; carcinoma, 26), mucinous (benign, 11; borderline malignancy, 16; carcinoma, 15), clear cell (borderline malignancy, 1; carcinoma, 25), or endometrioid (carcinoma, 17). The median age of the patients was 55 years old (range 22–79). Patients who had undergone neoadjuvant chemotherapy were excluded from the study. Informed consent was obtained from all patients prior to enrollment in the study. The ethics committee of Kyushu University Graduate School approved the study protocol.

Resected tumor tissues were immediately cut, frozen in liquid nitrogen, and kept at −80°C until RNA and DNA extraction. Total RNA was extracted from tissue specimens using an ISOGEN Kit (NIPPON GENE, Tokyo, Japan). Total RNA (1 μg) was reverse transcribed to cDNA using ReverTra Ace (Toyobo, Osaka, Japan), according to the manufacturer's protocol. Genomic DNA was extracted from frozen specimens using standard phenol/chloroform methods.

Mutation analysis. The *FBXW7*α sequence was amplified using cDNA and sequencing primers (Table S1). PCR was carried out with PrimeSTAR HS DNA Polymerase (Takara Bio, Shiga, Japan). Likewise, genomic DNA samples were used as templates to PCR amplify exons 4–9 of the *p53* gene with primers derived from intronic sequences (Table S1). Thermal cycling parameters were as follows: initialization for 5 min at 98°C followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 10 s, and elongation at 72°C for 1 min. These PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and purified with an Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Purified PCR products were sequenced using a Big-Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and an ABI3130xl sequencer (Applied Biosystems, Foster City, CA, USA).

Real-time quantitative reverse transcription–PCR (qRT-PCR). Real-time qPCR was performed using an Applied Biosystems 7500 Real-Time PCR System in a 20-μL reaction volume with SYBR Premix Ex Taq (Takara Bio). Each reaction was carried out under the following conditions: initialization for 30 s at 95°C followed by 40 cycles of 5 s at 95°C for denaturation and 34 s at 60°C for annealing and elongation. The expression of *FBXW7* mRNA is presented as the relative copy number normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The PCR primer sequences were as follows: *FBXW7*α, forward 5′-TTCACCAACTCTCCTCCC-CATT-3′ and reverse 5′-GCTGAACATGGTACAAGCCCA-3′, *GAPDH*, forward 5′-GCAAATTCCATGGCACCGT-3′ and reverse 5′-TCGCCCCACTTGATTTTGG-3′.

DNA methylation array. We performed DNA methylation array analysis to evaluate the methylation status of ovarian cancer specimens with Infinium HumanMethylation450 BeadChips (Illumina, San Diego, CA, USA) following by an Illumina Infinium HD MethylationAssay, according to the manufacturer's instructions. The gDNA (1500 ng) was bisulfite-converted using an Epitect Plus DNA Bisulfite Kit (Qiagen, Hilden, Germany) for use in an Infinium HumanMethylation450 assay. Bisulfite-converted DNA (300 ng) was used in the whole-genome amplification reaction. After amplification, the DNA was fragmented enzymatically, precipitated and re-suspended in hybridization buffer. Fragmented DNA was dispensed onto a HumanMethylation450 BeadChip, and hybridization was performed in a hybridization oven for 20 h. After hybridization, the array was processed through the primer detection step of a single-base extension reaction. Finally, BeadChips were coated and then imaged on an Illumina iScan. The methylation level of each CpG locus was calculated using GenomeStudio Methylation Module software version 1.0. (Illumina, San Diego, CA, USA) in which the methylation β-value (β-value = intensity of the methylated allele (M)/intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100) ranged from zero in the case of completely unmethylated loci to one in the case of complete methylation.

Bisulfite sequencing. Bisulfite PCR was carried out with EX Taq Polymerase (Takara Bio). Thermal cycling parameters were as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 2 min. Primer sets for bisulfite PCR were as follows: bi*FBXW7* forward 5′-TGTTGTAGAG-TAGGGGTTTATAAT-3′ and bi*FBXW7* reverse 5′-CCAAAA ACCATTTTTATAAAAAACAAT-3′. The PCR products were ligated into plasmids using a StrataClone PCR Cloning Kit (Stratagene, La Jolla, CA, USA), which were then transformed into competent bacteria. More than 10 individual clones were isolated and amplified with the Illustra Templiphi Amplification Kit (GE Healthcare). Templiphi products were sequenced using a Big-Dye Terminator version 3.1 Cycle Sequencing Kit

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(Applied Biosystems) and an ABI3130 Genetic sequencer (Applied Biosystems). At least 10 clones from each parental allele were sequenced. Sequence data were analyzed using the QUMA quantification tool (http://quma.cdb.riken.jp/) for methylation analysis.

Cell lines. The human ovarian cancer cell line SHIN-3 was purchased from Scienstaff (Nara, Japan). OVISE was purchased from JCRB Cell Bank (Osaka, Japan). The cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS GOLD (PAA, Pasching, Austria) with 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco, Palo Alto, CA, USA) in 5% CO_2 at 37°C.

Plasmids. pCMV-Neo-Bam p53 wt and pCMV-Neo-Bam p53 R175H were gifts from Bert Vogelstein (Addgene plasmid # 16434).[27] These plasmids were introduced into SHIN-3 and OVISE cells with the use of Lipofectamine 2000 transfection reagents (Invitrogen, Carlsbad, CA, USA).

Protein extraction and Western blotting. The cells were washed in ice-cold PBS and lysed using CelLytic M (Sigma) following the manufacturer's instructions. Total protein (30 μg) was electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Immobilon; Merck Millipore, Darmstadt, Germany). The membranes were blocked and incubated overnight at 4°C with primary antibodies targeting p53 (FL-393, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (#4967, 1:1000; Cell Signaling, Danvers, MA, USA). After washing, membranes were incubated for 1 h with anti-rabbit antibody (Sigma) diluted 1:4000. Specific protein bands were detected using the SuperSignal West Pico/Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis. For analysis of differences in two or more groups, Student's *t*-test, χ^2 analysis and ANOVA were used. When the results of ANOVA were significant, the Tukey–Kramer method was used. Overall survival curves were plotted according to the Kaplan–Meier method, with Wilcoxon analysis applied for comparison. Survival was measured from the day of the surgery. All differences were accepted as statistically significant at the level of $P < 0.05$. Statistical analysis was performed using JMP 9.0.2 (SAS Institute, Tokyo, Japan). Each *P*-value in the figure is indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

***FBXW7* mutations were rare in ovarian cancer.** According to previous reports, FBXW7α is ubiquitously expressed, whereas FBXW7β is expressed in the brain, and FBXW7γ is expressed in muscles.[16] Therefore, we focused on *FBXW7*α expression and mutational status in this study. First, we examined *FBXW7* and *p53* mutations in clinical samples of 57 ovarian cancer patients (26 serous carcinomas, 11 mucinous carcinomas, 10 endometrioid carcinomas and 10 clear cell carcinomas). The results are shown in Figure 1 and Table S2. We only detected a single (silent) mutation in *FBXW7*. This result was consistent with two previous reports demonstrating that *FBXW7* mutations are rare in ovarian cancer.[14,15] In contrast, *p53* mutations were detected in 31.6% of the samples (18/57). In particular, serous carcinomas showed a high frequency of *p53* mutations (16/26, 61.5%). It has been well established that *p53* mutations are frequent in high-grade serous carcinoma.[28,29]

***FBXW7* gene expression was low in serous carcinoma.** The results of mutational analyses demonstrated that mutations in

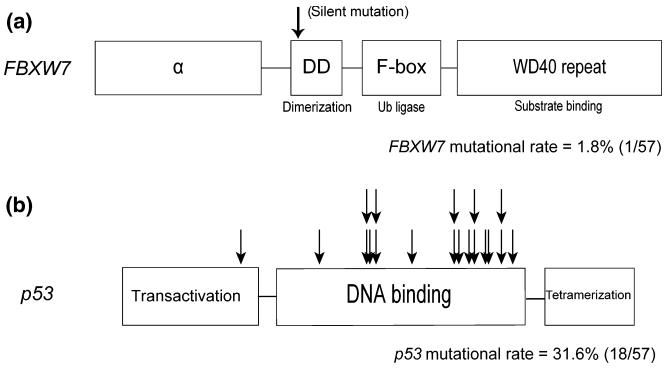

Fig. 1. *FBXW7* and *p53* mutations in 57 ovarian cancer clinical samples. Arrows designate points of mutation. (a) Structure and the observed mutation in the *FBXW7* gene in ovarian cancer patients. (b) Structure and the observed mutations in the *p53* gene in ovarian cancer patients.

FBXW7 did not contribute to the malignant potential of ovarian cancer. Therefore, we next examined *FBXW7* expression levels in 126 epithelial ovarian tumors (see Materials and Methods for pathological classification). The results are shown in Figure 2. *FBXW7* gene expression levels in ovarian cancer samples were significantly lower than those in borderline and benign tumors ($P < 0.01$; Fig. 2a), indicating that *FBXW7* expression levels were negatively correlated with malignant potential. Next, we examined *FBXW7* expression in different histological subtypes of ovarian cancer. *FBXW7* expression was the lowest in serous carcinomas, and increased in the following order: endometrioid carcinomas, clear cell carcinomas and mucinous carcinomas. In comparison to the average *FBXW7* gene expression levels of benign tumors, those in endometrioid carcinomas and serous carcinomas were significantly reduced ($P < 0.01$ and $P < 0.001$, respectively). Moreover, the expression levels of *FBXW7* in serous carcinomas were significantly lower than those in mucinous carcinomas and clear cell carcinomas ($P < 0.05$; Fig. 2b). In serous ovarian tumors, *FBXW7* expression in serous carcinomas was significantly lower than that in serous cystadenomas ($P < 0.01$; Fig. S1a). However, *FBXW7* expression levels in mucinous ovarian tumors did not significantly differ among benign, borderline and cancerous tumors (Fig. S1b). We could not compare the expression of *FBXW7* of benign, borderline and cancerous tumors in clear cell and endometrioid tumors because clear cell and endometrioid benign/borderline tumors were rare.

We also analyzed the clinicopathological features of ovarian cancer patients according to *FBXW7* expression (Table 1). We divided cancer patients into two groups (high or low expression) according to *FBXW7* expression levels. The high and low expression groups were defined according to an arbitrary value (i.e. patients were classified into the high expression group if they had higher *FBXW7* expression than this value and into the low expression group if they had lower *FBXW7* expression than this value). A total of 30 patients were included in the high expression group and 53 patients were included in the low expression group. Among clinicopathological features (menstruation status, clinical stage, lymph node metastasis, and histology) only histological subtype was significantly associated with *FBXW7* expression. That is to say, the *FBXW7* expression level in serous carcinoma was significantly suppressed. We thought that it might be due to the high frequency of *p53* mutations in serous

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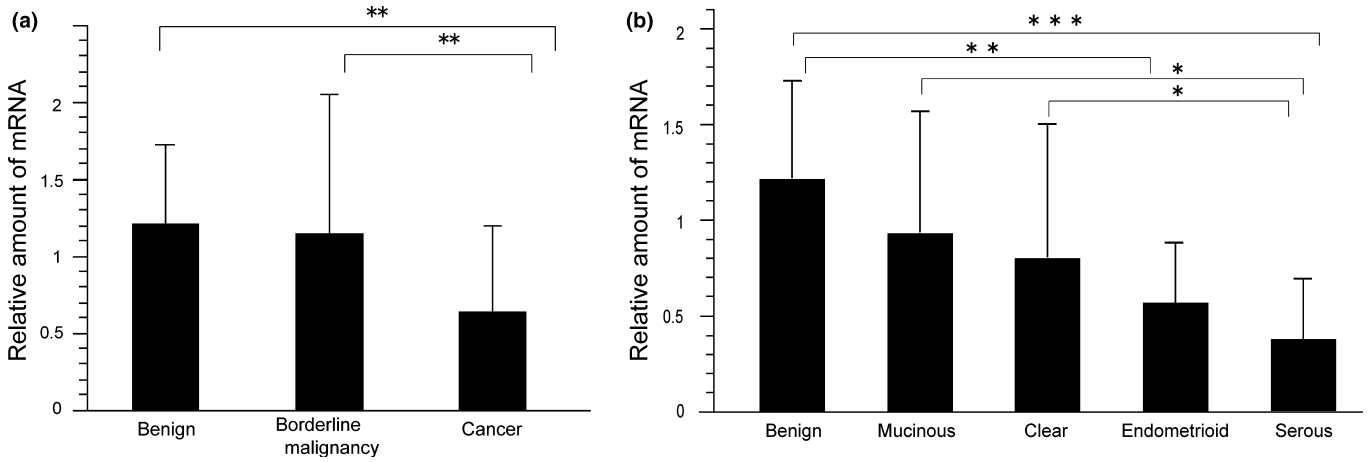

Fig. 2. *FBXW7* expression levels in 126 epithelial ovarian tumors. The data show the mean ± SD of values. *P*-values in the figures are indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (a) *FBXW7* mRNA expression levels in 126 epithelial ovarian tumors. (b) *FBXW7* mRNA expression levels in 17 epithelial ovarian benign tumors, 15 mucinous carcinomas, 25 clear cell carcinomas, 17 endometrioid carcinomas and 26 serous carcinomas.

Table 1. Clinicopathological features of ovarian cancer patients

	High expression (n = 30)	Low expression (n = 53)	*P*-value
Age (mean ± SD)	55.1 ± 10.6	55.5 ± 15.2	0.5538
Menstruation			
Premenopause	13	16	0.4486
Postmenopouse	14	32	
Unknown	3	5	
Clinical stage			
I	22	28	0.0536
II, III, IV	8	25	
Lymph node metastasis			
(+)	4	13	0.1486
(–)	23	39	
NX	3	1	
Histology			
Serous carcinoma	2	24	0.0005***
Clear cellcarcinoma	11	14	
Endometrioid carcinoma	7	10	
Mucinous carcinoma	10	5	

The criterion for validating high or low *FBXW7* expression groups was determined as greater or less than an arbitrary value.

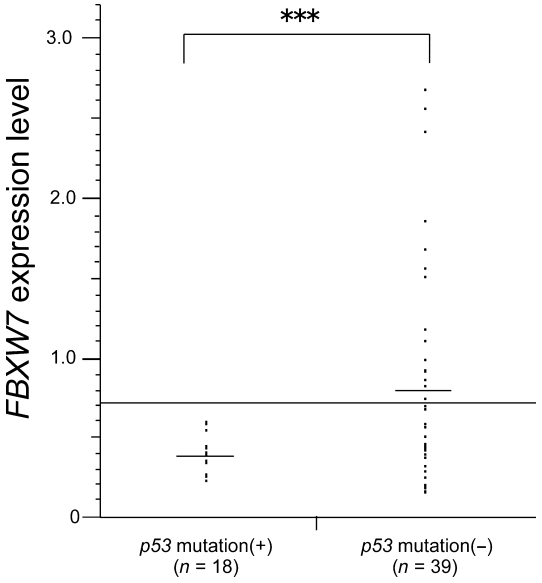

Fig. 3. Dot plot of *FBXW7* mRNA expression levels in ovarian cancer according to *p53* status. The transverse line represents the baseline value of *Fbxw7* expression dividing high and low expression groups. *P*-values are indicated ($***P < 0.001$).

carcinoma because *p53* influenced *FBXW7* expression, as described later.

***FBXW7* expression in the mutant *p53* group was significantly lower than that in the wild-type *p53* group.** Given that *Fbxw7* is a *p53*-dependent tumor-suppressor gene,[7] we examined the correlation between *FBXW7* expression levels and *p53* mutation status. A total of 57 ovarian cancer samples were divided according to *p53* status, and *FBXW7* expression levels were analyzed (Fig. 3). All samples with *p53* mutations were included in the low *FBXW7* expression group, and the *FBXW7* expression level in the *p53* mutation group was significantly lower than that in the *p53* wild-type group. However, there was no significant difference in overall survival between the two groups (Fig. S2).

***p53* mutations suppressed *FBXW7* expression by hypermethylation of the 5′-upstream region of *FBXW7*.** We found that the *FBXW7* expression levels in *p53*-mutated ovarian cancer samples were significantly lower than those in *p53* wild-type samples (Fig. 3). To identify the underlying mechanisms how *p53* alterations influenced *FBXW7* expression levels, we investigated DNA methylation status of 5′-upstream regions of *FBXW7*, because promoter hypermethylation of tumor-suppressor genes is one of the important causes of oncogenesis.[30,31]

To this end, we screened DNA methylation status of six samples using Infinium HumanMethylation450 BeadsChips. Among 25 probes surrounding the *FBXW7* gene, two probes at chr4, 153437913 (hg19) and 153454027 (hg19), both located

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in the 5′-upstream regions of *FBXW7* gene (Fig. 4a), were hypomethylated in the *FBXW7* high expression group and hypermethylated in the *FBXW7* low expression group. According to these results, we designed a primer set around the probe 153437913 (hg19) (see the Materials and Methods for the primer sequence) for bisulfite PCR sequencing experiments, and analyzed 21 ovarian cancer samples (13-*p53* wild-type samples and 8-*p53*-mutated samples). As shown in Figure 4(b,c), bisulfite PCR sequencing revealed that this region was significantly hypermethylated in *p53*-mutated samples compared with that in *p53* wild-type samples ($P = 0.006$). These data suggested that *p53* mutations suppressed *FBXW7* expression by hypermethylation of *FBXW7* 5′-upstream region in ovarian cancer.

Mutated p53 suppressed *FBXW7* expression in ovarian cancer cell. Finally, we examined whether forced expression of mutated p53 would affect *FBXW7* expression level in ovarian cancer cells. We introduced wild-type p53 or mutated p53 (R175H) into two types of cell lines, OVISE (with wild-type *p53* and wild-type *FBXW7*) and SHIN-3 (with mutated *p53* and wild-type *FBXW7*), and then carried out quantitative RT-PCR of *FBXW7* (Fig. 5). *FBXW7* expression was significantly suppressed by overexpression of p53 (R175H) compared with wild-type p53 overexpression in OVISE, whereas *FBXW7* expression was not suppressed by overexpression of p53 (R175H) in SHIN-3, in which cells *p53* was already mutated.

Discussion

In the present study, we found that the mRNA level of *FBXW7* was significantly suppressed in ovarian cancer compared with benign and borderline tumors. *FBXW7* gene expression varied according to the cancer subtype. Among the four major histopathological subtypes (serous, mucinous, endometrioid and clear cell carcinoma), the greatest decrease was observed in serous carcinoma. *FBXW7* was downregulated and the 5′-upstream regions of *FBXW7* was hypermethylated in *p53* mutational group. In ovarian cancer cells, overexpression of mutated p53 (R175H) suppressed *FBXW7* expression.

Functional loss of FBXW7 has been reported in many human cancers.[11–13,19–22] Two mechanisms suppressing FBXW7 function are known: *FBXW7* mutations and transcriptional suppression due to loss of p53. First, we examined *FBXW7* mutations because such mutations have been detected in many human malignancies. Moreover, whole exome sequence approaches recently identified *FBXW7* mutations in head and neck squamous cell carcinoma and uterine serous

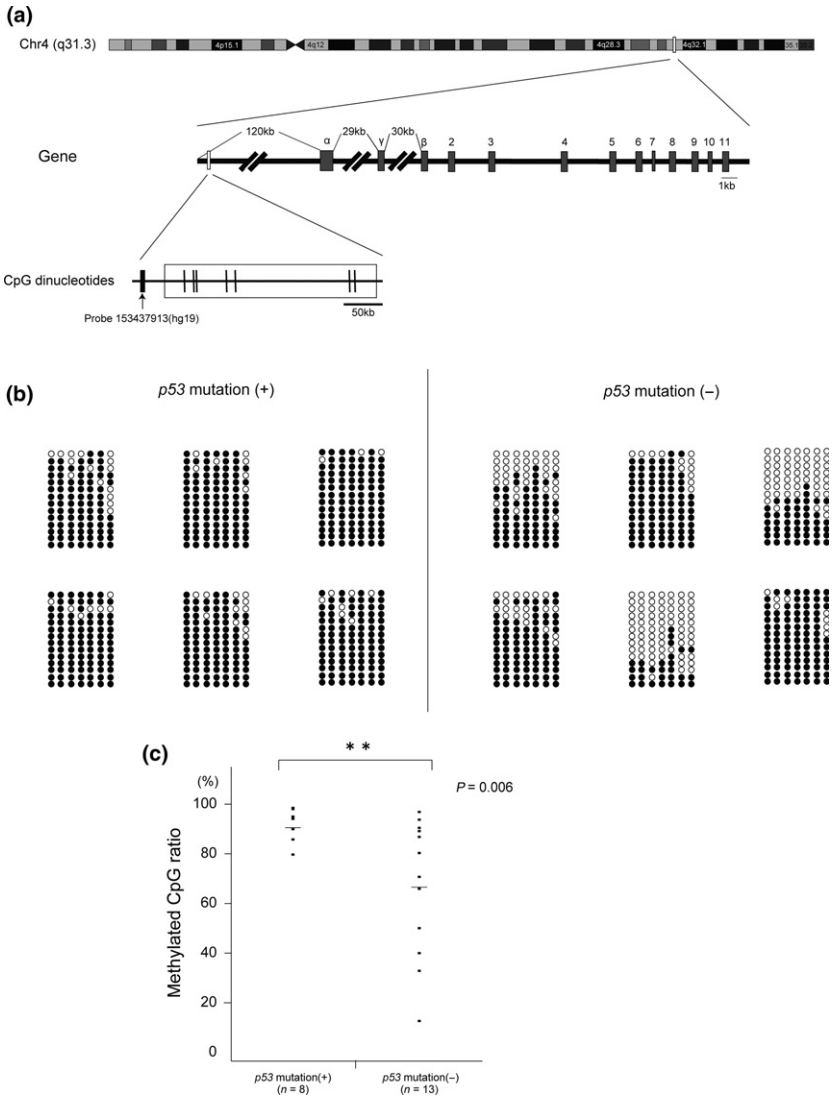

Fig. 4. Hypermethylation of the 5′-upstream regions of *FBXW7* in *p53*-mutated samples. The methylation status of seven CpG sites around the probe 153437913(hg19) was analyzed by bisulfite sequencing in 21 ovarian cancer samples (i.e. 8 *p53*-mutated samples and 13 *p53*-wild type samples). (a) Schematic map around the probe 153437913 (hg19). Vertical short lines represent CpG sites. (b) Bisulfite sequencing profiles of representative 12 samples. The circles correspond to CpG sites denoted by thin bars in Figure 5(a). Closed circles represent methylated CpG and open circles represent unmethylated CpG. (c) Dot plot of methylated CpG ratio of the 5′-upstream regions of *FBXW7* according to *p53* status.

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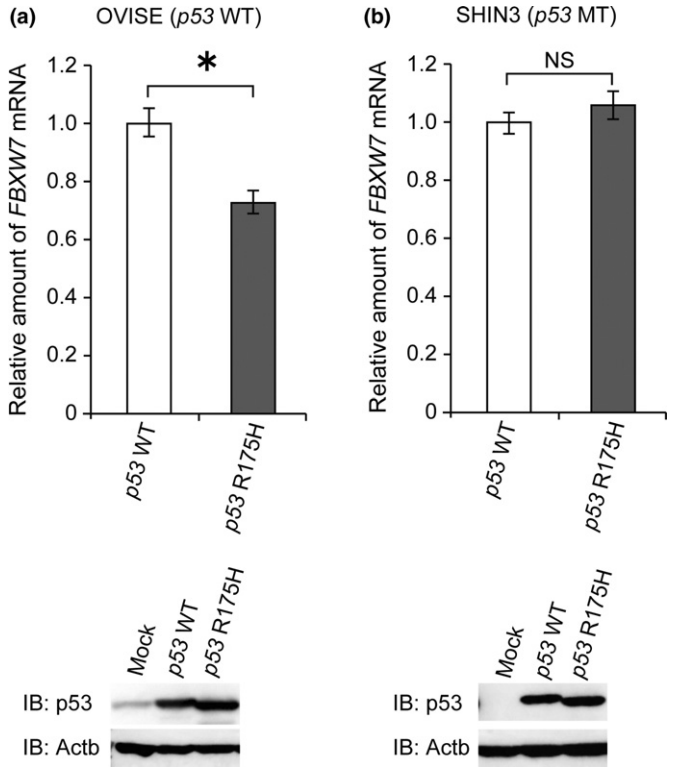

Fig. 5. Mutated p53 suppressed *FBXW7* expression in ovarian cancer cell. (a) Wild-type p53 or mutated p53 (R175H) was introduced in OVISE (wild-type *p53* and *FBXW7*). *FBXW7* mRNA expression level was suppressed by overexpression of mutated p53 compared with wild-type p53 overexpression. The data shows means ± SD. The experiments were carried out three times. *P*-values are indicated (**P* < 0.05). (b) Wild-type or mutated p53 (R175H) was introduced in SHIN-3 (mutated *p53* and wild-type *FBXW7*). *FBXW7* mRNA expression level was not affected by overexpression of wild-type or mutated p53. The data shows means ± SD. The experiments were carried out three times.

carcinoma.[32–34] However, in the present study, we detected only one silent *FBXW7* mutation in the ovarian cancer tissues. This result is consistent with those of previous reports demonstrating that *FBXW7* mutations are infrequent in ovarian cancer.[14,15]

Next we examined mRNA levels in ovarian cancer tissues, and found that *FBXW7* gene expression was significantly suppressed in ovarian cancer specimens compared with borderline and benign ovarian tumors. These data indicated that *FBXW7* expression was negatively correlated with the malignant potential of ovarian tumors. When we analyzed the *FBXW7* expression data in light of *p53* status, all *p53* mutations were found in the low *FBXW7* expression group. Consistent with this result, *FBXW7* expression was significantly suppressed in the presence of *p53* mutations. These data are consistent with the previous report that *FBXW7* expression levels were low in most gastric cancer samples with *p53* mutations.[19] These results strongly support the notion that p53 regulates the expression of *FBXW7*.

Among the four major subtypes of ovarian cancers *FBXW7* expression was most suppressed in serous carcinomas. It is widely known that each histological subtype is associated with distinct morphologic and molecular genetic alterations. High-grade serous carcinomas are considered to arise from the epithelium of the distal fallopian tube and/or ovary with *p53* mutations and dysfunction of *BRCA1* and *BRCA2*.[28,29] In this report, we used the term "serous carcinoma" to indicate high-grade serous carcinoma. High-grade serous carcinomas are thought to represent *de novo* cancer, while mucinous carcinomas arise via an adenoma-borderline tumor-carcinoma sequence. The differences in the molecular events occurring during oncogenesis might be related to the distinct *FBXW7* expression levels in these different types of ovarian cancers. In the present study, the *p53* mutation rate was high (61.5%) in serous carcinomas. As mentioned above, *p53* status was correlated with *FBXW7* expression, and this was thought to be one reason why the expression of *FBXW7* was low in serous carcinomas.

To investigate in detail the correlation between *p53* status and *FBXW7* expression, we focused on the DNA methylation status of the *FBXW7* promoter region. It is well known that DNA hypermethylation of the promoter region of tumor suppressor genes is one of the epigenetic characteristics of cancer. We performed DNA methylation array and bisulfite PCR sequencing experiments and showed that the methylation status of the 5′-upstream regions of *FBXW7* was associated with the *p53* status. These data suggested that *FBXW7* expression would be also suppressed by promoter hypermethylation, like other tumor suppressor genes such as *CDKN2A* and *MLH1*, although further studies with more samples are required. *p53* mutations were previously reported to be associated with DNMT1 protein overexpression, and DNMT1 overexpression is involved in hypermethylation of multiple tumor suppressor genes.[35,36] Our data suggested that *FBXW7* might be one of such tumor suppressor genes transcriptionally suppressed by *p53* mutations.

In conclusion, we demonstrated that dysfunctions of FBXW7 conferred malignant potential to ovarian tumors. This did not occur as a result of *FBXW7* mutation but as a consequence of transcriptional downregulation. We suggest that *FBXW7* expression would be suppressed by hypermethylation of *FBXW7* 5′-upstream regions, which might be induced by *p53* mutations. While chemotherapy is very useful in ovarian cancer treatment, *FBXW7* dysfunctions have recently been reported to correlate with drug resistance.[37–39] Moreover, FBXW7 degrades oncoproteins such as c-Myc, cyclin E, Aurora A and Notch, all of which are involved in carcinogenesis, proliferation and metastases. However, the key substrates of FBXW7 that play the most important role in ovarian cancer development and its mechanisms remain unclear. Further studies are necessary to elucidate the details of these processes.

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Disclosure Statement

The authors have no conflict of interest to declare.

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References

1 Jemal A, Bray F, Center MM *et al.* Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.
2 Ministry of Health, Labour, and Welfare. Mortality by sex in the Vital Statistics of Japan yearly survey in 2011 (for every 100,000 people). Tokyo: Ministry of Health, Labour, and Welfare, 2016. [Cited 03 Feb 2016]. Available from URL: http://www.mhlw.go.jp/.
3 Welcker M, Clurman BM. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* 2007; **8**: 83–93.
4 Fujii Y, Yada M, Nishiyama M *et al.* Fbxw7 contributes to tumor suppression by targeting multiple proteins for ubiquitin-dependent degradation. *Cancer Sci* 2006; **97**: 729–36.
5 Onoyama I, Nakayama KI. Fbxw7 in cell cycle exit and stem cell maintenance. *Cell Cycle* 2008; **7**: 3307–13.
6 Wang Z, Inuzuka H, Zhong J *et al.* Tumor suppressor functions of FBXW7 in cancer development and progression. *FEBS Lett* 2012; **586**: 1409–18.
7 Mao JH, Perez-losada J, Wu D *et al.* Fbxw7/cdc4 is a p53-dependent haploinsufficient tumour suppressor gene. *Nature* 2004; **432**: 775–9.
8 Onoyama I, Tsunematsu R, Matsumoto A *et al.* Conditional inactivation of Fbxw7 impairs cell-cycle exit during T cell differentiation and results in lymphomatogenesis. *J Exp Med* 2007; **204**: 2875–88.
9 Matsuoka S, Oike Y, Onoyama I *et al.* Fbxw7 acts as a critical fail-safe against premature loss of hematopoietic stem cells and development of T-ALL. *Genes Dev* 2008; **22**: 986–91.
10 Grim JE, Knoblaugh SE, Guthrie KA *et al.* Fbw7 and p53 cooperatively suppress advanced and chromosomally unstable intestinal cancer. *Mol Cell Biol* 2012; **32**: 2160–7.
11 Akhoondi S, Sun D, von der Lher N *et al.* FBXW7/hCDC4 is a general tumor suppressor in human cancer. *Cancer Res* 2007; **67**: 9006–12.
12 Spruck CH, Strohmaier H, Sangfelt O *et al.* hCDC4 gene mutations in endometrial cancer. *Cancer Res* 2002; **62**: 4535–9.
13 Malyukova A, Dohda T, von der Lehr N *et al.* The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. *Cancer Res* 2007; **67**: 5611–6.
14 Kwak EL, Moberg KH, Wahrer DC *et al.* Inflequent mutations of Archipelago (hAGO, hCDC4, Fbw7) in primary ovarian cancer. *Gynecol Oncol* 2005; **98**: 124–8.
15 Sgambato A, Cittadini A, Masciullo V *et al.* Low frequency of hCDC4 mutations in human primary ovarian cancer. *Gynecol Oncol* 2007; **10**: 553–5.
16 Welcker M, Orian A, Grim JE, Eisenman RN, Clurman BE. A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. *Curr Biol* 2004; **14**: 1852–7.
17 Matsumoto A, Onoyama I, Nakayama KI. Expression of mouse Fbxw7 isoforms is regulated in a cell cycle- or p53- dependent manner. *Biochem Biophys Res Commun* 2006; **350**: 114–9.
18 Kimura T, Gotoh M, Nakamura Y, Arakawa H. hCDC4b, a regulator of cyclin E, as a direct transcriptional target of p53. *Cancer Sci* 2003; **94**: 431–6.
19 Yokobori T, Mimori K, Iwatsuki M *et al.* p53-altered FBXW7 expression determines poor prognosis in gastric cancer cases. *Cancer Res* 2009; **69**: 3788–94.
20 Iwatsuki M, Mimori K, Ishii H *et al.* Loss of FBXW7, a cell cycle regulating gene, in colorectal cancer: clinical significance. *Int J Cancer* 2010; **126**: 1828–37.
21 Ibusuki M, Yamamoto Y, Shinriki S, Ando Y, Iwase H. Reduced expression of ubiquitin ligase FBXW7 mRNA is associated with poor prognosis in breast cancer patients. *Cancer Sci* 2011; **102**: 439–45.
22 Hagedorn M, Delugin M, Abraldes I *et al.* FBXW7/hCDC4 controls glioma cell proliferation in vitro and is a prognostic marker for survival in glioblastoma patients. *Cell Div* 2007; **2**: 9.
23 Akhoondi S, Lindstrom L, Widschwendter M *et al.* Inactivation of FBXW7/hCDC4-β expression by promoter hypermethylation is associated with favorable prognosis in primary breast cancer. *Breast Cancer Res* 2010; **12**: R105.
24 Jahid S, Sun J, Edwards RA *et al.* miR-23a promotes the transition from indolent to invasive colorectal cancer. *Cancer Discov* 2012; **2**: 540–53.
25 Kurashige J, Watanabe M, Iwatsuki M *et al.* Overexpression of microRNA-223 regulates the ubiquitin ligase FBXW7 in oesophageal squamous cell carcinoma. *Br J Cancer* 2012; **106**: 182–8.
26 Li J, Guo Y, Liang X *et al.* MicroRNA-223 functions as an oncogene in human gastric cancer by targeting FBXW7/hCdc4. *J Cancer Res Clin Oncol* 2012; **138**: 763–74.
27 Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 1990; **249**: 912–5.
28 Prat J. New insights into ovarian cancer pathology. *Ann Oncol* 2012; **23**: x111–7.
29 Koshiyama M, Matsumura N, Konishi I. Recent concepts of ovarian carcinogenesis: type I and type II. *Biomed Res Int* 2014; **2014**: 934261.
30 Jones PA. DNA methylation and cancer. *Oncogene* 2002; **21**: 5358–60.
31 Baylin SB. DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol* 2005; **2**: S4–11.
32 Agrawal N, Frederick MJ, Pickering CR *et al.* Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science* 2011; **333**: 1154–7.
33 Le Gallo M, O'Hara AJ, Rudd ML *et al.* Exome sequencing of serous endometrial tumors identifies recurrent somatic mutations in chromatin-remodeling and ubiquitin ligase complex genes. *Nat Genet* 2012; **44**: 1310–5.
34 Zhao S, Choi M, Overton JD *et al.* Landscape of somatic single-nucleotide and copy-number mutations in uterine serous carcinoma. *Proc Natl Acad Sci USA* 2013; **110**: 2916–21.
35 Lin RK, Wu CY, Chang JW *et al.* Dysregulation of p53/Sp1 control leads to DNA methyltransferase-1 overexpression in lung cancer. *Cancer Res* 2010; **70**: 5807–17.
36 Lin EK, Wang YC. Dysregulated transcriptional and post-translational control of DNA methyltransferases in cancer. *Cell Biosci* 2014; **4**: 46.
37 Inuzuka H, Shaik S, Onoyama I *et al.* SCF(FBW7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. *Nature* 2011; **471**: 104–9.
38 Wertz IE, Kusam S, Lam C *et al.* Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature* 2011; **471**: 110–4.
39 Wang Z, Fukushima H, Gao D *et al.* The two faces of FBW7 in cancer drug resistance. *BioEssays* 2011; **33**: 851–9.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. *FBXW7* expression levels in serous and mucinous ovarian tumors.

Fig. S2. Kaplan–Meier overall survival curves of ovarian cancer patients based on *FBXW7* expression levels.

Table S1. PCR primers for *FBXW7*/*p53* mutation analysis.

Table S2. *p53* mutation points in clinical samples from ovarian cancer patients.

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