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Legumain: A Biomarker for Diagnosis and Prognosis of Human Ovarian Cancer

Lina Wang,[1] Si Chen,[1] Mingna Zhang,[2] Na Li,[2] Yanan Chen,[1] Weijun Su,[1] Yanhua Liu,[1] Dan Lu,[1] Sanglin Li,[3] Yixuan Yang,[3] Zongjin Li,[4] Dwayne Stupack,[5] Pengpeng Qu,[2] Huaidong Hu,[3**] and Rong Xiang[1*]

[1]*Department of Immunology, Nankai University School of Medicine, Tianjin, China*

[2]*Department of Gynecology Oncology, Tianjin Central Hospital for Obstetrics and Gynecology, Tianjin, China*

[3]*Key Laboratory of Molecular Biology for Infectious Diseases of Ministry of Education of China, The Second Affiliated Hospital, Chongqing Medical University, Chongqing, China*

[4]*Department of Pathophysiology, Nankai University School of Medicine, Tianjin, China*

[5]*Department of Reproductive Medicine, Division of Gynecological Oncology, UCSD School of Medicine, San Diego, California*

ABSTRACT

Legumain is a member of the asparaginyl endopeptidase family that is over-expressed in response to hypoxic stress on mammary adenocarcinoma, colorectal cancer, proliferating endothelial cells, and tumor-associated macrophages (TAMs). Here, we demonstrate that elevated expression of legumain in ovarian cancer by a proteomic approach using isobaric tags for relative and absolute quantification (iTRAQ) followed by liquid chromatography–mass spectrometry (LC–MS/MS). To investigate the relationship between legumain expression and ovarian cancer development, we tested legumain expression in malignant human ovarian tumors (n = 60), borderline ovarian tumors (n = 20), benign ovarian tumors (n = 20), and normal ovary samples (n = 20) using immunohistochemical assay (IHC). A correlation between legumain expression, and clinocopathologic and biological variables was also established. Importantly, increased legumain expression was validated by real-time PCR and Western blots, correlated positively with an increased malignancy of ovarian tumors ($P < 0.01$). In fact, patients with strong legumain expression had a worse prognosis ($P = 0.03$). In addition, results of in vitro experiments revealed that over-expression of legumain correlates with increased cell migration and invasion of ovarian cancer cells. Although legumain's functional role and clinical utility remain to be established, our results indicated that a sensitive assay for early expression of legumain may serve as both a potential biomarker and a molecular target for treatment of ovarian cancer. J. Cell. Biochem. 113: 2679–2686, 2012.

KEY WORDS: BIOMARKER; ISOBARIC TAGS FOR RELATIVE AND ABSOLUTE QUANTIFICATION (ITRAQ); LEGUMAIN; OVARIAN CANCER; PROGNOSIS

Ovarian cancer is one of the most common gynecological malignant cancers and the leading cause of death from all types of gynecological cancers [Banerjee and Gore, 2009]. Most patients present with advanced disease, because of poor prognosis due to lack of effective methods for early diagnosis and new effective treatment strategies [Sehouli and Oskay-Ozcelik, 2009]. Although antigen CA125 has been used as a serum marker for ovarian cancer diagnosis and monitoring responses to chemotherapy, its application is severely limited due to poor sensitivity in early detection [van Haaften-Day et al., 2001; Bast et al., 2005]. Several

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*Correspondence to: Rong Xiang, MD, PhD, Nankai University School of Medicine, 94 Weijin Road, Tianjin 300071, China. E-mail: rxiang@nankai.edu.cn

**Correspondence to: Huaidong Hu, Key Laboratory of Molecular Biology for Infectious Diseases of Ministry of Education of China, The Second Affiliated Hospital, Chongqing Medical University, Chongqing, China. E-mail: huaidong.hu@gmail.com

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other tumor biomarkers, such as CEA and CA19-9, are currently utilized for detection of ovarian cancer in clinical practice. However, these detection methods also lack the sensitivity and specificity required for early detection of potentially curable lesions and are not suitable for screening of population [Akdogan et al., 2001; Yurkovetsky et al., 2006, 2007, 2010]. Therefore, it is essential to discover new or supplementary biomarkers to improve diagnosis and treatment of ovarian cancer.

The stable isotope-based quantitative proteomic approach has opened new approaches for the discovery of biomarkers. These include isotope-coded affinity tagging (ICAT) [Gygi et al., 1999], isobaric tagging for relative and absolute quantification (iTRAQ) [Ross et al., 2004], ^{18}O [Mirgorodskaya et al., 2000], and stable isotope labeling of amino acids in cell culture (SILAC) [Ong et al., 2002]. In addition, iTRAQ analysis followed by liquid chromatography–mass spectrometry (LC–MS/MS) is a gel-free mass spectrometric (MS) technique with isobaric amine-specific tags to compare the peptide concentrations between samples to establish quantitative values for corresponding proteins [Zhang et al., 2008].

Here, we used iTRAQ to compare expression levels of proteins between ovarian cancer and normal ovarian tissue. Our results indicate that legumain is over-expressed in ovarian cancer and upregulated expression of legumain increases ovarian cancer cell migration and invasion in vitro. These results suggest that legumain is a potential novel biomarker for detection and prognosis of ovarian cancer.

MATERIALS AND METHODS

PATIENTS AND TISSUE SAMPLES

In this study, paraffin-embedded or frozen ovarian tumor and normal ovary specimens of 120 patients, who received surgery between January 2004 and December 2008, were obtained from Tianjin Central Hospital for Obstetrics and Gynecology (Tianjin, China). Approval was obtained prior to this study from the Institutional Research Board at the Tianjin Central Hospital for Obstetrics and Gynecology. Information on the patients' pathologic type, histological grade, stage, with or without ascites, ascites cytology and age was obtained from pathology records of the Tianjin Central Hospital for Obstetrics and Gynecology. The patients' pathology type included serous cystadenoma, mucinous cystadenoma, endometrioid carcinoma, clear cell adenocarcinoma, and low differentiated adenocarcinoma. The tumors were graded as either well-differentiated (Grade 1), moderately-differentiated (Grade 2), or poorly-differentiated (Grade 3) according to histological grading system of the International Federation of Gynecology and Obstetrics (FIGO). The FIGO staging system was also used for ovarian cancer. Age of patients ranged from 25 to 67. Patients who did receive surgery between January 2004 and December 2006 were followed up to the end of 2011 ($n = 57$). Normal ovary specimens, diagnosed as normal by post-operative pathological examination, were sectioned from patients with uterine fibroids under total abdominal hysterectomy and bilateral salpingo-oophorectomy ($n = 20$).

REAGENTS AND CELL LINES

The iTRAQ kits were purchased from Applied Biosystems (Applied Biosystems, Framingham, MA). Sequence-grade modified trypsin and M-MLV reverse transcriptase were purchased from Promega (Madison, WI). Platinum® SYBR® Green qPCR Super Mix-UDG, PRMI-1640 and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA). The polyclonal antibody anti human legumain used for IHC was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), whereas monoclonal antibody anti human legumain used for Western blotting analysis was purchased from Sigma–Aldrich (St. Louis, MO). The enhanced chemiluminescence (ECL) detection kit and transwell chambers were purchased from Millipore (Bedford, MA). The SKOV-3 and ES2 human ovarian cancer cell lines were kindly provided by the Tianjin Central Hospital for Obstetrics and Gynecology.

PREPARATION OF PROTEIN SAMPLES AND QUANTITATIVE PROTEOMIC ANALYSIS

Tissue was lysed with lysis buffer (7 M urea, 1 mg/ml DNase I, 1 mM Na_3VO_4, and 1 mM PMSF) and centrifuged at 13,000 rpm for 30 min at 4°C. Supernatants were collected, and the concentration of total proteins determined using the BCA protein assay kit (Thermo Scientific, Rockford). From each sample, 100 μg of proteins were acetone-precipitated overnight at −20°C, dissolved in lysis buffer, and denatured. Cysteines were blocked as described in the iTRAQ protocol (Applied Biosystems). Each sample was digested with 20 μl of 0.1 μg/μl trypsin (Promega) solution at 37°C overnight and then labeled with iTRAQ tags according to the manufacturer's instructions. Profiles of protein levels in samples from ovarian cancer and normal ovarian tissue were established by tandem mass spectrometry (MS). To increase coverage of protein identifications and confidence of the generated data, two biological preparations were made and analyzed. Ovarian cancer and normal ovarian tissue samples (from eight patients) respectively were labeled with the iTRAQ reagents 115 and 114 tag, and a ratio of 115:114 indicated a relative abundance of proteins in the ovarian cancer tissue samples compared to the normal ovarian tissue samples.

IMMUNOHISTOCHEMICAL ASSAY

For IHC, fresh tissues were fixed in 10% neutral buffered formalin for 16 h at 4°C and then placed in a Thermo Shandon tissue processor, and embedded in paraffin. Sections were heated in a 60°C oven, and wax removed by three changes of xylene, followed by passage through graded ethanol (100%, 95%, and 70%) before being subjected to a final wash in double-distilled H_2O. After quenching of the endogenous peroxidase activity with 3% H_2O_2 for 10 min and blocking with BSA for 30 min, sections were incubated at 4°C overnight with primary antibodies against legumain at a dilution of 1:100. Detection of legumain was achieved with the Envision-horseradish peroxidase system (Dako Cytomation, Denmark). All slides were counterstained with Gill's Hematoxylin for 1 min, dehydrated, and mounted for light microscopic evaluation. A cutoff point 25% was used in our statistical analysis, and sections were classified as negative, weak (0% to <25%) or strong (>25%).

QUANTITATIVE REAL-TIME PCR (RT-PCR)

Quantitative RT-PCR was performed with the BioRad CFX96 real-time PCR machine (California) using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), which preferentially binds to double-stranded DNA. Specific primers for human β-actin were 5′-GGCATCCACGAAACTACCTT-3′ (sense primer) and 5′-CTCGTCA-TACTCCTGCTTGC-3′ (anti-sense primer). Specific primers for human legumain were 5′-GCAGGTTCAAATGGCTGGTAT-3′ (sense primer) and 5′-GGAGTGGGATTGTCTTCAGAGT-3′ (anti-sense primer). After denaturation at 95°C for 10 s, amplification was performed in 40–45 cycles at 95°C for 5 s, 52°C for 10 s, and 72°C for 10 s. Fluorescence data were acquired after the extension step in the PCR reaction. Once reactions were complete, a melting curve program was employed. The relative quantification of gene expression was analyzed with the $2^{-\Delta Ct}$ methods. Quantitative RT-PCR analysis was repeated at least three times.

WESTERN BLOT ANALYSIS

The protein samples (20 μg) were separated by 10% SDS–PAGE, and proteins were then transferred to PVDF membranes which were blocked for 1 h with 1% BSA in Tris buffered saline with tween-20 (TBST) buffer (20 mM/L Tris, pH 7.6, 100 mM/L NaCl, 0.1% Tween-20). This was followed by incubation with primary antibodies, including monoclonal antibody against legumain (1:1,000 dilution) and actin (1:5,000 dilution) in TBST buffer containing 1% BSA at room temperature for 2 h. After washing three times with TBST buffer, membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG as secondary antibodies (1:10,000 dilution) for 1 h at room temperature. After the membranes were washed three times in TBST buffer, the reactions were visualized with the ECL detection system. All Western blot analyses were repeated at least three times.

PRODUCTION OF LENTIVIRUS, INFECTION, AND POSITIVE CELL SELECTION

To facilitate a better understanding of the role of legumain in ovarian cancer progression, we established the SKOV3 and ES2 cells with legumain over-expression using the lentivirus system. The ES-2 cell line was established from a surgical tumor which was described as a poorly differentiated ovarian clear cell carcinoma [Lau et al., 1991]. SKOV3, is an ovarian adenocarcinoma that was derived from ascites [Fogh et al., 1977]. The legumain expression plasmid was generated by inserting the legumain fragment between *Bam*H I and *Xba* I sites of the multiple cloning site of pLV-EF1α-MCS-IRES-Bsd (Biosettia, San Diego, CA). Lentivirus production and infection was done according to the manufacturer's protocol, and positive cells were selected by blasticidin S at a concentration of 10 μg/ml.

TRANSWELL INVASION AND MIGRATION ASSAY

Cell invasion assays were performed in 24-well transwells (8-μm pore size, Millipore). Matrigel (BD Bioscience, San Jose, CA) was diluted to 1 mg/ml with serum-free culture medium and immediately applied to each membrane insert that formed the upper chambers of the multiwell invasion assay plate. Matrigel was incubated overnight in a sterile laminar flow tissue culture hood. The membranes were hydrated for 2 h with 250 μl serum-free medium, and excess medium removed by aspiration. A total of 1.0×10^5 cells in 100 μl of serum-free culture medium were seeded in the upper chambers of the wells. To induce cell invasion, 600 μl 10% FBS medium was added to the lower chamber. After incubation for 24 h at 37°C and 5% CO_2, membrane inserts were removed from the plate, and non-invading cells removed from the upper surface. Membrane-associated cells were stained with 0.09% crystal violet for 30 min and washed twice with PBS. Invading cells were counted in at least five random fields using a microscope (200×). Cell migration assays were performed in a similar manner except that membrane inserts were not coated with Matrigel.

STATISTICAL ANALYSIS

Data were analyzed with SPSS13.0 software. The significance of differences in legumain expression between ovarian cancer and normal ovary specimens was established by Fisher's exact test. A relationship between legumain expression and clinicopathological factors was examined with the χ^2 method or Fisher's exact test. The rank sum test was used to compare legumain expression levels between ovarian cancer and normal ovarian tissues for RT-PCR analysis. The correlation between legumain expression and patients' survival was tested by the Kaplan–Meier method. Two-sided *P*-values <0.05 were considered statistically significant.

RESULTS

IDENTIFICATION AND RELATIVE QUANTIFICATION OF PROTEINS IN OVARIAN CANCER AND NORMAL OVARIAN TISSUES

Our goal was to identify differentially expressed proteins that were related to ovarian cancer development by using the proteomic approach of iTRAQ. Here, we demonstrated that 119 proteins were down-regulated and 87 proteins were up-regulated in ovarian cancer tissue compared with those in normal ovarian tissue. Among these 206 proteins, legumain was up-regulated five fold in ovarian cancer tissues. Figure 1 illustrates the representative MS/MS spectrum of legumain peptides and information on quantitation.

VALIDATION OF LEGUMAIN EXPRESSION

iTRAQ data was verified by quantitative RT-PCR, Western blotting and IHC. Selected frozen primary ovarian tumor specimens (n = 28) and normal ovarian specimens (n = 8) were used for quantitative RT-PCR assay. Figure 2A indicates that legumain mRNA was upregulated in ovarian cancer tissue compared to that in normal ovarian tissue ($P < 0.05$). This trend was similar to that observed when using the iTRAQ approach. Furthermore, when Western blot analysis was performed to compare legumain protein expression in representative normal ovary specimens (n = 4) and ovarian cancer specimens (n = 4), it was found upregulated in ovarian cancer tissues compared to that in normal ovarian tissues. This trend was consistent with data obtained by the iTRAQ approach and quantitative RT-PCR (Fig. 2B,C). These results indicate that legumain expression is upregulated at both mRNA and protein levels in ovarian cancer tissues.

To assess the clinical relevance of legumain expression, we compared such levels with IHC in malignant ovarian tumors (n = 60), borderline ovarian tumors (n = 20), benign ovarian tumors

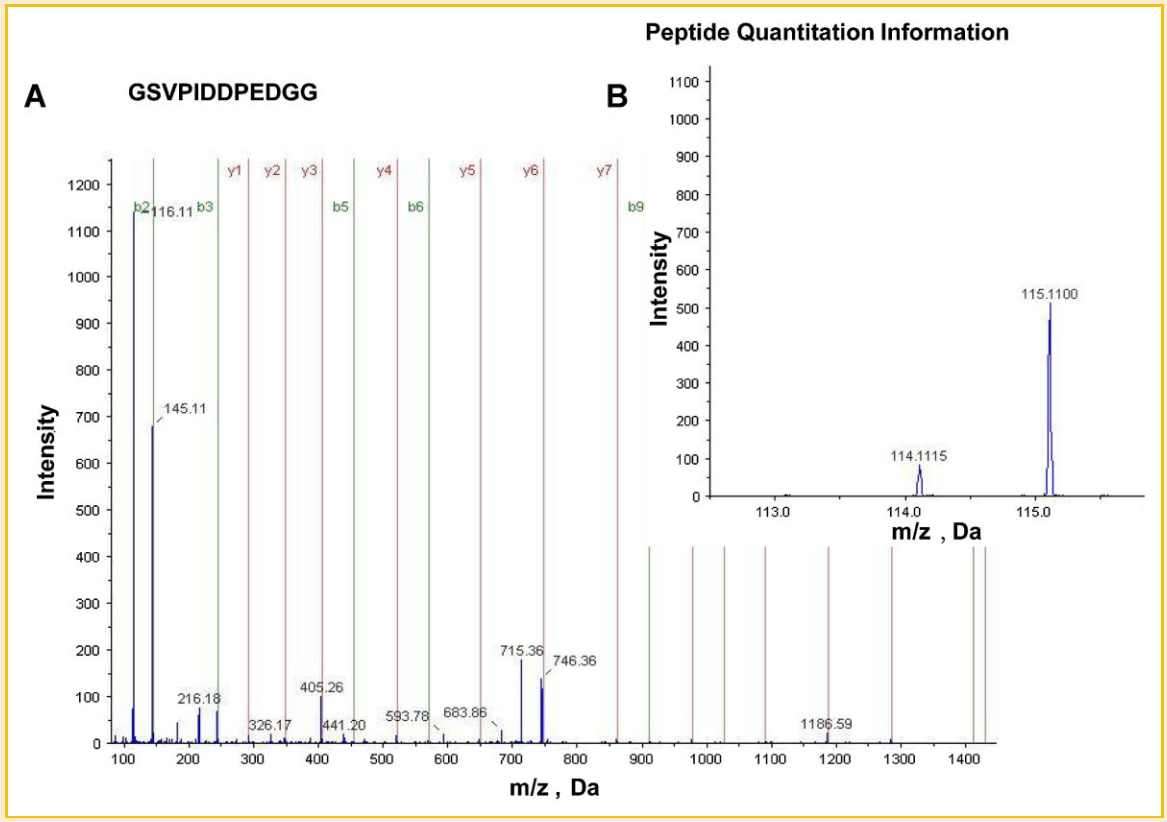

Fig. 1. MS/MS spectrum indicating peptides derived from legumain and peptide quantitation information. A: A representative MS/MS spectrum of the legumain peptide (GSVPIDDPEDGG). B: Relative quantity of legumain expression in ovarian cancer tissue labeled with 115 tag compared with that in normal ovarian tissue labeled with 114 tag. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(n = 20), and normal ovarian samples (n = 20). These data were examined and scored by a blinded investigator (S.C.), and a 25% cutoff point was used in our statistical analysis. Data were classified as negative, weak (0% to <25%) or strong (>25%). Legumain expression was absent in normal ovarian tissues, and only weakly expressed in benign ovarian tumors. In contrast, increased legumain expression was observed in both borderline ovarian tumors and malignant ovarian tumors. Legumain expression was found to be positively correlated with increased malignancy of tumors ($P < 0.01$) (Table I, Fig. 2D).

RELATIONSHIP OF LEGUMAIN EXPRESSION TO CLINICOPATHOLOGICAL VARIABLES

The relationship between expression of legumain and clinicopathological variables was analyzed by the χ^2 method or Fisher's exact test. Patients in stage III (n = 19) and stage IV (n = 1) exhibited stronger expression of legumain than those in stage I (n = 32) and stage II (n = 8; $P = 0.039$). Patients with tumor cells in ascites (n = 15) revealed increased legumain expression when compared to those without tumor cells in ascites (n = 19; $P = 0.038$). There was no significant relationship observed between legumain expression and other clinicopathological factors, such as pathologic type, histological grade, with or without ascites and age ($P > 0.05$; Table II). We next analyzed legumain expression in ovarian cancer in relation to the expression of CA125 and CA19-9. As shown in Table II, legumain expression was not associated with levels of CA125 or CA19-9 ($P > 0.05$). We also assessed any correlation between legumain expression and patients' survival. To this end, patients who received surgery at Tianjin Central Hospital for Obstetrics and Gynecology between January 2004 and December 2006 were followed up until the end of 2011. Importantly, Kaplan–Meier curve analysis indicated that patients with higher levels of legumain expression (n = 35) tended to have increased poor prognosis compared to patients with weak expression of legumain (n = 22; $P = 0.03$; Fig. 3).

LEGUMAIN EXPRESSION CORRELATES WITH INCREASED TUMORS CELL MIGRATION AND INVASION IN VITRO

To investigate if legumain was involved in tumor cell proliferation, apoptosis, migration, and invasion, we established SKOV3 and ES2 cell lines with legumain over-expression using the lentivirus system. RT-PCR analysis of legumain mRNA expression in these transduced cell lines indicated a robust increase in legumain in both of these cell lines compared to their respective control cells (Fig. 4A). Over-expression of legumain protein was also detected by Western blot analysis (Fig. 4B). However, legumain failed to affect cell proliferation and apoptosis (data not shown). When evaluating the effect of legumain over-expression on cell migration and invasion with the transwell assay, both cell lines exhibited a tendency for increased migration compared to their respective

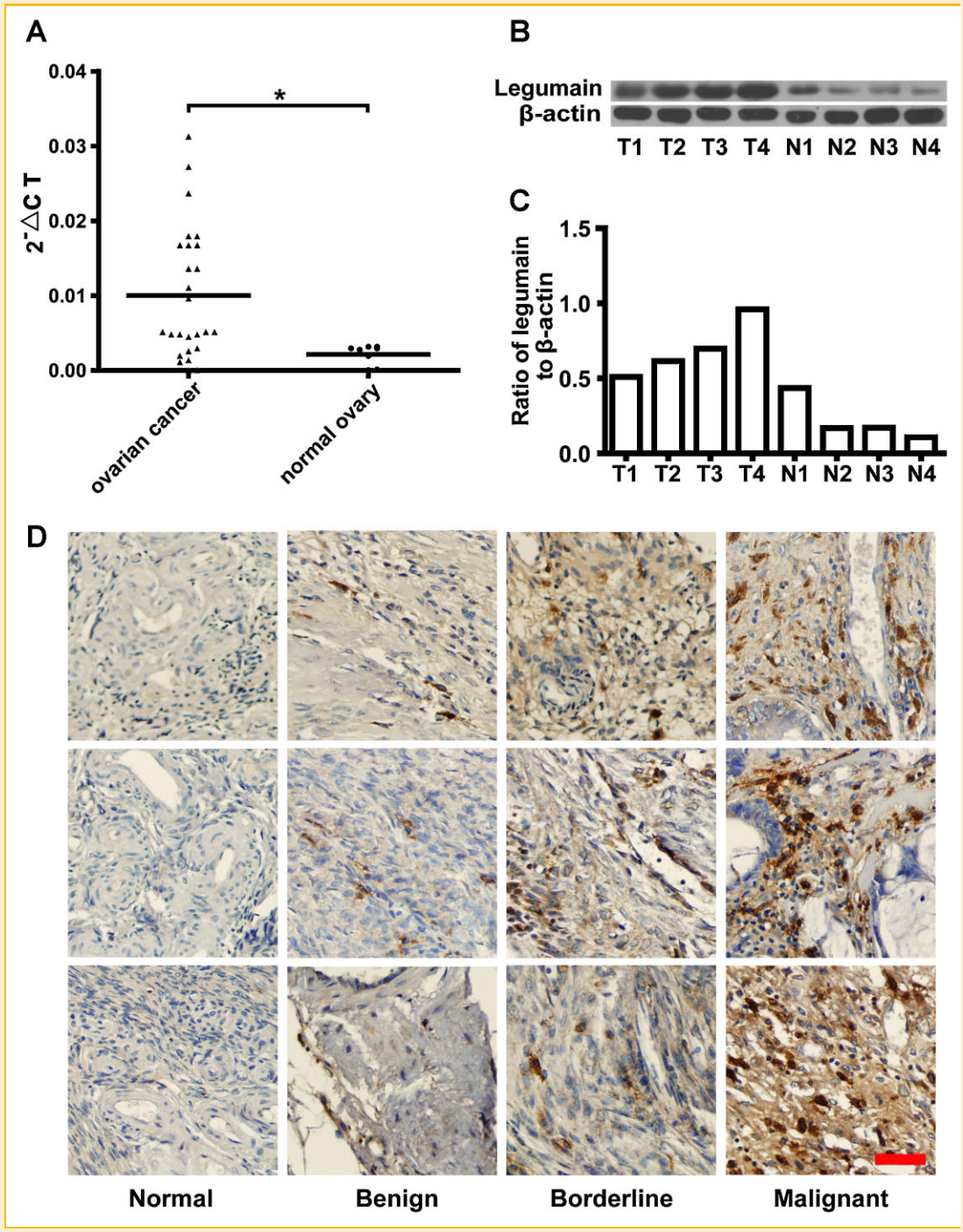

Fig. 2. Expression of legumain correlates with tumorigenesis and progression in human ovarian cancer. A: Expression of legumain mRNA in ovarian cancer tissues and normal ovarian tissues tested by real-time quantitative RT-PCR. B: Expression of legumain validated by Western blot analysis of four individual tumor samples and four individual normal samples (T, tumor samples; N, normal samples). C: Quantification of legumain expression in (B). D: Immunohistochemical staining of legumain in paraffin-embedded human ovarian cancer tissues and normal tissue samples. Legumain staining (brown) was recorded under a 20× objective and shown separately as three representatives of normal ovarian tissue samples, benign tumors, borderline tumors, and malignant tumors. Scale bar = 20 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

control cells (Fig. 4C). Results of the invasion assay suggested that over-expression of legumain in both cell lines enhanced the invasive ability in Matrigel compared to that of control cells (Fig. 4D). These experiments were repeated three times with similar results.

DISCUSSION

Ovarian cancer is one of the most common malignancies in adult females worldwide, which is associated with a high mortality. Thus

TABLE I. Legumain Expression in Relation to Increased Tumor Malignancy

	Total	Negative	Weak	Strong	*P* value
Normal	20	11	9	0	<0.01
Benign	20	7	13	0	
Borderline	20	2	13	5	
Malignant	60	0	23	37	

the discovery of novel protein biomarkers which are correlated with carcinogenesis and progression of ovarian cancer have the potential to improve clinical strategies and outcomes of this disease by increasing efficacy of diagnosis, prognosis, and treatment. Importantly, quantitative proteomics offer a powerful tool to identify novel cancer biomarkers that may be used for early detection or as therapeutic targets [Ross et al., 2004]. Here, we compared the expression profiles of legumain, an asparaginyl endopeptidase (AEP), between ovarian cancer and normal ovarian tissue with the iTRAQ approach. A total of 206 proteins were found to be differentially expressed by ovarian cancer tissues when compared to those in normal ovarian tissues. Of these 206 proteins, legumain was found up-regulated five fold in ovarian cancer.

Legumain is a member of the C13 family of cysteine proteases [Chen et al., 1997], and was first identified in plants as a processing enzyme of storage proteins during seed germination and was subsequently identified in parasites and then in mammals [Ishii, 1993; Dalton et al., 1995; Brindley et al., 1997; Chen et al., 1997, 1998]. Legumain as the only known AEP, has a restricted specificity requiring an asparagine residue at the P1 site of substrates [Chen et al., 1997; Liu et al., 2003]. Mammalian legumain also has been implicated in the processing of bacterial peptides and endogenous proteins in the lysosomal/endosomal systems for MHC class II presentation [Manoury et al., 1998, 2001; Watts et al., 2003].

TABLE II. Expression of Legumain in Relation to Clinicopathological and Biological Parameters

	Total	Weak	Strong	*P* value
Types				N.S.
Serous cystadenoma	17	5	12	
Mucinous cystadenoma	11	7	4	
Endometrioid carcinoma	13	5	8	
Clear cell adenocarcinoma	10	4	6	
Low differentiated adenocarcinoma	9	2	7	
Histological grade				N.S.
1	14	4	10	
2	12	5	7	
3	34	14	20	
Stage				0.039
I + II	40	19	21	
III + IV	20	4	16	
Ascites				N.S.
Yes	34	13	21	
No	26	10	16	
Ascitic cytology				0.038
Tumor cell+	15	3	12	
Tumor cell−	19	11	8	
Age				N.S.
≥50	29	10	19	
<50	31	13	18	
CA125				N.S.
≤35	18	7	11	
>35	42	16	26	
CA19-9				N.S.
≤37	43	19	24	
>37	17	4	13	

25% was used as a cutoff point in our statistical analysis. Sections were classified as negative, weak (0% to <25%) or strong (>25%).
Stage I: n = 32, Stage II: n = 8, Stage III: n = 19, Stage IV: n = 1.

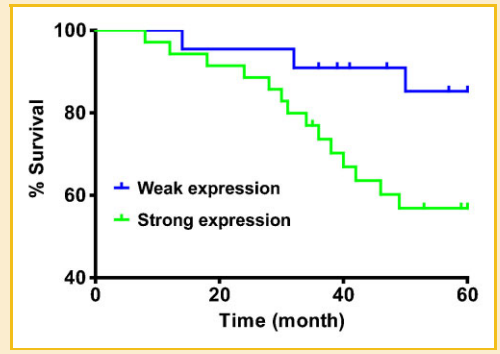

Fig. 3. Expression of legumain in relation to patients' survival. The Kaplan–Meier curve analysis of patients' tumors with strong legumain expression (n = 35) showed a worse prognosis when compared to patients who had tumors with weak legumain expression (n = 22; *P* = 0.03). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

More recently, a role for legumain in tumor biology has been reported that legumain found to be expressed in several types of human tumors including ovarian cancer [Liu et al., 2003]. Our present findings not only confirmed the upregulation of legumain was in ovarian cancer tissues compared to that in corresponding normal tissues, but also further suggested that this enzyme may also be involved in the development of ovarian cancers. In this regard, results of the iTRAQ assay indicated that the relative quantity of legumain in ovarian cancer tissues was about five fold higher than that found in normal tissues. These data were validated by RT-PCR, Western blot, and IHC, and found to be consistent with those obtained by iTRAQ analysis.

Expression of legumain has been linked to clinicopathological and biological variables in colorectal cancer [Murthy et al., 2005] and breast cancer [Gawenda et al., 2007]. Legumain is also over-expressed by endothelial cells in the breast tumor vasculature and by tumor-associated macrophages (TAMs) in the breast tumor microenvironment [Luo et al., 2006]. Although the possible involvement of legumain in several different types of cancer have been reported, our present study is the first to address expression and activity of legumain in ovarian cancer. Here, we further investigated any correlation between legumain expression and clinicopathological variables, including pathological type, stage, clinical grade, with or without ascites, ascitic cytology, age and such biological variables as biomarkers CA125 and CA19-9. We found the patterns of legumain expression to vary in different human ovarian cancer tissues and to be related to differentiation stages and ascitic cytology of the tumors. We further observed that legumain correlate significantly with survival of ovarian cancer patients, since high legumain expression was significantly associated with poor prognosis. Taken together, high expression of legumain was found to correlate with increased tumor grade, advanced disease stage and poor survival of patients with ovarian

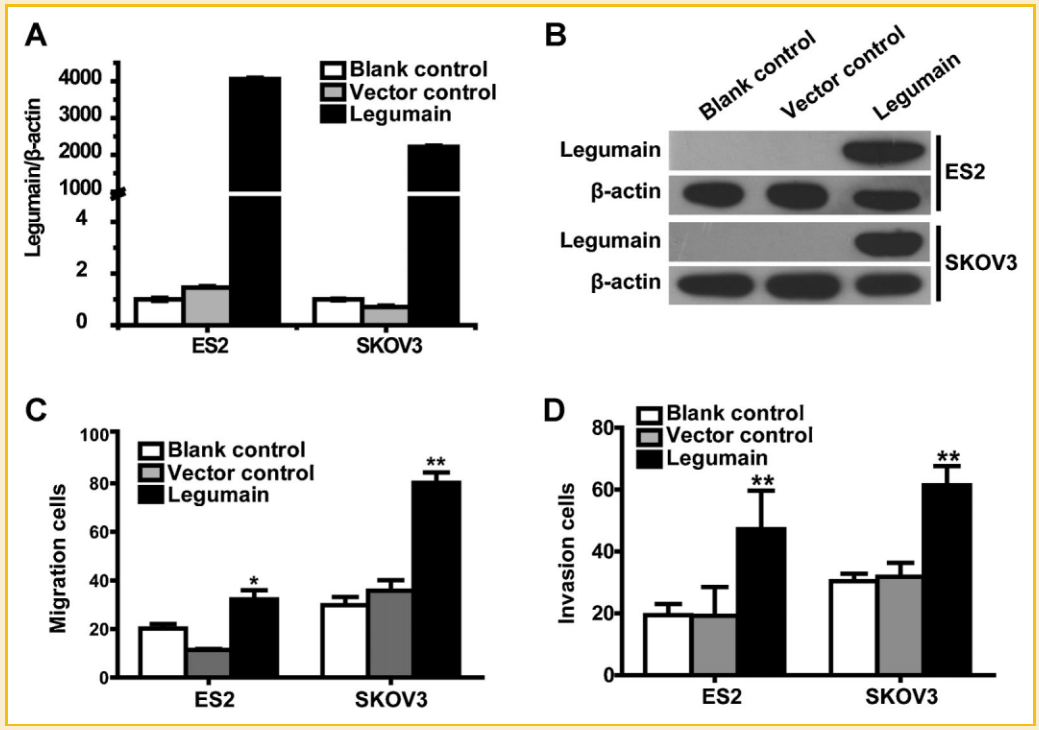

Fig. 4. Legumain affects migration and invasion of ovarian cancer cell lines in vitro. A: Expression of legumain was tested by quantitative RT-PCR in ovarian cancer cell lines (SKOV3 and ES2) with over-expressed legumain versus control cell lines. Legumain mRNA expression in these cell lines revealed a marked increase compared to their respective control cells. B: Over-expression of legumain was tested in ES2 cell and SKOV3 cells using Western blot analysis. C,D: Over-expression of legumain correlates with increase in cell migration and invasion of ovarian cancer cells in vitro. $^{*}P < 0.05$, $^{**}P < 0.01$.

cancer, indicating that legumain expression is an independent risk factor for ovarian cancer, particularly in terms of survival.

Thus far, little is known about the biological processes which involve legumain in cancer progression. However, correlation was observed between tumor invasion and metastasis and the presence of cysteine endopeptidases, such as cathepsins B and L [Mai et al., 2000]. Protease zymogen cathepsins B and L also may be activated by legumain-mediated hydrolysis of asparaginyl bonds [Chen et al., 1997, 1998]. Legumain was found to act as an asparaginyl endopeptidase in regulation of extracellular matrix remodeling through the activation of zymogen progelatinase A, which is an important mediator of extracellular matrix degradation [Itoh et al., 1998; Liu et al., 2003], or the degradation of fibronectin, which is a main component of the extracellular matrix [Morita et al., 2007]. Here, our results revealed that legumain over-expression in ovarian cancer cells did not affect cell proliferation and apoptosis, but increased cell migratory and invasive activities in vitro. Legumain could possibly affect ovarian caner progression through promoting cell migration and invasion by regulating extracellular matrix remodeling. However, this possibility requires further investigation.

Relatively, high levels of legumain expressed by tumor cells coupled with this enzyme's highly specific substrate requirement for catalytic function suggested legumain as an attractive candidate for prodrug conversion in chemotherapy. Thus, following legumain-induced activation, this prodrug proved effective to treat cancers with reduced toxicity [Liu et al., 2003; Bajjuri et al., 2011]. Moreover, Legumain-specific ligand-targeting nanoparticles have been effective for breast cancer chemotherapy in mouse model without toxicity [Liao et al., 2011]. Consequently, further studies need to be done to assess if legumain can serve as an effective molecular target for the treatment of ovarian cancer.

In conclusion, the expression of legumain was upregulated in ovarian cancer tissues when compared to normal tissues, and was related to patients' prognosis and found involved in increased migration and invasion of ovarian cancer cells in vitro. Taken together, legumain could be useful as an important predictive and prognostic factor in ovarian cancer. Furthermore, the increased expression of legumain may serve as a molecular target for the treatment of ovarian cancer.

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