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Downregulation of Proapoptotic Proteins Bax and Bcl-X_S in p53 Overexpressing Hepatocellular Carcinomas

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As the occurrence of structural p53 mutations in hepatocellular carcinoma (HCC) in Thailand was previously reported to be much lower than that found in other high-incidence HCC areas, we analyzed 16 HCC samples from Thailand to determine the expression and functionality of p53 protein. We observed the overexpression of p53 protein in 69% of HCC, despite the prevalence of the wild-type p53 gene. However, the overexpressed p53 protein was nonfunctional as suggested by its inability to modulate the expressions of several p53 effector proteins (p21 and Bcl-2 family proteins). In addition, we observed significant underexpression of two proapoptotic proteins, Bax and Bcl-X_S, in 81% ($P = 0.02$) and 64% ($P = 0.03$) of HCC, respectively. Consequently, the ratios of proapoptotic to antiapoptotic BCL-2 family proteins were reduced in 88% of the HCC tumor tissues when compared to normal tissues, such that the rheostat between BCL-2 family proteins is strongly skewed toward enhanced cell survival in the tumor cells.

Liver cancer, hepatocellular carcinoma (HCC), which is derived from hepatocytes, and cholangiocarcinoma (CCA), which is derived from the epithelial lining of the intrahepatic bile duct, is the most prevalent primary cancer for male and the third most abundant cancer in female in Thailand (1–5). In this country large regional differences are found for CCA, which is associated with the infection of the liver fluke, *Opisthorchis viverini*, in endemic areas such as northeastern Thailand which has also the highest incidence rates in the world for CCA (1, 6, 7). On the other hand HCC is found to have a more even geographical distribution in Thailand (4, 5).

Major risk factors associated with HCC in high incidence geographic zones, particularly China, South-East Asia and sub-Saharan Africa, are infection with hepatitis B virus (HBV) and exposure to the mycotoxin aflatoxin B_1 (8). However in Thailand, hepatitis B virus infection but not aflatoxin intake was identified to be the major risk factor for HCC (2). An association of aflatoxin exposure and specific mutations within the p53 tumor suppressor protein, a frequent G to T transversion at codon 249, was found in up to 50% of HCC tumors from Qidong, China, and South Africa (9, 10), but could only be found in 7% of Thai-HCC (11). In addition, the overall mutation frequency within p53 exons 5–8 was only 15% in Thai-HCC, compared to 50–60% in China or South Africa (11).

In general, it is known that p53 can transcriptionally modulate the expression of genes involved in cell cycle control, such as the cyclin-dependant kinase inhibitor, p21 (12). It was also found that structural p53 mutations were associated with the expression of the G1-S phase regulator, cyclin E, in HCC (13). Furthermore, diverse effects of p53 on the transcription of the BCL-2 family proteins that regulate apoptosis (also called programmed cell death), were reported. The transcription of Bax, a proapoptotic member of the BCL-2 family, was activated by p53 while Bcl-2, which functions to prevent apoptosis, was transcriptionally repressed by p53 (14–17). Here we analyzed the protein expression of p53 and several known p53 downstream effectors, p21 and BCL-2 family proteins, as well as of cyclin E, in HCC samples from Thailand in order to determine firstly the expression levels and functionality of the wild-type p53 protein and secondly, to examine the potential roles these cell cycle and apoptosis regulatory proteins may play in the development of HCC in Thailand. Finally, we determined telomerase activity since it has been detected in most cancers (18) and was discussed to play a crucial role in hepatocarcinogenesis (19).

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MATERIAL AND METHODS

Patients. Tumor tissues as well as adjacent non neoplastic tissues (referred to as "normal") were obtained from liver cancer patients, who were admitted to the National Cancer Institute of Thailand. All tissues were stored at −70°C within 30 min of surgical removal. Histological diagnosis of liver tumors was performed at the National Cancer Institute in Bangkok, Thailand, as well as the International Agency for Research on Cancer (IARC), Lyon, France. Most of the tissues were previously studied for p53 mutations, aflatoxin exposure and hepatitis B surface antigen (HBsAg) (11), aflatoxin metabolism (20), and rearrangements at minisatellite loci and integrated hepatitis B virus DNA (HBV-DNA) in the HCC genome (21). Some of these data are included in Table 1. Tissues from patients RN-35-00370 (sample 44 with liver adenocarcinoma), which was not previously studied, and RN-32-22581 (sample 42 with metastatic liver carcinoma) were grouped together with the HCC samples (Tables 1 and 2).

Extraction of proteins and Western analysis. Small sections of frozen tissue (~27-mm^3 volume), were cut and homogenized with individual micropestles (Eppendorf, Germany) in 200 μl of ice-cold lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% Chaps (Pierce Chemical Co., Rockford, IL), 10% glycerol) (18), that was also used for the telomerase assay. After incubation for 30 min on ice, the samples were centrifuged at 14,000 rpm, at 4°C for 20 min and the supernatant transferred to a new tube. Total protein was measured with a Bio-Rad Bradford kit (Bio-Rad Laboratories, Hercules, CA) and 30 μg of total protein was run on a 12% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and the membrane blocked with 5% nonfat dry milk in TBS-T (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20) overnight at 4°C. The membrane was then probed with a primary antibody, washed several times with TBS-T and then incubated with a horse radish peroxidase (HRP)-conjugated secondary antibody (Pierce). Finally, the membrane was washed and developed with an enhanced chemiluminescence system (Pierce). The membrane was stripped with stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8) for 30 min at 65°C, washed extensively and reprobed with another antibody. Typically, a blot was used for two to three times. The primary antibodies used were Bcl-2 (100, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-X_L/X_S (S-18, Santa Cruz Biotechnology), Bax (Ab-1, Calbiochem–Novabiochem Corp., CA), Bak (G-23, Santa Cruz Biotechnology), p53 (DO-1, Santa Cruz Biotechnology), p21 (Cip1, Transduction Laboratories, CA), cyclin E (HE12, Santa Cruz Biotechnology), Hsc70/Hsp70 (StressGen Biotechnologies Corp., CA) and cytochrome *c* (Ab-2, Calbiochem–Novabiochem Corp.). Densitometric quantification of the autoradiographs were performed with a Bio-Rad/GS700 imaging densitometer (Bio-Rad).

Telomerase assay. Telomerase activity was determined from 4, 10, and 20 μg of tissue protein lysate (see Extraction of proteins and Western analysis) using a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany) based on the telomeric repeat amplification protocol (TRAP) (18). Briefly, in the first step telomerase (presumably in the tissue lysate) adds telomeric repeats (TTAGGG) to a template primer and a primer-elongation process can take place. The amount of extended primer is correlated to the activity of telomerase in the lysate. In a second step these elongation products are amplified by PCR. The elongated and amplified products are detected by a nonradioactive method using an ELISA protocol. The photometric detection was performed at 450 nm with a reference wavelength of 630 nm. Lysate from 293 cells (provided in the kit) functioned as a positive control, whereas lysis buffer and heat inactivated cell lysate functioned as negative controls. Samples with values in the range of 2- to 41-fold the values of the negative control (lysis buffer) were regarded positive for telomerase or regarded negative for values less than 2-fold of the negative control value.

Statistical analysis. The *t* test was used to determine if the overexpression or underexpression of proteins or reduction in ratios of proteins in the tumors were significant when compared to normal tissues. The fold differences (in expressions or ratios) between tumor and normal tissues were used as a continuous variable for the *t* test (two-tailed) with a hypothesized mean = 2.5. The statistical analyses were performed using the Statview program (Abacus Concepts Inc., Berkeley, CA). $P < 0.05$ was considered significant.

RESULTS

Pairs of tumor and non-neoplastic (referred to as "normal") tissues from 16 HCC samples, of which 13 were previously studied for p53 mutation (11), were analyzed and the histopathological features are summarized in Table 1. Comparative analysis of multiple sections of tumor and normal tissue from the same sample as well as in different tissue lysis buffer, revealed only little variations in the described expression profiles (data not shown). The data described in this study therefore reflect the expression profiles of a representative piece of tissue.

Telomerase Activity in HCC from Thailand

In general, telomerase activity can be regarded as an established marker in liver cancer tissues (19). We examined telomerase activity in each pair of tumor and normal tissues of HCC patients from Thailand (Table 1). 14 of 16 HCC (88%) were positive for telomerase in the tumor tissues, whereas 12 of the 14 (86%) corresponding normal tissues for HCC showed no activity. No telomerase activity was found in the remaining two tumors as well as in their corresponding normal tissues. In summary, the data are consistent with other published studies for HCC (19, 22) and confirm telomerase activity in addition to the histological data, as a valid marker to distinguish between tumor and non-neoplastic tissues.

p53, p21, and Cyclin E Protein Expression in HCC from Thailand

The expressions of p53 and known p53 effector proteins, p21 and BCL-2 family proteins, as well as cyclin E were analyzed by Western analysis. The expression of a control protein, Hsp70, did not change significantly (i.e., less than 2.5-fold, an arbitrary cut-off level used in all our analysis) in all the pairs of HCC tumor and normal tissues, which indicated that there was no significant difference in total protein loaded for each pair of samples (Table 2). Therefore, we used Hsp70 expression to normalize the protein expression in each sample and then compared the fold differences in expression of proteins between tumor and normal tissues of each patient. The autoradiographs for a representative set of the samples are shown in Fig. 1 and the complete expression profiles of the analyzed proteins are summarized in Table 2.

TABLE 1

Patients, Tumor Characteristics,[a] and Telomerase Activity[b] in Hepatocellular Carcinomas from Thailand

Patient	Sex/age	HBsAg/HBV-DNA	Tumor grade	Associated cirrhosis	Telomerase activity (T/N)	p53-mut (codon)
			HCC			
6 RN:33-8564	M, 34	Pos/+	III	Yes	13.6	Mut (249)
7 RN:33-20968	M, 46	Pos/ND	I–II	Yes	176	None
8 RN:32-19873	M, 70	Neg/+	II	No	29.8	None
9 RN:34-07681	M, 57	Pos/+	II	Yes	19.6	None
10 RN:34-1666	F, 35	Neg/−	I–II	No	11.8	None
11 RN:32-17888	M, 17	Pos/+	III	Yes	196	Mut (254)
12 RN:33-12844	M, 37	Pos/+	II–III	No	17.7	None
24 RN:34-3158	M, 61	Neg/+	II	Yes	5.4	None
32 RN:33-2944	F, 37	Neg/+	III	No	1.0*	None
34 RN:32-14864	M, 39	Pos/+	III–IV	No	3.5	None
35 RN:34-0893	M, 43	Pos/+	II	No	Neg	ND
40 RN:33-2815	M, 73	Pos/+	III	Yes	Neg	None
41 RN:32-17091	M, 36	Pos/ND	III	No	2.6	None
42 RN:32-22581	F, 54	Neg/ND	Met	No	15.4	ND
43 RN:33-16736	F, 50	Neg/+	II–III	No	3.7*	None
44 RN:35-00370	M, 40	Neg/ND	Aden	No	10.9	ND

[a] Data are summarized and have been published elsewhere: hepatitis B surface antigen (HBsAg), integrated hepatitis B virus DNA in the HCC DNA (HBV-DNA), tumor grade, associated cirrhosis, and p53-mutation (p53-mut) (11, 20, 21). ND, not determined; HCC, hepatocellular carcinoma; Met, metastatic carcinoma; Aden, adenocarcinoma; T, tumor; N, nonneoplastic tissue.

[b] Telomerase activities were specifically found in the HCC tumor tissues but not in the corresponding normal tissues (except Neg and *). The relative activities were calculated after subtraction of the value for the negative control (lysis buffer) with 10 μg lysate analyzed (sample 32 with 20 μg lysate analyzed). *, telomerase activity in both tumor and normal tissues; sample 32 shows equal activities (1) in tumor and normal tissues, whereas sample 43 has higher activities in the tumor tissue (3.7). Neg, no telomerase activity in tumor and normal tissue.

Overexpression of p53 was found in 69% (11/16, $P = 0.06$) of the HCC tumor tissues including the two samples that were known to contain structural p53 mutations. 8 of 11 tumors, which were previously described to contain wild-type p53 gene, had p53 protein overexpression in the tumor tissues with levels of 2.5 (12T) to 86 (41 T) times higher than that in the normal tissues. We noted that 1 patient, sample 10, had particularly high expression of p53 in the normal tissue and this level is comparable to that found in most of the tumor tissues (Fig. 1).

However, despite the high incidence of p53 overexpression in tumor samples, only 19% (3/16, $P = 0.31$) of the tumors revealed weak (2.5- to 5-fold) overexpression of p21 when compared to normal tissues. Another 2 of the p53 overexpressing tumors had lower expression of p21 in the tumor than normal tissues. In summary, the data demonstrated no correlation between wild-type p53 overexpression and p21 expression in the HCC samples.

Cyclin E is overexpressed in 50% (8 of 16, $P = 0.18$) of the Thai HCC tumor tissues when compared to normal tissues, and the remaining samples did not show any significant change. One of the two tumor tissues that contained p53 mutation expressed higher amounts of cyclin E, while the other sample did not show any change. Another 6 of the tumors without p53 structural mutations also had overexpression of cyclin E. These data do not support an association between structural p53 mutation and cyclin E protein overexpression in contrast to the findings in another study where p53 mutation was associated with cyclin E overexpression (56%) of HCC from Taiwan (13).

BCL-2 Family Protein Expression in HCC of Thailand

Sixty-two percent (10/16) of the analyzed Thai-HCC samples expressed equal levels of Bcl-2 in tumor and normal tissues, 31% (5/16, $P = 0.98$) had lower expression in the tumor tissues and one sample had higher expression of Bcl-2 in the tumor tissue. In addition, only 4 of the 11 tumors that overexpressed p53 also showed lower expression of Bcl-2 in the tumor tissues compared to normal tissues. Another antiapoptotic protein, Bcl-X_L, was equally expressed in 87% (14/16 samples) and only two samples expressed lower levels in the tumor tissues (2/16, $P = 0.26$).

On the other hand, we found a dramatic and unexpected pattern of expression for the proapoptotic protein Bax. 81% (13/16, $P = 0.02$) of the tumor tissues had lower expression of Bax compared to the normal tissue (Table 2, Fig. 1). The underexpression of Bax ranges from 2.5-fold (12 T) to 127-fold (41 T) with 4 cases showing dramatic changes of 98- to 127-fold underexpression in the tumor tissues. This great difference was not found for any other analyzed protein, except two cases (sample 24 and 41) with 66- to 86-fold

TABLE 2

Expression of Proteins Involved in Cell Cycle and Apoptosis in Hepatocellular Carcinomas from Thailand

	p53	p21	Cyc E	Bax	Bcl-X_S	Bak	Bcl-2	Bcl-X_L	Cyt *c*	Hsp70
					HCC					
6*	++	0	+++	−−−	ND	0	0	−	0	0
7	++	−	+++	−−−	ND	0	−−	−−	0	0
8	0	0	+	0	0	0	0	0	0	0
9	++	0	+	−	0	0	0	0	0	0
10	0	0	0	−−	−−	0	0	0	0	0
11*	+	0	0	−−	−−−	−	0	0	0	0
12	+	−−	+	−	−−−	0	0	0	++	0
24	+++	0	0	−	−−	0	−−	0	0	0
32	+	0	0	0	−−−	0	0	0	0	0
34	++	0	+	−−−	0	0	−	0	0	0
35	−	0	0	−	0	−	−	0	++	0
40	0	−	0	0	0	−−	0	0	++	0
41	+++	+	+	−−−	−	−−	−−	0	0	0
42	++	+	++	−−	−	0	0	0	0	0
43	++	+	0	−−	−−−	−	0	0	0	0
44	0	−	0	−−	−−	−−	++	0	++	0

Note. Sixteen HCC were analyzed by Western analysis for protein expression in tumor and normal tissue: p53, p21, CycE, cyclin E; proapoptotic BCL-2 family proteins (Bax, Bcl-X_S, Bak); antiapoptotic BCL-2 family proteins (Bcl-2, Bcl-X_L); Cyt *c*, cytochrome *c*; Hsp70, heat shock protein 70. *Tumors 6 and 11 with known p53 mutation (11). Expression for Hsp70 protein was used to normalize the protein expression in each pair of tumor versus normal tissue. ND, not determined; 0, "no change" (values 0- to 2.5-fold overexpression or underexpression), 2.5- to 5-fold overexpression (+) or underexpression (−), 5- to 25-fold overexpression (++) or underexpression (−−); and >25-fold overexpression (+++) or underexpression (−−−) in tumor tissue compared to normal tissue.

higher p53 expression in tumor than normal tissues. Another proapoptotic member of the BCL-2 family, Bcl-X_S was also found to be underexpressed (up to 48-fold) in 64% (9 of 14, $P = 0.03$) of the tumor tissues when compared to normal tissues. The expression of Bak, another proapoptotic BCL-2 family protein, did not differ as much as Bax or Bcl-X_S between tumor and normal tissues and was underexpressed in only 37% (6 of 16, $P = 0.37$) of the tumors. Interestingly, two of the three tumors (samples 8, 32, 40) which did not show Bax underexpression revealed Bcl-X_S (sample 32) or Bak (sample 40) underexpression (Table 2). In summary, all but one of the analyzed HCC tumors showed underexpression of at least one of the proapoptotic proteins, Bax, Bcl-X_S and Bak, when compared to normal tissues.

To further clarify the specificity of our findings, we also analyzed the expression of cytochrome *c*, a protein that plays a role in apoptosis through changes in its intracellular distribution but does not belong to the BCL-2 family (23). Most of the samples (12 of 16) did not show any differences in cytochrome *c* expression between tumor and normal tissue and in 4 of 16 tumors overexpression was found ($P = 0.87$).

Ratios of Proapoptotic to Antiapoptotic Proteins in HCC of Thailand

As it is known that the relative expression of proapoptotic and antiapoptotic proteins determines the sensitivity of cells to apoptosis (24), we determined the ratios of these proteins in HCC samples (Fig. 2). We found significant reductions (2.5- to 119-fold) in the ratio of Bax/Bcl-2 in 63% (10/16, $P = 0.04$) of the tumor tissues when compared to normal tissues (Fig. 2a). In addition, the ratio of Bax/Bcl-X_L was also reduced in 69% (11/16, $P = 0.08$) of the tumor tissues (Fig. 2b). The ratios of Bcl-X_S/Bcl-X_L and Bak/Bcl-X_L were also calculated for the 5 samples (samples 8, 24, 32, 35, and 40) which did not show changes in Bax/Bcl-2 or Bax/Bcl-X_L (Fig. 2c). Here, 3 of the 5 samples had lower Bcl-X_S/Bcl-X_L or Bak/Bcl-X_L ratios in tumor tissue compared to normal tissue and another sample (tumor 8) had a weak reduction (2.3-fold) in the Bak/Bcl-X_L ratio. Only one sample (tumor 35) showed no change in the ratio for any of the described pairs of proteins. Interestingly, tumor 35 was one of the two tumors with no telomerase activity (Table 1).

DISCUSSION

p53 and Cancer

Alterations of the p53 gene are widely detected in human cancer malignancies (25–29). Such mutations can alter the posttranslational stability of the p53 protein, prolonging its half-life to many times longer than that of the wild-type protein and is one of the causes of the phenotypic overexpression of p53 protein found in many cancer tissues (30).

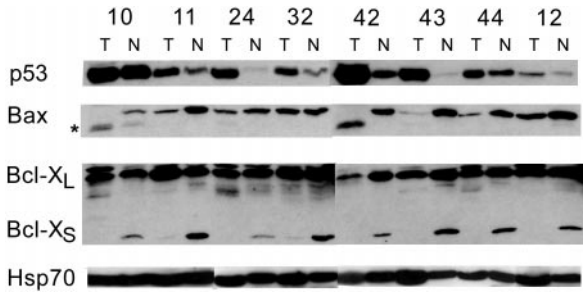

FIG. 1. Protein expression of p53, Bax, Bcl-X_S, Bcl-X_L, and Hsp70 in 8 pairs of tumor and normal tissues of hepatocellular carcinoma from Thailand. p53 overexpression in tumor tissues compared to normal tissues are found in 6 tumors. Sample 10 shows relatively high p53 expression in tumor as well as in normal tissue. Underexpression of proapoptotic Bax is found in 7 tumors and one tumor (sample 32) shows equal expression compared to normal tissue. Proapoptotic Bcl-X_S is found to be strongly underexpressed in all tumor samples, whereas expressions for antiapoptotic Bcl-X_L and Hsp70 are unchanged in all tumor tissues compared to normal tissues. *Tumors 10 and 42 show a shorter form of Bax, which may be a calpain cleavage product (see Discussion).

In spite of the wild-type p53 gene in most of our samples (11, and Table 1), we observed the overexpression of p53 protein in 69% of the tumors when compared to normal tissues (Table 2). The expressions of p21, Bax, and Bcl-2, which are known to be transcriptionally regulated by p53, were not modulated in a predicted manner, despite the overexpression of p53, which suggests the presence of non-functional wild-type p53 in the Thai-HCC (Table 2). p53 protein overexpression in tumors with wild-type gene has also been found in several other studies and functional inactivation but not structural mutation of p53 has been previously found to be crucial for liver carcinogenesis (31). Several mechanisms, such as association of p53 with nonfunctional Mdm2 protein, binding and inactivation of p53 by hepatitis B-X protein or environmental carcinogens, phosphorylation of p53, or factors affecting the location of p53, have been identified to contribute toward the stabilization and functional inactivation of p53 (30, 32, 33). Our findings on HCC of Thailand are consistent with a study on HCC from China, where conformationally altered and functionally inactive p53 protein, as suggested from its inability to induce p21 expression, was found in 93% of HCC and therefore at a much higher frequency than suggested by the presence of known p53 mutations in the gene (27).

BCL-2 Family Proteins

Bcl-2 expression was previously analyzed in HCC from different geographic origin and found not to play a role for hepatocarcinogenesis (34–37). In agreement with these studies, we found no difference in the expression of Bcl-2 in tumor and normal tissues for the majority of cases (62%). In contrast a total of 81% HCC (13/16, $P = 0.02$) had underexpression of Bax including 10 of the 11 HCC with overexpression of p53. Another proapoptotic protein, Bcl-X_S showed a similar underexpression (64%) in HCC. An inverse correlation between the Bax and p53 protein expression was also found in a study on primary non-small-cell lung cancer (38). Independent from the potential association with the p53 status, recent studies described other factors that might play a role in the underexpression of Bax in tumors. Full-length Bax (21 kilodalton) was reported to be cleaved to a 18 kilodalton fragment by calpain during drug-induced apoptosis in cell culture (39). We observed a shorter form of Bax in 2 of the HCC tumors (sample 10 and 42, Fig. 1) which suggests that underexpression of the full-length Bax may be caused by calpain activity in the tumors or increased sensitivity of Bax in the tumor to this protease. However, in all the other HCC with underexpression of Bax, no shorter form of Bax was detectable, therefore, other causes for the downregulation of Bax are suggested.

Recently, the Bax gene has been found to be frequently mutated in various cancers with the microsatellite mutator phenotype (40). Some of the mutations have resulted in undetectable Bax protein expression or altered the ability of Bax to participate in protein–protein interactions. In another case, a reciprocal relationship between p53 gene mutation and Bax gene mutation was observed in primary colorectal cancers (41). Since the frequency of p53 gene mutation is low in Thai-HCC and microsatellite instabilities have also been found in 54% of Thai-HCC (21), it is probable that mutation in Bax is one of the causes for the underexpression of Bax protein in the HCC tumors.

Reports of underexpression of Bcl-X_S in cancer tissues are rare and reasons for the underlying mechanisms are widely unknown. Since Bcl-X_S is a protein that originates from alternative splicing (42) it is possible that mutations or factors contributing toward the splicing of Bcl-X_S may be causative factors for its underexpression in HCC tumor tissues.

Significance of Bax and Bcl-X_S Downregulation

The ratio of antiapoptotic to proapoptotic protein expression, like Bax to Bcl-2, represents a rheostat that determines a cell's life or death response to an apoptotic stimulus (24, 43–45). When Bcl-2 is in excess, Bcl-2-Bax heterodimers predominate and cells are protected from apoptosis; when Bax is in excess, Bax homodimers or oligomers predominate and cells are susceptible to apoptosis (46, 47). In summary, 88% (14/16) of Thai–HCC expressed lower ratios of proapoptotic proteins (Bax, Bcl-X_S, Bak) to antiapoptotic proteins (Bcl-2 and Bcl-X_L) in the tumor than in normal tissues (Fig. 2). It is likely that this reduction should give the tumor cells a survival advantage. Two studies on breast cancers have reported similar observations

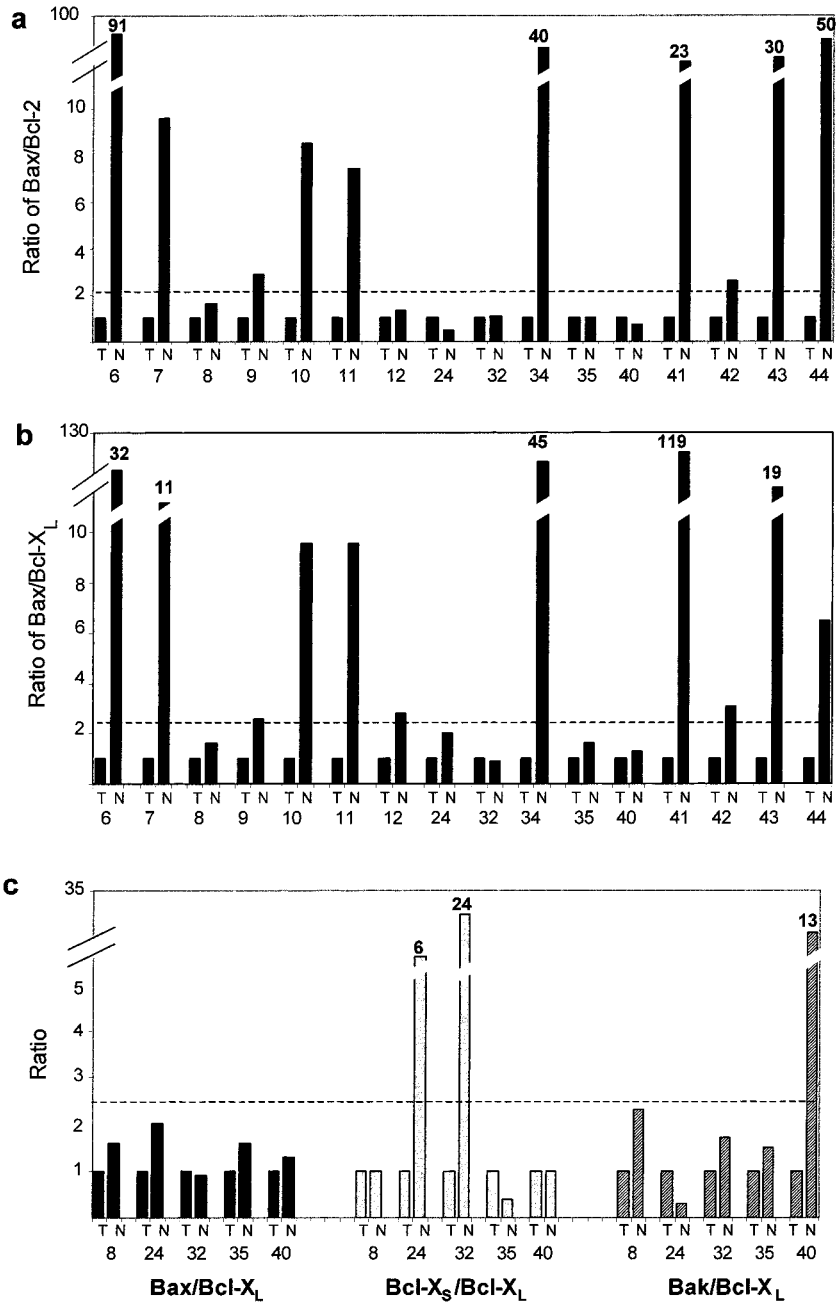

FIG. 2. Ratios of proapoptotic and antiapoptotic BCL-2 family proteins in hepatocellular carcinoma tissues from Thailand. (a) Reductions (at least 2.5-fold) in the ratio of Bax/Bcl-2 are found in 10/16 (63%) of the tumors when compared to the ratio in normal tissues. (b) Similarly, 11/16 (69%) of the tumors show reduction in the ratio of Bax/Bcl-X_L. (c) Five pairs of tumor and normal tissues that did not show changes for the ratio of Bax/Bcl-2 or Bax/Bcl-X_L were analyzed for changes in the ratios of Bcl-X_S/Bcl-X_L or BAK/Bcl-X_L. Three samples (24, 32, 40) show lower ratios in tumor tissue compared to normal tissue for Bcl-X_S/Bcl-X_L or BAK/Bcl-X_L and one sample (8) has a weak reduction for the ratio of Bak/Bcl-X_L. Only sample 35 does not show reductions in ratio for any of the described pairs of proteins.

and concluded that reduced expression of Bax is correlated with resistance toward apoptosis and poor response rates to chemotherapy (48, 49). Another study found that Bax deficiency promotes oncogenic transformation and drug resistance (50). Low expressions of Bax have recently also been found in several other types of cancers (51–53).

SUMMARY AND OUTLOOK

The loss of proapoptotic Bax and Bcl-X_S protein expression in tumor cells would confer resistance to apoptosis and could contribute to malignancy in several ways like allowing the cells to withstand additional genetic alterations. In addition, the loss of pro-

apoptotic protein function in the liver tumors may imply that the selective pressure for p53 mutations during tumorigenesis is relieved and hence the low incidence of p53 mutations in these HCC samples (11).

Interestingly, a similar trend in underexpression of the proapoptotic proteins Bax and Bcl-X_S in tumor tissues was found in our preliminary analysis of 4 CCA tissues from Thailand (unpublished data, Beerheide *et al.*), which might indicate a general role for the observed deregulation of Bax and Bcl-X_S in liver carcinogenesis. Future investigations on the underlying mechanisms for the observed underexpression of Bax and Bcl-X_S and the identification of the causative factors such as the role of Bax mutations are of particular importance.

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