Evaluation of annexin II as a potential serum marker for hepatocellular carcinoma using a developed sandwich ELISA method

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Abstract. Annexin II (Annexin A2, ANXA2) is a 36 kDa calcium-dependent phospholipid-binding protein that is located on the surface of most eukaryotic cells. ANXA2 is involved in several biological processes, including anti-inflammatory effects, Ca^{2+}-dependent exocytosis, immune responses, Ca^{2+} transport and phospholipase A2 regulation. In our previous study, ANXA2 was identified as an up-regulated gene in hepatocellular carcinoma (HCC) tissue by cDNA microarray. In the present study, we have evaluated ANXA2 as a tumor-associated marker of HCC. We determined the ANXA2 levels in human liver tissues with HCC using real-time RT-PCR and Western blot analysis. For quantitative analysis of the ANXA2 protein in body fluids, we developed a sandwich ELISA system in which a polyclonal antibody and a monoclonal antibody specific to ANXA2 were employed as a capture antibody and a probe antibody, respectively. We detected the ANXA2 concentration in human serum using our newly developed system and evaluated its usefulness as a tumor marker. Overexpression of ANXA2 in human liver tissue was confirmed by real-time RT-PCR and Western blot analysis. The sandwich ELISA system for ANXA2 was developed for the detection of ANXA2 in human samples. The dose-response relationship between ANXA2 and optical density was linear in the range of 0-10 μg/ml and the sensitivity was 0.02 μg/ml. We determined the ANXA2 concentration in serum specimens using the newly developed sandwich ELISA. The serum ANXA2 concentrations of the patients with HCC (53.38±36.23 μg/ml) were significantly elevated when compared with those of normal individuals (28.81±24.94 μg/ml). These results suggest that expression of ANXA2 may be increased in HCC patients and may play an important role in liver cancer progression. This new ELISA method can be used as a tool for the detection of ANXA2 in human serum, particularly for cancer diagnostics.

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver. HCC has become the 5th most common malignancy worldwide and the third leading cause of cancer-related death (1,2). Surgical resection is the most effective method of treating HCC, but many cases are not adapted to surgery because they involve metastases. Furthermore, the long-term survival of postoperative HCC patients is not satisfactory. Several methods including an ultrasonography, computed tomography (CT), imaging and serum test were used to diagnose of HCC. Among them, detection of tumor markers in human serum is the most effective method because it is convenient, inexpensive and accurate. Therefore, it is important to develop serological markers for HCC to enable early diagnosis, as well as monitoring of tumor aggressiveness, treatment responsiveness, recurrence and survival.

Annexins are a family of Ca^{2+}/lipid-binding proteins that contain Ca^{2+} binding proteins that differ from those of most other binding proteins. Twelve annexin subfamilies have been identified in vertebrates. Annexin II (Annexin A2, ANXA2) exists in two forms in cells, a 36 kDa monomer and a hetero-tetramer. The heterotetramer is composed of two subunits of ANXA2 that are linked together by a dimer of S100A10 (3,4).

ANXA2 is involved in various biological functions including exocytosis and endocytosis (5-7), cell-cell adhesion (8), cell proliferation (9), cell surface fibrinolysis (10), osteoclast formation and bone resorption (11) and cell growth regulation and apoptosis (12).

It has been reported that ANXA2 may be a tumor-related gene that participates in several types of cancer, including breast, renal and prostate cancer (13-19). In our previous study, we identified ANXA2 as an up-regulated gene in a HCC tissue by cDNA microarray (18).

In the present study, we evaluated ANXA2 as an HCC marker in human sources. We determined ANXA2 levels in human liver tissues with HCC using real-time RT-PCR and Western blot analysis. We also developed a method for measuring the quantity of ANXA2 protein and determined ANXA2 expression in human serum using a newly developed sandwich ELISA assay. Our newly developed sandwich ELISA will be useful for the diagnosis of HCC, as well as for biological studies conducted to evaluate ANXA2.

Materials and methods

Sample specimen. Primary HCC tissues were obtained from Kangnam St. Mary's Hospital (Seoul, Korea) and Chonbuk National University Hospital (Chunju, Korea). Normal liver tissues were obtained from patients who had a disease other than HCC, provided by Kangnam St. Mary's Hospital. Tissue specimens were frozen in liquid nitrogen and stored until use. Total RNA was extracted using an RNeasy midi kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Serum specimens were obtained from Dankook Medical School (Chunan, Korea). The serum was stored in a freezer until use. Consent from each patient was obtained for research use of the serum specimen and all procedures were conducted in accordance with the guidelines approved by Dankook University School of Medicine.

Real-time RT-PCR. Oligonucleotide sequences corresponding to the ANXA2 gene was designed using the Primer 3 software (http://frodo.wi.mit.edu). Aliquots of the first-strand cDNA mixture corresponding to 5 μg of total RNA served as the template for real-time RT-PCR, which was conducted using the SYBR® premix Ex Tag™ (Takara Bio, Otsu, Japan). PCR reactions were conducted using a real-time RT-PCR system (Takara Bio). The primers used for real-time RT-PCR were as follows: sense: 5'-ACAGCCATCAAGACCAAAGG-3', antisense: 5'-CAAAATCACCGTCTCCAGGT-3'. The optimized PCR conditions were as follows: 1 cycle at 95˚C for 10 sec; 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 95˚C for 15 sec; and final extension at 60˚C for 15 sec. Relative gene expression levels were normalized against ß-actin expression.

Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using the Pharmacia Phast System (Piscataway, NJ, USA) according to the manufacturer's instructions, using a 12% gel. Proteins were then transferred to a PVDF membrane and probed with polyclonal antibody (pAb) to ANXA2 (Genetex, San Antonio, USA) followed by anti-goat IgG-HRP (Sigma-Aldrich, St. Louis, MO, USA). Immunolabelled proteins were detected by incubation with enhanced chemiluminesence (ECL) substrate followed by exposure of the membrane to auto radiographic film.

Determination of ANXA2 using a developed sandwich ELISA to evaluate human sera. A 96-well microtiter plate was coated with 100 μl per well of 1 μg/ml of pAb (Genetex) to ANXA2 overnight at 4˚C, after which it was washed with PBST solution. The wells were then overcoated with 1% BSA solution. Then, 100 μl of serially diluted ANXA2 standard solution (Abnova, Taipei, Taiwan) and 5-fold-diluted samples with dilution buffer (10 mM EDTA, 1% BSA in PBST) were applied to each well in duplicate and allowed to react with the monoclonal antibody (mAb) (Abnova) for 2 h at room temperature. After washing the wells with PBST solution, 100 μl of appropriately diluted HRP-conjugated anti-mouse-IgG were applied to each well for 1 h. Subsequently, o-phenylenediamine (OPD) solution was added to the wells, after which the reaction was stopped with 1N H_2SO_4 solution. The absorbance was then measured at 490 nm on a spectrophotometer (Molecular Device, Sunnyvale, CA, USA).

ANXA2 assay evaluation. The dose-response effect and sensitivity of the ANXA2 assay were determined using serially-diluted ANXA2 samples. To determine the optimum dilution of serum for use in the assay, serum samples were diluted 2-20-fold with dilution buffer and assayed for ANXA2 following the previously described protocol. To test recovery, a known amount of ANXA2 was added to serum samples that had been diluted 5-fold. The total ANXA2 in the absence of the spike was then subtracted from the total in the presence of the spike and this difference was divided by the spike value. This ratio was then multiplied by 100 to give the % recovery of the spike. The intra-assay and inter-assay precision were then assessed using samples containing various amounts of ANXA2.

Statistical analysis. Samples were grouped into normal, acute hepatitis, chronic hepatitis, liver cirrhosis and HCC group. Statistical analyses were conducted using SPSS version 10.0 (SPSS Inc, Chicago, IL). The levels of ANXA2 in the sera were shown as the mean ± standard deviation (mean ± SD). Means among groups were compared using an unpaired student's t-test. $P<0.05$ was considered to be statistically significant. The receiver operating characteristics (ROC) were calculated to evaluate the diagnostic value of the ANXA2.

Results

ANXA2 expression in human tissues with HCC. In a previous study, ANXA2 was identified as an up-regulated gene in HCC tissues using cDNA microarray chips. In the present study, we analyzed the gene expression profiles of paired HCC and adjacent non-tumor tissues from 40 patients using cDNA microarray chips (Fig. 1A). ANXA2 expression was significantly overexpressed in 87.5% (35/40) of HCC samples relative to normal liver samples (Fig. 1B). Therefore, we randomly selected 10 paired tumor non-tumor samples of HCC

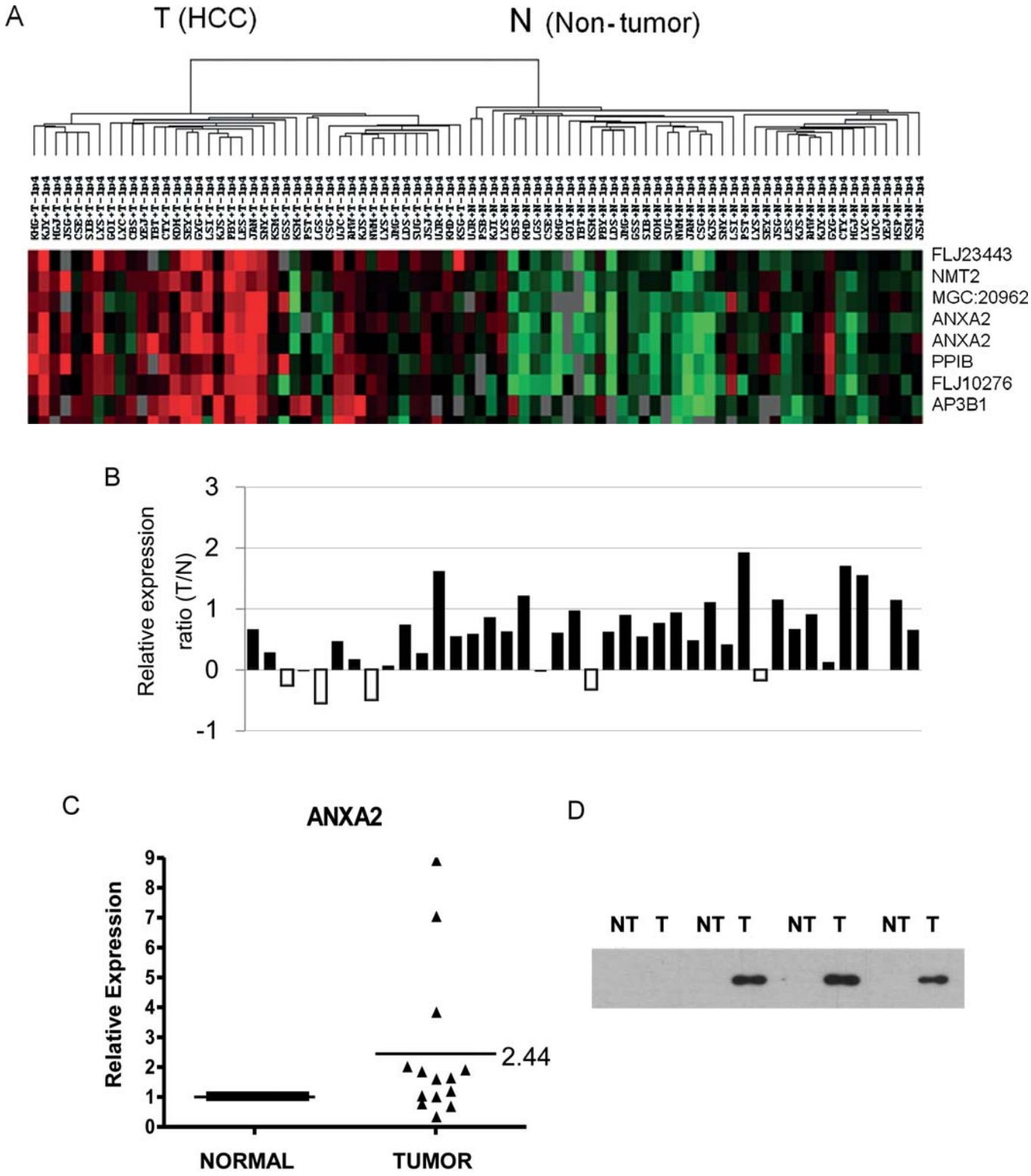

Figure 1. Expression of ANXA2 in human liver tissues. (A and B) cDNA microarray and relative expression ratio. Gene expression patterns of human HCC tissues were ascertained by cDNA microarray. (C) Real-time RT-PCR analysis. cDNA were synthesized from the mRNA in paired HCC tissues (T) and adjacent non-cancerous tissues (NT) were used as templates for reverse transcription. The level of relative expression of ANXA2 was determined by the ratio of ANXA2 to ß-actin. The value of tumor-originated ANXA2 was a relative value against ANXA2/ß-actin (value=1.0) of matched normal controls (n=14). (D) Western blot analysis. SDS-PAGE was conducted under reducing conditions (12% gels, 0.5% mercaptoethanol). Proteins were transferred onto a membrane and then probed with mouse anti-ANXA2 antibody.

and examined the RNA level by RT-PCR. RT-PCR analysis revealed that the ANXA2 expression levels were significantly higher in the HCC patients than the normal liver or adjacent non-tumor liver tissues (data not shown). To quantify the amount of mRNA in human tissues with HCC, we conducted real-time RT-PCR. Quantitative real-time RT-PCR analysis

Table I. Expression of ANXA2 in HCC tissues by real-time RT-PCR.

	Sample No.	Relative expression (mean ± SD)
Age		
≤50	5	1.2±0.67
>50	8	3.4±3.04
Gender		
Male	10	2.9±2.85
Female	3	1.3±0.81
Tumor size (cm)		
1-3	4	1.1±0.57
3-5	5	3.5±0.71
5-10	4	7.2±1.91
Edmond grade		
1	3	0.79±0.43
2	4	3.28±3.80
3	3	2.52±1.17
4	3	3.35±3.24
AFP		
≤23	4	3.6±2.56
>23	9	2.1±2.63

Clinical information from one sample could not be determined.

using 14 independent pairs of tumor and non-tumor tissues also revealed that the ANXA2 RNA levels were much higher (mean: 2.44, highest: 8.94) in most of the HCC (78.6%) than those of the corresponding non-tumors or the normal liver tissue (Fig. 1C). Furthermore, ANXA2 expression levels were dramatically higher in Edmond grade 2 (3.29±3.80) HCC tissues than in grade 1 (0.79±0.43) HCC tissues. No relationship was observed between the AFP value (R^2=0.13) and mRNA expression of ANXA2, but the ANXA2 expression was increased as the tumor size increased (Table I). We also examined the protein level of ANXA2 in human tissues with HCC by Western blot analysis using a monoclonal antibody. Specifically, cancer cells and normal liver cells were extracted from four patients with HCC who underwent surgery for treatment. The extracted cells were then lysed and analyzed by Western blot analysis. As shown in Fig. 1D, the ANXA2 levels in the tumor tissues (3 cases) were clearly higher than those in the non-tumor tissues. In one case, ANXA2 was not expressed in the non-tumor or tumor cells. These results indicate that the levels of ANXA2 expression are higher in patient tissues with HCC than in normal subjects.

Development of sandwich ELISA for ANXA2. For determination of ANXA2 in human serum specimens, we developed a sandwich ELISA method. Two kinds of antibodies were selected for sandwich ELISA and their reactivity to ANXA2 was evaluated and then utilized as either a capture protein or a probe protein in solid-phase-sandwich ELISA. To accomplish this, a micro-well plate was coated with one antibody (capture antibody) and then blocked with 1% BSA. Next, 100 μl of

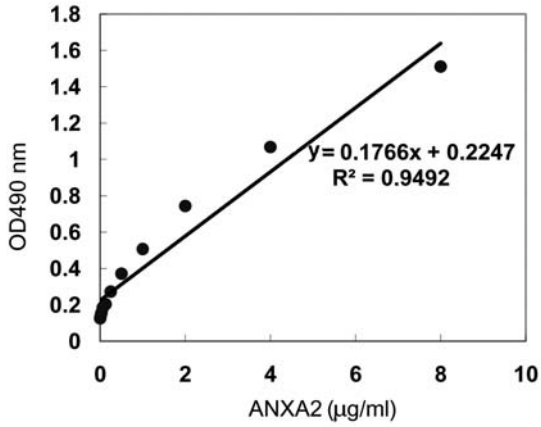

Figure 2. Dose-response relationship between ANXA2 and developed optical density. The solid-phase sandwich assay consisted of a microtiter plate and used pAb to ANXA2 as the capture protein and mAb as the probe protein. ANXA2 solutions were diluted appropriately to give the corresponding concentrations.

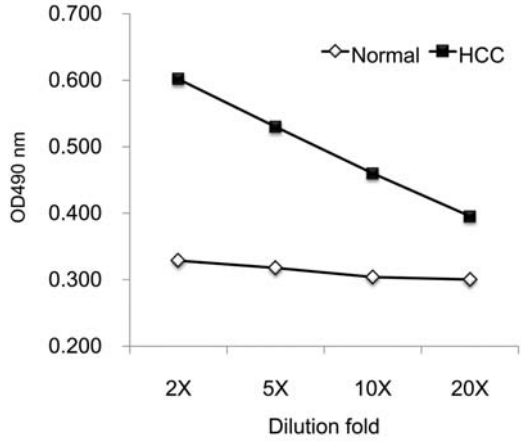

Figure 3. Effect of serum dilutions in the sandwich ELISA assay. The sandwich assay protocol was the same as the protocol described in Fig. 4. Serum specimens were diluted 2-20-fold with serum dilution buffer (10 mM EDTA, 1% BSA, 0.05% Tween-20 in PBS).

serially diluted ANXA2 samples were added to duplicate wells, after which the plates were incubated at RT for 2 h. Following incubation with another antibody (probe antibody) for 2 h, HRP-conjugated secondary antibody (appropriately diluted) was added for l h. The concentration of ANXA2 in the sample was then determined based on the color development in response to the addition of substrate (OPD) solution at 490 nm. A standard curve was established based on the colorimetric intensity of each ANXA2 standard solution. Fig. 2 shows the relationship between colorimetric intensity and ANXA2 concentration in the range of 0-10 μg/ml. We then performed a sensitivity test by evaluating serially diluted ANXA2 samples using the sandwich ELISA. The minimal concentration of the sample that could be detected with the new sandwich ELISA was 0.02 μg/ml.

The serum dilution effect in the sandwich assay was examined using serum specimens obtained from normal individuals and patients diagnosed with HCC. As shown in Fig. 3, the serum ANXA2 concentration was significantly

Table II. Serum ANXA2 concentration as measured by sandwich ELISA.

Clinical diagnosis	Total no.	Conc. (μg/ml) Mean ± SD	P-value (normal vs)
Normal	79	28.81±24.94	-
AH	55	30.84±20.97	0.50
CH	77	48.43±35.18	$5.5\ e^{-5}$
LC	85	48.57±30.26	$8.84\ e^{-6}$
HCC	86	53.38±36.32	$7.77\ e^{-7}$

AH, acute hepatitis; CH, chronic hepatitis; LC, liver cirrhosis and HCC, hepatocellular carcinoma.

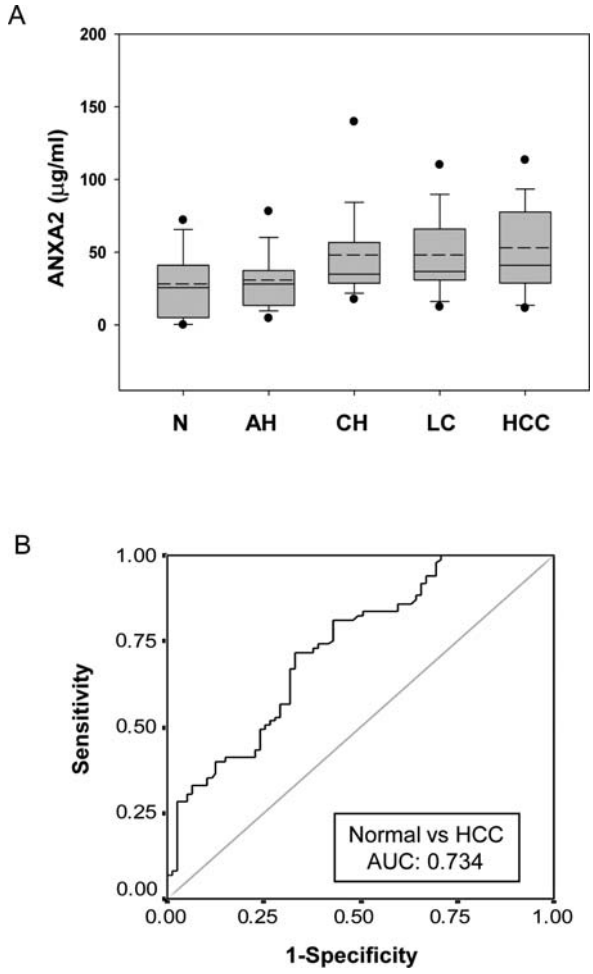

Figure 4. ANXA2 protein levels in the serum of HCC patients and normal serum. (A) The solid-phase sandwich ELISA assay utilized a goat anti-ANXA2 pAb as the capture antibody and a mouse anti-ANXA2 mAb as the probe antibody. The ANXA2 concentration in the serum of hepatic disease including HCC patients and normal serum were determined by the sandwich ELISA (N, normal; AH, acute hepatitis; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma). (B) ROC curve of ANXA2 (Normal vs. HCC).

elevated in patients with HCC when compared to normal subjects when samples that had been diluted 2-20-fold were evaluated.

Next, a recovery test was conducted by adding known amounts of ANXA2 (0.05, 0.25, 1 μg/ml) to normal serum. The total ANXA2 level in the absence of the spike was subtracted from the total in the presence of the spike and this difference was then divided by the spike value. This ratio was then multiplied by 100 to give % recovery of the spike. Generally, 84-98% of the added ANXA2 was recovered from 5-fold diluted specimens by sandwich ELISA.

To confirm the accuracy of the assay, within-run and between run assays were performed with three types of serum samples, which are high, medium and low levels of ANXA2. The within-run (n=10) and between-run (n=10) variations were 3.3-7.8% (coefficient of variation) and 10-12%, respectively.

Evaluation of the serum ANXA2 concentration by the sandwich assay. Serum specimens from 86 patients with HCC, 55 patients with acute hepatitis, 77 patients with chronic hepatitis, 85 patients with liver cirrhosis and 79 healthy subjects were evaluated in this study. The ANXA2 concentrations of serum samples that had been diluted 5-fold were determined by a sandwich ELISA assay. The results were analyzed and summarized in Table II and Fig. 4. The serum ANXA2 concentrations of the patients with HCC (53.3±36.3 μg/ml) were progressively increased when compared with those of those of acute hepatitis (30.8±20.9 μg/ml) and normal individuals (28.8±24.9 μg/ml). The P-value was $7.77e^{-7}$ and the value of area under the curves (AUC) in ROC analysis was 0.734 between normal and HCC patients, the accuracy of the test was considered to be fair. These results suggest that the concentration of ANXA2 may be elevated in liver cancer patients.

Discussion

HCC, which is the most common primary cancer of the liver, has a particularly high incidence in Asia (1). However, effective diagnosis and monitoring of HCC can often not be accomplished due to the lack of biomarkers that can characterize tumor formation and tumor progression. Currently, diagnosis of HCC relies on the observation of a liver mass on radiology imaging studies, although the detection of an elevated serum α-fetoprotein (AFP) level is still widely used as a serum marker for its diagnosis and monitoring. However, AFP levels have low sensitivity and specificity, particularly during the early stages of cancer. Several biomarkers, such as des-γ-carboxyprothrombin/prothrombin induced by vitamin K absence-II (PIVKA II), lens cularis agglutinin-reactive (AFP-L3) and glypican-3, have been examined for their ability to detect early HCC (2,20,21) but, these markers also have poor sensitivity and specificity.

Recently, ANXA2 was reported as an up-regulated gene in HCC tissue and HBV- and/or HCV-associated hepatocellular carcinoma cells (16). Using an analysis of EST frequency, ANXA2 was found to be a potential biomarker for HCC and a useful target for elucidating the molecular mechanisms associated with HCC (16). Mohammad *et al* (22) reported that ANXA2 expression and phosphorylation were up-regulated in hepatocellular carcinoma. In addition, in many

cases, more ANXA2 was expressed in the tumorous portion than in the non-tumorous portion of HCC. The expression of ANXA2 was mainly localized in cancer cells, especially in poorly differentiated HCC (22). In a previous study, ANXA2 mRNA was found to be preferentially overexpressed in HCC tissue samples by an immunohistochemistry assay. Furthermore, positive staining in the endothelial cells of the sinusoid was observed in HCC tissues (18).

In the present study, we evaluate the clinical usefulness of ANXA2 as tumor marker in human specimens. We analyzed the gene expression of paired HCC and adjacent non-tumor tissues using real-time RT-PCR. ANXA2 exhibited a significantly increased level of RNA expression in HCC samples when compared to normal liver samples. Additionally, the ANXA2 expression levels were increased from Edmond grade 2 in HCC. No relationship was observed between the AFP value and mRNA expression of ANXA2, but the mRNA expression of ANXA2 increased according to tumor size (Table I). These findings indicate that ANXA2 may be useful for the detection of early stage HCC.

To examine the expression of ANXA2 in hepatoma cell lines, we measured ANXA2 expression by RT-PCR and Western blot analysis. To accomplish this, 5 cell lines, HepG2, Hep3B, SK-Hep1, Huh-7 and Chang, were obtained and cultured. ANXA2 mRNA was slightly expressed in HepG2 and Chang, while it was highly expressed in Huh-7, SK-Hep-1 (data not shown).

Generally, the ANXA2 protein can be measured qualitatively in cells or tissues by Western blot analysis or immunohistochemical staining. However, these methods carry several limitations, such as a high background when conducted in the tissue, which can make quantitative analysis of the assay quite difficult. Even though a competitive ELISA of ANXA2 was described by Elling *et al* (23), competitive immunoassay is less specific and sensitive than sandwich ELISA. Our newly developed sandwich ELISA can overcome these shortcomings. From the ANXA2 assay in serum with newly developed ELISA, ANXA2 levels of the patients with HCC were elevated when compared with those of acute hepatitis and normal individuals.

The heterotetramer form of ANXA2 is composed of two subunits of ANXA2 linked together by a dimer of S100A10. It was reported that ANXA2 up-regulated the cellular levels of S100A10 by a post-translational mechanism (24). Even though we could not detect S100A10 with ANXA2 in this study, but combination of S100A10 and ANXA2 may increase the sensitivity and specificity of HCC diagnosis.

In summary, we found differential expression of ANXA2 in HCC tissues and serum. We have developed a quantitative assay system that employs a sandwich ELISA and the evaluated ANXA2 level in serum. The results of this study suggest that ANXA2 could be a useful biomarker for the diagnosis of HCC. However, further investigation of the molecular mechanism involved in ANXA2 action may offer a novel approach for the treatment of HCC.

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