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Expression of USP7 and MARCH7 Is Correlated with Poor Prognosis in Epithelial Ovarian Cancer

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Epithelial ovarian cancer (EOC) is one of the worst malignancies in females with poor overall survival due to the rapid metastasis and the absence of ideal biomarkers. Ubiquitin-specific protease 7 (USP7), an important deubiquitinating enzyme, was reported to be upregulated in several cancers, including liver, prostate and colon cancers. Membrane associated RING-CH protein 7 (MARCH7) belongs to the member of the E3 ubiquitin ligases. In addition, MARCH7 regulates T cell proliferation and the neuronal development and participates in the membrane trafficking and protein degradation. Importantly, MARCH7 itself is ubiquitinated and acts as a potential substrate of USP7. However, the roles of USP7 and MARCH7 in EOC remain to be investigated. We collected 121 EOC patients and analyzed the expression levels of USP7 and MARCH7 in tumor tissues with immunohistochemical staining. We found that the high expression of the two proteins was correlated with lymph node metastasis in EOC patients. Univariate and multivariate analyses revealed that the patients with high expression of the two proteins showed poorer prognosis compared with other patients. Subsequently, using SKOV3 human ovarian adenocarcinoma cells, we showed that either USP7 or MARCH7 enhanced the proliferation and invasion abilities. Moreover, USP7 could regulate the expression levels of E-cadherin and β-catenin through the MARCH7 signaling pathway. Our findings indicate that USP7 and MARCH7 are involved in the progression of EOC. In conclusion, analyzing the expression of USP7 and MARCH7 has high prognostic value in predicting EOC prognosis.

Keywords: epithelial ovarian cancer; lymph node metastasis; membrane associated RING-CH protein 7; prognosis; ubiquitin-specific protease 7
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Introduction

Ovarian cancer is a highly mortality gynecologic malignancy, ranking the 5th leading cause of cancer death in women, with 140,200 deaths reported annually worldwide (Jemal et al. 2011; Siegel et al. 2013). Epithelial ovarian cancer (EOC) makes up for 85-90% of the ovarian cancers, accounting for the 2nd most frequent gynecologic cancer (Ross et al. 2013; Chudecka-Glaz 2015). In most cases, ovarian cancers are diagnosed at stage III or IV and usually has a poor outcome due to the complete absence of specific symptoms as well as the strong metastatic capacity and high recurrence possibility, the 5-year overall survival rate of EOC patients with advanced stage was less than 30% (Lowe et al. 2013). Various prognostic factors for ovarian cancer can reflect its intrinsic biology (e.g., pathological grade and histological subtype), cancer stage, residual disease as well as the performance status (Holschneider and Berek 2000; Clark et al. 2001; Agarwal and Kaye 2005). Figuring out an ideal biomarker of ovarian cancer at the molecular level could be helpful to predict the prognosis of EOC patients and may provide novel therapies that are more appropriate.

Ubiquitination plays a critical role in diverse cellular functions, such as regulating the ubiquitin-mediated proteasomal degradation (Hershko and Ciechanover 1998) and regulates the sorting of substrates by the endocytic route to lysosomes (Hicke 1997). The E3 ubiquitin ligase contains either HECT domains or RING domains (Joazeiro and Weissman 2000). The RING domain is a kind of zinc-finger motif that is characterized by a conserved sequence of cysteines and histidines, such as the RING-finger (C3HC4) and RING-H2-finger (C3H2C3). The leukemia associated protein domain (LAP) or the plant homeodomain (PHD) is a motif related to the RING-finger structurally (Aasland et al. 1995; Saha et al. 1995), which is distinguished by the C4HC3 sequence. BKS domain is one subclass of the PHD/LAP domain, discovered in poxvirus, herpesviruses and several eukaryotic genomes (Nicholas et al. 1997). The

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sequence and function of the BKS is similar to the RING and RING-H2 fingers, so the BKS subtype of the PHD/LAP motifs is also called RING-CH (Swanson et al. 2001). Thus, the membrane associated RING-CH proteins (MARCH) belong to the E3 ubiquitin ligases, and MARCH family consist 11 members in mammals (Bartee et al. 2004; Lehner et al. 2005; Ohmura-Hoshino et al. 2006; Morokuma et al. 2007). Besides the ubiquitination, MARCH proteins play numerous cellular functions, including membrane trafficking, immune regulation, protein degradation, and spermatogenesis. As a member of MARCH family, MARCH7 is highly expressed in neurons, stem cells, and lymphocytes (Ramalho-Santos et al. 2002; Su et al. 2002; Szigyarto et al. 2010). It has been reported that MARCH7 plays a crucial role in the T cell proliferation and the neuronal development (Muthukumarana et al. 2006). Recently, it has been demonstrated that the MARCH7 is associated with the oncogenesis of the ovarian carcinoma (Hu et al. 2015), indicating this specific ubiquitin ligase can play important roles in the cancer.

The ubiquitin ligases can also be ubiquitinated as substrates and be degraded as a consequence, which keeps the balance of its quantity. The auto-ubiquitination of MARCH7 can decrease its protein level, and maintain normal metabolism. However, there also exist enzymes that can deubiquitinate proteins, for example, USP7 (the Herpesvirus Associated USP, HAUSP), the deubiquitinating enzyme, can deubiquitinate MARCH7 as reported by Nathan et al. (2008). USP7 can deubiquitinate various tumor suppressors (p53, PTEN), E3 ligases (MDM2/MDMX, viral proteins ICP0) and the chromatin-associated proteins (the histone H2B, UHRF1 and Tip60), therefore controlling important cellular functions involving in the tumorigenesis (Everett et al. 1997; Li et al. 2004; Song et al. 2008; Hussain et al. 2009; Faesen et al. 2011). It has been reported that USP7 was upregulated in many tumors, such as prostate cancer, colon cancer, hematoma, and lung cancer (Song et al. 2008; Hussain et al. 2009; Chen et al. 2015).

However, the expression of USP7 in EOC has not been investigated; there has been no study illustrating the role of USP7 in the tumorigenesis, metastasis, and the prognostic significance of EOC. In addition, the association of USP7-MARCH7 ubiquitin-regulation with EOC has not been reported. In the current study, we explored the expression of MARCH7 and USP7 in ovarian cancer cell lines and clinical tissues, and then analyzed the role of these two proteins in the EOC tumorigenesis, metastasis, and their prognosis value. The combination of these two biomarkers is helpful to identify the prognosis much better than either the only one. We further performed experiments to explore the underlying mechanism, showing that the expression level of MARCH7 can be regulated by USP7, and the function of USP7 in the cell proliferations and cell invasion capacity can be at least partially regulated by its downstream signal molecular MARCH7.

Patients and Methods

Patients

A total of 121 patients diagnosed with primary serous ovarian cancer between 2000 and 2014 from Yidu Central Hospital of Weifang and Women & Children's Health Care Hospital of Linyi were enrolled for this retrospective analysis. This study was reviewed and approved by the Clinical Research Ethics Committee of Women & Children's Health Care Hospital. All the resected tumor tissues were embedded with paraffin. Additional seven fresh paired tumor tissues and adjacent normal ovarian tissues were kept at −180°C liquid nitrogen freezers before use. Signed informed consent forms were obtained from all subjects who participated in the study. In all cases, the following parameters were collected: age, pathological grade, FIGO stage (International Federation of Gynecology and Obstetrics), serum CA-125 level and lymphatic invasion. None of the patients received pre-operative chemotherapy or radiotherapy. Patient outcome was evaluated as the months of survival from the date of tumor resection up to June 2014 or the date of last follow-up.

Immunohistochemistry

Paraffin-embedded samples were cut into sections and dewaxed and rehydrated using a graded series of ethanol, followed by microwave antigen retrieval. After blocked with 0.3% hydrogen peroxidase, sections were incubated overnight at 4°C with MARCH7 (1:100, bs-9341R, Bioss, Beijing, China) or USP7 primary antibody (ab4080, Abcam, USA). Fetal bovine serum (FBS) was used as the negative control. Immunostaining was conducted using the DAB kit. The sections were then followed with hematoxylin staining, dehydrated, cleared, and mounted.

Semi-quantitative analysis of immunohistochemistry results

All the immunostained slides were revised and scored by two pathologists blinded to the clinical parameters, separately. The MARCH7 positivity was defined as cytoplasm staining, while the USP7 positivity was defined as the nuclear staining with corresponding antigens. For the staining assessment, staining intensity was graded as score 0 (negative), 1 (weak, pale yellow), 2 (moderate, dark yellow), and 3 (strong, brown). The percentage of the positive cells was also scored as 0 (0-5%), 1 (5-25%), 2 (26-50%) and 3 (51-100%). The final immunoreactivity score (IRS) was calculated by multiplying the intensity and percentage scores (range: 0-9). Then we chose IRS = 4 as the cut-off score for the evaluation of MARCH7 or USP7 expression, and the following values were considered as the high expression group: MARCH7 $\geq$ 4.0 and/or USP7 $\geq$ 4.0.

Cell Culture and transfection

SKOV3 cells (the human ovarian adenocarcinoma cell line) were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% FBS, penicillin and streptomycin. The full length (FL, 1-1102) and low-activity truncation (1-1050) (Ma et al. 2010) of human origin USP7 gene was constructed in the pcDNA3.1 vector (pcDNA3.1-USP7 and pcDNA3.1-USP7-1-1050), respectively. Cells were transfected with pcDNA3.1-plasmids (pcDNA3.1-vector, pcDNA3.1-USP7, pcDNA3.1-MARCH7) or siRNAs using Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific, USA).

The siRNA for USP7 and MARCH7 were purchased from Shanghai Gene Pharma Co., Ltd. (Shanghai, China), the siRNA

sequences were as followed:

Scrambled siRNA: 5′-UUCUCCGAACGUGUCACGUdTdT-3′;

USP7: 5′-ACCCUUGGACAAUAUUCCUdTdT- 3′ (Chen et al. 2015);

MARCH7: 5′-GCACACGUGUCCGAUUUAU-3′ (Hu et al. 2015).

Cell Viability Assay

The SKOV3 cell viability was evaluated 24 hours after transfection (scrambled siRNA, pcDNA3.1-vector, pcDNA3.1-USP7, pcDNA3.1-MARCH7, USP7 siRNA, MARCH7 siRNA, respectively.) using the Presto-Blue Cell Viability Reagent (Invitrogen, Life Technologies, USA) according to the instructions. The fluorescence was then measured at wavelength of 560 nm and 590 nm using microplate reader. Higher fluorescence represents higher total metabolic activity.

Matrigel invasion assay

Invasion of SKOV3 cells was evaluated by the Matrigel invasion assays using 8 μm Matrigel-coated transwells (BD Biosciences, USA). A total of 2×10^5 cells were seeded into the upper chamber side, while the lower chambers were filled with 700 μl RPMI 1640 containing 10% FBS. After cultured for 12 hours at 37°C, the invasive cells on the lower chamber were fixed with 4% paraformaldehyde, stained with 0.05% crystal violet, and counted under microscope. Five fields were counted and photographed for each transwell chamber at 200× magnification.

Western blot analysis

The clinical tumor tissues and adjacent normal ovarian tissues were lysed with RIPA lysis buffer, while the cultured ovarian cancer cells were lysed with NP-40 lysis buffer (Beyotime Biotechnology, Shanghai, China), and then centrifuged at 12,000 rpm for 15 minutes at 4°C. Then quantified the concentration of supernatant with Bradford detection kit. Equal amount of protein (about 10 μg) was loaded in the SDS-PAGE gel, then transferred to NC membrane (PALL Company, USA) and incubated with primary antibody (1: 1,000) overnight at 4°C. After washed by Tris-buffered saline (TBS) for 3 times, the NC membrane was incubated in secondary antibody (1: 5,000) for 1 hours at 37°C and subsequently visualized by ECL (Santa Cruz biotechnology, USA).

Immunoprecipitation assay

For immunoprecipitation assay, after transfected (pcDNA3.1-vector, pcDNA3.1-HA-tagged-USP7-FL, pcDNA3.1-HA-tagged-USP7-1-1050) and cultured for 48 hours, cells were harvested and lysed in 4°C for 30 min. After centrifugation at 12,000 rpm for 15 min, anti-HA-Agarose antibody (Sigma, USA) were added to the supernatant and binding for 4 hours in 4°C. Then wash the beads with lysis buffer for three times, added 2 × SDS loading buffer onto the precipitated HA-beads and analyzed by immunoblotting.

Immunofluorescence

Briefly, cells (transfected with pcDNA3.1-MARCH7 or MARCH7 siRNA) were fixed with 4% formaldehyde and then permeabilized by 0.5% Triton-100. Expression of E-cadherin and β-catenin were detected with primary antibodies as well as the fluorophore-conjugated isotype-specific and affinity cross-adsorbed antibodies. Nucleus was finally stained with DAPI. Immunofluorescence were observed using the Olympus fluorescence microscope.

Statistical Analysis

Survival curves were plotted by the Kaplan-Meier analysis, and the significant differences between subgroups were calculated with the log-rank test. Cox proportional hazard model was used to perform realize the multivariate survival analysis. The correlation between MARCH7 and USP7 protein expression was determined using Spearman's test. Differences were considered significant when the P value was less than 0.05 (two-side). All statistical analyses were managed using the SPSS 22.0 statistical software package (Chicago, IL, USA).

Results

Patients characteristics

Of the 121 EOC cases aging from 46 to 75 years (median 54.0 years), 85 patients (70.2%) were diagnosed with pathological grade I or grade II, the other 36 patients (29.8%) with grade III; and 45 patients (37.2%) with FIGO stages I~II and 76 patients (62.8%) with stage III~IV. The lymph node metastasis was positive in 60 patients (49.6%). Overall survival time was defined as the length from the date of surgery to the date of death or the last visit. The 5-year overall survival rate of the cases was 52.0% and the median survival time was 72.0 months. Table 1 exhibits the parameters characteristics of these cases.

USP7 and MARCH7 proteins are up-regulated in human serous ovarian cancer tissues

We performed the Western blot analysis and immunohistochemical (IHC) staining to verify the expression patterns of USP7 and MARCH7 in ovarian cancer tissues. Increased USP7 and MARCH7 protein level was found in six of seven serous ovarian cancer tissues compared with adjacent non-tumorous ovarian tissue (from patient #1-#7, Fig. 1A). IHC results also demonstrated the immunoreactivities of USP7 in the nucleus while the MARCH7 protein in the cytoplasm. According to the IHC score criteria described in the methods, the protein level was divided into high expression (Fig. 1C, E, both from patient #4) and low expression (Fig. 1B, D). Of the 121 EOC tissues investigated, 56 cases (46.3%) were regarded as USP7-high expression and 51 cases (42.1%) with MARCH7-high expression (Table 1). Moreover, the Spearman correlation analysis revealed a corresponding correlation between USP7 expression and MARCH7 expression in EOC (R^2 = 0.577; $P < 0.001$, Fig. 1F).

Expression of USP7 and MARCH7 is associated with tumor progression

To further illustrate the significance of USP7 and MARCH7 expression in ovarian cancer, we analyzed the associations between upregulation of the two proteins with the clinicopathological characteristics of EOC (Table 1). We found that high expression of USP7 and MARCH7 were both significantly correlated with the lymph node

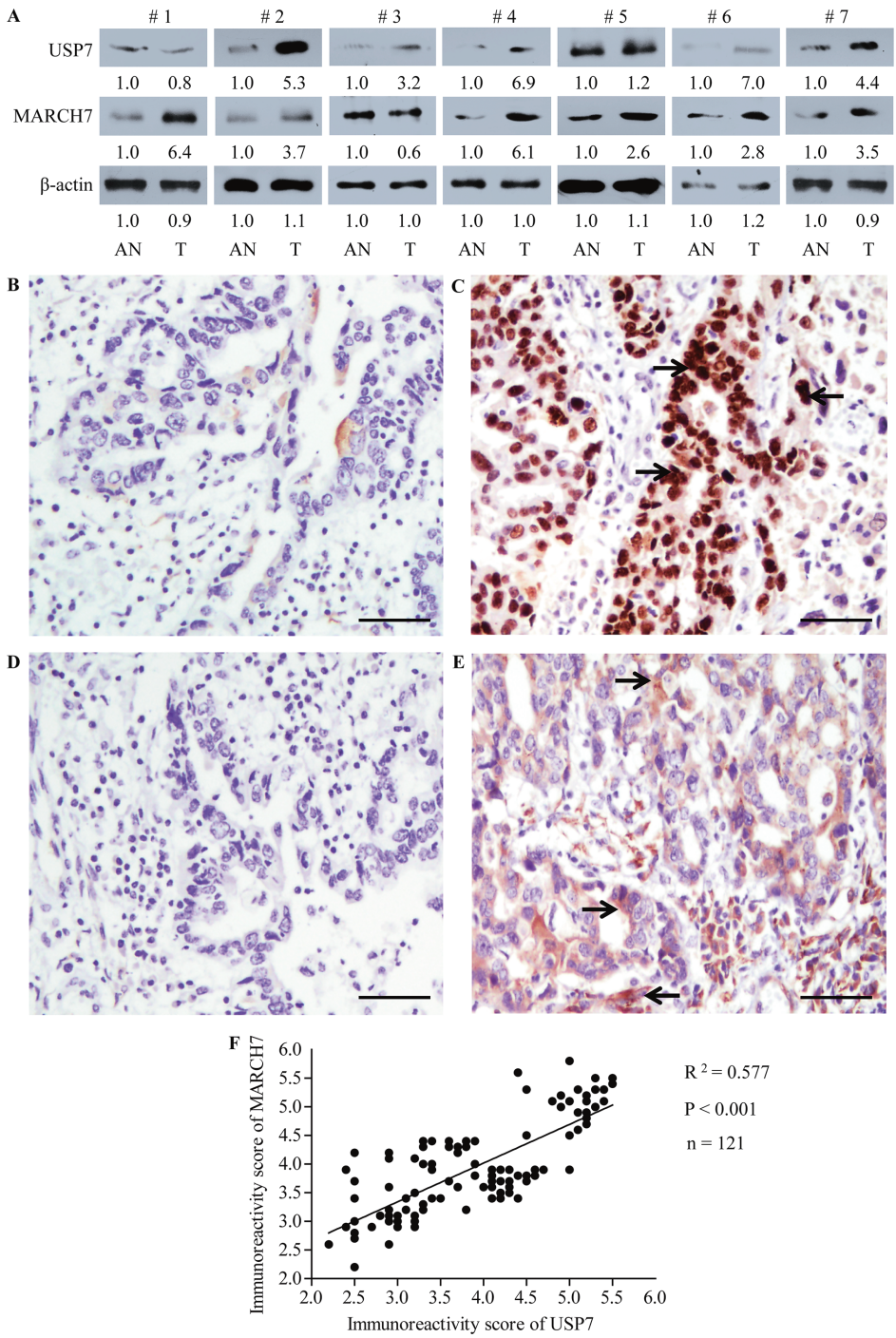

Fig. 1. Expression of USP7 and MARCH7 in clinical ovarian cancer samples.
(A) The expression of USP7/MARCH7 in seven paired samples of EOC tissues versus adjacent normal ovarian tissues was measured by western blot. AN: Tumor adjacent non-tumorous tissue; T: Tumor tissue. (B) Low USP7 expression: in this case, the score of staining intensity is 1 and the score of positive cell percentage is 0. So the total score is 0 (calculated by score of staining intensity multiplying the score of positive cell percentage), indicating USP7 low-expression. (C) IHC staining of USP7 from tumor tissues of patient #4 in (A): the score of staining intensity is 3 and the score of positive cell percentage is 3. Total score is 9 and indicated USP7 high-expression. Arrows pointed the strong IHC nucleus staining. (D) Low MARCH7 staining: the score of staining intensity is 0 and the score of positive cell percentage is 0. Total score is 0 and defined as MARCH7 low-expression. (E) IHC staining of MARCH7 from tumor tissues of patient #4 in (A): the score of staining intensity is 2 and the score of positive cell percentage is 3. Total score is 6 and indicated MARCH7 high-expression. Arrows pointed the strong IHC cytoplasm staining. (F) The relationship between USP7 and MARCH7 expression in EOC patients. The Spearman correlation analysis revealed a corresponding correlation between USP7 overexpression and MARCH7 overexpression ($n = 121$, $R^2 = 0.577$; $P < 0.001$).
Scale bar: 100 μm.

Table 1. Basic clinicopathologic parameters of patients and the correlation with USP7 and MARCH7 expression.

Variables	Cases	USP7 expression		P value	MARCH7 expression		P value
	(n = 121)	Low (n, %)	High (n, %)		Low (n, %)	High (n, %)	
Age (years)				0.794			0.259
≤ 60	85	45 (52.9%)	40 (47.1%)		52 (61.2%)	33 (38.8%)	
> 60	36	20 (55.6%)	16 (44.4%)		18 (50.0%)	18 (50.0%)	
Pathological grade				0.597			0.742
G1 ~ G2	85	47 (55.3%)	38 (44.7%)		50 (58.8%)	35 (41.2%)	
G3	36	18 (50.0%)	18 (50.0%)		20 (55.6%)	16 (44.4%)	
FIGO stage				0.290			0.023*
I ~ II	45	27 (60.0%)	18 (40.0%)		32 (71.1%)	13 (28.9%)	
III ~ IV	76	38 (50.0%)	38 (50.0%)		38 (50.0%)	38 (50.0%)	
CA-125				0.523			0.175
≤ 900	61	31 (50.8%)	30 (49.2%)		39 (63.9%)	22 (36.1%)	
> 900	60	34 (56.7%)	26 (43.3%)		31 (51.7%)	29 (48.3%)	
LN metastasis				0.008*			0.036*
No	61	40 (65.6%)	21 (34.4%)		41 (67.2%)	20 (32.8%)	
Yes	60	25 (41.7%)	35 (58.3%)		29 (48.3%)	31 (51.7%)	

The associations were evaluated with chi-square test.
*statistically significant
FIGO, International Federation of Gynecology and Obstetrics; USP7, ubiquitin-specific protease 7; MARCH7, membrane associated RING-CH protein 7.

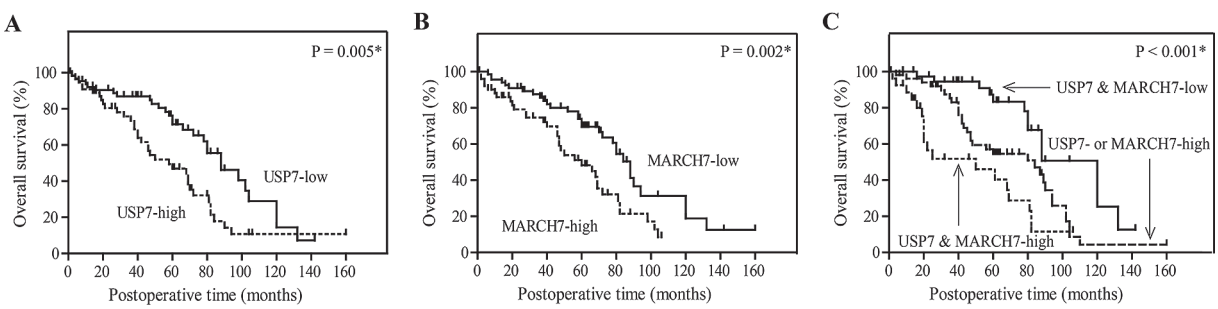

Fig. 2. The combination of USP7 and MARCH7 expression level in carcinoma tissues was found to enhance the accuracy of predicting prognosis for EOC patients. (A) Compared with USP7-high expression group, the overall survival was significantly better in the USP7-low expression group. (B) Positive expression of MARCH7 has significant negative association with overall survival. (C) Positive expressions of combined USP7/MARCH7 showed better prognostic value for EOC patients.

metastasis (P = 0.008, P = 0.036, respectively), and the MARCH7 higher expression level indicated more advanced FIGO stages (P = 0.023, Table 1). There was no statistically significant association of USP7 or MARCH7 with patients' age, pathological grade or the CA-125 level.

Univariate and multivariate analyses

The overall survival rate was calculated to find prognostic factors by univariate analysis. The Kaplan-Meier analysis demonstrated that patients with high USP7 overexpression showed poorer overall survival than those with low USP7 expression tumors (P = 0.005, Fig. 2A, Table 2). On the other hand, the high expression of MARCH7 was also significantly associated with poor overall survival (P = 0.002, Fig. 2B, Table 2). With regard to the combined expression of USP7 and MARCH7 proteins, we divided the patients into three subgroups: group 1, patients exhibiting high expression of USP7 and MARCH7 in the EOC tissues

Table 2. Kaplan-Meier survival analysis.

Variables	Cases	Overall survival time (months)		P value
	(n = 121)	Mean	5-year survival rate	
Age (years)				0.030*
≤ 60	85	82.7	68.7%	
> 60	36	61.3	49.5%	
Pathological grade				0.003*
G1 ~ G2	85	83.9	72.6%	
G3	36	55.2	41.7%	
FIGO stage				< 0.001*
I ~ II	45	104.1	92.5%	
III ~ IV	76	57.9	44.8%	
Preoperative CA125				0.662
≤ 900	61	80.3	57.5%	
> 900	60	74.8	67.5%	
Lymph node metastasis				< 0.001*
No	61	96.8	86.9%	
Yes	60	55.3	40.3%	
USP7 expression				0.005*
Low expression	65	87.0	70.6%	
High expression	56	63.3	52.0%	
MARCH7 expression				0.002*
Low expression	70	88.9	72.5%	
High expression	51	58.7	48.5%	
Both USP7 and MARCH7 high expression				< 0.001*
No	94	88.0	66.6%	
Yes	27	48.4	46.1%	

The overall survival rate was evaluated with Kaplan-Meier survival analysis; the univariate results was performed by log-rank test.
*statistically significant.
FIGO, International Federation of Gynecology and Obstetrics; USP7, ubiquitin-specific protease 7; MARCH7, membrane associated RING-CH protein 7.

(USP7-high/MARCH7-high, 27 patients); group 2, patients with tumors showing different expression of USP7 and MARCH7 (USP7-high/MARCH7-low or USP7-low/MARCH7-high, 53 patients); and group 3, patients with low expression of the both proteins (USP7-low/MARCH7-low, 41 patients). Notably, there was a significant trend of the worst overall survival in patients with high expression of both USP7 and MARCH7 ($P < 0.001$, Fig. 2C, Table 2), and the overall survival for patients in group 2 (USP7-high/MARCH7-low or USP7-low/MARCH7-high) was worse than those in group 3, and were also statistically different from those in group 1 (Fig. 2C). Besides, the USP7 and MARCH7 expression levels, other clinicopathological factors including age, pathological grade, FIGO stage, and the

Table 3. Cox multivariate analysis of the clinicopathological parameters for overall survival.

Variables	Hazard ratio	95% Confidence Interval	P value
Age	1.494	0.852 ~ 2.619	0.161
> 60 vs. ≤ 60 years			
Pathological grade	1.670	0.929 ~ 3.000	0.086
G3 vs. G1~G2			
FIGO stage	2.655	1.322 ~ 5.331	0.006*
III ~ IV vs. I ~ II			
Lymph node metastasis	1.994	0.985 ~ 4.034	0.055
Positive vs. Negative			
USP7 expression	2.957	1.356 ~ 6.448	0.006*
High vs. Low			
MARCH7 expression	3.069	1.377 ~ 6.840	0.006*
High vs. Low			
Both USP7 and MARCH7 high expression	3.181	1.108 ~ 9.135	0.032*
Yes vs. No			

Multivariate analysis was performed with the hazards regression model.
*statistically significant.
FIGO, International Federation of Gynecology and Obstetrics; USP7, ubiquitin-specific protease 7; MARCH7, membrane associated RING-CH protein 7.

lymph node metastasis were all dramatically associated with the overall survival, as indicated by the Kaplan-Meier survival analysis (Table 2).

In multivariate analysis, the Cox proportional hazards model showed that advanced FIGO stages (P = 0.006), high USP7 protein expression (P = 0.006), high MARCH7 protein expression (P = 0.006), as well as the combination of USP7 and MARCH7 protein level (P = 0.032) were all the independent prognostic factors for the overall survival in EOC patients (Table 3).

USP7 and MARCH7 can regulate the cell proliferation and invasion

We performed the overexpression and knock-down experiments to evaluate the functions of USP7 and MARCH7 in ovarian cancer cells. The transfection efficiency was checked with Western blot analysis (Fig. 3A). As shown in Fig. 3B, the knock-down of either USP7 or MARCH7 can inhibit the viability of the SKOV3 cells, while the overexpression of USP7 or MARCH7 can enhance their proliferation. Similar results were observed on the aspect of the cell invasion ability, showing both USP7 and MARCH7 can positively regulate the cell invasion (Fig. 3C).

The signaling axis of USP7-MARCH7-E-cadherin/β-catenin

In addition, we carried out the experiments to investigate the underlying signaling pathways. Through *in vitro* studies, we found that the expression levels of MARCH7 and *β*-catenin can be positively regulated by USP7, but E-cadherin expression was downregulated (Fig. 4A). Interestingly, when MARCH7 was knocked-down by siRNA, the effects of USP7 on E-cadherin and *β*-catenin expression was attenuated (Fig. 4A). To verify the interaction between USP7 and MARCH7, we constructed a low-activity USP7 truncation (pcDNA3.1-HA-tagged-USP7-1-1050) (Ma et al. 2010) without changing its substrate-binding affinity and over-expressed it in the SKOV3 cells. Immunoprecipitation results showed that both USP7-FL and USP7-1-1050 could pull-down MARCH7 (Fig. 4B). In addition, the immunofluorescence experiments confirmed that overexpression of MARCH7 can increase the expression level of *β*-catenin but down-regulate the expression of E-cadherin. On the other hand, MARCH7-siRNA showed the opposite effects (Fig. 4C).

Discussion

The ubiquitin-mediated protein down-regulation plays critical roles in maintaining protein level and the normal cellular process, such as cell cycle and tumor progression.

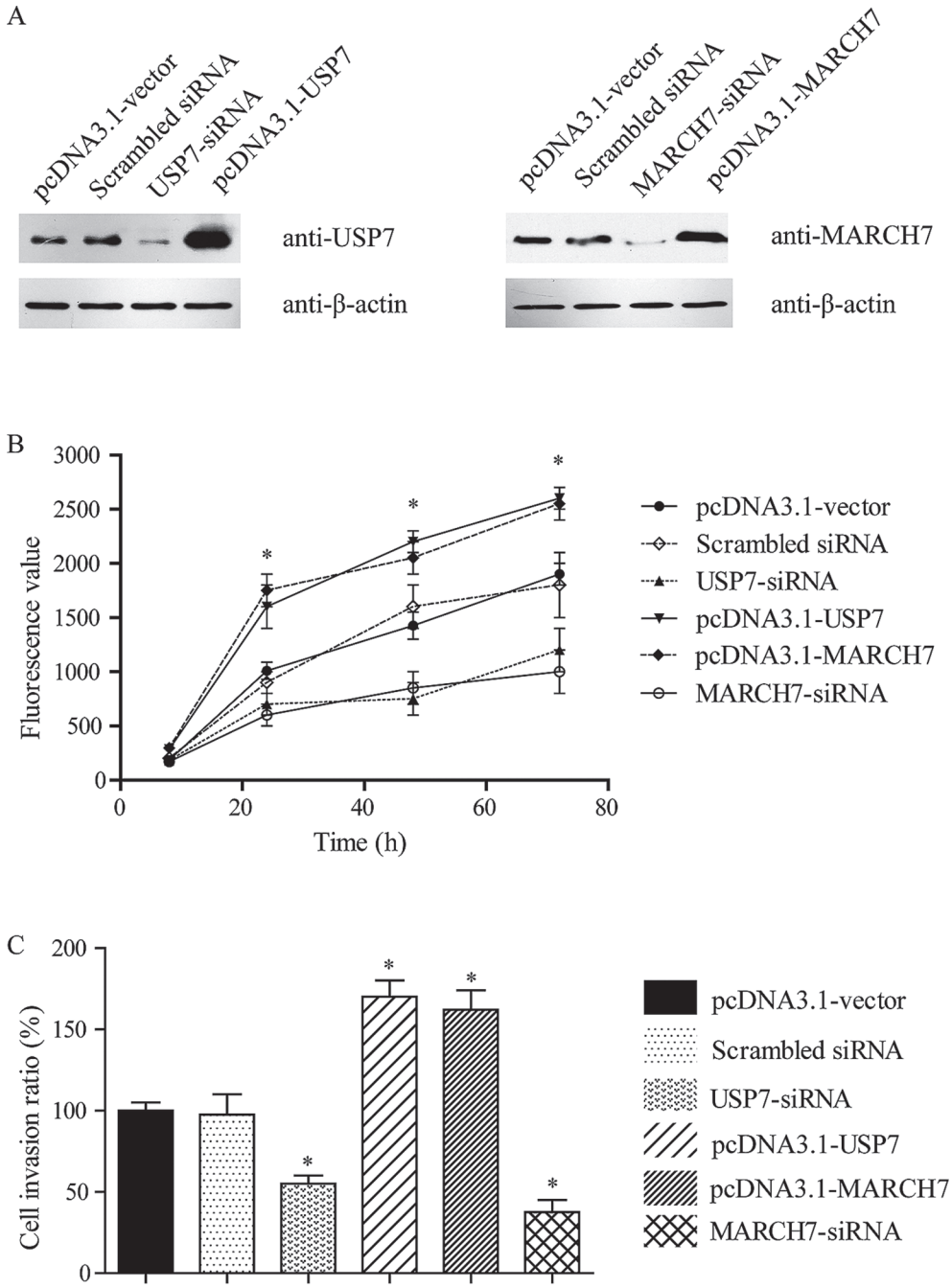

Fig. 3. USP7 and MARCH7 can regulate the cell proliferation and invasion.
(A) The efficiency of USP7 and MARCH7 knock-down and overexpression was detected by western blot. (B) Either USP7 or MARCH7 knock-down can down-regulate the proliferation capacity of SKOV3 cells, while their overexpression can significantly increase the cell viability. (C) USP7-siRNA or MARCH7-siRNA can inhibit the cell invasion, while the overexpression of these two proteins can up-regulate the invasion of SKOV3 cells through Matrigel invasion assay.

Negative regulation of ubiquitin-mediated protein degradation can cause development of various cancers (Hoeller et al. 2006). The MARCH7, a novel E3 ubiquitin ligase participating in the regulation of neuronal development and the T cell proliferation (Muthukumarana et al. 2006), was recently reported to function in the development of the ovarian cancer (Hu et al. 2015). Moreover, the removal of ubiquitin from the ubiquitylated proteins is also significant on determining the ubiquitylation state of E3-ubiquitin ligase substrates (including some E3-ubiquitin ligases themselves, which could be auto-ubiqutylated), thus the de-ubiquitylating (DUB) enzymes have also emerged as important regulators in controlling various cell functions. Several DUBs have been reported to contribute in the DNA

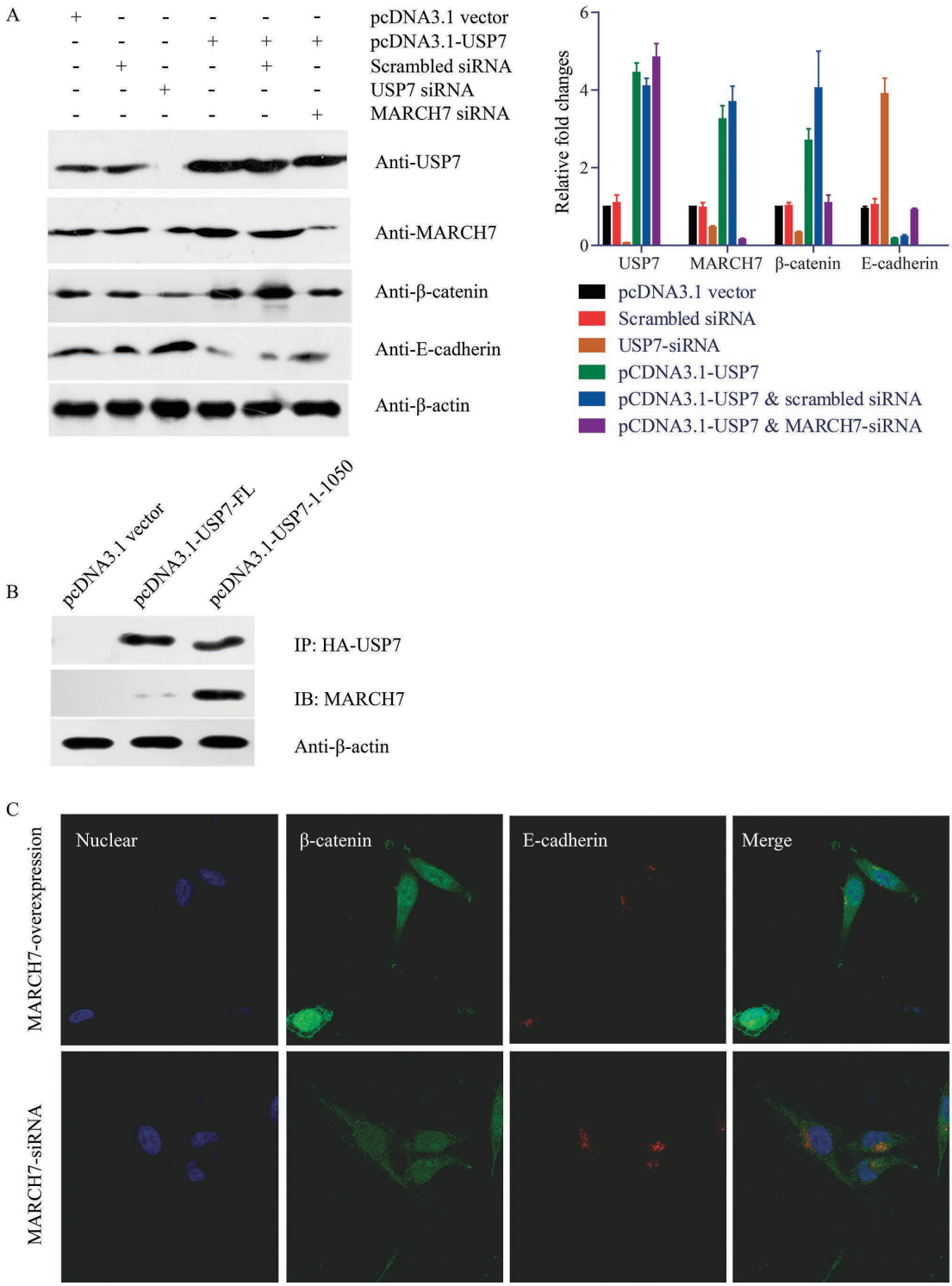

Fig. 4. Identification of the signaling axis USP7-MARCH7-E-cadherin/*β*-catenin.
(A) USP7-siRNA can down-regulate the expression of MARCH7 and *β*-catenin, while up-regulate the protein level of E-cadherin; the overexpression of USP7 showed the opposite effects. However, when knock-down the MARCH7 after the USP7 overexpression, the regulating effects of USP7 on E-cadherin and *β*-catenin expression was attenuated, showing no statistical difference with the control groups (pcDNA3.1-vector or scrambled siRNA group). (B) Immunoprecipitation results showed both HA-tagged USP7-FL and USP7-1-1050 can interact with protein MARCH7, indicating MARCH7 may be the substrate of the deubiquitinating enzyme USP7. (C) Immunofluorescence experiments indicated that MARCH7 overexpression can positively regulate the expression level of *β*-catenin, while inhibit the expression of E-cadherin; the MARCH7-siRNA showed opposite effects.

damage response, such as USP1, USP7 and USP28 (Nijman et al. 2005; Zhang et al. 2006; Morra et al. 2015). The USP7 can de-ubiquitinate several E3 ligases (MDM2/MDMX, viral proteins ICP0) and tumor suppressors (p53, FOXO, PTEN, and claspin), therefore regulates important cellular process correlated with the tumorigenesis (Everett et al. 1997; Li et al. 2004; Song et al. 2008; Hussain et al. 2009). Consistent with its functions, USP7 is upregulated in many cancers, including prostate, colon, liver and lung cancers (Song et al. 2008; Hussain et al. 2009; Chen et al. 2015). Most interestingly, the MARCH7 was reported to be upregulated by the USP7 in embryonic stem cells, according to a previous report (Nathan et al. 2008), but there were no studies about the roles of USP7 and MARCH7 in the ovarian cancer and the relationship between the two proteins in the corresponding cells.

Although concentrated studies on the molecular mechanisms of EOC have been conducted, the prognosis can be quite different even with similar pathological grades and FIGO stages. Nowadays, treatment of human ovarian cancer has been increasingly informed by various biomarkers that predict patient prognosis and a number of candidate prognostic biomarkers have already been discovered for human EOC. However, previously clinical trials showed that the vast majority of them perhaps be poorly suited to the significance of identifying treatments, but might effective only in certain selected subsets of patients. Therefore, identifying novel and efficient biomarkers for EOC with better clinical application is still in urgent demand. In the present study, our data demonstrated that the abnormal expressions of USP7 and MARCH7 proteins appeared to be associated with the FIGO stage of EOC, its clinicopathological features and patient survivals. These strong correlations suggest that USP7 and MARCH7 overexpression promotes tumor development and that USP7 and/or MARCH7 could possibly serve as a biomarker for a more advanced phenotype of EOC. To our knowledge, our results firstly demonstrated the prognostic significance of the co-expression of USP7 and MARCH7 in EOC.

Since accumulating studies have suggested that the combined biomarkers may be more efficient than the single one in the prognosis of various human carcinomas, we hypothesized that the prognostic significance of the combination of USP7 and MARCH7 (USP7/MARCH7) might be better than USP7 or MARCH7 alone. To validate this assumption, we analyzed the correlations of USP7/MARCH7 combined expression, USP7 expression, and MARCH7 expression with overall survival of EOC patients, respectively. Our data showed that USP7/MARCH7 combined expression, USP7 expression, and MARCH7 expression were all independent predictive factors for overall survival of EOC patients. More interestingly, USP7/MARCH7 combined expression could be more powerfully in predicting the prognosis of EOC patients, indicating that the detection of co-expression of USP7 and MARCH7 could be used to design appropriate, individualized treatment and be helpful to characterize patients who may benefit from close following up after surgery.

We further investigated the functions of these two proteins in the human ovarian cancer cell line, SKOV3. We found that both USP7 and MARCH7 can regulate the proliferation and invasion of SKOV3 cells. In addition, USP7 can regulate the expression of E-cadherin and β-catenin by altering the expression of MARCH7. Considering the report that MARCH7 can act as the substrate of USP7 (Nathan et al. 2008), our results suggest the involvement of the USP7-MARCH7-E-cadherin/β-catenin axis in the progression of EOC. Additional molecular and clinical research would be necessary to confirm our findings.

In summary, USP7 and MARCH7 proteins are differentially expressed in EOC and closely connected with the biological characteristics of this malignancy. Combination of USP7 and MARCH7 expression may function as a promising biomarker for prognostication of the EOC.

Conflict of Interest

The authors declare no conflict of interest.

References

Aasland, R., Gibson, T.J. & Stewart, A.F. (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.*, **20**, 56-59.

Agarwal, R. & Kaye, S.B. (2005) Prognostic factors in ovarian cancer: how close are we to a complete picture? *Ann. Oncol.*, **16**, 4-6.

Bartee, E., Mansouri, M., Hovey Nerenberg, B.T., Gouveia, K. & Fruh, K. (2004) Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. *J. Virol.*, **78**, 1109-1120.

Chen, S.T., Okada, M., Nakato, R., Izumi, K., Bando, M. & Shirahige, K. (2015) The Deubiquitinating Enzyme USP7 Regulates Androgen Receptor Activity by Modulating Its Binding to Chromatin. *J. Biol. Chem.*, **290**, 21713-21723.

Chudecka-Glaz, A.M. (2015) ROMA, an algorithm for ovarian cancer. *Clin. Chim. Acta*, **440**, 143-151.

Clark, T.G., Stewart, M.E., Altman, D.G., Gabra, H. & Smyth, J.F. (2001) A prognostic model for ovarian cancer. *Br. J. Cancer*, **85**, 944-952.

Everett, R.D., Meredith, M., Orr, A., Cross, A., Kathoria, M. & Parkinson, J. (1997) A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J.*, **16**, 566-577.

Faesen, A.C., Dirac, A.M., Shanmugham, A., Ovaa, H., Perrakis, A. & Sixma, T.K. (2011) Mechanism of USP7/HAUSP activation by its C-terminal ubiquitin-like domain and allosteric regulation by GMP-synthetase. *Mol. Cell*, **44**, 147-159.

Hershko, A. & Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.*, **67**, 425-479.

Hicke, L. (1997) Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *FASEB J.*, **11**, 1215-1226.

Hoeller, D., Hecker, C.M. & Dikic, I. (2006) Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. *Nat. Rev. Cancer*, **6**, 776-788.

Holschneider, C.H. & Berek, J.S. (2000) Ovarian cancer: epidemiology, biology, and prognostic factors. *Semin. Surg. Oncol.*, **19**, 3-10.

Hu, J., Meng, Y., Yu, T., Hu, L. & Mao, M. (2015) Ubiquitin E3

ligase MARCH7 promotes ovarian tumor growth. *Oncotarget*, **6**, 12174-12187.

Hussain, S., Zhang, Y. & Galardy, P.J. (2009) DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. *Cell Cycle*, **8**, 1688-1697.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. & Forman, D. (2011) Global cancer statistics. *CA Cancer J. Clin.*, **61**, 69-90.

Joazeiro, C.A. & Weissman, A.M. (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell*, **102**, 549-552.

Lehner, P.J., Hoer, S., Dodd, R. & Duncan, L.M. (2005) Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases. *Immunol. Rev.*, **207**, 112-125.

Li, M., Brooks, C.L., Kon, N. & Gu, W. (2004) A dynamic role of HAUSP in the p53-Mdm2 pathway. *Mol. Cell*, **13**, 879-886.

Lowe, K.A., Chia, V.M., Taylor, A., O'Malley, C., Kelsh, M., Mohamed, M., Mowat, F.S. & Goff, B. (2013) An international assessment of ovarian cancer incidence and mortality. *Gynecol. Oncol.*, **130**, 107-114.

Ma, J., Martin, J.D., Xue, Y., Lor, L.A., Kennedy-Wilson, K.M., Sinnamon, R.H., Ho, T.F., Zhang, G., Schwartz, B., Tummino, P.J. & Lai, Z. (2010) C-terminal region of USP7/HAUSP is critical for deubiquitination activity and contains a second mdm2/p53 binding site. *Arch. Biochem. Biophys.*, **503**, 207-212.

Morokuma, Y., Nakamura, N., Kato, A., Notoya, M., Yamamoto, Y., Sakai, Y., Fukuda, H., Yamashina, S., Hirata, Y. & Hirose, S. (2007) MARCH-XI, a novel transmembrane ubiquitin ligase implicated in ubiquitin-dependent protein sorting in developing spermatids. *J. Biol. Chem.*, **282**, 24806-24815.

Morra, F., Luise, C., Merolla, F., Poser, I., Visconti, R., Ilardi, G., Paladino, S., Inuzuka, H., Guggino, G., Monaco, R., Colecchia, D., Monaco, G., Cerrato, A., Chiariello, M., Denning, K., et al. (2015) FBXW7 and USP7 regulate CCDC6 turnover during the cell cycle and affect cancer drugs susceptibility in NSCLC. *Oncotarget*, **6**, 12697-12709.

Muthukumarana, P.A., Lyons, G.E., Miura, Y., Thompson, L.H., Watson, T., Green, C.J., Shurey, S., Hess, A.D., Rosengard, B.R. & Metcalfe, S.M. (2006) Evidence for functional interrelationships between FOXP3, leukaemia inhibitory factor, and axotrophin/MARCH-7 in transplantation tolerance. *Int. Immunopharmacol.*, **6**, 1993-2001.

Nathan, J.A., Sengupta, S., Wood, S.A., Admon, A., Markson, G., Sanderson, C. & Lehner, P.J. (2008) The ubiquitin E3 ligase MARCH7 is differentially regulated by the deubiquitylating enzymes USP7 and USP9X. *Traffic*, **9**, 1130-1145.

Nicholas, J., Ruvolo, V., Zong, J., Ciufo, D., Guo, H.G., Reitz, M.S. & Hayward, G.S. (1997) A single 13-kilobase divergent locus in the Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genome contains nine open reading frames that are homologous to or related to cellular proteins. *J. Virol.*, **71**, 1963-1974.

Nijman, S.M., Huang, T.T., Dirac, A.M., Brummelkamp, T.R., Kerkhoven, R.M., D'Andrea, A.D. & Bernards, R. (2005) The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Mol. Cell*, **17**, 331-339.

Ohmura-Hoshino, M., Goto, E., Matsuki, Y., Aoki, M., Mito, M., Uematsu, M., Hotta, H. & Ishido, S. (2006) A novel family of membrane-bound E3 ubiquitin ligases. *J. Biochem.*, **140**, 147-154.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C. & Melton, D.A. (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science*, **298**, 597-600.

Ross, J.S., Ali, S.M., Wang, K., Palmer, G., Yelensky, R., Lipson, D., Miller, V.A., Zajchowski, D., Shawver, L.K. & Stephens, P.J. (2013) Comprehensive genomic profiling of epithelial ovarian cancer by next generation sequencing-based diagnostic assay reveals new routes to targeted therapies. *Gynecol. Oncol.*, **130**, 554-559.

Saha, V., Chaplin, T., Gregorini, A., Ayton, P. & Young, B.D. (1995) The leukemia-associated-protein (LAP) domain, a cysteine-rich motif, is present in a wide range of proteins, including MLL, AF10, and MLLT6 proteins. *Proc. Natl. Acad. Sci. USA*, **92**, 9737-9741.

Siegel, R., Naishadham, D. & Jemal, A. (2013) Cancer statistics, 2013. *CA Cancer J. Clin.*, **63**, 11-30.

Song, M.S., Salmena, L., Carracedo, A., Egia, A., Lo-Coco, F., Teruya-Feldstein, J. & Pandolfi, P.P. (2008) The deubiquitinylation and localization of PTEN are regulated by a HAUSP-PML network. *Nature*, **455**, 813-817.

Su, A.I., Cooke, M.P., Ching, K.A., Hakak, Y., Walker, J.R., Wiltshire, T., Orth, A.P., Vega, R.G., Sapinoso, L.M., Moqrich, A., Patapoutian, A., Hampton, G.M., Schultz, P.G. & Hogenesch, J.B. (2002) Large-scale analysis of the human and mouse transcriptomes. *Proc. Natl. Acad. Sci. USA*, **99**, 4465-4470.

Swanson, R., Locher, M. & Hochstrasser, M. (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes Dev.*, **15**, 2660-2674.

Szigyarto, C.A., Sibbons, P., Williams, G., Uhlen, M. & Metcalfe, S.M. (2010) The E3 ligase axotrophin/MARCH-7: protein expression profiling of human tissues reveals links to adult stem cells. *J. Histochem. Cytochem.*, **58**, 301-308.

Zhang, D., Zaugg, K., Mak, T.W. & Elledge, S.J. (2006) A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response. *Cell*, **126**, 529-542.