Original Article

Assessment of XAF1 as A Biomarker to Differentiate Hepatocellular Carcinoma from Nonneoplastic Liver Tissues

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ABSTRACT

Objective: XIAP-associated factor 1 (XAF1) expression has been shown to be related with apoptosis in hepatocellular carcinoma (HCC). However, the correlation of XAF1 expression with HCC tumor grade has not been intensively assessed. XIAP-associated factor-1 (XAF1) is an important apoptosis inducer in human HCC. The aim of this study is to determine the correlation between XAF1 expression and HCC histopathological grades.

Methods: The mRNA levels of *XAF1* in 24 paired HCC-nonneoplastic specimens were quantified by real-time reverse transcription PCR (RT-PCR). Protein levels of XAF1 in 110 paired HCC-noncancer tissues were investigated by immunostaining specimens on a tissue microarray (TMA). Correlations between *XAF1* mRNA levels or protein expression and clinicopathological features were assessed by statistical analysis.

Results: Both *XAF1* mRNA and protein were significantly under-expressed in HCC tissues compared to their non-neoplastic counterparts. No significant relationship was found between *XAF1* mRNA or protein expression and histological tumor grade.

Conclusion: All these data suggest that XAF1 is a potential biomarker for differentiating HCC with noncancerous tissues.

Key words: XAF1, Biomarker, HCC, Tumor grade, Tissue microarray

INTRODUCTION

The inhibitor of apoptosis (IAP) family proteins are important cellular regulators of apoptosis[1, 2, 3]. X-linked IAP (XIAP) is the prototype member of the IAP family, and is critically involved in a number of cellular functions such as suppressing apoptosis[4, 5, 6], modulating receptor-mediated signal transduction[7] and protein ubiquitination[8, 9]. XIAP has also been proved to be implicated in cancer formation, progression and resistance to radiation or chemotherapy[10-14].

XIAP-associated factor 1 (XAF1) is a zinc finger protein that strongly antagonize the apoptosis-inhibiting activities of XIAP1[15]. XAF1 exerts pro-apoptotic effects through caspase-dependent and caspase-independent pathways[16, 17]. Allelic loss and down-regulation of *XAF1* gene have been observed in most of the cancer cell lines[18]. Reduced expression of *XAF1* mRNA was also found in several cancer biopsies including melanoma, colon cancer, gastric cancer, renal cancer, bladder cancer, prostate cancer and pancreatic cancer[19-26]. XAF1 protein has been shown to mediate tumor necrosis factor (TNF) and interferon (IFN)-induced cellular apoptosis as well[27-29]. All of these suggest that aberrant silence of *XAF1* gene constitutes one of the potential mechanisms of cancer cell survival[30].

Liver cancer is one of the most prevalent and death-causing cancers in China. The most common form of malignant liver tumor is hepatocellular carcinoma (HCC). More than 80% of HCC patients are hepatitis B virus (HBV) carriers. Recent studies have shown that XAF1 protein level is significantly related with apoptosis in HCC[31]. XAF1 expression level in primary liver tumors is also a useful independent factor to

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predict recurrence-free survival after liver resection and transplantation[32, 33]. So far, the relationship between XAF1 expression and HCC tumor grade has not been addressed. In this study, we compared the *XAF1* mRNA and protein expression in HCC biopsies and paired adjacent nonneoplastic liver tissues by quantitative real-time reverse transcription PCR (RT-PCR) and immunohistochemical staining. We also assessed the correlation of XAF1 expression with clinicopathological features of HCC. This is an important step to evaluate the potential use of XAF1 as a biomarker for diagnosis or prognosis of HCC.

MATERIALS AND METHODS

Clinical Specimens

For relative quantification of *XAF1* mRNA, 30 cases of diagnosed HCC specimens and paired nonneoplastic tissues were obtained from the tissue bank of Shandong Cancer Hospital. Resected HCC specimens were graded by a certified pathologist using the Edmonson and Steiner nuclear grading scheme. Grade I was defined as well-differentiated, grade II as moderately-differentiated and grade III as poorly-differentiated HCC respectively. The specimens included 10 grade I, 10 grade II, and 10 grade III HCC. All tumor specimens were collected according to the established procedures and approved by the review board of the hospital. All usage of these specimens was approved by the ethics committees of Donghua University and Shanghai Jiaotong University.

Reagents

XAF1 antibody was purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody and 3,3'-diaminobenzidine (DAB) substrate kit were KPL products (Gaithersburg, MD, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). RNeasy mini kit was Qiagen product (Hilden, Germany). cDNA reverse transcription kit, XAF1 TaqMan probe and primers, and Mastermix were ordered from Applied Biosystems (Foster City, CA, USA). Chemical reagents were analytical grade and obtained from Sigma (St. Louis, MO, USA).

Construction of Tissue Microarrays (TMAs)

The medium-density TMAs were custom-made by Shanghai biochip Co., Ltd. Briefly, 80 cases of HCC and paired nonneoplastic liver tissues were fixed, dehydrated and embedded in paraffin. Sections from donor blocks were deparaffinized in xylene, stained by hematoxylin and eosin, and examined by a certified pathologist. The area of interest was identified, marked and then aligned with the recipient block. Sampled tissues were arranged in 16 columns of 10 rows for a total of 160 individual cores (1 mm, 5 μm) to construct the TMAs. Clinical informations including gender, age, and tumor grade based on pathological diagnosis were provided for all cases.

Real-time PCR Assay

For real-time PCR experiments, 50 milligrams of each dissected tissue was cut into pieces, suspended into TRIzol reagent, and disrupted in a tissue-lyser (Qiagen, Hilden, Gemany). Total RNA was extracted according to the protocol provided by manufacturer and purified using the RNeasy mini kit. RNA concentration was determined by absorbance at 260 nm on a NanoDrop 2000 instrument (NanoDrop Technologies, Wilmington, DE, USA). Two-step quantitative PCR was performed on a 7500 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) as we previously reported[34]. The mRNA levels were quantified relatively to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls. Thirty cases including 10 respective cases in grade I, grade II and grade III were analyzed.

Immunohistochemical Analysis

Immunohistochemical staining was performed according to our published protocol[35] with appropriate controls. After staining, all slides were reviewed, protein expression and intracellular localization (nuclear and/or cytoplasmic) were recorded and averaged by observing 10 high power fields under a Zeiss upright microscope. Staining was scored based on the percentage of XAF1 positive signal observed in cytoplasm and/or nucleus of the cells. Semiquantitative scores were designated as follow: 0, no staining; 1, 1%−30% of the cells; 2, 31%−50% of the cells; 3, >50% of the cells. Staining for 3 paired tissue samples including 1 grade I and 2 grade III were not scored because of sample detachment or missing tissue components.

Statistical Methods

SPSS software (version 14.0, SPSS Inc., Chicago, IL, USA) was employed for statistical analysis. Spearman's Rank correlation was used to calculate the relationship between XAF1 expression and HCC tumor grade. *P*-values less than 0.01 were considered to be statistically significant. Different criteria based on the scores obtained from the immunohistochemical staining were set to calculate the sensitivity and specificity of using the percentage of XAF1 positive cells to differentiate nonneoplastic tissues from tumors. The sensitivity is defined as the percentage of positive cells (either cytoplasmic or nuclear) that meets certain criteria in tumors. The specificity is defined as the percentage of positive cells (either cytoplasmic or nuclear) that meets certain criteria in nonneoplastic tissues.

RESULTS

Relative Quantification for *XAF1* mRNA

The mRNA levels in HCC biopsies were compared with that of their adjacent noncancerous tissues. The relative levels of *XAF1* mRNA normalized to *GAPDH* mRNA in each HCC case are represented in Figure 1. All HCC specimens had lower *XAF1* mRNA levels as compared with their adjacent noncancerous tissues. The numbers of cases that have more than one fold decrease of mRNA were 6 (60%) in grade I, 9 (90%) in grade II and 9 (90%) in grade III, respectively. This indicates that downregulation of *XAF1* gene expression is a common event in HCC. The fold changes of mRNA expression in each tumor grades varied with individual HCC cases. No significant correlation between the relative *XAF1* mRNA levels and tumor grades was noted. It is obvious that using ≥1 fold decrease in mRNA expression relative to adjacent noncancerous tissues as a criterion, the specificity for diagnosing HCC is 80%.

Immunohistochemical Analysis for XAF1 Protein Using TMAs

The immunohistochemical staining of XAF1 protein in cells were reviewed and scored by a certified pathologist. Representative photomicrographs of XAF1 protein staining and histology are shown in Figure 2. XAF1 protein was detected both in the cytoplasm and nucleus of nonneoplastic liver cells and HCC cells. The percentage of positive cells with cytoplasmic or nuclear XAF1 protein distribution was calculated and summarized in Table 1. The extent of XAF1 staining varied between cases as well as within an individual case. All non-HCC cells express XAF1 protein but the number of cases that do not express XAF1 showed significant differentiation-related differences in HCC. The percentages of cases that showed no cytoplasmic XAF1 expression were 21.1% for grade I, 41.5% for grade II and 60.0% for grade III, respectively. Similarly, 26.3% of grade I, 54.7% of grade II and 60.0% of grade III HCC did not show XAF1 signal in nucleus. As shown in Figure 3, the percentages of HCC that had a score of 3 for cytoplasmic XAF1 staining decreases significantly with increasing tumor grades. Interestingly, the percentages of HCC that showed no nuclear XAF1 localization were positively correlated with differentiation status as well.

For statistical analysis, we grouped all of the tissue samples in two ways: 1. Grouping nonneoplastic tissues and HCC into 2 categories. 2. Grouping grade I, II and III tumors into 3 categories. The results showed that Spearman's correlation coefficient based on the percentage of cytoplasmic XAF1 positive cells was –0.785 (P<0.001) between nonneoplastic tissues and HCC and –0.315 (P>0.05) among different tumor grades, respectively. Correspondingly, Spearman's rank correlation coefficient based on the percentage of

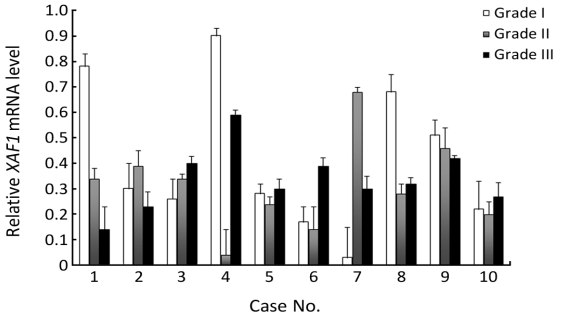

Figure 1. Relative quantification of *XAF1* mRNA levels in HCC as compared with their adjacent tissues.

Table 1. Immunoreactivity of XAF1 protein in nonneoplastic liver tissues and HCC specimens in different tumor grades

Grade	Number of cases	Percentage of positive cells	Cytoplasmic XAF1	Nuclear XAF1
N*	77	0	0	14 (18.2%)
		1%–30%	0	48 (62.3%)
		31%–50%	2 (2.6%)	14 (18.2%)
		>50%	75 (97.4%)	1 (1.3%)
I	19	0	4 (21.1%)	5 (26.3%)
		1%–30%	3 (15.8%)	10 (52.6%)
		31%–50%	3 (15.8%)	4 (21.1%)
		>50%	9 (47.4%)	0
II	53	0	22 (41.5%)	29 (54.7%)
		1%–30%	4 (7.5%)	22 (41.5%)
		31%–50%	15 (28.3%)	2 (3.8%)
		>50%	12 (22.6%)	0
III	5	0	3 (60.0%)	3 (60.0%)
		1%–30%	1 (20.0%)	2 (40.0%)
		31%–50%	1 (20.0%)	0
		>50%	0	0
Spearman's rho correlation coefficient with grade**			–0.766, P<0.01	–0.370, P<0.01

*N represents noncancerous tissues. **Spearman's rho correlation coefficient calculated by designating N as variant 1 and I, II and III as variant 2.

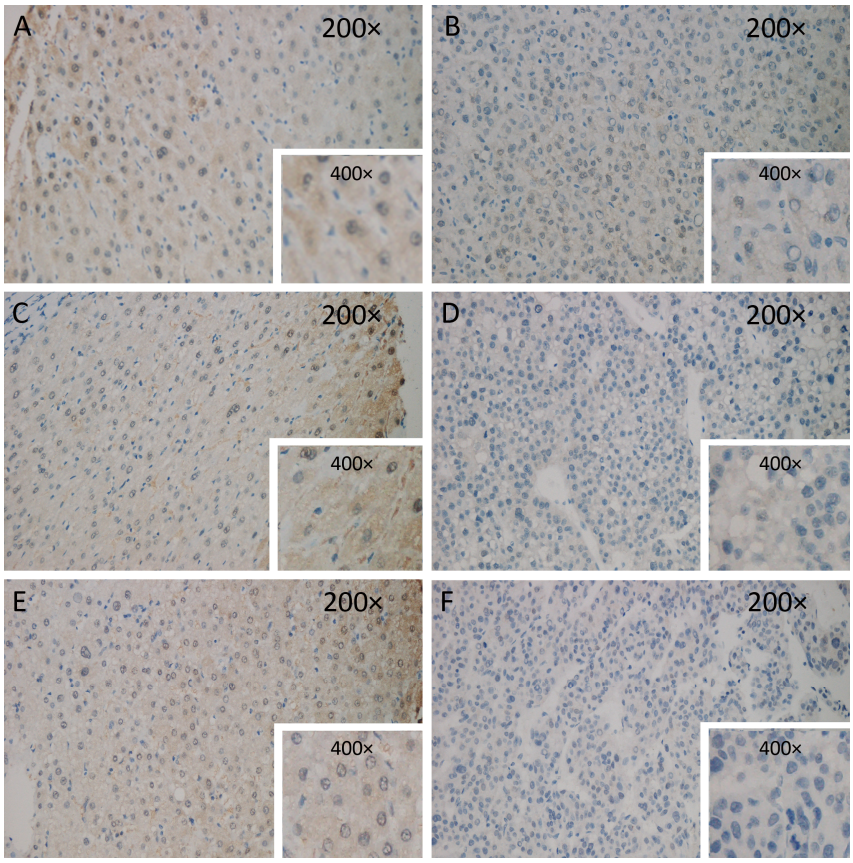

Figure 2. Representative immunohistochemical staining of XAF1 protein in grade I, grade II, grade III HCC as compared with nonneoplasmic tissues (main image, 200×; insert, 400×). **A:** Normal tissue adjacent to B; **B:** Grade I HCC; **C:** Nonneoplastic tissue adjacent to D; **D:** Grade II HCC; **E:** Nonneoplastic tissue adjacent to F; **F:** Grade III HCC.

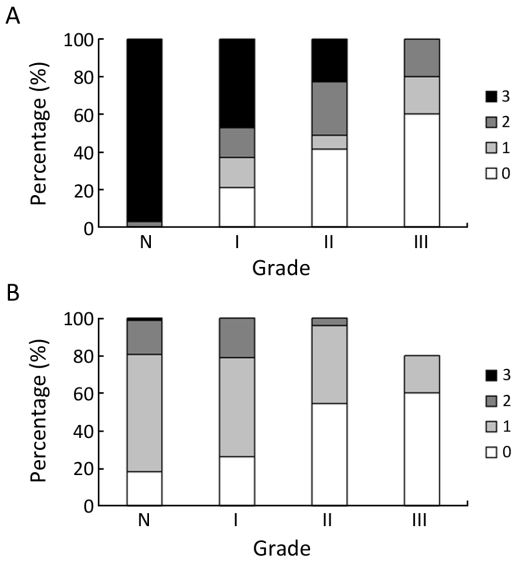

Figure 3. Trend changes of the XAF1 protein staining scores in nonneoplastic tissues and HCC in different grade. **A:** Percentages of cytoplasmic XAF1 staining; **B:** Percentages of nuclear XAF1 staining. 0, 1, 2, and 3 represent protein staining score.

nuclear XAF1 positive cells was –0.370 (P<0.001) between nonneoplastic tissues and HCC and –0.195 (P>0.05) among different tumor grades, respectively. These results indicate that both the percentage of cytoplasmic XAF1 positive cells and the percentage of nuclear XAF1 positive cells are useful for differentiating nonneoplastic tissues and HCC but not applicable for differentiating tumor grades. Three criteria as shown in Table 2 were then set to determine the sensitivity and specificity of these parameters in differentiating nonneoplastic tissues and HCC. As a result, all the three criteria based on the cytoplasmic staining for XAF1 had high specificity (>95%) but only the ≤50% criterion had a reasonable sensitivity of 72.7%. The results obtained by using the criteria based on the nuclear staining of XAF1 were not satisfactory for potential clinical applications.

Table 2. Sensitivity and specificity of using the percentage of cells expressing XAF1 protein to differentiate HCC and nonneoplastic tissues

Percentage of positive cells	Sensitivity	Specificity
Cytoplasmic XAF1		
0	29/77 (37.7%)	100%
≤30%	37/77 (48.1%)	100%
≤50%	56/77 (72.7%)	97.40%
Nuclear XAF1		
0	37/77 (48.1%)	77/77 (100%)
≤30%	71/77 (92.2%)	15/77 (19.48%)
≤50%	77/77 (100%)	1/77 (1.30%)

DISCUSSION

XIAP1 is an intrinsic cellular regulator of apoptosis, and *in vitro* studies have shown that it is the most potent caspase inhibitor in the IAP family; XIAP1 inhibits both the initiator caspase-9 and the effector caspase-3 and -7[36]. Two negative regulators of XIAP1 including XAF1 and second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO) have been identified[37, 38]. In contrast to XIAP, which is mainly localized in the cytoplasm, XAF1 is distributed in the nucleus and Smac/DIABLO is normally found in mitochondria[4]. Overexpression of XAF1 triggers translocation of XIAP1 from cytoplasm to the nucleus and neutralizes the activity of XIAP1 to inhibit cell death[15]. XIAP1 and XAF1 can form complex with survivin, which triggers survivin ubiquitination and degradation[16]. Following an apoptotic insult, Smac/DIABLO is released from mitochondria and proteolytically processed to generate an active form to antagonize the caspase-binding activity of XIAP1[37, 38].

XAF1 has been demonstrated to be a tumor suppressor; it is unique in the control of IAP function and in the sensitization of cancer cells to apoptosis. *XAF1* gene has been shown to be significantly down-regulated in several human liver cancer cell lines relative to normal human liver[18]. Downregulation of XAF1 in HCC has been found to be caused by promoter methylation[32]. A relevant study showed that the ratio of *XIAP*/*XAF1* mRNA is significantly higher in HCC than in cirrhotic liver tissues, and this ratio is also correlated with the overall clinical survival rate of patients[32, 33]. Our results confirmed the downregulation of XAF1 in HCC and demonstrated that ≥1 fold decrease in *XAF1* mRNA expression can be used as a valuable factor to identify neoplastic transformation of human hepatocytes. The correlation of *XAF1* mRNA level with clinical prognosis of HCC patients remains to be studied.

Substantial evidences have suggested that down-regulation or loss of XAF1 contributes to the tumorigenesis in numerous cancers[30]. *In vivo* studies have shown that XAF1 protein is a potent tumor suppressor[15, 28, 39]. In a previous report, a Japanese group compared the immunohistological XAF1 staining in 7 well-, 10 moderately-, and 7 poorly-differentiated HCC cases in Japan and found that the score of overall XAF1 protein staining was significantly lower in poorly-differentiated HCC than that in well or moderately-differentiated HCC[31]. However, the sample size they analyzed is so small that it is difficult to make definitive conclusions. We performed XAF1 immunostaining on a larger specimen scale (77 cases including 19 well-differentiated, 53 moderately-differentiated and 5 poorly-differentiated Chinese HCC) but failed to find significant correlation between decrease in XAF1 protein expression and tumor differentiation status. This may be attributed by the heterogeneity of the HCC population or the difference in etiology of HCC between the Japanese group [19/24 hepatitis C virus (HCV) positive] and Chinese group (77/77 HBV positive). When considering high specificity in differentiating HCCs and non-neoplasms, the percentage of cells expressing no XAF1 protein in cytoplasm or nucleus and ≤30% XAF1 positive cells can be used as criteria. All of these criteria have theoretical 100% specificity. When both reasonably high specificity and sensitivity are considered, ≤50% cytoplasmic XAF1 positive cells may be a good criterion. Further studies are needed to validate the practicability of these criteria in clinical use by employing larger sample size (1,000−3,000 cases).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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