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Decreased expression of Bid in human hepatocellular carcinoma is related to hepatitis B virus X protein

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Abstract

As a mitochondrial membrane death ligand, Bid oligomerises Bak to release cytochrome C and its deficiency renders hepatocytes resistant to apoptosis induced by Fas. The Bid level in hepatocellular carcinoma (HCC) is unknown. In this report, we examined the expression of Bid protein and mRNA in HCC cancerous tissues and their corresponding non-cancerous ones. The effect of the hepatitis B x protein (HBx) on the expression of Bid was also evaluated by transfecting hepatoma cells with the *HBx* gene. The results showed that the expression of Bid was significantly lower in cancerous tissues than that in their corresponding non-cancerous tissues. Immunohistochemical study revealed that Bid molecule was mainly localised in hepato-cytoplasm. Some nuclei were also positive for Bid antigen though to a lesser degree. *In vitro* experiments demonstrated that the expression of Bid in cells transfected with HBx was significantly lower than that in the cells without HBx transfection. This finding suggests that HBx may play a causative role in the reduction of Bid expression in HCC. This *in vitro* result is, to some degree, supported by clinical data that all the HCC examined are positive for hepatitis B virus (HBV). We conclude from this data that the expression of Bid in HCC is significantly decreased and the reduction of Bid may result from a mechanism associated with HBx, a major hepatocarcinogenic product from HBV. The imbalance of increased anti-apoptosis and decreased pro-apoptosis seen in HCC is a critical mechanism leading to the uncontrolled growth of tumour cells. Therefore, this study suggests that a deficiency in the expression of Bid may contribute to the development of such an imbalance in HCC.

Keywords: Hepatitis B virus; X protein; Bid; Hepatocellular carcinoma

1. Introduction

The Bcl-2 family of proteins are important regulators of apoptosis. They can be divided into three subgroups: (a) anti-apoptotic proteins such as Bcl-2 and Bcl-xL with multiple Bcl-2 homology (BH) domains, BH1, BH2, BH3 and BH4; (b) pro-apoptotic proteins such as Bax and Bak with BH1, BH2 and BH3 domains; (c) pro-apoptotic proteins such as Bid and Bik with the BH3 domain only. Bid, as a BH3 domain-containing molecule, is a proximal caspase-8 substrate of the tumour necrosis factor receptor 1 and Fas signalling pathways [1,2]. Upon receptor activation, Bid is cleaved by caspase-8 into a truncated form and translocated from the cytosol to the mitochondria [3]. Bid is believed to exert its pro-apoptotic effect by at least two mechanisms. The first is a BH3-dependent interaction in which Bid induces the release of pro-apoptotic factors (cytochrome C and procaspase 9) from the mitochondria after oligomerising mitochondrial Bak [3–5]. The second is a BH3-independent membrane destabilisation. Bid can permeabilise the outer mitochondrial membrane and cause the release of several proteins from the intermembrane space [6,7]. Recently, Bid-deficient mice have been shown to be resistant to Fas-induced hepatocellular apoptosis [8].

Hepatocellular carcinoma (HCC) is one of the most common cancers in Asia and approximately 90% of HCC cases in this region are associated with hepatitis B virus (HBV) infection. How HBV infection leads to the development of HCC is complicated and its pathway is still not clear. The HBV genome is a partially double-stranded 3.2Kb-circular DNA molecule. It contains

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four overlapping open reading frames that encode the structural and non-structural viral proteins. Among them, the x protein (HBx) encoded by the ORF-x plays a central role in liver oncogenesis [9]. It has been documented that HBx is involved in the induction of cell cycle progression and the modulation of apoptosis [10–12]. Binding to and partially sequestering p53 in the cytoplasm, HBx abrogates p53-mediated apoptosis in human hepatocytes [10]. In addition, HBx can efficiently block caspase 3 activity in rat fibroblast and hepatoma cells and inhibit transforming growth factor-β-induced apoptosis [11,12]. HBx is also able to transactivate transcription through multiple *cis*-acting elements including nuclear factor kappa β (NF-κβ) [13]. A number of reports have shown that HCC expresses a low level of pro-apoptotic molecules, but a high level of anti-apoptotic ones. Among the former are Caspase 3 and Fas [14,15], the latter Bcl-2 and Fas-associated phosphatase-1 [16,17].

2. Materials and methods

2.1. Tumour specimens and cell line

Human HCC tumours (15 cases) and their corresponding non-cancerous liver tissues were obtained from Prince of Wales Hospital, Hong Kong. Informed consent for tissue collection was obtained from patients prior to operation. Patients were tested for HBsAg as a marker for HBV infection and were all positive. All specimens were frozen immediately after surgical resection and stored in liquid nitrogen before extraction of RNA, protein and immunohistochemical analysis. All cancerous and non-cancerous tissue specimens were confirmed by pathological examinations. A hepatoma cell line, Hep G2, was purchased from American Type Culture Collection (Rockville, MD, USA) and used in the *in vitro* experiment. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum, penicillin and streptomycin in a humidified incubator with 5% CO_2.

2.2. HBV infection

Hepatitis B surface protein/antigen (HBsAg) and HBx protein were examined as markers for HBV infection and they were detected in the peripheral blood and liver tissue, respectively. Briefly, patient's serum was obtained before the operation and HBsAg was determined by an enzyme immunoassay kit employing a mouse monoclonal Anti-HBs antibody (AUSZYME MC Dynamic, Abbott Diagnostics, Abbott Park, IL, USA). The assay was performed according to the instruction of the kit. The sensitivity of the assay was 0.15 and 0.08 ng/ml ad and ay HBsAg and the specificity was 99.99%. Specimens with absorbance values equal to or greater than the cut-off value (Negative control plus the factor 0.25) were considered initially positive for HBsAg. The positive specimens were further verified by an FDA-licensed neutralisation confirmatory test (Abbott HBsAg Confirmatory Assay, Abbott Diagnostics, Abbott Park, IL, USA). The detection of HBx protein is detailed in the section of Immunohistochemical staining.

2.3. DNA transfection

pCMV-X/F contains the HBV coding region (HBV nucleotides 1372-1833) with the nucleotide sequence encoding the flag polypeptide (DYKDDDDK) at the C-terminal end inserted into the multiple cloning site (*EcoRI*/*EcoRV*) of the pCDNA3 vector. Using TransFast transfection reagent (Promega, Madison, WI, USA), Hep G2 cells (80% confluence) were transfected with the HBV expression vector in serum-free conditions. After 24 h, the medium was replaced with DMEM with 10% heat-inactivated fetal bovine serum, and the cells were cultured for another 48 h, after which total RNA and protein were extracted.

2.4. Western blot analysis

Tissue or cell samples were homogenised with ice-cold phosphate-buffered solution (PBS) and then lysed in a solution containing 8 M urea, 0.1 M $Na_2H_2PO_4$ and 0.01 M Tris–HCl. Supernatants were obtained after centrifugation at 10 000 *g*. After boiling, equal amounts of proteins were loaded and separated on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels. Proteins were then electrophoretically transferred from the gel onto nitrocellulose membranes and the membranes were blocked for 1 hour in PBS-Tween buffer containing 5% dry milk powder (fat free) at room temperature. The membranes were then incubated with an anti-human Bid antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. After washing, the membranes were incubated with a secondary antibody, anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, they were treated with the reagents in the chemiluminescent detection kit (ECL system, Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Anti-human β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect human β-actin, which was used as a control for equal loading.

2.5. Immunohistochemical staining

Tissues stored in liquid nitrogen were thawed in 10% neutral buffered formalin. After paraffin embedding,

tissues were sectioned at 4 μm thick. Tissue sections were blocked and incubated with an anti-human Bid antibody (15:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-HBx antibody (1:200) (Affinity BioReagents, Inc., Golden, CO, USA) overnight at 4°C. The tissue sections were then washed with PBS twice and incubated with a biotinylated-labelled secondary antibody for 30 min. Bid antigen staining was detected using the horseradish peroxidase ABC kit and NovaRED or DAB substrates (both from Vector Laboratories, Burlingame, CA, USA). Vector Gill's haematoxylene was used to counterstain the sections. After dehydration, the tissue sections were mounted with the DPX permanent agent. Negative controls were prepared by replacing the primary antibody with PBS. Immunoreactivities for Bid and HBx in the liver tissues were scored according to the standard described in Table 1.

2.6. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from liver tissues and Hep G2 was isolated using TRIzol Reagents (Life Technologies, Grand Island, NY, USA). First-strand cDNA synthesis was performed using oligo(dT)12-18 priming in the presence of Moloney murine leukaemia virus reverse transcriptase (RT). Polymerase chain reaction (PCR) conditions were optimised such that only the desired product was produced. Initial denaturation was done at 94°C for 2 min followed by 30–32 cycles of amplification. Each cycle consisted of 35 s of denaturation at 94°C, 35 s of annealing at 60°C, and 45 s for enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 10 min to allow reannealing of the amplified products. PCR products were then size-fractionated through a 1.5% agarose gel and the bands visualised using ethidium bromide. PCR primers were designed according to the published sequence of human *Bid* [18]. The sequences of the primers were: 5′-5′ ATG GAC TGT GAG GTC AAC AAC GG 3′-3′ (sense) and 5′ CAC GTA GGT GCG TAG GTT CTG GTT A 3′ (antisense). The PCR product was 588 bp. Human *G3PDH* (glyceraldehyde-3-phosphate dehydrogenase), a constitutively expressed gene, was chosen as a control gene. In order to better quantitate the level of mRNA, *G3PDH* and *Bid* were co-amplified in the same reaction tube. The densities of the bands corresponding to the amplification products were determined with the Molecular Imager and Imaging Densitometer from Bio-Rad Laboratories (Hercules, CA, USA) and the ratio of *Bid* to *G3PDH* was calculated. In some instances, the authentic fragments generated by the RT-PCR were confirmed by DNA sequencing using an automatic DNA sequencer.

Table 1
Scoring standard for Bid staining

Grade	Scoring by tissue	Scoring by cell
0	None	None
1	Slight	Part of the cytoplasm
2	Moderate	Whole cytoplasm without reticular deposits
3	Strong	Reticular deposits in $<1/2$ of cytoplasm
4	Extremely strong	Reticular deposits in $>1/2$ of cytoplasm

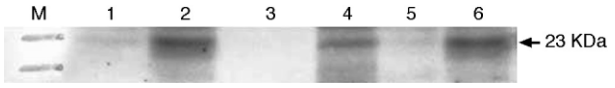

Fig. 1. Expression of Bid protein in liver tissues of hepatocellular carcinoma (HCC). Bid protein was detected by western blotting. Samples in lanes 1, 3 and 5 were from the cancerous liver tissues and those in lanes 2, 4 and 6 were from the non-cancerous liver tissues. M, marker.

2.7. Statistical analysis

All values were expressed as means ± standard error. Statistical comparisons were analysed by the two-tailed

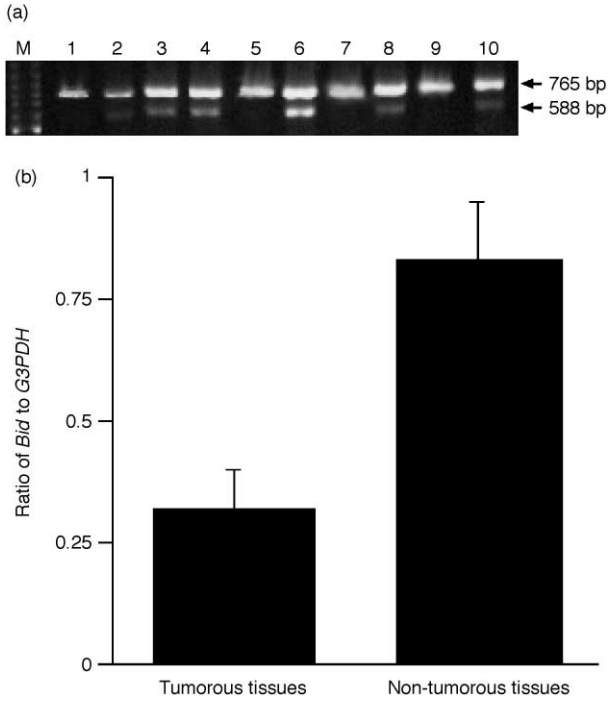

Fig. 2. Expression of *Bid* mRNA in liver tissues of HCC. *Bid* mRNA was analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) (588 bp) with *G3PDH* as an internal control gene (765 bp). (a) A typical experiment of RT-PCR is shown. Five pairs of samples were assayed. Samples from lanes 1, 3, 5, 7 and 9 were from the cancerous liver tissues and those in lanes 2, 4, 6, 8 and 10 were from the non-cancerous liver tissues. (b) Bands of RT-PCR products were quantitated by measuring the density. The ratio of *Bid* to *G3PDH* was calculated. The ratio for the samples from cancerous liver tissues is much lower than that for the samples from the non-cancerous liver tissues ($P<0.01$, $n=15$).

Student's *t*-test and were performed using InStat software (GraphPad Software, San Diego, CA, USA). A P value of less than 0.05 was taken as statistically significant.

3. Results

3.1. Expression of Bid protein in HCC tissues

Compared with non-cancerous liver tissues, samples from cancerous tissues expressed very low levels of Bid protein and in some of them Bid was undetectable as demonstrated by Western Blotting analysis. Fig. 1 shows typical results obtained from three pairs of samples. Bid protein was low/ absent in all tumour samples (lanes 1, 3 and 5). All of the non-cancerous liver tissues expressed a high level of Bid (lanes 2, 4 and 6).

3.2. Expression of Bid mRNA in the HCC tissues

The data in Fig. 2(a) show the results of a representative experiment with five pairs of samples. The

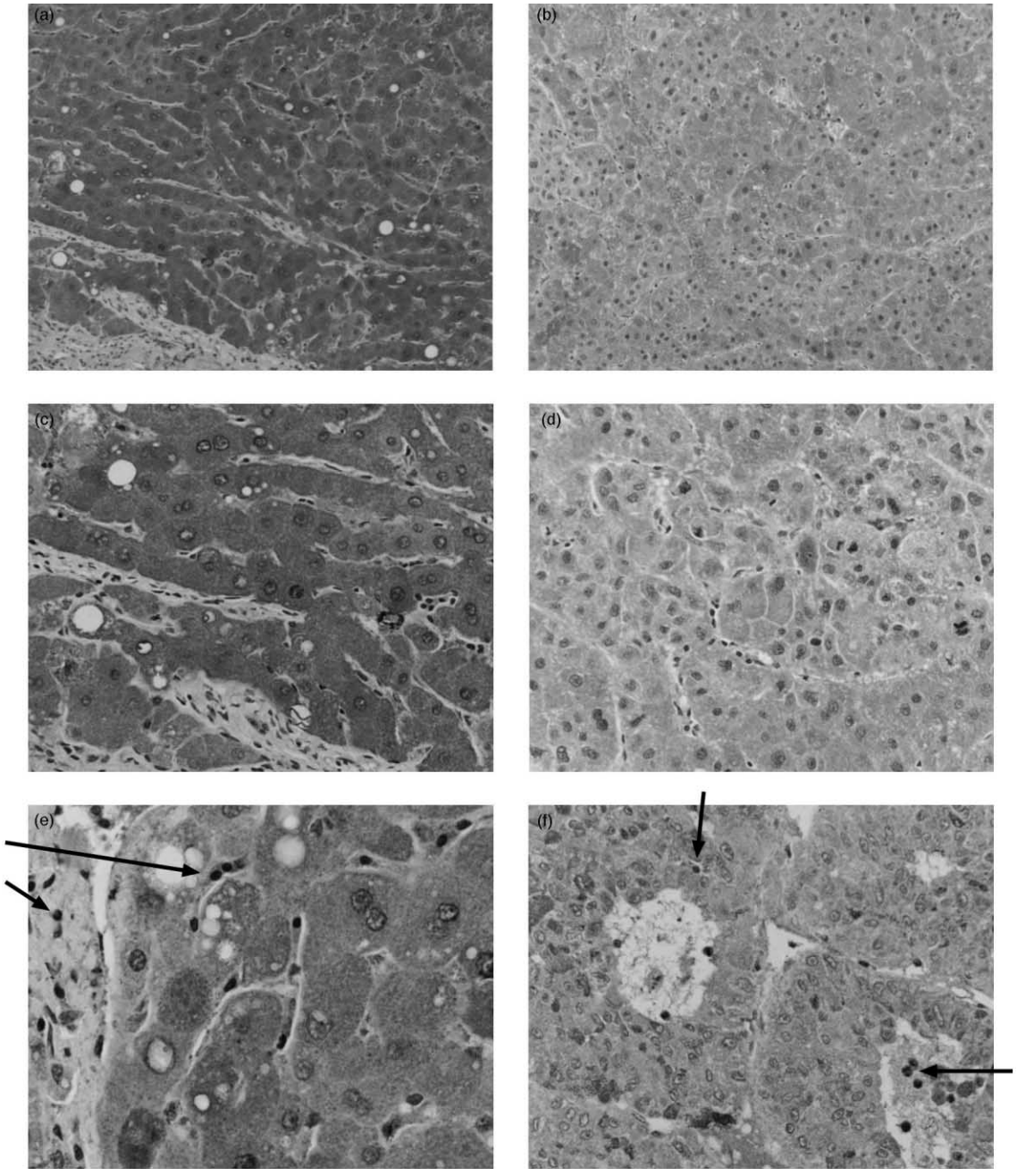

Fig. 3. Immunohistochemical staining of Bid in liver tissues: (a) Non-cancerous tissue (100×); (b) cancerous tissue (100×); (c) non-cancerous tissue (200×); (d) cancerous tissue (200×) with lymphocytes and Kupffer cells; (e) non-cancerous tissue (400×) with lymphocytes and Kupffer cells; (f) cancerous tissue with necrosis and haemorrhage (200×).

expression of *Bid* mRNA in the tissues from the non-cancerous areas was much stronger than that in the samples from the cancerous regions. Four samples from cancerous regions did not express detectable *Bid* mRNA (lanes 1, 5 7 and 9), while the other sample had low levels of Bid expression (lane 3). The density of bands was quantified by ImageQuant software (Molecular Dynamics). The ratio of *Bid* to *G3PDH* was calculated and results are presented in Fig. 2(b). The expression of *Bid* mRNA was significantly lower in the tissues from the cancerous regions than in those obtained from the non-cancerous regions ($P < 0.01$, $n = 15$).

3.3. Bid staining and its localisation

Immunostaining for Bid was very strong in hepatocytes from non-cancerous liver tissues and Bid antigen was mainly confined in the cytoplasm (Fig. 3a, c and e). The staining was homogeneous, reticular and uniformly distributed. According to the scoring system described in Table 1, the scores were 3–4 in all cases of non-cancerous liver tissues. Cancerous liver tissues also expressed Bid antigen, but to a much lesser degree compared with the hepatocytes of the non-cancerous liver tissue and the average scores were between 1 and 2 in most of the tumour cells (Fig. 3b, d and f). The staining in cancerous regions was in a diffuse pattern with some heavy staining mostly around peri-cancerous centre areas (Fig. 3d and f). In some instances, both paranuclear and nuclear reaction products were visualised and this happened in both cancerous and non-cancerous tissues. In non-cancerous tissues, the intensity of Bid protein staining in hepatocytes was similar to that observed in the cytoplasm of infiltrating lymphocytes among hepatocytes, but much stronger than lymphocytes and Kupffer cells presented in portal tracts (Fig. 3e). Contrary to the non-cancerous tissues, lymphocytes and Kupffer cells in the cancerous areas were stained heavier than hepatocytes (Fig. 3f). Inflammatory cells in the cancerous centre with necrosis and haemorrhage were strongly stained by the Bid antibody (Fig. 3f). Vessels, periportal tissues, biliary duct cells and replacing lipocytes in both cancerous and non-cancerous tissues were, however, free from significant staining.

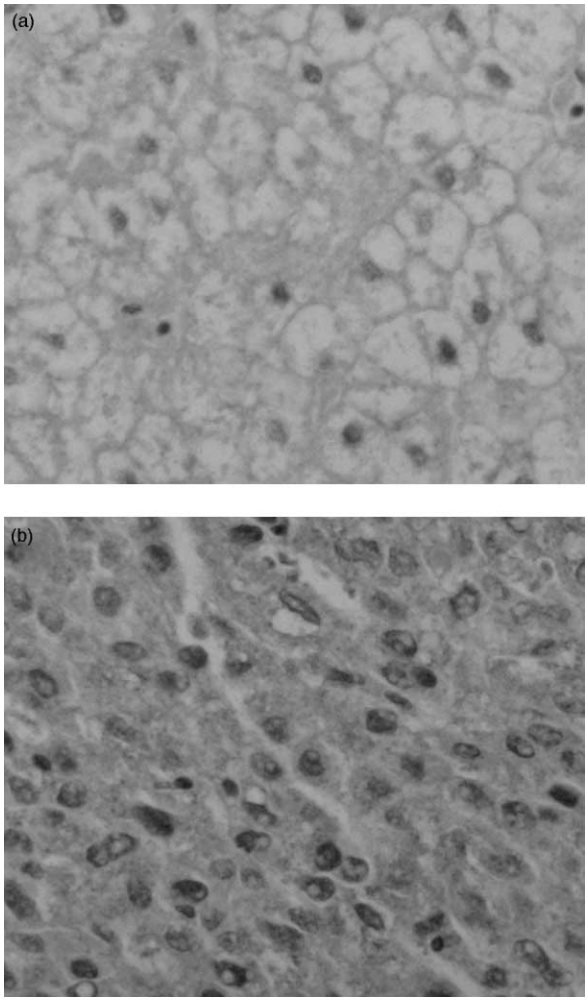

Fig. 4. Immunohistochemical staining of HBx protein in liver tissues: (a) non-cancerous tissue (400×); (b) cancerous tissue (400×).

3.4. Serum HBsAg and liver tissue HBx staining

HBsAg levels in sera from all 15 patients were well above the cut-off value defined by the value of the negative control plus the factor 0.25 and all were confirmed to be positive for HBsAg. Both cancerous liver tissues and non-cancerous ones had HBx expression. However, the intensity of HBx protein expression was much stronger in the former than in the latter (Fig. 4). Quite often, HBx protein staining as homogeneous and uniformly distribution in cancerous liver tissues, whereas its staining was usually scattering. HBx protein was mainly located in cytoplasm, but the nuclei of hepatocytes located in cancerous areas were also frequently positive for HBx. Lymphocytes and Kupffer cells among the hepatocytes were generally negative for HBx.

3.5. HBx and Bid expression

The successful transfection and expression of HBx in Hep G2 cells was demonstrated by RT-PCR. The cells transfected with HBx showed a corresponding band (227 bp), whereas the control (vector only) had no detectable HBx (Fig. 5). The expression of Bid in the cells with HBx was significantly lower than that in the cells without HBx, as evidenced by both RT-PCR (Fig. 6a and b) and western blotting analyses (Fig. 7). It was noted that the expression of Bid could not be completely inhibited by HBx in all of the experiments carried out.

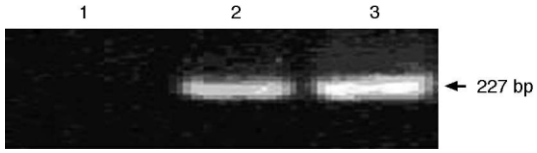

Fig. 5. Reverse transcriptase-polymerase chain reaction (RT-PCR) to confirm the successful transfection of HBx into hepatoma cells. The corresponding band for HBx is 227 bp. Lane 1 is the control; lanes 2 and 3 are transfections of 1:1 and 1:2 (TransFast Reagent:DNA), respectively.

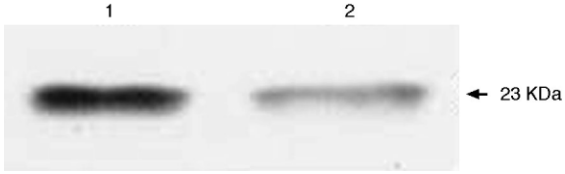

Fig. 7. Expression of Bid protein in samples isolated from Hep G2 cells with or without HBx transfection. Bid protein was detected by western blotting. Lane 1 is the sample transfected with vector only and lane 2 is the sample transfected with HBx.

4. Discussion

The process of apoptosis is a fundamental mechanism for the deletion of unwanted, senescent or damaged cells. Insufficient apoptosis of DNA-damaged and malignant cells has now been recognised as a major determinant in hepatocarcinogenesis [10–14]. Located in BH3 of the Bcl-2 domains, Bid serves as a critical death member in the pro-apoptotic family. However, Bid has not yet been investigated in liver cancers. In the present study, we found that the expression of Bid was significantly downregulated in cancerous liver tissues compared with those from their corresponding non-cancerous areas. Therefore, a partial or complete loss of Bid, as found in this study, may contribute to the imbalance between anti-apoptosis and pro-apoptosis in the development of HCC. This concept is further supported by both *in vitro* and *in vivo* experiments which have indicated that Bid-deficient hepatocytes are resistant to Fas-induced hepatocellular apoptosis [8]. The Fas-apoptotic pathway is one of the most important mechanisms in the induction of apoptosis and under physiological conditions, the liver is highly sensitive to Fas-mediated apoptosis compared with other organ tissues [19]. Upon contact with cross-linking anti-Fas antibodies or Fas ligand (Fas L), the Fas-bearing cells rapidly undergo apoptosis. Despite the fact that hepatoma cells express both Fas and FasL [17,20,21], apoptosis occurring in hepatoma tissues remains much lower than that in normal liver tissues. The reason for this contradictory and interesting phenomenon is not clear. Our results suggest that a partial or complete loss of Bid may serve as one of elements to counteract the action of the Fas/FasL system, which would help to explain why apoptosis remains low in Fas- and FasL-expressing HCC. The role of the decreased expression of Bid in the downregulation of apoptosis seen in the development of HCC is further supported by the findings that the activities of caspase 3 and caspase 8 are inhibited in Bid-deficient hepatocytes [8] and that the expression of caspase 3 is significantly lower in HCC tissues [14]. The activation of both caspase 3 and caspase 8 is a critical signal transduction step in the apoptotic pathway of Bid [2–4].

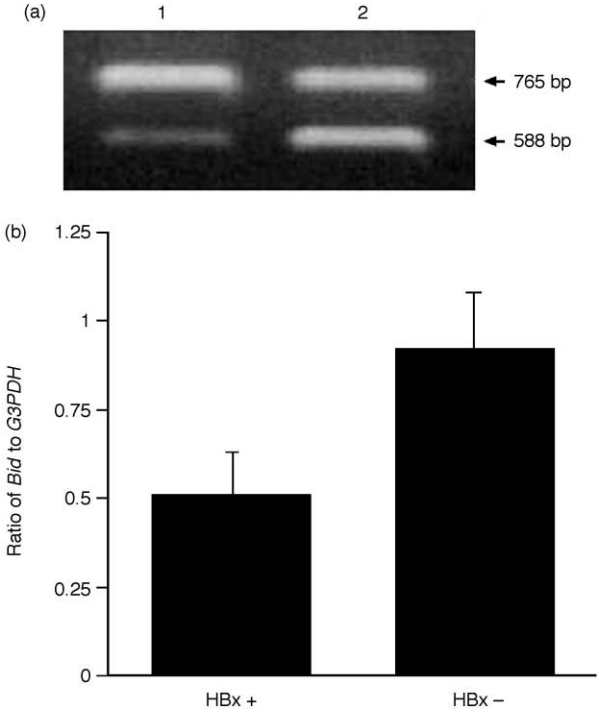

Fig. 6. Expression of *Bid* in mRNA samples isolated from Hep G2 cells with or without HBx transfection. *Bid* mRNA was analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) (588 bp) with *G3PDH* as an internal control gene (765 bp). (a) A typical RT-PCR experiment is shown. Lane 1 is the sample with HBx transfection and lane 2 is transfected with vector only. (b) Bands of RT-PCR products were quantitated by measuring the density. The ratio of *Bid* to *G3PDH* was calculated. The ratio for the samples from cells transfected with HBx was significantly lower than that for the samples from cells without HBx ($P<0.05$, $n=8$).

The distribution and the expression pattern of Bid in cancerous liver tissues and non-cancerous areas may also have pathogenic significance. Considering that most HCCs occur after chronic hepatitis and cirrhosis, the non-cancerous liver tissues from HBV-positive HCC usually has a certain degree of cirrhosis or chronic hepatitis and are often denoted as premalignant lesions [34]. The strong expression of Bid, characterised by homogeneous, reticular and uniform distribution, in non-cancerous regions may indicate that in these premalignant areas Bid expression may act as an active

compensatory mechanism against the potential apoptosis induced by the Fas/FasL system. The activity of Bid may be induced by various inflammatory mediators associated with chronic hepatitis and cirrhosis, such as factalkine, a CX3C-family chemokine [22] and tumour necrosis factor (TNF)-alpha [23]. Liver tissues are known to be relatively abundant in factalkine and the level of TNF-alpha is significantly increased in chronic hepatitis and cirrhosis [24,25]. The reason for the scattering of strong reactive Bid antigens around peri-cancerous centre areas is not clear at present. However, it might indicate a progression of tumour cell phenotypic and tumorigenic alterations between peritumoral regions and tumour centres.

The development of HCC is well known to be associated with HBV infection, especially in Asian countries such as Hong Kong. In fact, all HCC samples examined in this study were positive for HBV. Among the various pathogenic components of HBV, HBx transactivates several cellular genes regulating cell cycle and growth [26]. We hypothesised that the overexpression of HBx decreased the level of Bid in HCC. In order to test this hypothesis, Hep G2, a hepatoma cell that is negative for HBV/HBx, was transfected with HBx and the level of Bid was determined after transfection. A significantly lower level of Bid was found in the cells transfected with HBx, suggesting that there may be a causative role for HBx in the downregulation of Bid. Suppression of Bid expression by HBx is probably associated with another Bcl-2 homologue gene *A1/Bfl-1*. A1/Bfl-1 is localised to and functions at the same cellular location as Bid, i.e. in mitochondria [27]. Furthermore, it is able to block the activity of Bid and thus prevent cells from apoptosis [27]. The expression of *A1/Bfl-1* is induced by a transcription factor NF-κβ, which is known to be stimulated by HBx [28–30] and whose level has been shown to increase in HCC [31,32]. Therefore, it is likely that the pathway by which HBx inhibits Bid may be via the stimulation of NF-κβ and the induction of A1/Bfl-1. However, further experiments are warranted to prove any associations between Bid and A1/Bfl-1 or NF-κβ in HCC. In addition to the inhibition of Bid, HBx has been known to suppress p53- and transforming growth factor beta (TGFβ)-induced apoptosis [10,12,33]. Thus, HBx acts as an important hepatocarcinogenic element in the alteration of the balance between anti-apoptosis, and pro-apoptosis tipping that balance in favour of the former. Consequently, hepatocytes are allowed to grow without undergoing apoptosis and could finally develop into HCC.

The discovery that the expression of Bid is reduced in cancerous liver tissues compared with in non-cancerous areas which are chronic hepatitis or cirrhotic in nature, is consistent with the distribution of HBx in liver cancer as found in the present study. The result of increased HBx protein in cancerous liver tissues compared with in non-cancerous ones was consistent with previous studies [35,36]. A study by Su and colleagues indicated that although both cancerous liver tissues and non-cancerous ones might be positive for HBx, its expression was stronger in the former than in the latter [35]. It has also been reported that HBV genome was predominantly detected in HCC cancerous tissues, compared with non-cancerous liver tissues [36]. Although our present study suggests that an aberrant expression of Bid plays a role in the development of HCC, it is certainly not a sole factor determining tumour formation, as Bid-deficient mice do not show any sign of liver tumours [8]. It is well known that HCC is a multifactorial and multi-stage disorder. Among various carcinogenetic factors, HBx is particularly important because malignant transformation has been observed in X gene-transfected cell lines and HCC develops at high frequency in an X gene-transgenic mouse model [37–40]. Therefore, the finding of decreased Bid by HBx can be considered as one of the pathogenic steps in the development of HCC.

Taken together, we have demonstrated that the expression of Bid is significantly reduced in the liver tissues of HCC at both the mRNA and protein levels. Furthermore, the decreased expression of Bid is, at least, partially related to HBx in HBV infection-related HCC.

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