Assessment of HB-EGF Levels in Peritoneal Fluid and Serum of Ovarian Cancer Patients using ELISA

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Abstract. *Background: Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a rational target for ovarian cancer therapy. The aim of this study was to examine HB-EGF levels in the peritoneal fluid and serum of ovarian cancer (OVCA) patients. Patients and Methods: Samples were collected from six healthy women, 21 OVCA patients, and 21 ovarian cyst patients. HB-EGF levels were measured using a sandwich ELISA kit and calculated using a parallel line assay. Results: No significant difference between the slopes of the standard and sample curves was observed at an anti-HB-EGF antibody concentration of 1.6 μg/ml. HB-EGF levels in the peritoneal fluid and serum of OVCA patients were significantly higher than those in patients with ovarian cysts or controls. Serum HB-EGF levels were also significantly correlated with levels in peritoneal fluid in OVCA patients. Conclusion: We developed an assay for the exact measurement of HB-EGF levels in peritoneal fluid and serum.*

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), an EGF family ligand, is an autocrine/paracrine growth factor that binds to the epidermal growth factor receptor (EGFR) and plays a pivotal role in intracellular signaling pathways that promote tumor progression (1, 2). HB-EGF is initially synthesized as a type I transmembrane protein (3). The transmembrane form of HB-EGF (pro-HB-EGF) is cleaved at the cell surface by a disintegrin and metalloprotease (ADAM) 9, ADAM10, ADAM12, or ADAM17, in a process called ectodomain shedding. This releases soluble HB-EGF (sHB-EGF) into the extracellular space (4).

We previously reported the validation of HB-EGF as a therapeutic target for ovarian cancer (OVCA) therapy (5-9). Cross-reacting material 197 (CRM197) is a non-toxic mutant of diphtheria toxin (DT) that shares immunological properties with the native molecule. CRM197 binds to human HB-EGF and blocks its mitogenic activity by prohibiting EGFR binding (10). A clinical study showed that CRM197 is useful in patients with OVCA and is a promising agent for OVCA therapy (11). The authors themselves have performed a phase I clinical trial of CRM197 administration for incurable advanced or recurrent OVCA patients under the approval of an Ethical Committee at Fukuoka University.

Previous reports have used several different methods to detect sHB-EGF in body fluids (*e.g.* binding assays using iodine-125-labeled DT, or competitive enzyme-linked immunosorbent assays (ELISA)) (7, 12). However, a radioisotope-based assay required a large sample volume and was labor-intensive, demanding a rapid turnaround time due to the short half-life of radioisotope. Additionally, nonspecific reactions are frequently found in competitive ELISA systems. The development of a novel convenient and precise ELISA method for measurement of HB-EGF in serum and peritoneal fluid would allow us to determine the exact HB-EGF levels of patients with ovarian cancer in clinical practice. Therefore, the aim of this study was to accurately measure HB-EGF levels in peritoneal fluid or serum *ex vivo* by modifying a commercially available HB-EGF-specific ELISA kit.

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Patients and Methods

Sample collection. Peritoneal fluid or serum was obtained from 21 OVCA patients and 21 patients with ovarian cysts (diagnosed as a benign ovarian tumor) who had undergone surgery at the Department of Obstetrics and Gynecology, School of Medicine, Fukuoka University (Fukuoka, Japan) between April 2005 and August 2010. Serum of control patients was also collected from six healthy female volunteers. The ages (mean±SD) of the OVCA patients, ovarian cyst patients and healthy controls were 59.6±9.6, 49.7±16.3 and 58.3±5.8 years, respectively. None of the patients had received chemotherapy before surgery. Diagnosis was based on conventional morphologic examination of paraffin-embedded tumors, which were classified according to the International Federation of Gynecology and Obstetrics criteria. All of the patients provided written informed consent to participate in the study and the study was approved by the Institutional Review Board of Fukuoka University Hospital. Supernatants from the peritoneal fluid and serum samples were collected immediately after centrifugation (3000 × *g* for 15 min) and stored at −80˚C until required.

Sandwich ELISA for human HB-EGF. HB-EGF levels in the peritoneal fluid and serum samples were determined using a commercially available sandwich ELISA (DuoSet Kit; R&D Systems, Minneapolis, MN, USA). A goat anti-human HB-EGF antibody (stock solution 72 μg/ml) was diluted in phosphate-buffered saline (PBS) to yield working solutions of 0.4, 0.8, 1.6 and 3.2 μg/ml, which were used as the solid-phase capture antibody (SCA). Each well of a 96-well plate (Greiner Bio-one, Frickenhausen, Germany) was coated with 100 μl of the SCA overnight at 20˚C. After washing the plate three times with 400 μl of Wash Buffer (0.05% Tween 20 in PBS), the plate was blocked with 300 μl of Reagent Diluent (PBS containing 1% bovine serum albumin) for 1 h at 20˚C. After washing the plate three times with 400 μl of Wash Buffer, 100 μl of sample was added to each well. The plate was then incubated for 2 h at 20˚C. After washing three times with 400 μl of Wash Buffer, the plate was coated with 100 μl of biotinylated goat anti-human HB-EGF antibody diluted in Reagent Diluent (final concentration 100 ng/ml) for 2 h at 20˚C. After washing three times with 400 μl of Wash Buffer, 100 μl of streptavidin conjugated to horseradish-peroxidase diluted in 1% BSA in PBS (1:200) was added to each well. The plate was incubated for 15 min at 20˚C, washed and developed with 100 μl of Substrate Solution (a 1:1 mixture of H_2O_2 and tetramethylbenzidine). After incubation for 20 min at 20˚C, color development was stopped by adding 50 μl of Stop Solution (2 N H_2SO_4). The absorbance was read at 450 nm with a background subtraction at 570 nm. All samples were assayed in triplicate. Recombinant human HB-EGF was serially diluted 2-fold in Reagent Diluent (from 2,000 pg/ml) to yield a seven-point standard curve. The highest standard concentration (2,000 pg/ml) was regarded as 1, and the other six points were estimated to be 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625. The values of the fold dilutions were logarithmically estimated and plotted on the transverse axis. The optical density at each fold dilution was logarithmically converted and plotted on the vertical axis. Each sample was also serially diluted (2-fold) in Reagent Diluent. The converted optical density of undiluted sample was regarded as 1, and the other six points were taken to be 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625. The values of the fold dilutions were logarithmically estimated and plotted on the transverse axis as described for the standard curve. Each optical density value was logarithmically converted and then plotted on the vertical axis. A 96-well plate was coated with 100 μl of SCA at concentrations of 0.4, 0.8, 1.6 or 3.2 μg/ml according to the modified manufacturer's protocol. The slopes of the standard and sample curves at each concentration of SCA were estimated and statistically compared using analysis of covariance (ANCOVA). When no significant difference was observed between the standard and sample slopes, the concentration of HB-EGF in the peritoneal fluid or serum was calculated using a parallel line assay method.

Statistical analysis. Statistical analysis was performed using analysis of covariance (ANCOVA), the Mann-Whitney *U*-test and Spearman's correlation test. A *p*-value less than 0.05 was considered statistically significant.

Results

Validation of the DuoSet ELISA Development assay kit. To accurately measure HB-EGF expression levels in peritoneal fluid or serum, validation of HB-EGF levels was performed using a DuoSet ELISA Development kit, which was developed to measure the concentration of HB-EGF in cell culture supernatants. No significant difference between the sample and standard slopes was observed for the six healthy control serum samples at any concentration of SCA (0.4, 0.8, 1.6 or 3.2 μg/ml) (Figure 1 and 3A and Table I). No significant difference between the sample and standard slopes was observed for the serum and peritoneal fluid samples from six OVCA patients at 1.6 and 3.2 μg/ml goat anti-human HB-EGF (Figure 2, 3B, and 3C and Table I), and no significant difference was observed between the standard and sample slopes for any of the samples at 1.6 μg/ml (Table I). Therefore, the parallel line assay method was used to calculate the HB-EGF concentration in peritoneal fluid and serum at an SCA concentration of 1.6 μg/ml.

HB-EGF values in peritoneal fluid and serum of OVCA and ovarian cyst patients. HB-EGF levels in the serum of 10 OVCA patients (305.3±174.1 pg/ml) were significantly higher than those in 12 patients with ovarian cysts (160.7±67.2 pg/ml) and the six controls (112.7±94.8 pg/ml) (Figure 4A). HB-EGF levels in the peritoneal fluid of 17 OVCA patients (541.5±306.3 pg/ml) were significantly higher than those in 9 patients with ovarian cysts (217.0±99.1 pg/ml) (Figure 4B).

Relationship between HB-EGF levels in peritoneal fluid and in serum in OVCA patients. Six sets of peritoneal fluid and serum samples were obtained from the same OVCA patient. The correlation between HB-EGF levels in the peritoneal fluid and serum was analyzed to evaluate the clinical significance of serum HB-EGF levels in OVCA. The results showed a significant correlation between HB-EGF levels in peritoneal fluid and serum (r=0.886, $p<0.05$; Figure 5).

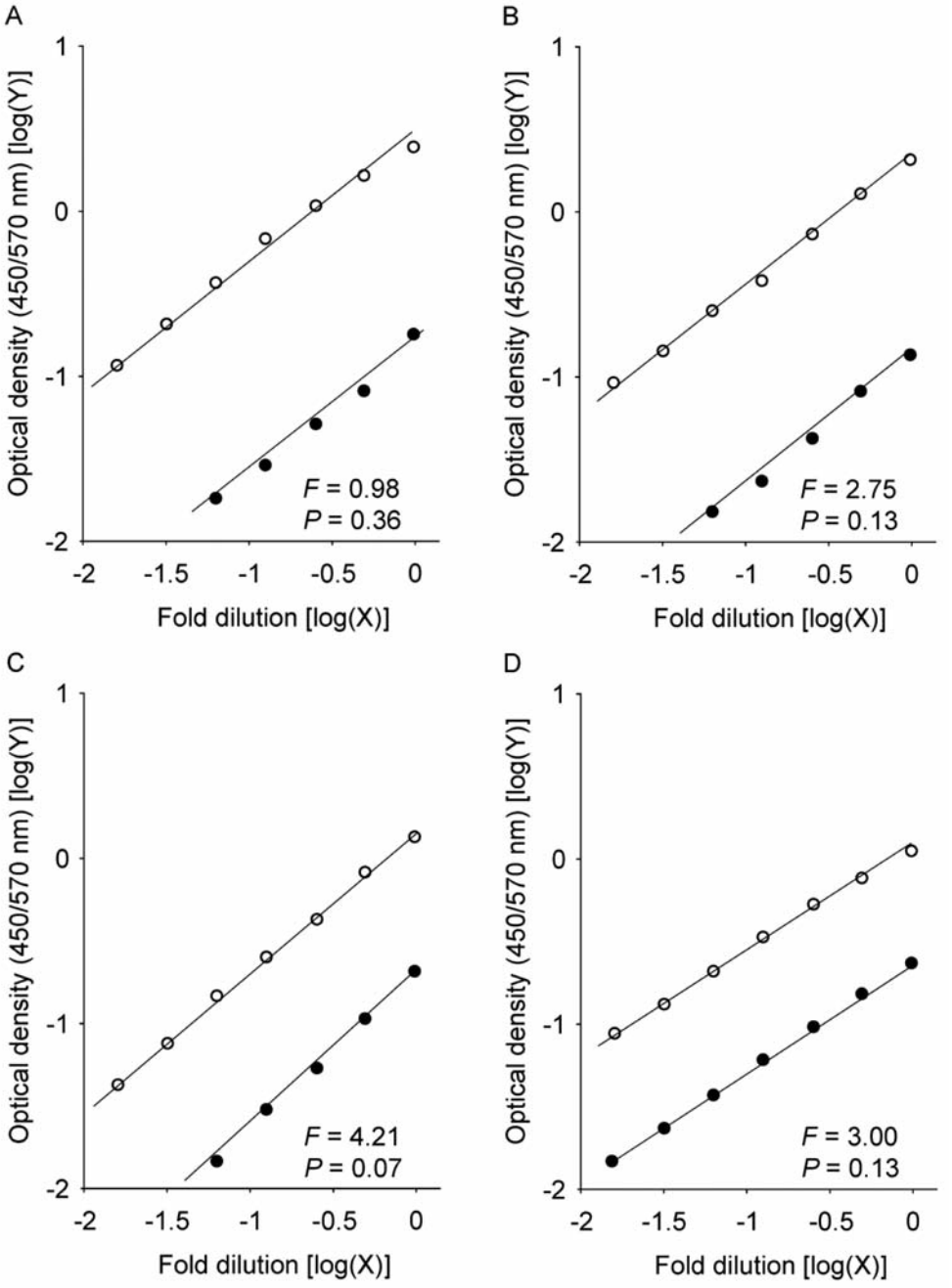

Figure 1. *Measurement of HB-EGF levels in the serum of a control patient (patient no. 1) using the sandwich ELISA method. The concentration of solid-phase capture antibody was (A) 0.4 μg/ml, (B) 0.8 μg/ml, (C) 1.6 μg/ml, and (D) 3.2 μg/ml. Open and closed circles indicate the standard and sample curves, respectively. The F-value is shown in each figure. The P-value represents the comparison between the standard and sample slopes using analysis of covariance (ANCOVA).*

Discussion

In this study, an increasing concentration of SCA resulted in a reduction in the level of nonspecific binding in this ELISA system, leading to parallel standard and sample slopes being obtained for peritoneal fluid and serum. Measuring the angle of both slopes enabled the calculation of HB-EGF levels using the parallel line assay method. As a result, a method of accurately measuring HB-EGF concentrations in peritoneal fluid and serum was established using the DuoSet ELISA Development kit and the parallel line assay method at a SCA concentration of 1.6 μg/ml.

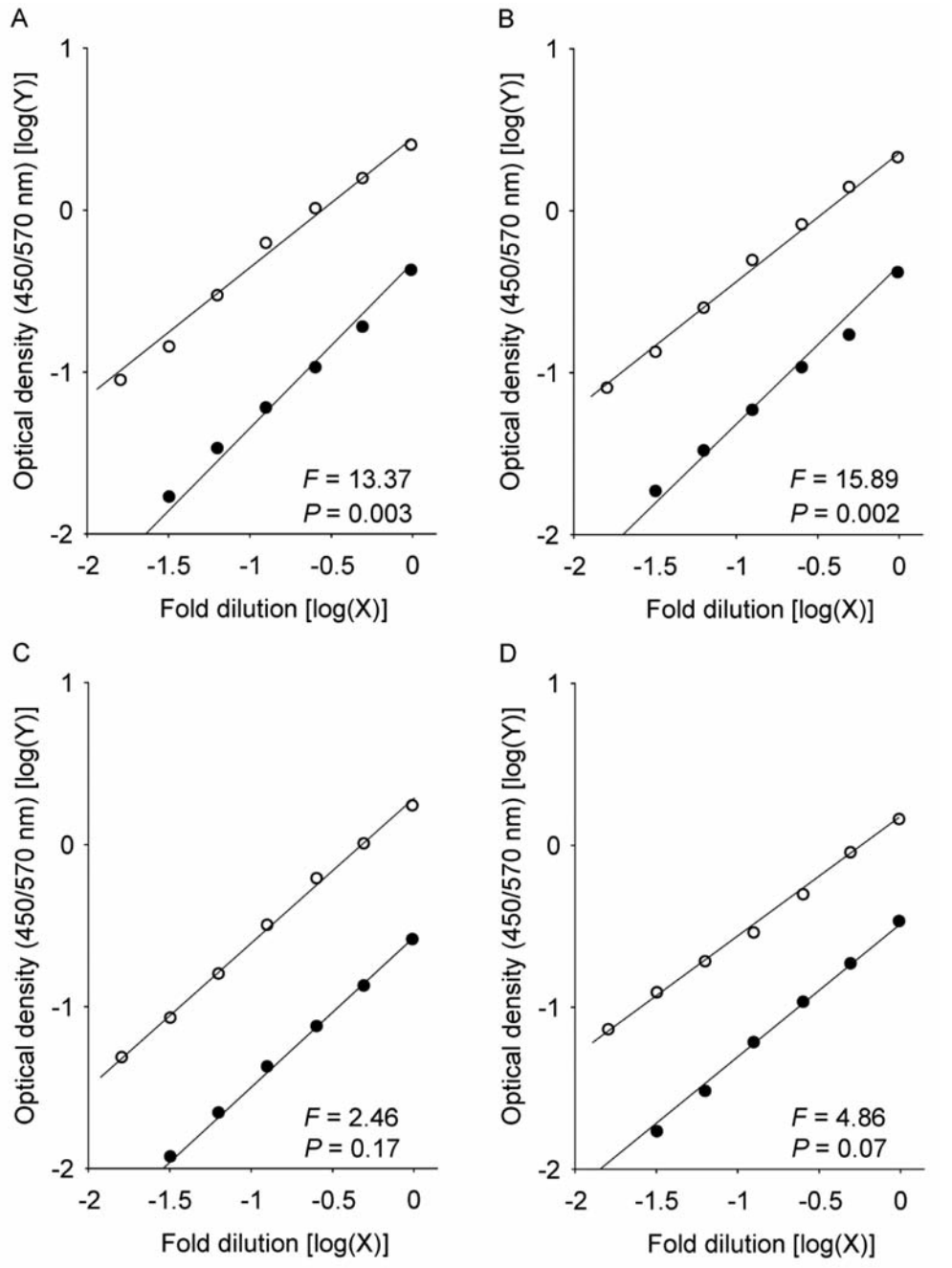

Figure 2. *Measurement of HB-EGF levels in serum of a patient with ovarian cancer (patient no. 10) using the sandwich ELISA method. The concentration of solid-phase capture antibody was (A) 0.4 μg/ml, (B) 0.8 μg/ml, (C) 1.6 μg/ml, and (D) 3.2 μg/ml. Open and closed circles indicate the standard and sample curves, respectively. The F-value is shown in each figure. The P-value represents the comparison between the standard sample slopes using analysis of covariance (ANCOVA).*

The transmembrane form of HB-EGF (pro-HB-EGF) forms complexes with several molecules including CD9 (motility-related protein 1), integrin-α3β1 and heparin-sulfate proteoglycans (HSPGs) (13). sHB-EGF, which is released from the cell membrane by ectodomain shedding of pro-HB-EGF, also contains a heparin-binding motif. Therefore, sHB-EGF can associate with HSPGs, a class of glycosaminoglycan-modified proteins that control diverse patterning events *via* regulation of growth-factor signaling and morphological changes (14-16). In particular, perlecan (also known as HSPG2) is secreted into the extracellular space and has a high affinity for HB-EGF (17). In addition to

Table I. *Comparison of the standard and sample slopes from ovarian cancer (OVCA) patients and controls at 0.4 μg/ml, 0.8 μg/ml, 1.6 μg/ml and 3.2 μg/ml solid-phased capture antibody (SCA).*

	Patient no.	SCA (μg/ml)							
		0.4		0.8		1.6		3.2	
		Slope	F-value	Slope	F-value	Slope	F-value	Slope	F-value
Control serum	1	0.980	0.98	0.988	2.75	0.653	4.21	0.677	3.00
	2	1.350	3.26	1.091	2.98	0.735	2.64	0.731	5.89
	3	0.801	4.00	0.635	3.91	0.695	2.57	0.802	7.07*
	4	0.563	2.39	0.568	3.39	0.693	2.82	0.850	8.37*
	5	0.614	3.39	0.597	3.95	0.644	2.86	0.752	8.23*
	6	1.027	2.91	0.795	2.57	0.720	3.22	0.799	9.61*
OVCA serum	7	1.089	23.02*	1.086	16.30*	0.963	3.70	0.473	4.62*
	8	0.704	16.41*	0.831	19.73*	0.759	3.63	0.594	7.08*
	9	0.837	25.13*	0.918	22.08*	0.816	3.65	0.576	6.41*
	10	0.710	13.37*	0.854	15.89*	0.684	2.46	0.492	4.86
	11	0.690	1.63	0.602	6.21*	0.868	2.46	0.994	3.57
	12	0.942	24.85*	0.735	26.81*	0.781	4.87	0.819	8.39*
OVCA peritoneal fluid	7	1.132	21.72*	0.936	23.04*	0.847	5.48	0.733	4.62
	8	0.428	5.65*	0.384	9.50*	0.639	4.42	0.710	8.61*
	9	0.716	10.81*	0.448	11.48*	0.665	3.79	0.732	6.79*
	10	0.399	6.36*	0.423	9.80*	0.641	3.97	0.715	6.82*
	11	0.671	1.54	0.515	6.55*	0.768	5.10	0.772	4.76
	12	1.250	11.20*	0.772	15.19*	0.623	2.57	0.723	5.10

*$p<0.05$.

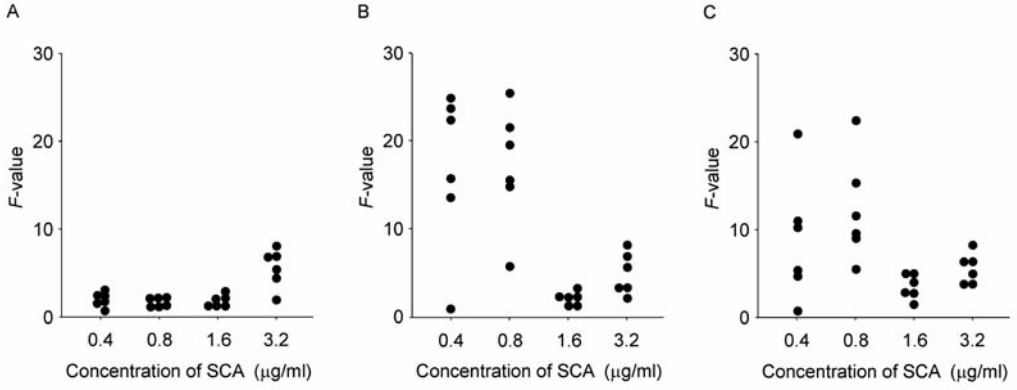

Figure 3. *F-Values calculated for (A) the serum from six control patients, and (B) the serum and (C) the peritoneal fluid of six ovarian cancer patients. Each closed circle indicates the F-value at 0.4, 0.8, 1.6 or 3.2 μg/ml of solid-phase capture antibody (SCA).*

perlecan, HB-EGF forms complexes with adiponectin, an adipocyte-specific secretory protein in serum (18, 19). This presents a problem in that complexes between HB-EGF and these giant modular proteins may influence the measurement of HB-EGF expression levels in serum or other body fluids. In general, OVCA cells express a variety of molecules, including cytokines and structural molecules, and secrete them into the abdominal cavity and the tissue microenvironment (20). Therefore, in this study, to accurately measure HB-EGF levels in the peritoneal fluid and serum of OVCA patients, a higher concentration of SCA was needed, suggesting the possibility that many unknown molecules may bind to sHB-EGF in patients with OVCA, which may reduce the affinity of HB-EGF for the SCA.

The levels of HB-EGF in the peritoneal fluid and serum of OVCA patients was markedly increased, and a positive correlation between HB-EGF levels in peritoneal fluid and serum was observed for OVCA patients. This suggests that the expression level of HB-EGF in the serum directly reflects the amount of HB-EGF that cancer cells secrete into the

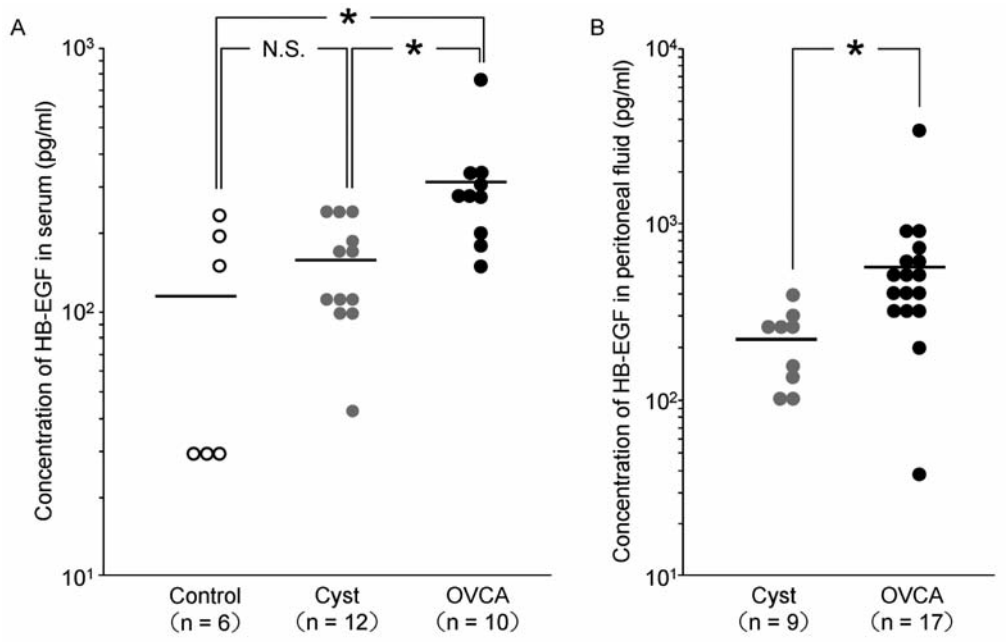

Figure 4. *HB-EGF levels in (A) serum and (B) peritoneal fluid of patients with ovarian cancer (OVCA), ovarian cysts, and healthy controls. Horizontal lines indicate the mean values. *p<0.05; N.S.; not significant.*

peritoneal cavity. In conclusion, the results of the present study show that we developed an accurate method for HB-EGF determination in peritoneal fluid and serum samples.

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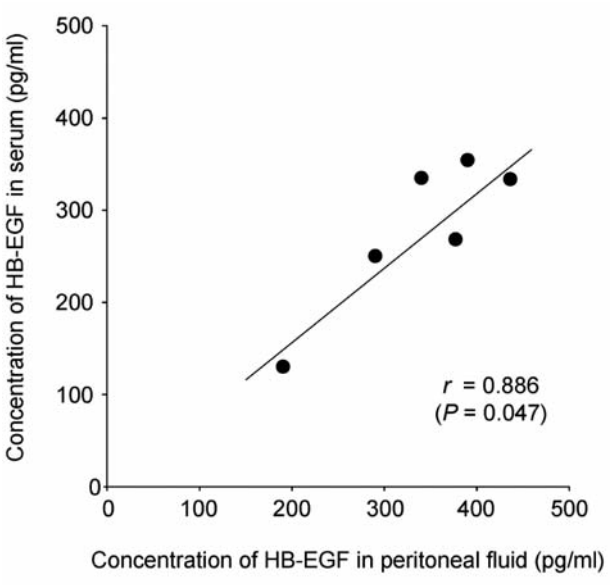

Figure 5. *Correlation between HB-EGF concentrations in peritoneal fluid and serum from same patient with ovarian cancer. r: Spearman's correlation coefficient*

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