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ARTICLE

Lipocalin2 Expressions Correlate Significantly With Tumor Differentiation in Epithelial Ovarian Cancer

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SUMMARY We recently identified lipocalin2 (LCN2) as being upregulated in ovarian cancer cell lines. The purpose of this study was to validate LCN2 upregulation in ovarian cancers and to investigate its potential as a serum biomarker. We assayed LCN2 expression in ovarian cancers using real-time PCR and IHC. To evaluate the potential of LCN2 as a biomarker, we measured serum LCN2 levels in 54 ovarian cancers, 15 borderline and 53 benign ovarian tumors, and 90 healthy controls. SYBR green PCR and IHC showed LCN2 overexpression in ovarian cancers. LCN2 immunoreactivity was significantly associated with tumor differentiation ($p=0.009$), as well-differentiated tumors showed the highest LCN2 expression. Serum LCN2 level in ovarian cancer was significantly higher than in the other study groups ($p<0.001$), and in accordance with IHC results, it also correlated with tumor differentiation, with well-differentiated tumors having the highest value. The sensitivity and specificity of LCN2 in detecting ovarian cancer was 72.2% and 50.4%, respectively. By Cox univariate analysis, LCN2 positivity was an independent prognostic factor for overall survival (hazard ratio = 1.47, $p=0.012$). In conclusion, LCN2 expressions are upregulated and related to tumor differentiation in ovarian cancers and should be included in future research assessing potential biomarkers for ovarian cancer. **(J Histochem Cytochem 57:513–521, 2009)**

KEY WORDS
ovarian cancer
tumor marker
lipocalin2
NGAL

OVARIAN CANCER is the fifth leading cause of cancer deaths among women, and it is the most common cause among gynecological malignancies (Jemal et al. 2007). The high mortality rate of ovarian cancer results from the high percentage of cases diagnosed at an advanced stage, which is because of the relatively asymptomatic nature of early-stage disease and the lack of adequate screening tests. When ovarian cancer is diagnosed in its early stage and is still organ confined, the 5-year survival rate exceeds 90%. Unfortunately, only 19% of all ovarian cancers are diagnosed at this stage. Therefore, an adequate early detection screening for ovarian cancer could greatly improve patient survival.

Use of serum markers for early detection of ovarian cancer has largely focused on CA125, a heavily glycosylated high-molecular-weight mucin (MUC16) (Yin et al. 2002). However, the usefulness of CA125 as a biomarker for early diagnosis is limited by the fact that CA125 exhibits a sensitivity of <60% in early-stage disease (Jacobs and Menon 2004). Aside from limited sensitivity, serum CA125 is elevated by benign gynecological conditions such as benign ovarian tumors, uterine fibroids, adenomyosis, and inflammation of the peritoneum. In recent years, numerous potential biomarkers of ovarian cancer have been identified and evaluated alone or in combination with CA125 and/or other markers (Gagne et al. 2005; Mok et al. 2007; Cho et al. 2009). Microarray technology permits analysis of expression levels of thousands of genes and is widely used to identify new biomarkers for the early detection of cancer (Wong et al. 2001; Raetz and Moos 2004). In a previous cDNA microarray analysis (Macrogen; Seoul, Korea) using serous ovarian cancer cell line, YDOV-157, and three human ovarian surface epithelial (HOSE) cells, we showed that lipocalin2 (LCN2) had an ovarian cancer/HOSE ratio of 160, suggesting its expression is upregulated in ovarian cancers (Cho et al. 2008).

LCN2, also known variously as neutrophil gelatinase-associated lipocalin (NGAL), oncogene 24p3, and neu-

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related lipocalin (NRL), is a 24-kDa secretory glycoprotein that was originally identified in mouse kidney cells and is stored in human neutrophil granules (Kjeldsen et al. 1993). Although the primary function of lipocalin is thought to relate to the transport of small ligands, they have been implicated in a variety of functions such as iron trafficking and induction of apoptosis (Devireddy et al. 2001; Yang et al. 2002). Recently, it was suggested that LCN2 may scavenge bacterial products at sites of infection. Several inflammatory stimuli, such as lipopolysaccharides and interleukin (IL)-1β, can markedly induce LCN2 expression and secretion in tissues exposed to microorganisms (Liu and Nilsen-Hamilton 1995). LCN2 limits bacterial growth by sequestering the iron-laden sideophore. Furthermore, LCN2 has become of interest to cancer researchers because its expression changes in colorectal (Nielsen et al. 1996), breast (Stoesz et al. 1998), and pancreatic cancers (Furutani et al. 1998), and LCN2 was identified as an independent poor prognostic factor in breast cancer patients (Bauer et al. 2008). In this study, we investigated LCN2 overexpression in ovarian cancer cell lines and cancer tissues. We also measured serum LCN2 levels and evaluated the clinical relevance of LCN2 as a diagnostic and prognostic marker for ovarian cancer.

Materials and Methods

Cell Lines

A total of six ovarian cancer cell lines were developed either from malignant ascites or from tissues of solid tumors. Eight HOSE cell lines were obtained by scraping the surfaces of healthy ovaries. The ovarian cancer cell lines used in this experiment were YDOV-13 (which originated from a malignant Brenner tumor); YDOV-105, YDOV-139, YDOV-157, and YDOV-161 (which originated from serous cystadenocarcinomas); and YDOV-151 (which originated from a mucinous cystadenocarcinoma). This study was approved by the institutional review board of Yongdong Severance Hospital, and informed consent was obtained from each patient before sample collection. All cell lines were established in the laboratory of Obstetrics and Gynecology, Yongdong Severance Hospital, Seoul, Korea.

Biosamples

Paraffin-embedded samples of ovarian cancer (n=61), borderline ovarian tumors (n=9), benign ovarian tumors (n=11), and healthy tissue (n=10) were collected between April 2001 and May 2007 and stored at the Yongdong Severance Hospital pathology department archives. Serum samples (n=122) and fresh frozen tissues (n=12) from a different group of patients were obtained from women who underwent elective surgery for an ovarian tumor at the Yongdong Severance Hospital between May 2004 and July 2007. Blood samples of case groups (n=122) were collected 24 hr or less before surgery by peripheral venous puncture. Control serum specimens (n=90) were obtained from patients undergoing a routine health examination at Yongdong Severance Hospital between October 2005 and June 2006. All blood samples were centrifuged at 1500 × g at 4C for 15 min. The separated serum was removed, aliquoted, and stored at −80C for future analysis. Fresh tumor specimens were obtained at the time of surgery, were snap frozen in liquid nitrogen, and were stored at −80C. None of the included patients had a prior diagnosis of cancer or had received chemotherapy or surgery for the present disease. Healthy controls had no history of cancer or gynecological disease and no abnormalities as assessed by laboratory examinations or gynecological sonography. All ovarian cancer patients were surgically staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. All FIGO Stage I/II ovarian cancer patients had pelvic and para-aortic lymph node dissection according to the National Comprehensive Cancer Network (NCCN) clinical practice guidelines.

SYBR Green Real-time PCR

The SNU840 cell line was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea), and SKOV3, TOV112D, OVCA429, and RMUG-S cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained in DMEM/F12 supplemented with 10% FBS in the presence of 5% CO_2 at 37C in a humidified incubator. SYBR green real-time PCR was used to analyze cell lines and fresh tissues. Total RNA was extracted from 8 HOSE cell lines (HOSE 10, 15 186, 198, 201, 213, 216, and 225), 4 borderline ovarian tumor tissues (3 serous and 1 mucinous), 11 ovarian cancer cell lines (SKOV3, TOV112D, OVCA429, RMUG-S, SNU840, and YDOV-13, -105, -139, -151, -157, and -161), and 7 ovarian cancer tissues (7 serous) using the RNeasy Mini kit (Qiagen; Valencia, CA). The RNA samples were treated with DNase I before reverse transcription processing to remove genomic DNA contamination. A total of 2 μg RNA from each sample was reverse transcribed into cDNA with the SuperScript III first-strand synthesis system (Invitrogen; Carlsbad, CA) according to the manufacturer's suggested protocol. The expression of candidate gene mRNA was measured by SYBR green real-time PCR using an ABI 7300 instrument (Applied Biosystems; Foster, CA). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), a housekeeping gene, was used as an internal control. The specific forward primer 5′-GGAGCTGACTTCGGAACTAAAGG-3′ and reverse primer 5′-TGTGGTTTTCAGGGAGGCC-3′ for LCN2 was used. The PCR was performed in 20 μl buffer containing 2 μl cDNA, 5 pM of each

primer, and power SYBR green PCR master mix (Applied Biosystems). The thermal cycling conditions consisted of a preincubation for 2 min at 50C and denaturation for 10 min at 95C, followed by 40 cycles of denaturation for 15 sec at 95C and annealing/extension for 1 min at 60C. All experiments were done in triplicate to verify the results. The normalization formula was as follows: target amount = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = [Ct (Candidate gene) − Ct (Candidate gene *GAPDH*)] − [Ct (HOSE186) − Ct (HOSE 186 *GAPDH*)].

IHC

The paraffin-embedded specimens used in this study were archived tissue samples and not from patients contributing fresh specimens and consisted of tissue from 10 healthy ovaries, 11 mucinous cystadenomas, 9 borderline ovarian tumors (5 serous and 4 mucinous), and 61 epithelial ovarian cancers (38 serous, 12 mucinous, 6 endometrioid, 2 transitional cell, 2 mixed, and 1 clear cell).

IHC studies were performed using the avidin-biotin technique with the LSAB+ System-HRP (DakoCytomation; Glostrup, Denmark). Paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol, and treated for 10 min with 3% H_2O_2 in methanol to block endogenous peroxidase. Sections were incubated in a moist chamber with primary anti-human LCN2 goat IgG (5 μg/ml; R&D Systems, Minneapolis, MN) for 30 min at room temperature, followed by incubation with biotinylated secondary antibody (DakoCytomation) for 30 min. The reaction product was visualized using a DAB chromogen solution (DakoCytomation). Sections were counterstained with hematoxylin and mounted in Paramount aqueous mounting medium (DakoCytomation). Representative photomicrographs were recorded using a digital camera (Nikon; Tokyo, Japan).

To evaluate IHC expression of LCN2, we applied a four-grade scoring system corresponding to the sum of staining intensity (0 = negative; 1 = weak; 2 = moderate; 3 = strong) and the percentage of positive cells (0 = 0%; 1 = 1–25%; 2 = 26–50%; 3 = 51–100% positive cells), as described elsewhere (Shibusa et al. 1998). Slides were scored in the absence of any clinical data, and the final immunostaining score was the average score of two observers.

ELISA

Serum LCN2 level was quantified with a solid phase sandwich ELISA using the Human Lipocalin-2/NGAL Immunoassay kit (R&D Systems). Serum used in this assay was derived from 90 healthy controls, 53 patients with benign tumors (19 mucinous cystadenomas, 16 mature teratomas, 7 serous cystadenomas, 5 endometriotic cysts, 3 tubo-ovarian abscesses, and 3 hemorrhagic corpus luteal cysts), 15 patients with borderline ovarian tumors (10 mucinous and 5 serous), and 54 patients with epithelial ovarian cancers (38 serous, 9 mucinous, 4 clear cell, and 3 endometrioid).

Microplates were precoated with rat anti-LCN2 monoclonal antibody (100 μl of 20 μg/ml in 0.1 M carbonate buffer; pH, 9.5) and blocked with 1% BSA and 0.05% Tween 20. Serum and 640 ng/ml (9850 pM) of human LCN2 standard were diluted with Calibrator Diluent RD5-25 (provided by the manufacturer) and added to the plates for 2 hr at 4C. After four washes with diluted wash buffer, a volume of 200 μl horseradish peroxidase conjugated to anti-LCN2 monoclonal antibody was added and incubated for 2 hr at 4C. After four additional washes, color reagents A (hydrogen peroxide) and B (tetramethyl benzidine) were added, and the signal was allowed to develop for 30 min at room temperature. The reaction was stopped with 50 μl of 1 N sulfuric acid, and the absorbance at 450 nm was measured by an automatic ELISA reader.

Results were converted from mean absorbance of duplicate wells after subtraction of background values. Recombinant human LCN2 protein (R&D Systems) was used as a standard. The standard curve was prepared simultaneously with the measurement of test samples. A reagent blank, a test sample blank, and internal controls of serum samples were used to normalize LCN2 values obtained from each experiment.

Statistical Analysis

Relative serum LCN2 levels were compared using an unpaired *t*-test on log-transformed values. Serum LCN2 levels ranged over multiple orders of magnitude; thus, a logarithmic transformation was used to change the data to an arithmetic scale. The transformed data complied more accurately with the assumption of a Gaussian distribution for residuals in general linear models. Data were summarized based on the number of observations, the geometric mean [*p* values were applied appropriately to differences in the log (LCN2) levels], the 95% CI for the geometric mean, and the range of the data.

Comparison of groups was performed using the Mann-Whitney *U* test, one-way ANOVA, and Kruskal-Wallis tests where appropriate. To report specificity and sensitivity estimates, we used full receiver operating characteristics (ROC) curves with cut-off values that maximized the sum of sensitivity and specificity. The Cox proportional hazards model was used to determine the prognostic significance of the variables for predicting overall and disease-free survival. Predictive variables were selected by stepwise (forward and backward) selection procedures. All statistical tests were two sided, and significance was defined at a level of $p<0.05$. Statistical analyses were performed using SPSS version 12.0 (SPSS; Chicago, IL).

Results

SYBR Green Real-time PCR Analysis of LCN2

To study variation in transcript LCN2 levels, SYBR green real-time PCR analysis was applied to an expanded series of epithelial ovarian cancer cell lines, cancer tissues, and borderline ovarian tumor tissues (Figure 1). The mean $2^{-\Delta\Delta Ct}$ value of borderline tumors (615-fold), cancer cell lines (4727-fold), and cancer tissues (1058-fold) was significantly higher than that of healthy HOSE cells ($p=0.042$). Except for TOV112D, the other 10 ovarian cancer cell lines had the higher levels of LCN2. There was no significant difference in LCN2 expression among the tumor tissues of different histological subtypes.

IHC

The mean ages of women involved in the IHC study were 53 years for those with ovarian cancer, 45 years for those with borderline ovarian tumors, and 37 years for those with benign ovarian tumors. LCN2 immunoreactivity was not evident in normal ovarian surface epithelium. However, 98.3% (60/61) of ovarian cancers, 100% (9/9) of borderline ovarian tumors, and 72.7% (8/11) of benign ovarian tumors stained positive for LCN2. Most staining was observed in the cytoplasm of tumor cells (Figure 2).

The immunostaining scores from healthy ovaries, benign ovarian tumors, borderline ovarian tumors, and epithelial ovarian cancers were 0.00 (95% CI, 0.00–0.00), 2.36 (95% CI, 1.36–3.36), 3.38 (95% CI, 2.41–4.36), and 4.44 (95% CI, 4.09–4.78), respectively (Table 1). Differences between diagnostic groups ($p<0.001$) and differences between tumor grades ($p=0.002$) were statistically significant, with well-differentiated cases having higher staining scores (Figure 3). For those with ovarian cancers, there was no significant difference in LCN2 immunoreactivity among different stages or histological types.

Pretreatment Serum LCN2 Levels in Patients With Ovarian Cancer

In real-time PCR and IHC, we found that LCN2 was overexpressed in borderline and malignant tumors but very weakly expressed in benign tumor tissues. Therefore, we next examined the LCN2 levels in the pretreatment serum samples using a solid phase sandwich ELISA. FIGO staging was available for all 54 ovarian cancer cases. There were 5 Stage I samples, 3 Stage II samples, 38 Stage III samples, and 8 Stage IV samples from epithelial ovarian cancers included in the ELISA studies. The mean ages for the groups by diagnostic category were 50.3 years for healthy controls, 39.2 years for patients with benign ovarian tumors, 37.7 years for patients with borderline ovarian tumors, and 52.6 years for patients with ovarian cancers. Because the age difference between the study group was significant

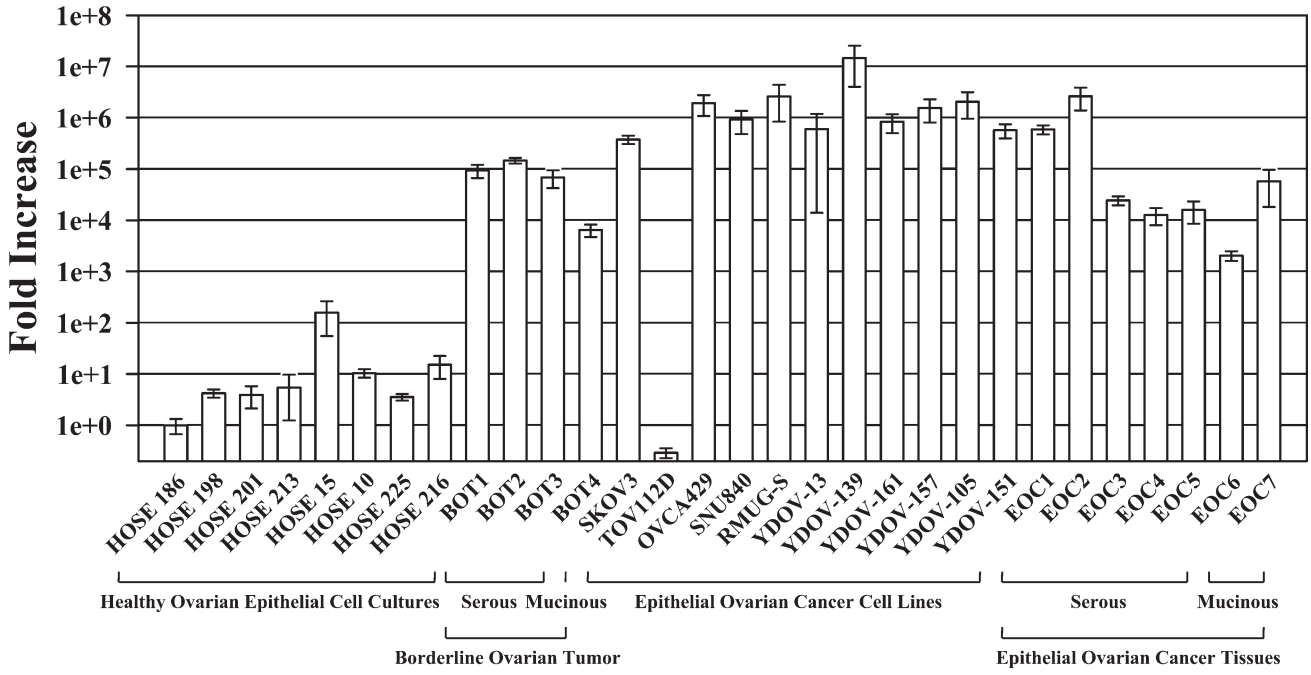

Figure 1 Relative quantitation of lipocalin2 (LCN2) in healthy ovarian epithelial cell cultures, borderline ovarian tumor tissues, ovarian cancer cell lines, and cancer tissues. Independent *t*-tests showed statistically significant differences between study groups ($p<0.001$). Each value is expressed as the mean of duplicate. The reference tissue, HOSE 186, was considered to have a value of 1.

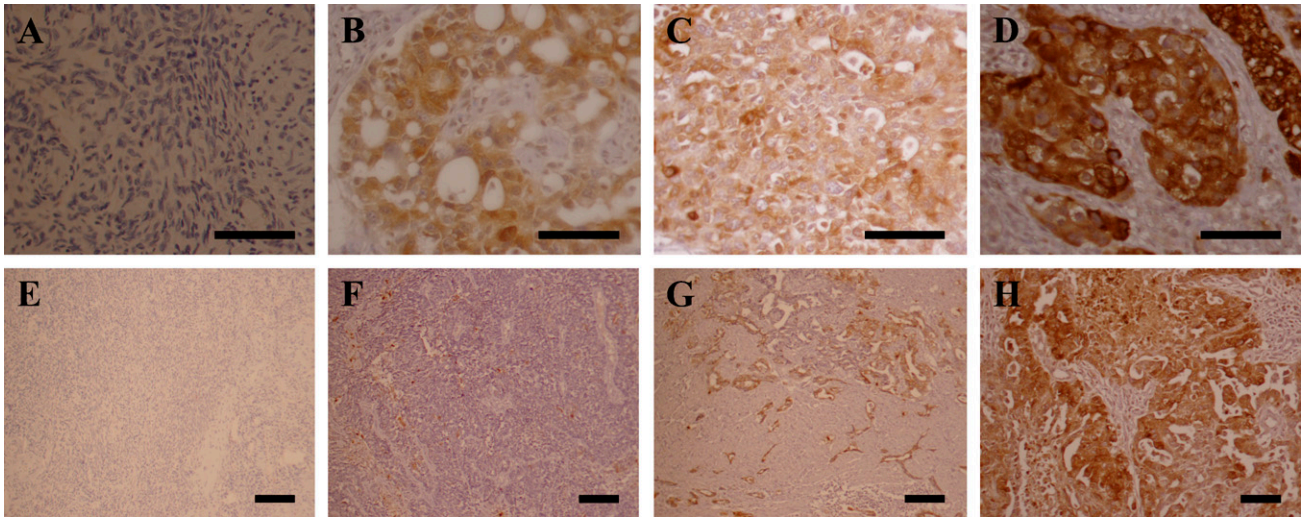

Figure 2 Evaluation of LCN2 IHC staining. The staining intensity (**A**, no evidence of staining, 0; **B**, weak staining, 1+; **C**, moderate staining, 2+; **D**, strong positive staining in most cells, 3+) and the percentage of positive cells (**E**, no cells staining positive, 0; **F**, <25% of cells staining positive, 1+; **G**, 25%–50% of cells staining positive, 2+; **H**, >50% of cells staining positive, 3+) were scored. Representative fields were photographed in serous type. Bars: **A–D** = 50 mm; **E–H** = 100 mm.

(p=0.001), p values for the differences in mean LCN2 levels are presented from linear models that include a term for age.

In healthy controls, the mean serum LCN2 level was 61.9 ng/ml (95% CI, 29.3–121.6). The corresponding LCN2 values were 67.1 ng/ml (95% CI, 14.3–238.2) for patients with benign ovarian tumors, 72.1 ng/ml (95% CI, 33.2–111.0) for patients with borderline ovarian tumors, and 87.4 ng/ml (95% CI, 67.5–107.3) for patients with ovarian cancers. Serum LCN2 levels were significantly higher in ovarian cancer patients compared with healthy controls (p=0.012). We also compared the relationship of serum LCN2 and CA125 levels with clinicopathological characteristics in ovarian cancer patients (Table 2). There were significant differences in LCN2 levels among tumor grade (p=0.038) and histological type (p=0.001) of ovarian cancer, with well-differentiated tumors and mucinous cases having higher LCN2 expression (Figure 4). However, serum CA125 levels did not correlate with these clinicopathological characteristics.

Table 1 Expression of LCN2 in relation to clinicopathological characteristics in IHC analysis

		Scores	
	No. of patients	Geometric mean (95% CI)	Range
All study subjects	91	3.59 (3.19–4.00)	0.0–6.0
Diagnostic category			
Healthy	10	0.00 (0.00–0.00)	0.0–0.0
Benign	11	2.36 (1.36–3.36)	0.0–4.5
Borderline	9	3.38 (2.41–4.36)	2.0–5.5
Cancer	61	4.44 (4.09–4.78)	0.0–6.0
p value		<0.001	
FIGO stage of cancer			
I/II	13	4.15 (3.47–4.83)	2.5–6.0
III/IV	44	4.48 (4.04–4.92)	0.0–6.0
Recurrence	4	4.87 (3.51–6.23)	4.0–6.0
p value		0.597	
Histology of cancer			
Serous	38	4.43 (4.00–4.86)	0.0–6.0
Mucinous	12	4.54 (3.55–5.53)	1.0–6.0
Endometrioid	6	4.08 (2.65–5.50)	2.0–6.0
Others	5	4.70 (2.80–6.59)	2.0–5.5
p value		0.885	
Grade of cancer			
Borderline	9	3.38 (2.41–4.36)	2.0–5.5
Well	9	5.05 (4.16–5.94)	3.0–6.0
Moderate	21	4.80 (4.41–5.20)	2.5–6.0
Poor	26	3.92 (3.26–4.58)	0.0–6.0
p value		0.009	

The Kruskal-Wallis ANOVA was used to compare the staining score among the groups. LCN2, lipocalin2; FIGO, International Federation of Gynecology and Obstetrics.

Diagnostic and Prognostic Significance of Serum LCN2 Levels

The ROC curve was used to analyze the ability of LCN2 to identify patients with ovarian cancer for all possible cut-off values. The area under the curve (AUC) for serum LCN2 levels in ovarian cancer patients was 0.622 (95% CI, 0.526–0.717). On the basis of an optimal cut-off value (55.2 ng/ml) that maximized the sum of sensitivity and specificity in the ROC curve, the sensitivity and specificity of serum LCN2 level for detecting ovarian cancer was 72.2% and 50.4%, respectively. For CA125, a fixed cut-off value of 35 U/ml was used for the analysis of diagnostic power. The AUC for CA125 was 0.917 (95% CI, 0.873–0.960), with a sensitivity of 79.6% and specificity of 79.1%.

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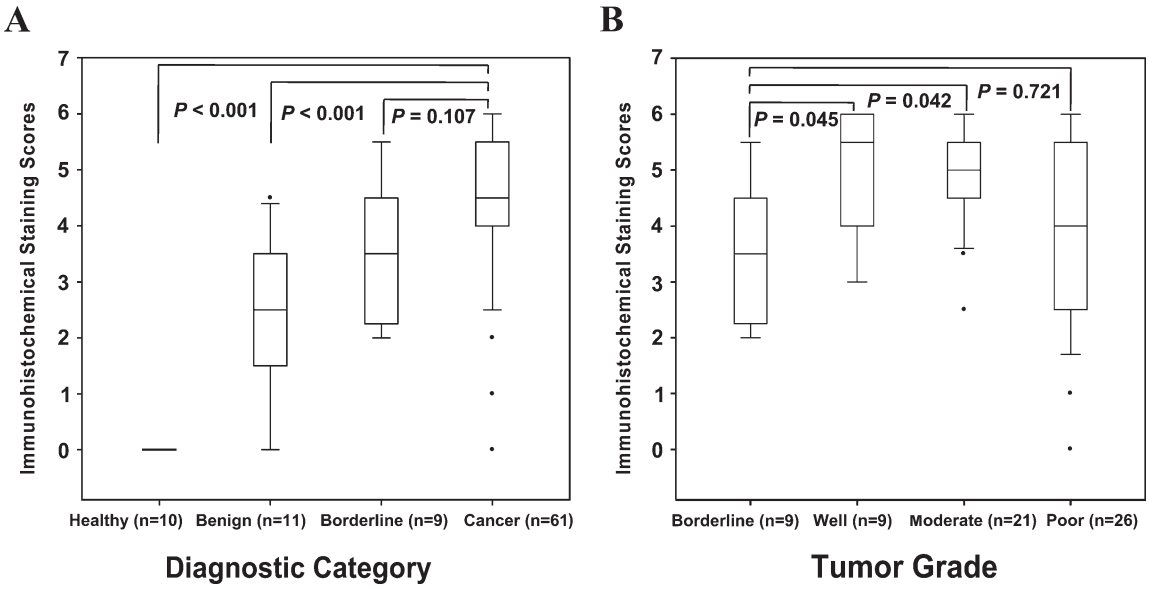

Figure 3 IHC staining score of LCN2 in ovarian cancer samples. (**A**) IHC staining score of LCN2 in ovarian cancer samples was significantly higher than that in benign ovarian tumors and healthy controls. (**B**) The mean scores associated directly with tumor grade; well-differentiated tumors stained more strongly than poorly differentiated tumors. The Kruskal-Wallis ANOVA and a post hoc Dunn method were used to compare the staining score among the groups.

Clinicopathological and outcome information and marker values for LCN2 and CA125 were available for 50 ovarian cancer patients who were monitored for survival and recurrence. The mean follow-up time was 22.1 months. Nine patients (18.0%) died within this period, 12 (24.0%) survived but suffered recurrence, 4 (8.0%) survived but suffered persistent disease, and 25 (50.0%) showed no evidence of disease after treatment. For the patients with recurrent disease, the mean time to recurrence after initial treatment was 14.6 months.

Cox proportional hazards analysis was performed to compare the impact of LCN2 expression on survival

Table 2 Pretreatment serum levels of LCN2 and CA125 in ovarian cancer patients

		LCN2 level (ng/ml)		CA125 level (U/ml)	
	No. of patients	Geometric mean (95% CI)	Range	Geometric mean (95% CI)	Range
All study subjects	212	70.4 (63.8–77.0)	14.3–414.3	239.6 (138.1–341.0)	5.2–6899.5
Diagnostic category					
Healthy	90	61.9 (57.2–66.5)	29.3–121.6	13.6 (12.3–14.9)	5.2–41.1
Benign	53	67.1 (55.4–78.7)	14.3–238.2	32.9 (21.6–44.2)	6.3–215.5
Borderline	15	79.6 (40.2–119.0)	27.8–281.7	272.2 (4.8–539.7)	12.2–1482.0
Cancer	54	87.4 (67.5–107.3)	21.1–414.3	810.6 (454.9–1166.3)	9.9–6899.5
p value		0.021		<0.001	
FIGO stage of cancer					
I/II	8	72.5 (41.2–103.9)	32.1–151.0	243.0 (−237.3 to 723.3)	9.9–1663.0
III/IV	46	90.0 (67.0–113.0)	21.1–414.3	909.3 (502.0–1316.7)	25.9–6899.5
p value		0.536		0.184	
Histology of cancer					
Serous	38	72.8 (62.9–82.7)	21.8–147.9	997.9 (532.6–1463.2)	14.0–6899.5
Mucinous	9	167.1 (53.2–281.0)	32.1–414.3	472.8 (−413.3 to 1359.1)	9.9–3539.5
Others	7	64.6 (42.1–87.0)	35.8–103.8	227.9 (−35.9 to 491.9)	25.9–825.0
p value		0.001		0.252	
Grade of cancer					
Borderline	15	79.6 (40.2–119.0)	27.8–281.7	272.2 (4.8–539.7)	12.2–1482.0
Well	6	155.7 (7.7–303.6)	49.1–414.3	1027.6 (−728.1 to 2783.3)	124.0–3539.5
Moderate	23	79.3 (63.7–95.0)	21.1–151.0	807.3 (326.5–1288.1)	9.9–4141.0
Poor	25	78.5 (49.1–108.0)	29.9–401.1	771.8 (175.2–1368.4)	14.0–6899.5
p value		0.038		0.495	

The Kruskal-Wallis ANOVA and the Mann-Whitney *U* test was used to compare the serum LCN2 level among the groups. LCN2, lipocalin2; FIGO, International Federation of Gynecology and Obstetrics.

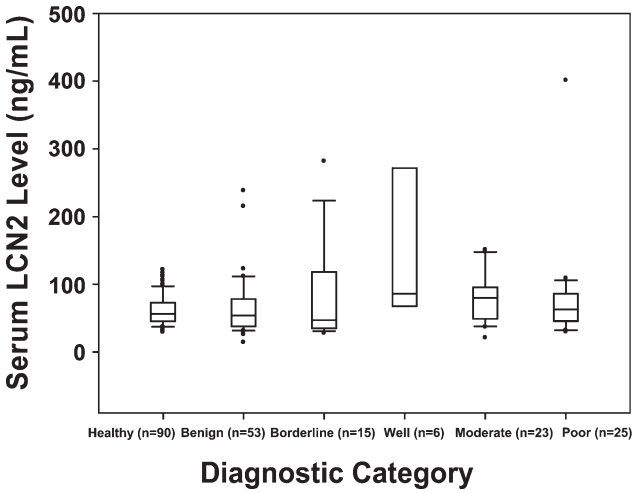

Figure 4 Pretreatment serum LCN2 levels in study subjects. The differences in diagnostic categories were statistically significant ($p=0.021$), as were the differences in tumor differentiation ($p=0.038$). The Kruskal-Wallis ANOVA and a post hoc Dunn method were used to compare the serum LCN2 level among the groups.

with those of currently used clinicopathological prognostic factors (CA125, age, stage, grade, and histological type). On univariate Cox survival analysis, we found that LCN2 (hazard ratio = 1.47, $p=0.012$), CA125 (hazard ratio = 2.46, $p=0.041$), and stage (hazard ratio = 3.46, $p=0.017$) were significantly associated with overall survival. However, no variables were independent predictors of poor prognosis on multivariate analysis.

Discussion

In this study, we validated the use of LNC2 as a potentially relevant ovarian cancer serum biomarker. LCN2 was identified in our previous study as being upregulated in ovarian cancer cell lines using cDNA microarrays. In this study, we examined the potential of LCN2 as a novel biomarker using SYBR green real-time PCR in normal and ovarian cancer cell lines and in ovarian cancer tissues. We also used IHC to study LCN2 expression in cancer and normal tissues. Finally, we measured and compared the LCN2 levels in sera from healthy controls and case patients with ovarian cancers, borderline ovarian tumors, and benign ovarian tumors. We showed that serum LCN2 levels were significantly elevated in our cohort of ovarian cancer patients.

Although LCN2 was identified more than a decade ago, the physiological functions of this protein remain poorly understood. LCN2 is the human homolog of the murine molecule known as oncogene 24p3 (mouse) and neu/HER2-related lipocalin (rat) (Hraba-Renevey et al. 1989). LCN2 is released from activated neutrophils and exists in monomeric and homo- and heteromeric forms; the latter forms a dimer with human neutrophil gelatinase B (pro-MMP-9) (Kjeldsen et al. 1993). A number of functions have been postulated for LCN2. For instance, LCN2 is involved in the inflammatory response, and high concentrations of LCN2 expression are found in tissues that are often exposed to microorganisms, indicating a role for this protein in the defense against bacteria (Xu and Venge 2000). Chronic inflammation was recently recognized as a risk factor for epithelial-derived malignancies (Brower 2005). In a previous study on the inflammatory response in epithelial ovarian cancer, we found that the neutrophil count was significantly elevated in ovarian cancers (Cho et al. 2009). As cancer and inflammation are related, it is reasonable to expect an upregulation of LCN2 expression in premalignant and early-stage ovarian malignancies when the inflammatory process is heightened. LCN2 also serves as an iron-transporting protein (Yang et al. 2002). Because iron is a component of enzymes involved in DNA synthesis, metabolism, oxygen response, and regulation of gene expression at the transcriptional and post-transcriptional level, the delivery of iron to cells is crucial for development, cell growth, and survival (Cooper and Porter 1997). In addition, abnormal expression of LCN2 has been shown in several types of cancers including colon, pancreas, and breast cancer (Nielsen et al. 1996; Stoesz et al. 1998; Moniaux et al. 2008).

In this study, we showed, through SYBR green real-time PCR, that LCN2 mRNA expression is increased in ovarian cancer cell lines, ovarian cancer tissues, and borderline ovarian tumor tissues compared with healthy ovarian surface epithelial cells ($p<0.001$). With the exception of only 1 ovarian cancer cell line, TOV112D, the other 10 ovarian cancer cell lines and 7 ovarian cancer tissues had significantly higher LCN2 levels than the HOSE cell lines. The TOV112D comes from a Grade 3 ovarian endometrioid tumor, and the histological type may reflect the low expression of LCN2 in the TOV112D cell line, because endometrioid cell types had significantly lower LCN2 expression than other histological types by real-time PCR, IHC, and ELISA. Although the mechanisms underlying histological type-specific expression of LCN2 are unclear, endometrioid tumors display morphological and molecular genetic alterations that are different from those seen in other types of ovarian tumors, and it may be different expression of LCN2. Furthermore, the small number of patients with endometrioid tumors in this study may have influenced the results.

To further validate LCN2 expression in actual tumor tissues, we examined the LCN2 expression by IHC staining. We observed the immunoreactivity exclusively in tumor cells (60 of 61 cases, 98.3%). Significantly stronger cytoplasmic staining was detected in cancer tissues than in benign ovarian tumors ($p<0.001$) and healthy ovarian tissues ($p<0.001$). Normal ovarian epithelia and ovarian stroma were negative for LCN2

expression. These results strongly suggest that the source of elevated serum LCN2 level in ovarian cancer is the cancer tissue itself. IHC results also showed a grade-specific pattern of LCN2 expression. It has been previously reported that LCN2/NGAL expression correlated strongly with poor histological grade in the IHC study of breast cancer patients (Bauer et al. 2008). However, in our study, significantly increased LCN2 immunoreactivity was observed in well-differentiated ovarian tumors compared with moderately and poorly differentiated tumors (Table 1; Figure 3). Furthermore, these immunostaining data correlated with the ELISA results of this study. Partly consistent with the this study, Lim et al. (2007) reported that LCN2 expressions were evident in borderline and Grade 1 ovarian tumors and explored the clinical usefulness of LCN2 as a marker of premalignant lesions in ovarian cancer. Moniaux et al. (2008) also found a gradient of LCN2 expression in pancreatic tumors, from the strongest staining in well-differentiated tumors to no staining in poorly differentiated tumors. Our study suggests that well-differentiated epithelial ovarian cancers stain intensely for LCN2 and that such staining reliably reflects the amount of epithelial differentiation. LCN2 expression is linked with the epithelial phenotype of ovarian tumors and is lost as cancer progresses and epithelial tumors become poorly differentiated.

For tumor marker discovery, it is essential to show that changes in mRNA expression are reflected at the protein level and that these proteins are shed into body fluid where they can be sampled conveniently. To determine whether the profile of LCN2 expression found in tissues was reflected in peripheral blood, we examined serum LCN2 level by ELISA from patients with ovarian cancer, borderline ovarian tumors, benign ovarian tumors, and from control subjects. LCN2 serum levels in ovarian cancers were significantly higher than those of other study groups ($p=0.021$). In addition, LCN2 serum levels were significantly higher in the patients with well-differentiated tumors than other grades of tumors ($p=0.038$), which is consistent with the IHC analysis. When analyzed in according to the histological subtypes, LCN2 serum levels in mucinous-type tumors (167.1 ng/ml) were considerably higher than in other histological types (serous: 72.8 ng/ml, other types: 64.6 ng/ml). The molecular basis for this observation is unclear but may reflect fundamental differences in histogenesis between non-mucinous and mucinous ovarian carcinomas. Several published studies have reported molecular differences in mucinous and serous type ovarian cancer and suggested that mucinous tumors should be regarded as separate entities (Pieretti et al. 2002; Fujita et al. 2003). Several biomarkers, such as mesothelin and N-cadherin, have been found to show differing expression between non-mucinous and mucinous ovarian cancers (Peralta Soler et al. 1997; Ordonez 2003). We also suspected that this discrepancy between IHC and ELISA result could be because of the fact that the samples were from a different cohort of patients.

Finally, we analyzed the diagnostic and prognostic power of LCN2 serum levels and found that serum levels of this protein may be a useful discriminative marker for ovarian cancer. However, the approximate area under the ROC curve for LCN2 as an independent diagnostic tool for ovarian cancer detection was 0.622, which was inferior to that of CA125, suggesting that LCN2 alone is unlikely to be sufficiently sensitive to detect all cases of ovarian cancer. Furthermore, no variables were independent predictors of poor prognosis by Cox proportional multivariate analysis. Nevertheless, a large study with more cases and controls needs to be performed to confirm the clinical relevance of LCN2 in combination with CA125 or other potential tumor markers. Considering the heterogeneity of ovarian cancers from different patients, it is unlikely that any single marker will be sufficiently sensitive to provide an optimal initial screen. Adding one or several markers to CA125 for use as a combined marker could improve diagnostic performance if sensitivity were improved with no loss in specificity.

Gene expression analysis has the potential to guide the treatment of ovarian tumors, help diagnose the subtypes of disease, and predict the patient survival. Our study provides a case of validation, which is necessary once a differentially expressed gene has been identified through microarray analysis. We report that serum LCN2 levels may serve as a possible circulating biomarker for epithelial ovarian cancers. Future studies are needed to assess whether serum LCN2 levels, either alone or in combination with other markers, could be used as a serum biomarker to improve the sensitivity and specificity of identifying early-stage ovarian cancer or subgroups of such cancers.

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