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Annexin A2 is a discriminative serological candidate in early hepatocellular carcinoma

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To date, the useful markers of hepatocellular carcinoma (HCC) remains incompletely developed. Here, we show that annexin A2 complement alpha-fetoprotein (AFP), a widely used liver cancer marker, in the serologically surveillance and early detection of HCC. First, differentially expressed proteins in HCC were identified using a subcellular proteomic approach. Annexin A2 was then selected for further verification. It was found to be overexpressed in HCC tissues (60.7%, 136/224). Using a self-estabished sandwich enzyme-linked immunosorbent assay, we found that annexin A2 significantly increased in the sera of HCC (n = 175, median, 24.75 ng/μl) compared with the healthy (n = 49, median, 16.69 ng/μl), benign tumors (n = 19, median, 19.92 ng/μl), hepatitis (n = 23, median, 6.48 ng/μl) and cirrhosis (n = 51, median, 7.39 ng/μl) controls and other malignant tumors (n = 87). Importantly, raised concentrations of annexin A2 were observed in 83.2% (79/95) of early stage (median, 24.32 ng/μl) and 78.4% (58/74) of AFP-negative (median, 24.09 ng/μl) patients. Annexin A2 alone had a better area under the receiver-operating characteristic curve (AUC = 0.79, 95% confidence interval: 0.73–0.85) in comparison with AFP (AUC = 0.73, 95% confidence interval: 0.66–0.80) in detecting of early stage HCC. Combining both markers notably improved the diagnostic efficiency of early HCC with an achieved sensitivity of 87.4%. Additionally, the expression characteristics of annexin A2 during hepatocarcinogenesis were detected in *p21-HBx* gene knockin transgenic mice model. The results showed that annexin A2 expression was substantially elevated in HCC-bearing mice, in accordance with the finding in human samples. In conclusion, annexin A2 may be an independent serological candidate for hepatitis B virus–related HCC, especially in the early stage cases with normal serum AFP.

Introduction

Liver cancer is the fifth common fatal cancer worldwide with more than 90% mortality. Hepatocellular carcinoma (HCC) represents approximately 85% of all primary liver cancer (1). Overwhelmingly, chronic infection with hepatitis B virus (HBV) is recognized important risk factors of HCC in many countries, including China. It is estimated that almost 20% chronic-infected cases would progress to cirrhosis, and above 80% of tumors developed from cirrhotic livers. Therefore, cirrhosis is considered the premalignant condition of HCC (2,3). Due to the asymptomatic nature of early HCC and lack of effective screening strategies, most patients (>80%) present with overt advanced disease. Currently, the most utilized surveillance methods for patients with cirrhosis are serum alpha-fetoprotein (AFP) level and ultrasonography with some limits (3–5). For example, about 30% HCC cases with normal serum AFP levels are hardly diagnosed before clinical manifestations appear. Meanwhile, AFP is limited for early HCC without convincing evidence for significant improvement in the early diagnosis and overall survival rate (6). So far, except AFP, the potential early detection biomarkers of HCC include des-γ-carboxyprothrombin, lectin-bound AFP (AFP-L3), α-l-fucosidase, glypican-3 and so on (6–9). However, recent studies showed that des-γ-carboxyprothrombin and AFP-L3 were not superior to AFP for the diagnosis of early HCC (5,10). Therefore, it highlights the need for new early detection biomarkers for HCC.

In the present study, we adopted a subcellular proteomic approach to identify the differentially expressed proteins between normal and liver cancer cells. Among the 49 identified proteins, annexin A2 was selected to further histological and serological validation. Annexin A2 belongs to a widely distributed, calcium-dependent, phospholipid-binding protein family. It is located on the surface of endothelial cells and most epithelial cells (11,12). Annexin A2 has been implicated in many functions, for example, exocytosis, endocytosis, vesicle transport, regulating ion channels, immune response, cell–cell adhesions, mitogenic- and lipid-messenger-mediated signalling and fibrinolysis(11–15). The aberrated expression of annexin A2 was observed in several malignant tumors, such as colon, lung, gastric, esophageal, and breast (16–20). It is also concerned with cell proliferation, apoptosis, morphology control, transcriptional regulation, motility, invasion, metastasis and angiogenesis and may play key roles in tumorigenesis (12,21,22). Regarding normal liver tissue, annexin A2 is consistently negative in hepatocytes but expressed in the biliary epithelial cells and endothelial cells (23–25). During hepatocarcinogenesis, it is expressed in limited hepatocytes of cirrhotic liver tissues and obviously elevated in the malignant hepatocytes (24–28). Moreover, it was found to upregulate in HCC tissues at the messenger RNA and protein levels (24,25,27). Recently, adding annexin A2 to the established histological diagnostic marker panel has been considered to improve the diagnostic accuracy in HCC (29). In addition, serum annexin A2 concentrations were frequently elevated in HCC patients (30).

Here, we validated annexin A2 in a larger number of 224 HCC tissues. Meanwhile, serous annexin A2 protein was quantitatively measured in 404 samples, including early (n = 95) and late (n = 80) stage HCC, non-cancerous controls (n = 142) and five other cancers (n = 87) using an established enzyme-linked immunosorbent assay (ELISA). The diagnostic potential of serum annexin A2 was compared with the widely used HCC marker AFP. Subsequently, the tissue and serum samples from multiple time points of *p21-HBx* knockin transgenic mice were collected and detected. These data suggested that annexin A2 may serve as a discriminative serological marker for HBV-related HCC, especially in the early stage HCC with normal serum AFP levels.

Materials and methods

Cell lines and protein extraction

Human liver cancer cell lines, HepG2, Hep3B, SK-HEP-1, Bel-7404, SMMC-7721 and HLE and normal liver cell HL-7702 were from the sources described previously and maintained in recommended media at 37°C with 5% CO_2 (31).

Subcellular protein extraction and clean-up were performed using ProteoExtract™ Subcellular Proteome Extraction Kit and Protein Precipitation Kit (Calbiochem, Germany) according to the manufacturer's guidelines.

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; ROC, receiver-operating characteristic curve; 2-DE, two-dimensional gel electrophoresis.

2-DE separation and protein identification

A total of 100 µg of proteins from each fraction were rehydrated to immobiline™ DryStrip gels (24 cm, pH 3–10 non-linear, Amersham Bioscience, Sweden) for 12 h, and 2-DE separation and image analysis were performed as described previously (32). To ensure the reproducibility of the results, each gel was run in triplicate. Quantitative analysis was performed using the Student's *t*-test. The confidence level was 95%.

The gel slices in which spot volume changed by 2-fold between liver cancer cells and normal hepatocytes were cut, digested with trypsin, and then identified as described previously (32). All mass data were searched using GPS Explorer Software v3.6 (with MASCOT search engine) with a peptide and fragment ion-mass tolerance of 0.2 Da. The database used was the Uniprot-Human v1.4.

Sample collection and preparation

Surgical tissue and serum specimens were collected after obtaining informed consent and approval from the Institutional Review Board of the Cancer Institute and Hospital of Chinese Academy of Medical Sciences (Beijing, China). All patients were diagnosed by two senior pathologists without chemo/radiotherapy before surgical operation. A total of 237 HCC tumor and paired adjacent non-tumor liver tissue samples, including 29 fresh and 208 formalin-fixed paraffin-embedded tissues, were collected from HCC patients undergoing resection during the period from May 2004 to November 2007. The tissue samples were collected and proteins were extracted as described previously (31).

Serum samples were collected from patients with HCC (n = 175; median age, 54 ± 12 SD; range 15–83 years), hepatitis (n = 23; median age, 41 ± 14 SD; range 20–63 years), posthepatitic cirrhosis (n = 51; median age, 53 ± 13 SD; range 28–90 years), liver benign tumors (n = 19, including two liver focal nodular hyperplasia, two polycystic liver and 15 haemangioma; median age, 48 ± 12 SD; range 23–72 years) and healthy controls (n = 49; median age, 49 ± 9.1 SD; range 31–70 years). The histological grade of differentiation and Barcelona Clinic Liver Cancer staging (BCLC) were determined as previously described criteria (5,33). Early stage of HCC were defined as BCLC stage 0 and A, and late stage was defined as combination of BCLC stage B and stage C (5). Their clinical features are shown in Supplementary Table S1 (available at *Carcinogenesis* Online) The clinically acceptable normal serum AFP was defined as <20 ng/ml. In addition, serum of 87 patients (male/female: 51/36; median age, 55 ± 10.5 SD; range 30–78 years) with other cancer were also collected, including 15 breast cancer, 14 lung cancer, 20 esophageal cancer, 19 gastric cancer and 19 colorectal cancer.

ELISA analysis

For detection of annexin A2 protein in human serum, a double-antibody sandwich ELISA was developed, referred to the approach described previously (31). The coating antibody was goat anti-annexin A2 antibody (sc-1924, Santa Cruz Biotech.) at a final concentration of 2 µg/ml. Gradient diluted human recombinant annexin A2 protein (H00000302-P02, Abnova, Taiwan) or 50 µl of serum (diluted 1:10 with Phosphate Buffered Saline with 0.05% Tween 20.) were used as antigens and incubated at 37°C for 1 h. Each sample was analyzed in triplicate. The detecting antibody was anti-annexin A2 monoclonal antibody (sc-28385, Santa Cruz Biotech.). The CV of each sample was controlled to less than 15%. Additionally, serum AFP was measured by a commercially available chemiluminescence detection method (Roche, Germany). The levels of annexin A2 and AFP were additionally evaluated by receiver-operating characteristic curve (ROC) analysis to determine the diagnostic performance. The optimal cutoffs were determined using the maximum sum of sensitivity and specificity.

Pepscan analysis

In order to identify core linear epitopes of anti-annexin A2 antibodies, 162 18-mer polypeptides with 16 amino acid residues overlap along each peptide, which represents the entire sequence of annexin A2 (NP_001002857) was separately synthesized (Supplementary Table S2, available at *Carcinogenesis* Online) and bound covalently to cellulose membranes (Whatman; Maidstone, UK) using a pipetting robot (Intavis; Köln, Germany) following the manufacturer's guidelines (34). After gradient ethanol hydration, peptide membranes were blocked in 5% non-fat dry milk in phosphate-buffered saline containing 0.2% Tween-20 and then they were performed according to the routine western blot processes. The capture (GTX22242, GeneTex Inc., CA) and detecting (H00000302-M01, Abnova, Taiwan) antibodies in a previous report (30) were also analyzed.

Immunohistochemical and immunofluorescence staining

Annexin A2 expression was checked on the tissue microarrays with 208 HCC cases by immunohistochemistry (IHC) staining. First, the tissue microarrays were incubated with annexin A2 antibody (sc-9061, Santa Cruz Biotech., CA) or control IgG (5 µg/ml) at 4°C, overnight, and then visualized using an ultrasensitive streptavidin-peroxidases system (Maxim Biotech, China). After calculating the informative cases, the intensity of annexin A2 staining in individual case was quantified as described previously (35). A score of 4~12 was defined as 'positive expression'and a score of 0~3 as 'negative'.

For immunofluorescence staining, liver cancer cells were grown in 0.01% poly-L-Lysine coated slices for 24 h. After fixed with cool methanol and incubated with anti-annexin A2 antibody (sc-9061, Santa Cruz Biotech.) and then Alexa-488-conjugated secondary IgG (Invitrogen, Carslbad, CA), the fluorescence signals were observed under a TCS SP2 laser confocal microscope (Leica Microsystems, Bensheim, Germany).

Western blot analysis

Approximately 15 µg of protein lysate was separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with anti-annexin A2 (sc-9061, Santa Cruz Biotech.) and anti-β-actin (Sigma-Aldrich, MO) antibodies, and then developed using an enhanced chemiluminescence system (Santa Cruz Biotech.). The upregulation or downregulation of annexin A2 was defined as higher or lower relative band intensity in tumors compared with their paired adjacent normal liver tissues.

Annexin A2 expression analysis in p21-HBx knockin transgenic mice

The liver tissues and sera of wild-type and *p21-HBx* transgenic mice were collected at the ages of 6, 12, 18 and 24 months. Among them, *p21-HBx* transgenic mice harboring HCC were killed at the ages of 24 months (36). Each group at different time point included at least three mice. Then, IHC staining and ELISA of annexin A2 were evaluated using the same approaches above.

Statistical analysis

We used SPSS software v16.0 (SPSS Inc., IL) to calculate all statistical comparisons. All comparisons were two tailed and P values less than 0.05 were considered significant.

Results

Annexin A2 as a differentially expressed protein of liver cancer cells

Cytosol, membrane/organelle and nuclear proteins fractions were extracted in liver cancer and normal liver cells (HepG2, Hep3B, SK-HEP-1 and HL-7702) to partially enrich moderate- and low-abundance candidates. The separation efficiencies were checked by western blot assay (Supplementary Figure S1, available at *Carcinogenesis* Online) and total proteins of the three fractions were separated via 2-DE in triplicate, ensuring the spot reproducibility of more than 80%. Compared with normal liver cells, a total of 49 differentially expressed proteins were identified (Figure 1A, Supplementary Table S3, available at *Carcinogenesis* Online), which were involved in apoptosis, RNA binding and splicing, transport, regulation of metabolism, regulation of cytoskeleton, cell proliferation and signal transduction processes based on the Gene Ontology annotation. A spot that was apparently upregulated in the cytosol fraction of SK-HEP-1 with molecular weight (MW) approximately 30kDa was identified as annexin A2 (Figure 1B). It is expressed in almost all of liver cancer cells except for HepG2 with multiple low MW bands confirmed by western blotting (Figure 1C). Additionally, the subcellular protein analysis further showed that annexin A2 was detected in the cytosol, membrane/organelle and nuclear fractions of liver cancer cells, and faintly expressed in the cytoplasm of HL-7702 cell by western blotting (Figure 1D) and immunofluorescence staining (Figure 1E).

Upregulation of annexin A2 in human HCC samples

First, the clinical association of annexin A2 expression in HCC patients was analyzed using western blotting. Compared with the non-tumorous corresponding tissues, the overexpression of annexin A2 was observed in 33.3% (4/12) and 94.1% (16/17) of neoplastic liver samples of AFP-positive and AFP-negative cases, respectively (Figure 2A and B). It was significantly associated with serum AFP levels (Fisher's exact test, P = 0.0009; Supplementary Table S4, available at *Carcinogenesis* Online).

Furthermore, the expression of annexin A2 in HCC was verified on tissue microarrays using IHC staining. Among the 208 analyzed HCC cases, 195 paired specimens were informative. Annexin A2 was positively expressed in 59.5% (116/195) of HCC tissues but in 12.8%

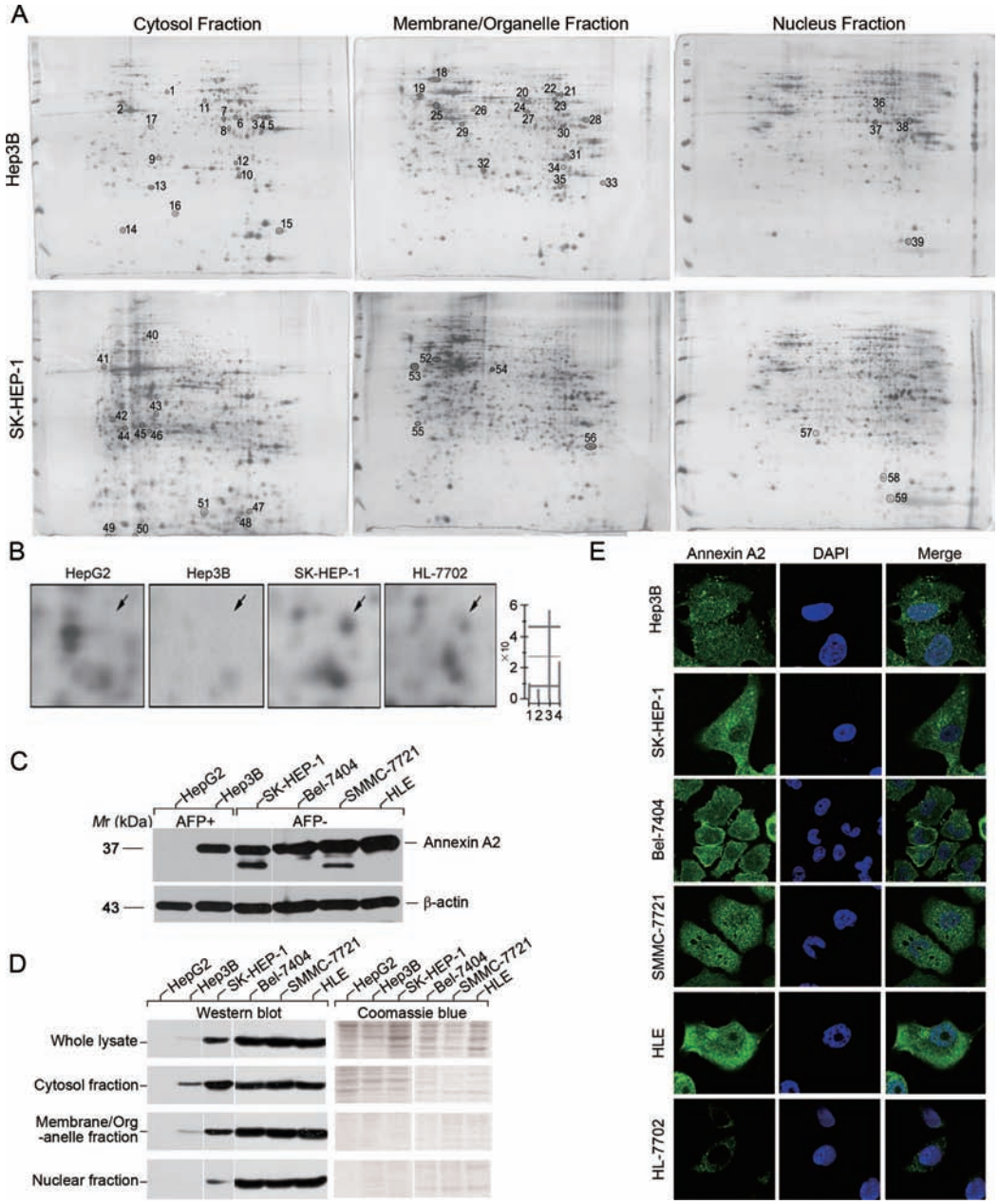

Fig. 1. Identification of annexin A2 in human liver cancer cells. (A) Representative two-dimensional gels of liver cancer cells. Protein identification of selected overexpressed spots in Hep3B and SK-HEP-1 is indicated. The position of each spot is marked by circles. The spot numbers correspond to those in Supplementary Table S3 (available at *Carcinogenesis* Online). (B) Annexin A2 expressed in the cytosol fractions of HepG2 (1), Hep3B (2) and SK-HEP-1 (3) and HL-7702 (4) cells revealed by a 2-DE analysis. The right histogram represents the expression levels of annexin A2 in different cells. (C) Protein levels of annexin A2 in six liver cancer cells. The β-actin protein was used as a loading control. (D) Subcellular protein levels of annexin A2 in six liver cancer cells were revealed by Western blots. Coomassie blue-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel was used as the loading control. (E) Confocal visualization of annexin A2 in five liver cancer cells and HL-7702.

(25/195) of matched adjacent non-cancerous tissues. The upregulation of annexin A2 in HCC tumor tissues was statistically significant (chi-square test, $P < 0.0001$). Its staining was faint and confined to the cytoplasm in some hepatocytes of non-tumorous tissues, whereas it was intense in the cytoplasm of most cancer cells with 20.0% (39/195) positive rate of plasma membrane staining (Figure 2C). In addition, it was observed in the cytoplasm of some sinusoidal endothelial and bile duct cells.

Overall, annexin A2 expression was validated in a total of 224 HCC cases, of which 60.7% (136/224) patients showed apparently upregulated annexin A2 expression in HCC tissues (chi-square test, $P < 0.0001$). However, it was not correlated with gender, age, tumor size, differentiation degree, American Joint Committee on Cancer, BCLC staging and AFP levels (Supplementary Table S4, available at *Carcinogenesis* Online).

Technical setup of a new ELISA to measure serum annexin A2 levels

Using pepscan analysis, we sieved multiple anti-annexin A2 antibodies and developed a new sandwich ELISA. The coating antibody recognized at least eight linear epitopes, including aa 51–68, 73–90, 129–152, 163–182, 196–206, 213–230, 283–294 and 289–302 (Figure 3B), whereas the detecting antibody bound preferentially to peptides 25–36 (Figure 3C). The dose-response relationship between recombinant annexin A2 protein and our developed optical

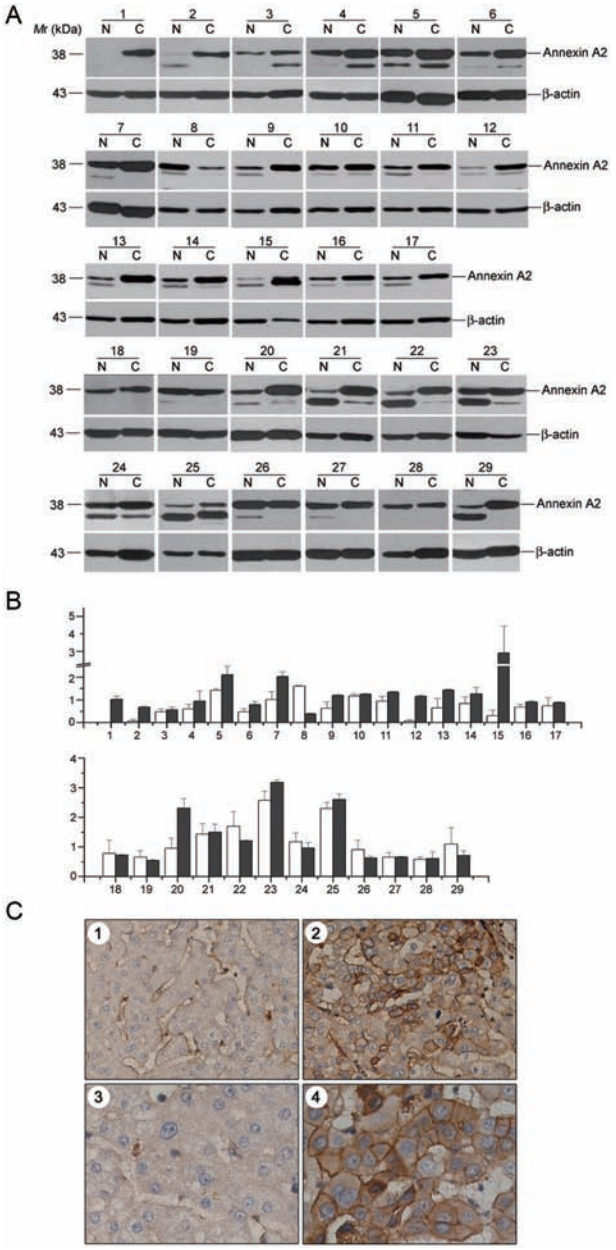

Fig. 2. Histological validation of annexin A2 in human HCC specimens. (A) Western blot analysis of tumor (C) and matching adjacent non-tumorous liver tissues (N) of 29 HCC patients. β-actin was used as a loading control. The experiments were independently repeated three times with equivalent results. (B) Densitometric analysis of 29 HCC cases. The gray and black bars represent the relative band intensity of annexin A2 in non-tumorous and tumorous liver tissues, respectively. Each data point represents the mean ± standard deviation (SD) derived from three independent experiments. AFP-negative and AFP-positive HCC cases were numbered as 1–17 and 18–29, respectively. (C) Representative immunohistological staining of annexin A2 in HCC specimens. Annexin A2 was mainly localized to the cytoplasm and plasma membrane of tumor cells. 1 and 3, adjacent non-tumorous liver tissues (1, ×200; 3, ×400); 2 and 4, tumor tissues (2, ×200; 4, ×400).

density was linear in the range of 0–20 ng/μl and the lowest detected concentration of the sample was about 0.04 ng/μl.

Comparing serum levels of annexin A2 and AFP in HCC patients and non-cancerous controls

Subsequently, serum annexin A2 was measured in healthy controls (n = 49) and patients with benign tumors (n = 19), hepatitis (n = 23), posthepatitic cirrhosis (n = 51) and HCC (n = 175, including 95 early stage and 80 late stage patients). The annexin A2 levels were significantly higher in the HCC patients (median, 24.75 ng/μl) compared with the healthy controls (median, 16.69 ng/μl), hepatitis (median, 6.48 ng/μl), cirrhosis (median, 7.39 ng/μl) (Mann–Whitney test, $P < 0.0001$) and liver benign tumors (median, 19.92 ng/μl, Mann–Whitney test, $P = 0.0024$). Importantly, it was elevated in the early stage HCC (median, 24.32 ng/μl) compared with healthy, hepatitis, cirrhosis (Mann–Whitney test, all of $P < 0.0001$) and benign tumors (Mann–Whitney test, $P = 0.0048$; Figure 4A; Supplementary Table S5, available at *Carcinogenesis* Online). It was not age and gender dependent among the healthy donors (Spearman's rank correlation test for age, $P = 0.9274$; Mann–Whitney test for gender, $P = 0.9492$).

Serum AFP were significantly higher in the HCC patients (median, 44.42 ng/ml) compared with the healthy controls (median, 3.26 ng/μl), benign tumors (median, 3.32 ng/μl), hepatitis (median, 9.00 ng/μl) and cirrhotic controls (median, 8.00 ng/μl) (Mann–Whitney test, $P < 0.0001$; Supplementary Table S5, available at *Carcinogenesis* Online). The patients with hepatitis and cirrhosis backgrounds also had higher serum AFP compared with liver benign tumor cases (Mann–Whitney test, $P < 0.0001$). However, AFP levels for early stage HCC (median, 22.01 ng/ml) were not increased and similar to hepatitis subjects (Mann–Whitney test, $P = 0.1517$, Figure 4B). Meanwhile, AFP abundance was gender, histological grade, tumor size and AJCC staging dependent (Supplementary Table S6, available at *Carcinogenesis* Online); they were higher in women than in men (median, 381.35 ng/ml vs. 29.91 ng/ml, respectively, $P = 0.0463$) in accordance with the previous report (10). The AFP values were dramatically higher in patients with moderately and poorly differentiated HCC (median, 106.70 ng/ml) as compared with well-differentiated HCC (median, 5.49 ng/ml; (Mann–Whitney test, $P < 0.0001$). The patients with larger tumor size (>3 cm in diameter, median, 84.17 ng/ml) had increased serum AFP levels (≤ 3 cm ones, median, 17.07 ng/ml; Mann–Whitney test, $P = 0.0166$). Moreover, the serum levels of AFP in patients with III–IV stages (median, 215.5 ng/ml) were higher than I–II stages (median, 25.54 ng/ml) (Mann–Whitney test, $P = 0.0071$).

Serum levels of annexin A2 was not elevated in patients with other cancers

Additionally, serum levels of annexin A2 were measured in other patients with breast, lung, esophageal, gastric and colorectal cancers, the results showed that annexin A2 levels from those tumors were not increased (median, 3.75 ng/μl; range 0.46–233.31 ng/μl) compared with HCC cases and healthy controls (Mann–Whitney test, $P < 0.0001$, Figure 4C). There were no significant differences among different cancers (Kruskal–Wallis test, $P = 0.6453$).

Annexin A2 expression in HBx knockin transgenic mice

To explore the expression characteristics of annexin A2 during hepatocarcinogenesis, the *p21-HBx* transgenic mice were used. Over half of the mice developed spontaneous HCC at 18 months or even much later. We found that annexin A2 was strongly expressed in the cytoplasm of malignant hepatocytes, whereas it barely existed or slightly increased in the normal liver tissues of wild-type and transgenic mice at the ages of 6, 12 and 18 months, as well as in the adjacent non-cancerous liver tissues of tumor-bearing mice (Figure 5A and B). Similarly, the transgenic mice harboring HCC had significantly higher serum annexin A2 than those without tumors at the ages of 6, 12, 18 and 24 months (Mann–Whitney test, all of $P < 0.01$, Figure 5C). However, there was no significant difference in wild-type mice among different age groups (analysis of variance test, $P = 0.1071$).

Annexin A2 and AFP as complementary tumor markers

The correlation coefficient between serum annexin A2 and AFP values was not significant (Pearson's correlation test, $P = 0.1984$), indicating that measuring both markers in serum can improve the reciprocally holistic diagnostic value.

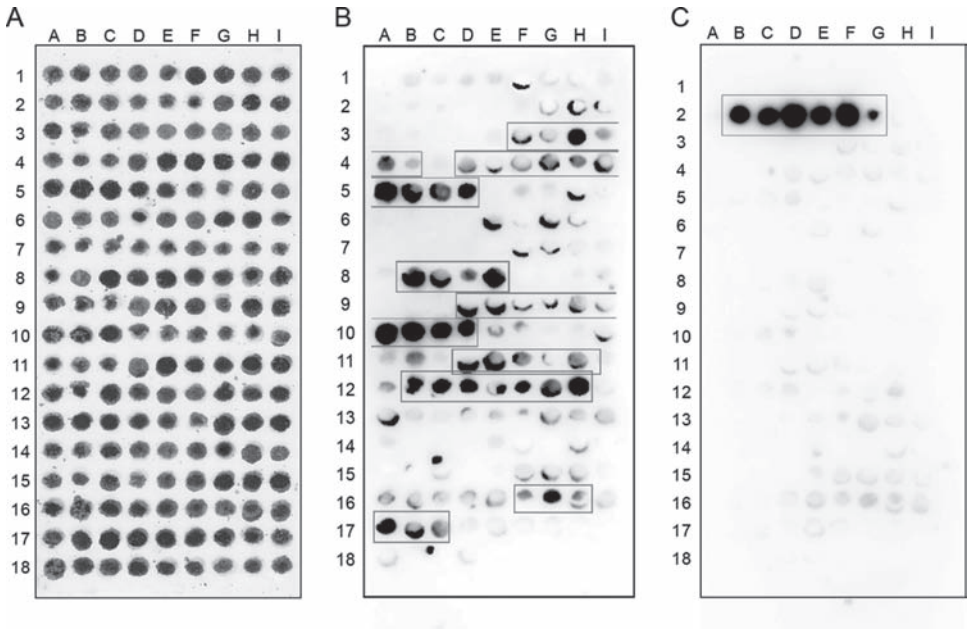

Fig. 3. Pepscan analysis of designated annexin A2 antibodies. The frame represented the potential linear epitopes. (A) Pepscan membrane was stained with Coomassie Blue R250. The amino acid sequence represented by each spot was shown in Supplementary Table S2 (available at *Carcinogenesis* Online). (B–C) The epitopes recognized by the capture and detection antibodies in our ELISA.

The specificity and sensitivity of serum annexin A2 and AFP measurements were represented by ROC curves. When only healthy individuals and HCC patients were evaluated, the AUC was 0.79 (95% confidence interval [CI]: 0.73–0.85) for annexin A2 and 0.87 (95% CI: 0.82–0.92) for AFP (Figure 6A). However, when HCC was compared with only hepatic and cirrhotic controls, annexin A2 (AUC = 0.80, 95% CI: 0.73–0.88) had a significantly better performance than AFP (AUC=0.69, 95% CI: 0.62–0.75; Figure 6B; chi-square test, $P = 0.0154$). Furthermore, when healthy individuals and all of HCC precancerous controls (hepatitis and cirrhosis) were compared with HCC, annexin A2 still had slightly higher AUC (0.80, 95% CI: 0.74–0.85) than AFP (0.76, 95% CI: 0.70–0.81; chi-square test, $P = 0.2651$). When both were combined in a logistic regression model, it was found that annexin A2 and AFP were independent biomarkers for HCC. The AUC for the combination of annexin A2 and AFP (0.86, 95% CI: 0.81–0.90) achieved the best performance (Figure 6C; chi-square test, $P = 0.0005$ for annexin A2 and combination, $P = 0.0001$ for AFP and combination). In addition, when only the early stage HCC (BCLC stages 0 and A) was compared with healthy, hepatitic and cirrhotic controls, the AUC for the combination of annexin A2 and AFP improved to 0.85 (95% CI: 0.79–0.90) from 0.79 (95% CI: 0.73–0.85) for annexin A2 alone and 0.73 (95% CI: 0.66–0.80) for AFP alone (chi-square test, $P = 0.0068$ for annexin A2 and combination, $P = 0.0003$ for AFP and combination; Figure 6D).

When healthy, hepatitic and cirrhotic controls were compared with HCC, the sensitivity and specificity of AFP alone (optimal cutoff of 14.88 ng/ml) were 63.4% and 79.7%, respectively; those of annexin A2 (optimal cutoff value of 17.43 ng/μl) were 81.7% and 68.3%, whereas those of the combination of annexin A2 and AFP were 76.0% and 80.5% (Supplementary Table S7, available at *Carcinogenesis* Online).

For early stage HCC, the optimal annexin A2 cutoff value was 17.3 ng/μl, which led to a sensitivity and specificity of 83.2% and 67.5%, respectively, and those of AFP were 54.7% and 81.3% (cutoff of 15.64 ng/ml). The combination of annexin A2 and AFP improve the sensitivity and specificity for early stage HCC to 87.4% and 68.3% (Supplementary Table S7, available at *Carcinogenesis* Online).

Importantly, in cases of HCC with low AFP levels ($n = 74$), annexin A2 can add to the diagnosis performance with the AUC of 0.77 (95% CI: 0.70–0.84, Figure 6E). In the instance, the sensitivity and specificity of annexin A2 (optimal cutoff value of 15.5 ng/μl) were 89.2% and 58.5%. When the cutoff value was set to 17.3 ng/μl, those of the values were 78.4% and 67.5%.

When the analysis was limited to early stage HCC cases and cirrhosis controls, the AUC for annexin A2 was 0.80 (95% CI: 0.70–0.90) compared with 0.66 for AFP (95% CI: 0.56–0.76). As shown in Figure 6F, annexin A2 had a significantly better AUC compared with AFP (chi-square test, $P = 0.03$) with the sensitivity and specificity of 86.4% and 73.5% at the cutoff value of 17.3 ng/μl.

Discussion

We identified a group of differentially expressed proteins in HCC through a subcellular proteomic approach and then systemically validated annexin A2 in large sample size. It pronounced upregulated in HCC tissues. Serum annexin A2 was significantly increased in HCC patients compared with healthy controls, benign tumors, hepatitis and cirrhosis subjects, especially for early stage HCC, but not in the other five malignant tumors. Annexin A2 alone had better diagnostic performance for early HCC than AFP. The further analyses in *p21-HBx* transgenic mice model also suggested that the elevation of annexin A2 might be an actual event during hepatocarcinogenesis.

We established a new indirect sandwich ELISA to evaluate serum annexin A2 levels and to assess complementary abilities for HCC diagnosis of annexin A2 and AFP. Overall, compared with AFP, annexin A2 has some advantages to distinguish HCC, non-cancerous and cirrhosis controls. At first, 42% (74/175) of our analyzed cases were considered AFP-negative using a clinically acceptable threshold <20 ng/ml. Among them, serum annexin A2 levels were elevated in 78.4% (58/74) of the cases at a cutoff value of 17.3 ng/μl. We found that AFP values were significantly higher in women, larger tumor size, moderately poorly differentiated and late AJCC stage of HCC, whereas serum annexin A2 levels were not influenced by age, gender, pathological differentiation, tumor size and tumor stages. Therefore, annexin A2 was helpful to AFP, particular for small, well-differentiated tumors and AFP-negative cases. Second, increased AFP values were observed in 30.4% (7/23) hepatitis and 21.6% (11/51) cirrhosis cases. The accuracy of AFP in detecting early stage tumors was not satisfying. The median levels of AFP in late-stage cases were approximately four

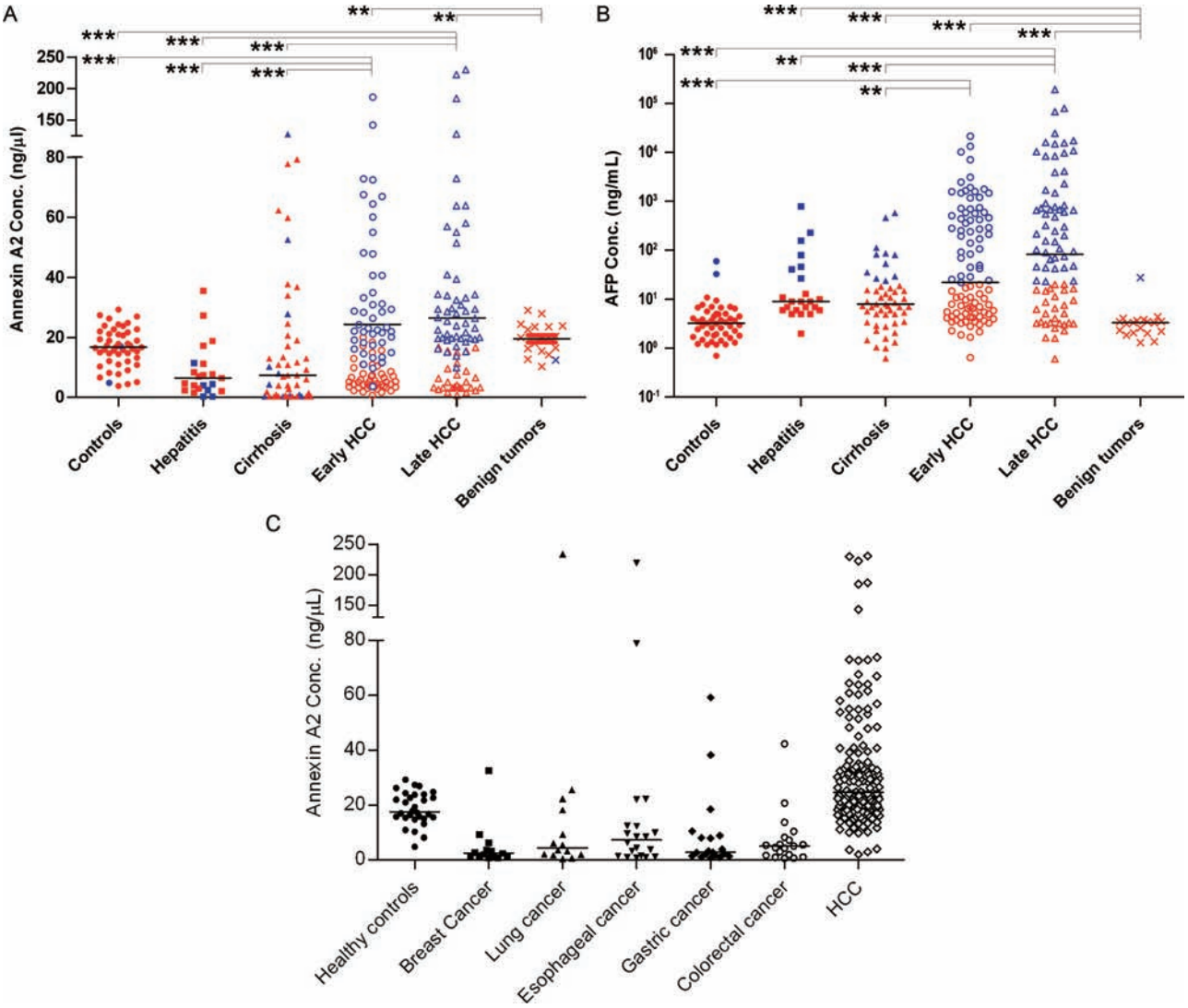

Fig. 4. Serum levels of annexin A2 and AFP among those with healthy controls, hepatitis, cirrhosis, early stage HCC, late-stage HCC and liver benign tumor patients. (A and B) The distribution of serum annexin A2 and AFP concentrations. (C) Serologic concentration distribution of annexin A2 from healthy controls and patients with breast cancer ($n = 15$), lung cancer ($n = 14$), esophageal cancer ($n = 20$), gastric cancer ($n = 19$), colorectal cancer ($n = 19$) and HCC ($n = 175$). The median of its serum levels in each group of individuals was shown as black lines. In (A) and (B), the AFP levels of each HCC patients were labeled with red (AFP-negative) and blue (AFP-positive). The comparisons between any two groups were performed using Mann–Whitney test and P values were labeled. ***, $P < 0.001$; **, $P < 0.01$.

times higher than those in early stage. They all suggested that AFP was not an ideal marker for early detection of HCC (10). However, annexin A2 abundance was elevated in 83.2% (79/95) of early stage patients and it was no significant difference between early stage and late-stage cases. Consequently, annexin A2 had the better diagnostic performance than AFP, especially in detecting early stage HCC. The sensitivity of annexin A2 was 83.2%, whereas this of AFP was only 54.7%. Third, due to the distinct expression characteristics of AFP and annexin A2, the combination of both markers enhanced the sensitivity and specificity, indicating that these two markers are complementary. At an annexin A2 cutoff value of >17.3 ng/μl or AFP value of >15.64 ng/ml, early detection rate of HCC increased from 83.2% and 54.7% for each marker alone to 87.4% for both markers. Finally, as shown as our ROC analysis, although the discriminating ability of annexin A2 was relatively limited for healthy controls and HCC compared with AFP, it is more helpful for distinguishing the patients with chronic liver diseases from HCC. The combination of AFP and annexin A2 improved the diagnostic performance of various non-cancerous controls and HCC. In addition, because serum ALT levels were not correlated with annexin A2 (Spearman's rank correlation test, $P = 0.3096$), it was suggested that liver injury was not the major cause to release annexin A2 in HCC.

Importantly, it was reported that HBV-related HCC cells express approximately 3-fold higher annexin A2 compared with hepattitis C virus–related HCC (37). A recent work also showed that annexin A2 upregulated in viral *HBx* gene transfected HepG2, suggesting that it might play roles in HBV-induced HCC (38). Therefore, we analyzed annexin A2 expression in *p21-HBx* transgenic mice model. The serological examination showed that the annexin A2 levels significantly increased in HCC-bearing mice, suggesting that the elevation of serum annexin A2 may serve as an index of hepatocarcinogenesis. In addition, HCC in this model was not developed via fibrosis and cirrhosis stage, but usually accompanied with hepatic steatosis and inflammation (36). So it is difficult to evaluate the HCC surveillance performance of annexin A2 in this model.

Various proteolytic cleavage forms of annexin A2 were observed in several cells at physiological and pathological conditions. For example, limited chymotrypsin digestion of annexin A2 results in the cleavage between residues 23 and 24, as well as between residues 27 and 28 (39). The larger 33–34 kDa fragment was subsequently

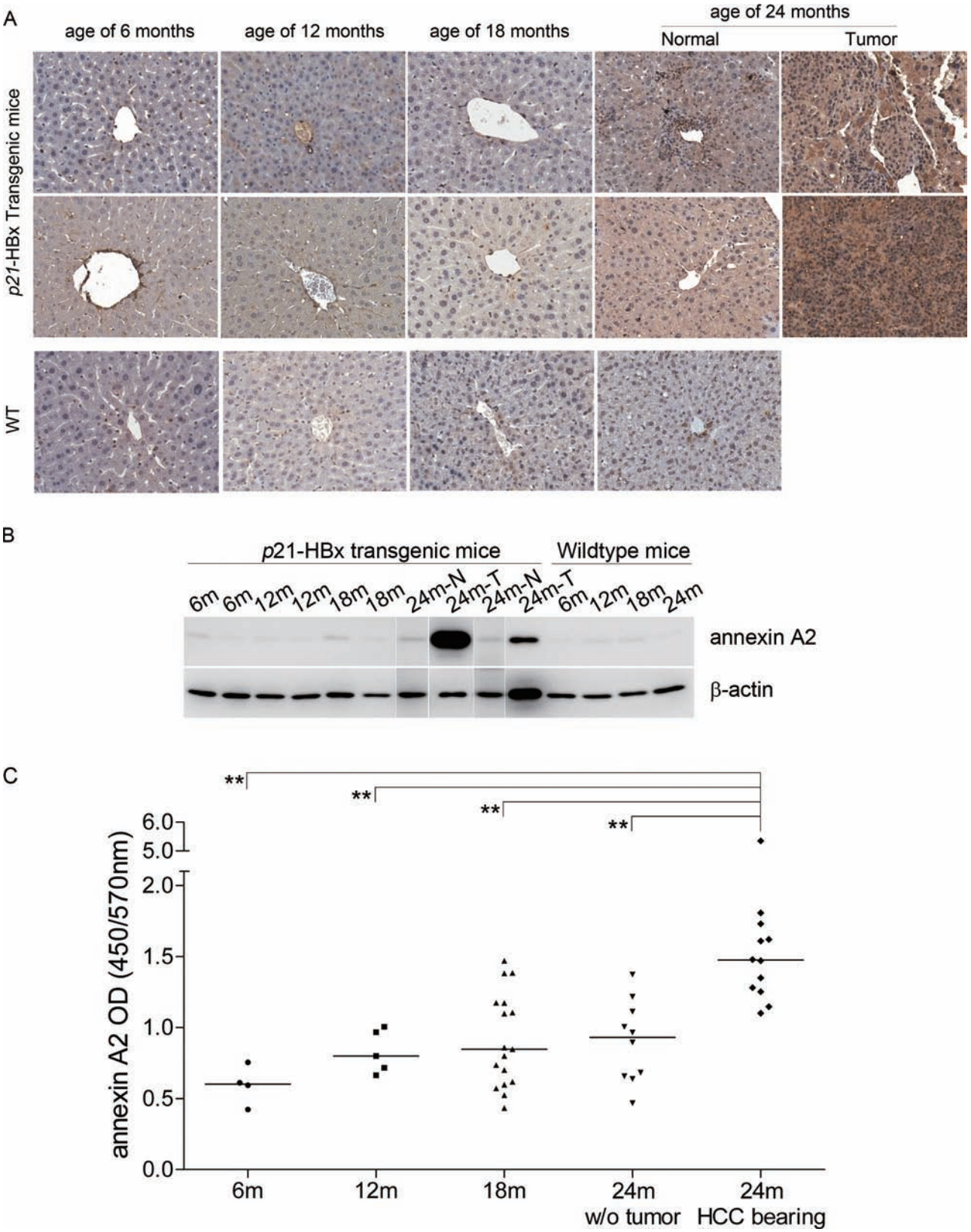

Fig. 5. The expression of annexin A2 in the liver tissues and sera of *p21-HBx* transgenic and wild-type mice. (A) The representative IHC staining of normal liver tissue of *p21-HBx* transgenic and wild-type mice at the ages of 6, 12, 18 and 24 months. The adjacent normal liver tissues and tumor tissues of transgenic mice at the ages of 24 months were included. (magnification ×200). (B) Western blot analysis of liver tissues of *p21-HBx* transgenic and wild-type mice at the ages of 6, 12, 18 and 24 months. The adjacent normal liver tissues and tumor tissues of HCC-bearing mice were marked as 'N' and 'T', respectively. (C) Serum levels of annexin A2 in the *p21-HBx* mice with different ages. The mice at the ages of 24 months were further divided into two groups: without tumors and HCC-bearing mice. The comparisons between any two groups were performed using Student's *t*-test or Mann–Whitney test, and *P* values were labeled. **, $P < 0.01$.

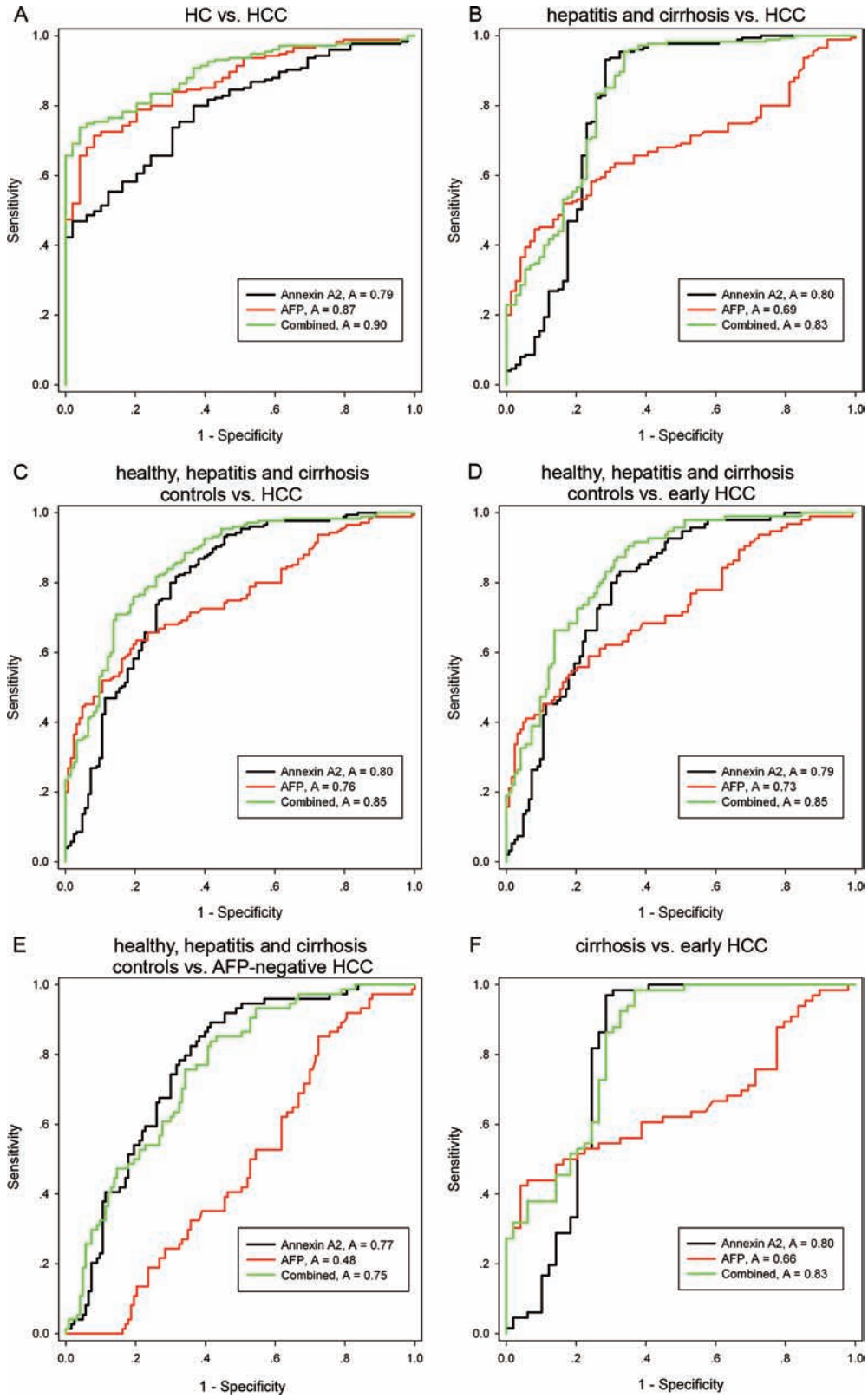

Fig. 6. ROC curves of annexin A2 and AFP. ROC curves were derived by plotting the relationship between specificity and sensitivity at various cutoff levels. Annexin A2 is black, AFP is red and combination of annexin A2 and AFP is green. (A) ROC curve evaluating those with only healthy controls (n = 49) and HCC (n = 175). (B) ROC curve evaluating those with only hepatic (n = 23) and cirrhotic (n = 51) controls and HCC (n = 175). (C) ROC curve evaluating those with healthy (n = 49), hepatitc (n = 23) and cirrhotic (n = 51) controls and HCC (n = 175). (D) ROC curve evaluating those with healthy (n = 49), hepatitc (n = 23) and cirrhotic (n = 51) controls and early stage HCC (n = 95). (E) ROC curve evaluating those with healthy (n = 49), hepatitc (n = 23) and cirrhotic (n = 51) controls and AFP-negative HCC (n = 74). (F) ROC curve evaluating those with cirrhotic (n = 51) controls and early stage HCC (n = 95).

cleaved between Arg-204 and Lys-205 by trypsin (40). Plasmin may cleave annexin A2 at Lys-307 (41) or Lys-27 (42) in human endothelial cells and monocytes. MMP-7 hydrolyzed annexin A2 at Lys-9 enhancing tPA-mediated pericellular proteolysis by cancer cells (43). Annexin A2 contains KFERQ motif that can bind to endosomes and then enter lysosomes. These organelles had persisted even within highly proteolytic microenvironments to generate truncated forms (44). In addition, annexin A2 cleavage induced by multiple proteins or proteases, such as glycogen synthase-3, calpain etc. were also reported (45,46). These potential proteases or proteins had the abnormal expression in various tumors showed in a public database Human Protein Atlas (http://www.proteinatlas.org/, Supplementary Figure S2, available at *Carcinogenesis* Online). Furthermore, annexin A2 was mainly cleaved in the C-terminus in normal colon mucosa but not in colorectal cancer (47). Therefore, extracellular annexin A2 may exist in various proteolytic forms.

Previously, a sandwich ELISA system for annexin A2 was reported (30). We analyzed the system using pepscan experiments. Their coating and detecting antibodies recognized single epitopes of aa 3–13 and aa 61–72 near to the N-terminus of annexin A2, (Supplementary Figure S3, available at *Carcinogenesis* Online). However, the coating antibody of our assay recognized at least eight sequential epitopes with different affinity. Given its immunogen characteristic, we speculated that it might also recognize conformational epitopes. The bioinformatics prediction (48) also mapped the conformational epitopes of annexin A2 to the C-terminus (Supplementary Table S8, available at *Carcinogenesis* Online). Herein, our assay clearly distinguished from the previous report with distinct recognized epitopes (Figure 3, Supplementary Figure S3, available at *Carcinogenesis* Online). Due to the complex proteolytic cleavages of serum annexin A2, it was possible that these two assays detected diverse forms of the proteins/peptides or their combination in serum and may complement each other.

Because of the complicated proteolytic cleavages of annexin A2, we also observed that serum annexin A2 levels fluctuated along with the progression of liver diseases. Annexin A2 is a ubiquitious expressed protein in human body, so its baseline serum concentrations are not low in healthy controls. In liver disease (chronic hepatitis and cirrhosis), it did not significantly increase (24–28). In HCC, however, malignant hepatocytes conspicuously overexpressed annexin A2. Inflammation, abnormal coagulation and fibrinolysis activity, impaired liver function and fibrosis may all increase the extracellular proteolytic activity (49,50). Thus, increased proteolytic activity might be responsible for the decrease of serum annexin A2 in liver disease when the samples were detected by our system. When it is insufficient to counteract the increase of annexin A2, it shows the significant elevation of serum proteins. Notably, the observed MW of annexin A2 spot in our 2D gel was also lower than its theoretical value. The peptide sequence identified by mass spectrometry covered only the N-terminal half of annexin A2 (Supplementary Table S3 and Figure S4, available at *Carcinogenesis* Online). It hinted that this form of annexin A2 might undergo proteolytic cleavage in C-terminus in liver cancer cells. In addition, although annexin A2 had been reported overexpressed in multiple tumors, including stomach, breast and colon, we did not observe its relevant elevation in sera of 87 patients with five kinds of solid cancers using our ELISA. We speculated that large amounts of proteins were proteolytically cleaved because some principal proteases induced the cleavage of annexin A2–existed abnormal expression in these tumors (Supplementary Figure S2, available at *Carcinogenesis* Online). Perhaps, there are some other mechanisms leading to the inconsistent expression at the histological and serological levels. Our limited sample size is still insufficient to draw a conclusion. In-depth analysis of the dynamic changes of serum annexin A2 peptidome under normal and disease conditions will help better understand the mechanisms of the protein.

In conclusion, our results demonstrated upregulation of annexin A2 in both of tissues and sera of HCC patients and also in both AFP-positive and -negative cases. Remarkably, its serum levels were significantly elevated in early stage of HCC. Combination of conventional serum marker AFP with annexin A2 protein may complement and benefit for early HCC detection. Therefore, given HCC epidemiological characteristics in China, our results strongly showed that annexin A2 is an independent and discriminative serological candidate marker of HBV-related HCC with the potential clinical application prospect. Further validation with a larger sample size in multiple clinical centers may help to systematical evaluation of annexin A2 and develop novel diagnostic and prognostic markers for liver cancer.

Supplementary material

Supplementary Tables S1–S8 and Figures S1–S4 can be found at http://carcin.oxfordjournals.org/.

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