Down-Regulation of Annexin A10 in Hepatocellular Carcinoma Is Associated with Vascular Invasion, Early Recurrence, and Poor Prognosis in Synergy with p53 Mutation

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Annexins (ANXs) are a large group of calcium-binding proteins participating in diverse important biological processes. ANXA10 is the least expressed new member of unknown function. We showed that ANXA10 mRNA was expressed in adult liver and hepatocellular carcinoma (HCC), but not in multiple adult and fetal tissues, cholangiocarcinoma, and several other common carcinomas. Of 182 unifocal primary HCCs, ANXA10 mRNA was dramatically reduced in 121 (66%), and the down-regulation correlated with p53 mutation ($P = 0.024$), early intrahepatic tumor recurrence ($P = 0.0007$), and lower 4-year survival ($P = 0.0014$). Down-regulation of ANXA10 was twofold more frequent in large than small HCCs ($P = 0.0012$), in grade II to III than grade I HCC ($P < 0.00001$), and in stage IIIA to IV than stage I to II HCC ($P < 0.00001$). Moreover, ANXA10 down-regulation and p53 mutation acted synergistically toward high-grade ($P < 0.00001$), high-stage HCC ($P < 0.00001$), and poorer prognosis ($P = 0.0025$). Our results indicate that the expression of the tissue- and tumor-restricted ANXA10 is a marker of liver cell differentiation and growth arrest, and its down-regulation associated with malignant phenotype of hepatocytes, vascular invasion, and progression of HCC, leading to poor prognosis. Thus, ANXA10 might serve as a new potential target of gene therapy for HCC. (*Am J Pathol* 2002, 160:1831–1837)

The annexin (ANX) family is the largest single category of eukaryotic calcium-binding proteins without EF hands (the helix-loop-helix calcium-binding motif).¹ The ANXs play an important role in a broad range of physiological processes, including anti-coagulation,² endocytosis,

exocytosis,^{3,4} immunosuppression,⁵ differentiation,^{6–8} proliferation,^{9,10} and inhibition of calcium channels, phospholipase A₂, and protein kinase C.^{7,11–14} There are at least 13 human ANX members, designated A1, A2, A3, A4, A5, 5'-A6, 3'-A6, A7, A8, A9, A10, A11, and A13.¹⁵ The ANXs share a similar structure characterized by the presence of four or eight repeats of a 70-amino acid motif and a highly variable N-terminal end.^{1,14,16} Unlike the conserved repeats, the amino-terminal domains of ANXs are highly variable, and may mediate the specific functions of individual ANXs.¹

Several ANXs are involved in tumorigenesis. ANXA1 is overexpressed in breast cancer and hepatocellular carcinoma (HCC),^{17–19} ANXA2 in brain glial tumors and pancreatic cancer,^{20–23} and ANXA8 in acute promyelocytic leukemia.^{13,24} Overexpression of ANXA1 correlates positively with metastatic potential of breast cancer,¹⁹ and increased growth of tumor cells inoculated in nude mice.²⁵ In contrast, ANXA6 has tumor suppressive activity in squamous cell carcinoma,²⁶ and a decrease in ANXA6 and ANXA7 proteins is associated with malignant phenotype and the metastatic potential of melanoma.^{27,28} Moreover, ANXA4 plays a role in chemoresistance.²⁹ Hence, there are at least two categories of ANXs implicated in tumor progression and tumor suppression, and different ANXs play important and complex roles in cancer.

ANXA10, a novel member of the ANX family, has several distinct features, including rare expression, codon deletion in conserved repeat 3, and an unusual ablation of the type II calcium-binding sites in tetrad core repeats.¹⁵ The loss of type II calcium-binding sites is a fundamental deviation from ANX structure conservation and undoubtedly has functional consequences for membrane association and aggregation properties.³⁰ ANXA10 is mapped to chromosome 4q33.³¹ Chromosome 4q is one of the most common regions with a high

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frequency of allelic loss in HCC.³²⁻³⁵ Introduction of normal human chromosome 4 leads to suppressed tumorigenicity of teratocarcinoma PA-1 cells in nude mice, suggestive of a putative tumor suppressor gene on chromosome 4.³⁵ The biological function of ANXA10 and its role in HCC are not known. Using differential display, we found a cDNA clone identical to ANXA10¹⁵ that was expressed in liver and often down-regulated in HCC. In this study, we correlate the mRNA expression of ANXA10 in HCC with clinicopathological features. We show that ANXA10 might be a potential tumor suppressor gene because its down-regulation is associated with malignant phenotype of liver cells, and correlates with vascular invasion and progression of HCC.

Materials and Methods

Study Group

Between 1987 and 1997, 909 surgically resected primary and 127 recurrent HCCs were pathologically assessed at the National Taiwan University Hospital. The tissue samples were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored in deep freezers. Of these, 187 patients who already had RNA samples taken from resected HCCs were analyzed for ANXA10 mRNA levels, including 182 unifocal and 5 multifocal primary HCCs. To elucidate the clinical implication of ANXA10 expression, the 182 cases of unifocal primary HCC, as previously defined,³⁶⁻³⁹ were selected for clinicopathological analysis. Multifocal HCC was excluded from this correlation because of incomplete sampling of the tumor nodules for analysis and their variation in pathological features. To study the tissue and tumor distribution, we also examined the mRNA expression of ANXA10 in multiple adult tissues and several common tumor tissues obtained from surgical pathology specimens, and multiple fetal tissues obtained from autopsy.

The intrahepatic tumor recurrence (IHTR) was based on imaging diagnosis with ultrasonography and/or computed tomography, supplemented with elevated serum α -fetoprotein (AFP). Until the end of October 2001, tumor recurrence was detected in 113 patients. Among the 182 patients studied, 156 were eligible for the evaluation of early IHTR (≤ 1 year). Twenty-six patients who died within 1 year after resection and had no information about or were negative for IHTR were excluded from the evaluation of early recurrence.

Histological Study and Tumor Staging

The tumor grade was simply divided into three groups: well (grade I), moderately (grade II), and poorly differentiated HCC (grades III and IV). Resected unifocal HCC was staged as stage I, II, IIIA, IIIB, and IV as previously described.⁴⁰ Briefly, stage I HCC is encapsulated and without parenchymal invasion. Stage II HCC has liver invasion, but no vascular invasion. Stage IIIA HCC has invasion of small vessels in the tumor capsule. Stage IIIB and IV

HCCs have invasion of portal vein branches. The pathological stage of HCC correlates closely with survival.⁴⁰

Differential Display Analysis

Differential display was performed according to the recommendation of the manufacturer using the RNImage kit (GenHunter Corp., Nashville, TN). Total RNA (0.2 μ g) was reverse-transcribed in a mixture containing 100 U MMLV reverse transcriptase, 20 μ mol/L dNTP, and 0.2 μ mol/L anchor primer H-T11C (5'-AAGCT11C-3'). The reverse transcription products (2 μ l) were subjected to polymerase chain reaction (PCR) amplification in a PCR mixture containing 2 μ mol/L dNTP, 0.2 μ mol/L H-T11C (5'-AAGCT11C-3'), 25 mCi/mmol [α -³³P]dATP (Amersham, Buckinghamshire, England), 1 U Vent DNA polymerase (New England Biolabs, Inc. Beverly, MA), 0.2 μ mol/L arbitrary primer HAP35 (5'-AAGCTTCAGGGCA-3'). PCR was done in Perkin-Elmer's 9600 thermocycler (94°C for 30 seconds, 40°C for 2 minutes, 72°C for 1 minute for 40 cycles, and a final elongation at 72°C for 5 minutes). The amplification products were separated by 6% denaturing polyacrylamide gel electrophoresis. The dried gel was then exposed by film autoradiography. The differentially expressed band was cut, eluted, and sequenced.

Reverse Transcriptase (RT)-PCR

RT-PCR assays were used for large-scale quantitative measurements for ANXA10 mRNA levels, as previously described,⁴¹ using S26 ribosomal protein mRNA as internal control.⁴² Briefly, 2 μ l of RT product, 1.25 U Pro Taq DNA polymerase (Protech Technology Enterprise Co, Taipei, Taiwan), Pro Taq buffer, and 200 μ mol/L each dATP, dTTP, dGTP, and dCTP were mixed with primer pairs for ANXA10 and S26 in a total volume of 30 μ l. The PCR was performed in an automated DNA thermal cycler 480 (Perkin-Elmer/Cetus). The PCR was stopped at the exponential phases, 29 cycles for ANXA10 and 22 cycles for S26. These experiments were repeated when the S26 loading showed inappropriate template amount for PCR. The primers used included the sense primer ANXA10-A: 5'-TTGTTCTCTGTGTTTCGAGAGAAACC-3', and anti-sense primer ANXA10-F: 5'-GTAGGCAAATTCAGGATAGTAGGC-3'.

The products were electrophoresed on 2% agarose gel. The ANXA10 mRNA expression level was determined by the ratio of signal intensity of ANXA10 to that of S26 measured by 1D Image Analysis Software (Kodak Digital Science, Rochester, NY), and scored as high (ratio ≥ 1.0), intermediate ($0.5 \leq \text{ratio} < 1.0$), trace (ratio < 0.5), and negative (or immeasurable). An ANXA10/S26 ratio < 0.5 was regarded as down-regulation, because ANXA10 mRNA level in liver was rarely less than a ratio of 0.5.

Analysis of p53 Mutation

The analysis of p53 mutations was done as described.^{37,39}

Figure 1. Northern blot for ANXA10 using a cDNA fragment cloned from differential display as probe. ANXA10 mRNA (A10) of 1.4 kb was detected in the livers (L) and dramatically decreased in HCC (T) in five cases.

Follow-Up Observation

To minimize the influence of second and third recurrent primary HCC, the endpoint for follow-up was set at the 4th year. Of the 182 cases studied, 179 (98%) had been followed for >4 years or until death.

Statistics

The analyses were performed using the Statistica for Windows software (Statsoft, Inc., Chicago, IL). Two-tailed chi-square and Fisher exact tests were used for univariate analysis. The cumulative survival after tumor removal was calculated with the log-rank test. *P* values <0.05 were considered statistically significant.

Results

Cloning and Expression of ANXA10 mRNA

We used differential display for the analysis of genes aberrantly expressed in HCC, and cloned a 192-bp cDNA fragment that was consistently expressed in liver and often down-regulated in HCC. This cDNA clone was identical to ANXA10¹⁵ (GenBank GI no. 9625245). A nearly full-length cDNA of 1268-bp long was subsequently cloned from Hep3B cell line by RT-PCR amplification; the sequence was identical to ANXA10.¹⁵ The preferential down-regulation of ANXA10 in HCC was verified by Northern blotting (Figure 1). Using RT-PCR for large-scale analysis, ANXA10 mRNA was expressed abundantly in liver, and in low level in only 12 (6.4%) of 176 livers harboring benign (focal nodular hyperplasia) and malignant tumors (HCC, cholangiocarcinoma, metastatic carcinoma). In contrast, ANXA10 mRNA was dramatically decreased in 121 of 182 unifocal primary HCCs (66%) as compared to paired nontumor livers (Table 1; Figure 2).

Figure 2. RT-PCR of ANXA10 mRNA in paired HCC and liver. ANXA10 mRNA (A10) was dramatically decreased in HCC (T) in 16 of 22 representative cases. Stage, tumor stage (I to IV); size, tumor size (cm).

Tissue-Specific Expression of ANXA10 mRNA in Fetal and Adult Tissues

To elucidate the tissue-specific expression of the ANXA10, RNA samples taken from multiple fetal and adult tissues were analyzed. Using polyA⁺ RNA blot obtained from multiple adult tissues (RPN4800; Amersham Pharmacia Biotech, Buckinghamshire, UK), a single ANXA10 transcript of ~1.3 kb was identified in adult liver alone (Figure 3). By RT-PCR analysis, ANXA10 mRNA was expressed only in a very limited number of adult tissues, abundant in liver and stomach, low in pancreas, and not expressed in other adult tissues, such as lung, heart, breast, kidney, and spleen (Figure 4). ANXA10 mRNA was also undetectable in most fetal tissues, such as brain, thyroid, lung, heart, liver, kidney, adrenal gland, and testes. ANXA10 mRNA was expressed abundantly in fetal stomach (Figure 4). Among three fetal livers taken from fetuses less than gestation age of 22 weeks and without congenital anomalies, ANXA10 mRNA was expressed in low level in one and not detected in two (Figure 4).

Expression of Annexin A10 in Benign and Malignant Liver Tumors

ANXA10 mRNA was expressed in benign liver lesions such as focal nodular hyperplasia, and decreased dramatically in hepatoblastoma (Table 1). To clarify the tumor-specific distribution, we analyzed the ANXA10 mRNA expression in several common carcinomas of

Table 1. ANXA10 mRNA Expression in Benign and Malignant Human Tumors

Tumor type	ANXA10 mRNA expression	
	Expressed	Not expressed or dramatically decreased
Hepatocellular carcinoma (<i>n</i> = 182)	61	121 (66%)
Hepatoblastoma (<i>n</i> = 5)	0	5 (100%)
Cholangiocarcinoma (<i>n</i> = 2)	0	2
Metastatic colorectal carcinoma (<i>n</i> = 10)*	0	10 (100%)
Focal nodular hyperplasia (<i>n</i> = 9)	8	1 (11%)
Ovarian carcinoma (<i>n</i> = 16)	0	16 (100%)
Lung carcinoma (<i>n</i> = 3)	0	3
Breast carcinoma (<i>n</i> = 10)	0	10 (100%)

*Includes one ovarian and one breast, and eight metastatic colorectal carcinomas.

Figure 3. Northern blot analysis of ANXA10 in multiple adult tissues. The commercially obtained Hybond Northern blot (Amersham) containing polyA⁺ RNAs was hybridized with a 1.2-kb cDNA fragment containing the whole coding region and 3'-UTR of ANXA10. A single ANXA10 transcript of ~1.3 kb (arrowhead) was identified in adult liver alone.

other anatomical sites. As shown in Table 1, ANXA10 mRNA was undetectable or weakly expressed in carcinomas of the ovary, breast, colon, and lung, and metastatic carcinomas of the liver (Figure 5).

Clinicopathological Correlations of ANXA10 mRNA Expression

To elucidate the biological significance of its expression, we correlated the ANXA10 expression with clinicopathological features. As shown in Table 2, the decreased ANXA10 expression was closely associated with serum AFP elevation ($P < 0.00001$). The ANXA10 expression did not correlate with age, gender, hepatitis B virus (HBV) infection, and liver cirrhosis (data not shown). Histopathologically, down-regulation of ANXA10 was found more often in large HCCs than in HCCs <3 cm ($P = 0.0012$), in grade II and III than in grade I HCCs ($P < 0.00001$), and in HCCs with vascular invasion (stage IIIA to IV) than in stage I to II HCCs that has no vascular invasion ($P < 0.00001$).

Down-regulation of ANXA10 was associated with higher frequency of early intrahepatic tumor recurrence ($P = 0.0007$). Furthermore, HCC with down-regulation of ANXA10 was associated with a lower 4-year patient sur-

Figure 4. Expression of ANXA10 in multiple adult and fetal tissues. ANXA10 mRNA (A10) was expressed in abundance in adult and fetal stomach, in low level in adult pancreas, but undetectable in most adult tissues and fetal tissues.

Figure 5. Expression of ANXA10 in other malignant tumors. ANXA10 mRNA (A10) was detected in focal nodular hyperplasia (FNH), but not in hepatoblastoma (HB), cholangiocarcinoma (CH), metastatic colon carcinoma (M), lung carcinoma (399, adenosquamous cell carcinoma; 544, squamous cell carcinoma; 546, spindle cell cancer), breast carcinoma, and ovarian carcinoma (926, 950, endometrioid carcinoma; 916, serous cystadenocarcinoma; 922, 928, adenocarcinoma; 924, yolk sac tumor; 945, mucinous cystadenocarcinoma; 933, clear cell carcinoma).

vival rate than HCC with normal ANXA10 expression, 54% versus 33%, $P = 0.0014$ (Figure 6).

Correlation and Synergy between ANXA10 Expression and p53 Mutations

Because p53 mutation correlates closely with tumor aggressiveness and unfavorable prognosis of HCC,^{37,39} we analyzed the potential interplay between these two important unfavorable prognostic factors. In this study, p53 mutation was found in 77 (49%) of 156 HCCs examined. Down-regulation of ANXA10 correlated with p53 mutation, $P = 0.024$ (Table 2). HCC with both ANXA10 down-regulation and p53 mutation had the highest frequencies of grade II to III and stage IIIA to IV tumors than other groups, $P < 0.00001$ and $P < 0.00001$, respectively

Table 2. Correlation of ANXA10 mRNA Expression with Clinicopathological Features in 182 Patients with Unifocal Primary Hepatocellular Carcinoma

	ANXA10 mRNA expression		
	Normal expression (n = 61)	Down-regulation (n = 121)	P value
AFP			
<320 ng/ml (n = 97)	51	46 (47%)	<0.00001
≥320 ng/ml (n = 85)	10	75 (88%)	
p53 mutation			
Yes (n = 77)	19	58 (75%)	0.024
No (n = 79)	33	46 (58%)	
Tumor size			
≤3 cm (n = 45)	24	21 (47%)	0.0012
>3 cm (n = 137)	37	100 (73%)	
Tumor grade			
I (n = 39)	25	14 (36%)	<0.00001
II–III (n = 142)	36	106 (75%)	
Tumor stage			
I–II (n = 83)	42	41 (49%)	<0.00001
IIIA–IV (n = 99)	19	80 (81%)	
Early recurrence (≤1-yr)*	14/52 (27%)	58/104 (56%)	0.0007

*Tumor recurrence in the liver was detected ≤12 months after resection of the primary HCC.

Figure 6. Cumulative survival in HCC in relation to ANXA10 mRNA expression. A decreased ANXA10 (D) expression had a lower 4-year survival than normal expression (N), $P = 0.0014$ (log-rank test).

(Table 3). Moreover, HCCs with both ANXA10 down-regulation and p53 mutation also had the most frequent early intrahepatic tumor recurrence, $P = 0.0001$ (Table 3), and the worst 4-year survival, $P = 0.0025$ (Figure 7).

Discussion

Using differential display analysis in paired HCC and liver tissue, a cDNA band preferentially decreased in HCC was cloned. The partial cDNA sequence was identical to liver ANXA10, a novel member of the ANX family with unique loss of the calcium-binding site and of unknown function. In this study, we demonstrated that ANXA10 mRNA was expressed in a tissue-restricted and developmentally regulated pattern. In contrast to fetal liver, ANXA10 mRNA was expressed abundantly in adult liver. Among the more than 13 ANX family members, different ANXs have unique tissue and cell distribution in the vertebrates. ANXA2, -A5, -A6, and -A11 are ubiquitous, whereas ANXA3, -A8, and -A13 have more restricted tissue distribution.^{1,43,44} These findings indicate their functions are tissue-specific.^{45,46} Although the function of ANXA10 remains to be clarified, our results indicate that ANXA10 is developmentally regulated in liver, and may serve as a potential marker of differentiation, maturation, or growth arrest of hepatocytes. This suggestion is

Figure 7. Cumulative survival in HCC in relation to normal (N) or decreased (D) expression of ANXA10 and presence (+) or absence (-) of p53 mutation. HCC with decreased ANXA10 expression and p53 mutation had the lowest 4-year survival, $P = 0.0025$ (log-rank test).

strengthened by the finding that ANXA10 mRNA was expressed abundantly in benign liver lesions such as focal nodular hyperplasia. ANXA10 mRNA was also expressed in HCC and hepatoblastoma, although often decreased. In contrast, ANXA10 mRNA was not detected in cholangiocarcinoma, and in several common carcinomas of other anatomical sites, where ANXA10 mRNA was also not expressed in their normal tissues, such as colorectal, breast, and ovarian carcinomas. These findings suggest that ANXA10 mRNA expression is a marker of hepatocellular origin. The ANXA10 mRNA expression in adult liver and malignant liver cell tumors seems paradoxical. However, we found a close association of normal ANXA10 expression with grade I HCC, 64% versus 25%, $P < 0.00001$. This finding supports the suggestion that ANXA10 expression indeed might represent tumor cell differentiation and growth arrest in HCC.

Human ANXA10 is mapped to chromosome 4q33.³¹ Chromosome 4q is one of the most common regions with a high frequency of allelic loss in HCC.^{33-35,47} Chromosomal alterations at 4q are of great interest because it is a unique trait for HCC,^{32,33} and chromosome 4q contains genes encoding growth factors or genes expressed predominantly in the liver. These include albumin, alcohol dehydrogenase (ADH3), fibrinogen, AFP, and UDP-glucuronyl-transferase. In this study, we showed that

Table 3. Synergistic Effect between ANXA10 Down-Regulation and p53 Mutation in Unifocal Hepatocellular Carcinoma

Feature	Down-Regulation of ANXA10/p53 mutation				P value
	No/No n = 33	Yes/No n = 46	No/Yes n = 19	Yes/Yes n = 58	
Tumor grade					
I	16 (48%)	9 (20%)	4 (21%)	4 (7%)	<0.00001
II-III	17	37	15	54	
Tumor stage					
I-II	26 (79%)	23 (50%)	10 (53%)	13 (22%)	<0.00001
III-IV	8	23	9	45	
Early intrahepatic tumor recurrence*	7/30 (23%)	15/38 (39%)	6/15 (40%)	36/53 (68%)	0.0001

*Tumor recurs in the liver ≤ 12 months after resection of the primary HCC.

ANXA10, a new member of genes at 4q, was expressed predominantly in liver. To elucidate its role in HCC, we demonstrated for the first time that HCC with ANXA10 mRNA expression was dramatically down-regulated in 121 (66%) of 182 unifocal primary HCCs. The down-regulation was associated with more frequent AFP elevation ($P < 0.00001$), and grade II and III tumors ($P < 0.00001$). These results are in accord with our previous observation that poor differentiation of HCC correlates with AFP mRNA expression and serum AFP elevation.³⁸ During the tumor progression, HCC tends to spread throughout the liver via the portal vein branches even at the advanced stage, and the extent of portal vein invasion is the most crucial factor for tumor staging and prognosis.⁴⁰ However, the molecular mechanisms for portal vein spread remain primarily unknown. In this study, down-regulation of ANXA10 was nearly twofold more frequent in large than in small HCCs ≤ 3 cm ($P = 0.0012$), in grade II to III than in grade I HCC ($P < 0.00001$), and in stage IIIA to IV HCC that has various extents of vascular invasion than in stage I to II HCC that has no vascular invasion ($P < 0.00001$). These results suggest that down-regulation of ANXA10 contributes to the progression and metastasis of HCC. Furthermore, down-regulation of ANXA10 was associated with early IHTR ($P = 0.0007$), which is mostly because of true recurrence of HCC and associated with more unfavorable prognosis.⁴⁸ Indeed, HCC with ANXA10 down-regulation had a worse 4-year survival ($P = 0.0014$). Hence, down-regulation of ANXA10 may serve as a potential molecular marker to identify a subset of HCCs with high risk for early IHTR. In this study, our observations provide evidence that ANXA10 is a new member of ANXs that may possess metastasis suppression activity.^{26–28} However, the molecular mechanisms for the metastasis-suppressive activity of ANXA10 in HCC warrants more studies for better understanding.

Mutation of p53 occurs frequently in HCC and is associated with tumor invasion and unfavorable outcome.^{37,39} In this study, down-regulation of ANXA10 correlated with p53 mutation, $P = 0.024$. The molecular mechanism for this association is currently unknown, but the potential interplay between these two factors in HCC needs to be clarified. We showed that HCC with both ANXA10 down-regulation and p53 mutation had the highest frequencies of grade II to III tumor and vascular invasion (stage IIIA to IV HCC), significantly higher than other groups ($P < 0.00001$ and $P < 0.00001$, respectively). Moreover, HCC with ANXA10 down-regulation and p53 mutation had more frequent early IHTR ($P = 0.0001$), and worse 4-year survival ($P = 0.0025$). These results indicate that combined alterations of the two molecular markers act synergistically toward poorly differentiated and more aggressive HCC. Our results also suggest that a combinatorial analysis of multiple genetic alterations may help to identify subsets of HCC with different biological course and prognosis. The tissue- and tumor-restricted expression makes ANXA10 a potential ideal target of gene therapy for HCC.

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