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Functional Characterization of Hepatitis B Virus X Protein Based on the Inhibition of Tumorigenesis in Nude Mice Injected with CCL13-HBx Cells

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Key Words
HBx · Inhibition of tumorigenesis · Apoptosis · Myosin light chain

Abstract

Objective: This study aimed to determine the effects of HBx on the inhibition of tumorigenesis in nude mice injected with CCL13-HBx cells. Therefore, the characteristics of the induced tumors and the phenomenon of apoptosis were assessed. ***Methods:*** The induced tumors were identified using the specific marker of hepatocellular carcinoma (HCC), anti-α-fetoprotein (AFP), and their characteristics were pathologically examined. Apoptosis was detected by DNA fragmentation, and the expression of the proapoptotic proteins p53, Bax, Bad, caspase-3, and caspase-8 and the anti-apoptotic protein Bcl-2 was detected by Western blotting. To identify possible molecules involved in the inhibition of tumorigenesis, extracts of the induced tumors were separated by 2D-PAGE, and the proteins were identified by MS. ***Results:*** The tumors of the nude mice injected with CCL13 and CCL13-HBx cells were identified as HCCs. Moreover, HBx was found to suppress tumor growth via apoptosis in the nude mice injected with CCL13-HBx cells. The MS findings revealed that phosphorylated myosin light chain was a candidate molecule involved in the inhibition of tumorigenesis. ***Conclusion:*** HBx suppressed tumorigenesis in the nude mice injected with CCL13-HBx cells, which proved to be a good animal model for the in vivo study of the effects of HBx on tumorigenesis.

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Introduction

Hepatitis B virus (HBV) infection plays a critical role in the development of cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV infection is largely endemic in Asia, Oceania, and Africa [2]. The hepatitis B virus X (HBx) protein is one of the major causative factors of HCC. It has been reported as a multifunctional protein that is involved in cell cycle regulation, responses to apoptotic stimuli, genotoxic stress, protein degradation, cell transformation, carcinogenesis, and signaling transduction cascades [3, 4]. HBx regulates cell growth via proliferation or apoptosis, depending on different signaling pathways, which include the Ras/Raf/mitogen-activated protein kinase (MAPK) [5], Janus kinase (JAK)/signal

The contribution of J.-C. Wang is equivalent to that of the first author.

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transduction and activator of transcription (STAT) [6], and protein kinase B (PKB)/Akt [7]. Moreover, HBx is also a transcriptional activator associated with a wide variety of transcription factors, including AP-1, AP-2, NFκB, cyclic adenosine 3′,5′-monophosphate responsive element-binding protein (CREB)/activating transcription factor (ATF), Est, and serum response factor (SRF) [4]. However, the biological roles of HBx in various experimental systems are controversial. Sirma et al. [8] reported that HBx inhibits the clonal outgrowth of cells by blocking the cell cycle and subsequently triggering apoptosis via the p53-independent pathway. Chirillo et al. [9] demonstrated that HBx sensitizes the NIH3T3 polyclonal cell line to death by programmed cell death. Many other studies have reported that HBx mediates apoptosis in cells or sensitizes cells to apoptosis [9–19]. In contrast, some studies have reported that HBx inhibits apoptosis and enhances cell proliferation [7, 20–22]. Although HBx is considered to play an important role in hepatocarcinogenesis, its specific role remains unclear and controversial.

To study the role of HBx in hepatocarcinogenesis, we detected the expression of the HBx antigen and anti-HBx antibodies in HCC patients by performing Western blotting and enzyme-linked immunosorbent assay (ELISA). The results of the analyses indicated that the sera from 70% of the HCC patients and 5% of chronic hepatitis (CH) patients contained antibodies that specifically bound to HBx. Further, the tissues obtained from the liver of 85% HCC patients contained HBx [23]. With regard to the effects of HBx on cell growth in vitro and in vivo, we demonstrated that HBx downregulates Wnt-3/β-catenin expression and suppresses cell growth by repressing cell proliferation and/or triggering apoptosis in the CCL13-HBx stable cell line, and that it inhibits tumorigenesis in nude mice injected with CCL13-HBx cells [11, 24]. However, the details of the mechanism underlying the inhibition of tumorigenesis in these mice remain unclear. In this study, we injected nude mice with CCL13-HBx cells and assessed the expression of apoptotic proteins in their tumor cells by Western blotting. The results indicated that the expression of the proapoptotic proteins p53, Bax, Bad, caspase-3, and caspase-8 was upregulated, while that of the anti-apoptotic protein Bcl-2 was slightly downregulated. Apoptosis in the tumors of the nude mice injected with CCL13-HBx cells was detected by the DNA ladder assay. The results of the assay demonstrated that the expression of glycogen synthase kinase (GSK-3β) was upregulated, while that of β-catenin was downregulated; these results were consistent with those of our in vitro experiments [11]. Further, to identify possible molecules that are involved in the inhibition of tumorigenesis in the nude mice injected with CCL13-HBx and CCL13 cells, a candidate molecule – phosphorylated myosin light chain (pMLC) – was identified and separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Materials and Methods

Transplantation of Cells into BALB/c Nude Mice

Tumorigenicity test in nude mice was performed in male BALB/c nude mice [24]. Briefly, 1×10^7 CCL13 and CCL13-HBx cells in 0.2 ml PBS were subcutaneously injected into the flank region of nude mice. Tumor size was analyzed once a week until 10 weeks [24].

DNA Fragmentation

Tissues obtained from the tumors of the nude mice injected with CCL13-HBx or CCL13 cells were dissected into small pieces and washed with phosphate-buffered saline (PBS). DNA fragmentation analysis was performed using the Tissue and Cell Genomic DNA Purification Kit (GeneMark, Taiwan) according to the manufacturer's instructions. The extracted DNA was subjected to 2% agarose gel electrophoresis.

Western Blotting

Proteins extracted from the tumor cells of the nude mice injected with CCL13-HBx or CCL13 cells were subjected to Western blot analysis. Membranes were immunodetected using anti-HBx (Abcam), anti-p53 (Novocastra), anti-caspase-3, anti-caspase-8, anti-GSK-3β (Cell Signaling), anti-Bax, anti-Bad, anti-Bcl-2, anti-β-catenin (Santa Cruz), anti-α-fetoprotein (AFP; Zymed), anti-pMLC (GeneTex), or anti-actin (Sigma) antibody. The ECL kit (Millipore) was used according to the manufacturer's instructions.

2D-PAGE

2D-PAGE is a good method for proteomic analysis by high-resolution protein separation, which is the best method for the monitoring of protein modification [25]. The total proteins extracted from the tumor cells of the nude mice injected with CCL13-HBx and CCL13 cells were purified with the ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories), applied to 11-cm ReadyStrip IPG Strips (Bio-Rad Laboratories), and analyzed according to the manufacturer's instructions. The protein spots in the gels were visualized using the Bio-Rad Silver Stain Kit; the target protein spots were cut, and the proteins were identified according to the methods described by Lee et al. [26].

Mass Spectrometry

The protein spots were visualized by silver staining after separation by 2D-PAGE. The target protein spots were cut and sent to the Proteomic Research Core Laboratory at National Cheng-Kung University, Tainan, Taiwan. The target protein was then analyzed by electrospray ionization mass spectrometry (ESI-MS)/mass spectrometry (MS; Finnigan MAT, San Jose, Calif., USA) and searched with the Mascot computer program (Matrix Science).

Results

Inhibition of Tumorigenesis in the Nude Mice Injected with CCL13-HBx Cells

In our previous study, CCL13 and CCL13-HBx cells were subcutaneously injected in nude mice. Examination revealed significantly larger tumors in the nude mice injected with CCL13 cells than in those injected with CCL13-HBx cells [24]. As shown in figure 1, the tumors of the nude mice injected with the 2 types of cells were considerably different in size. Further, tumorigenesis was suppressed in the nude mice injected with CCL13-HBx cells (fig. 1b). However, the details of the underlying mechanism remain unclear.

Development of HCC in the Nude Mice Injected with CCL13 and CCL13-HBx Cells

To study the characteristics of the induced tumors, pathological examination was conducted. The tumors of the nude mice injected with CCL13 cells grossly measured 14–32 mm in diameter, and those of the nude mice injected with CCL13-HBx cells grossly measured 5–12 mm in diameter (fig. 2, upper panel). Well-differentiated early-stage HCCs are characterized by an increased cell density with a high nuclear/cytoplasmic ratio, an increased cytoplasmic eosinophilia, an irregular, thin, trabecular pattern, and a frequently observed pseudoglandular pattern [27]. These morphological characteristics were observed in the tumors of the nude mice injected with CCL13 cells (fig. 2, left panel; magnifications, ×40 and ×200). However, poorly differentiated HCCs were observed in the tumors of the nude mice injected with CCL13-HBx cells (fig. 2, right panel; magnifications, ×40 and ×200). Further, tumor cells with hyperchromatic nuclei and an increased cell density with a high nuclear/cytoplasmic ratio were observed (fig. 2, lower panel, enlarged images; magnification, ×400). However, the diagnosis of HCCs based on their pathological and morphological characteristics is very difficult. Prognostic molecular markers in HCC have recently been identified [28]. Suzuki et al. [29] reported that the β-catenin expression was increased in well-differentiated tissues, and was decreased in the poorly differentiated tissues. The results of Western blotting showed that the expression of β-catenin was decreased in CCL13-HBx-injected nude mice tumors (see fig. 3c, middle panel). The results suggested that poorly differentiated HCC was determined in the tumors of the nude mice injected with CCL13-HBx cells. Furthermore, AFP has been considered as a marker protein of hepatocellular carcinogenesis [30, 31]. The results of Western blotting revealed the expression of AFP in the tumor cells of the nude mice of both groups but not in the cervical tissues of patients with cervical cancers (CX19 and CX22; fig. 4). Further, AFP expression was also detected in the tumorous liver tissues of an HCC patient but not in the normal liver tissue (fig. 4). Thus, the tumors of the nude mice injected with CCL13 and CCL13-HBx cells were identified as HCCs.

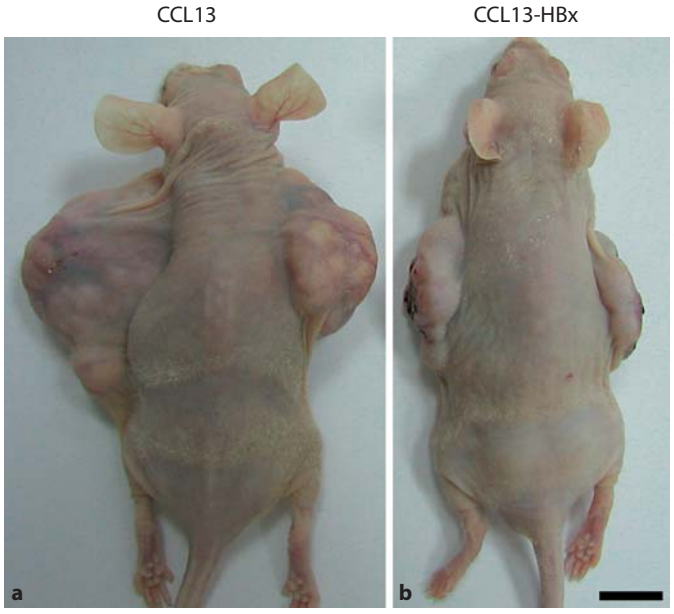

Fig. 1. In vivo testing of tumorigenesis. **a** Tumors of a volume of approximately 1 cm^3 were found at the 6th week after CCL13 cells were subcutaneously injected into the nude mice. **b** Tumors of a volume of approximately 0.1 cm^3 were found at the 6th week after CCL13-HBx cells were subcutaneously injected into the nude mice. Bar = 10 mm.

Effect of HBx on the Inhibition of Tumorigenesis via Apoptosis in the Nude Mice Injected with CCL13-HBx Cells

Cell death or survival depends on the balance between apoptosis and proliferation; an imbalance between these 2 processes is associated with the development of HCC [32]. In addition, HBx plays an important role in hepatocellular carcinogenesis [1, 3, 4]. HBx has been reported to inhibit cell growth by triggering apoptosis or inhibiting proliferation [9–19]. However, contrasting results have also been reported [7, 20–22]. As shown in figure 1, the size of the tumors in the nude mice injected with CCL13 and CCL13-HBx cells was considerably different. To determine the effects of HBx on the inhibition of tumorigenesis in the nude mice infected with CCL13-HBx cells, the

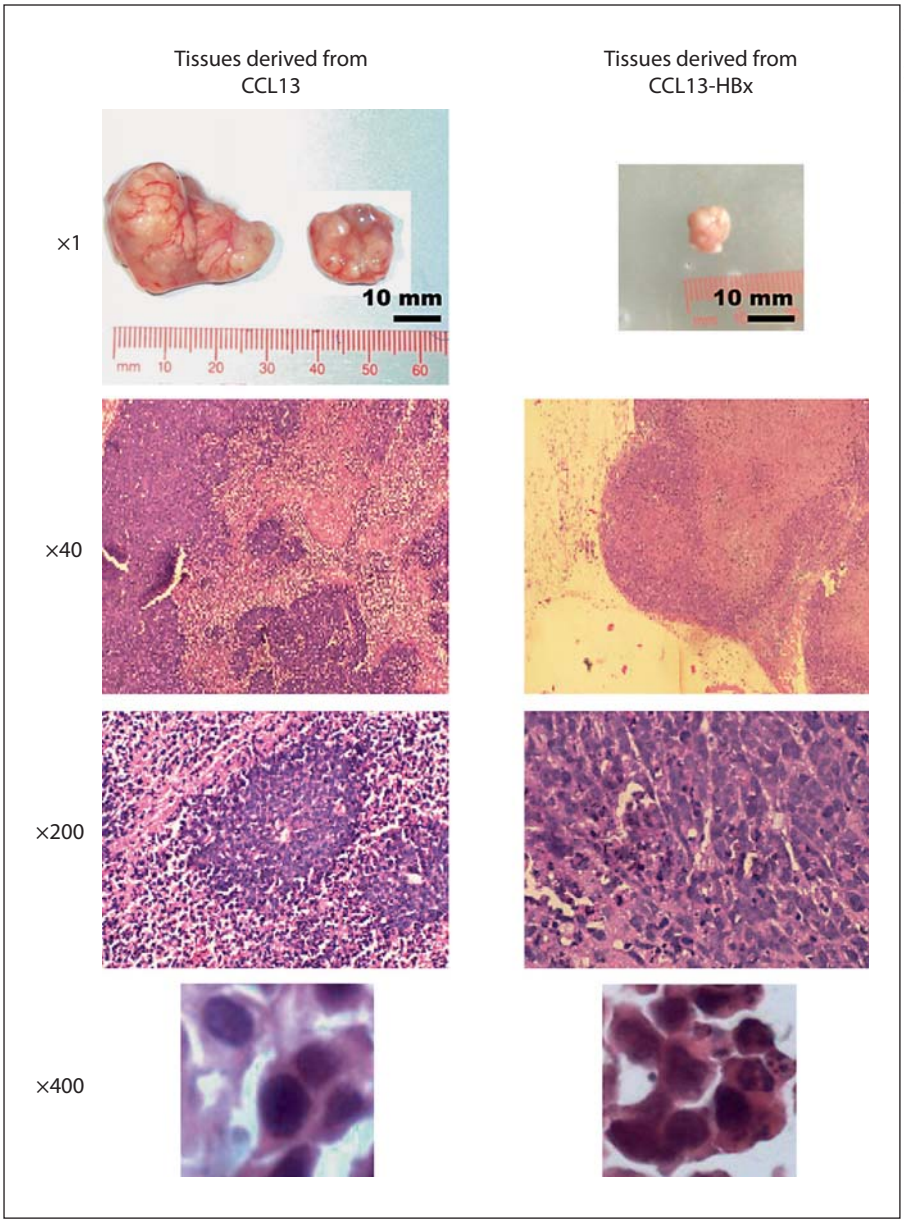

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Fig. 2. Pathological examination of the induced tumors. The upper panel shows the gross view of the tumors induced by the CCL13 and CCL13-HBx cells. The morphological characteristics of well-differentiated HCCs were observed in the tumor cells of the nude mice injected with CCL13 cells (left panel; magnifications, ×40 and ×200), while those of poorly differentiated HCCs were observed in the tumor cells of the nude mice injected with CCL13-HBx cells (right panel; magnifications, ×40 and ×200). The enlarged image shows tumor cells with hyperchromatic nuclei (lower panel; magnification, ×400).

expression of the proapoptotic proteins p53, Bax, caspase-3, and caspase-8, and the anti-apoptotic protein Bcl-2 was analyzed by Western blotting. The results indicated that the expression of the proapoptotic proteins was upregulated (fig. 3a), while that of the anti-apoptotic protein was slightly downregulated on HBx induction (fig. 3a). In addition, the phenomenon of apoptosis was assessed by the DNA ladder assay (fig. 3b). Interestingly, the expression of GSK3β was found to be upregulated, while that of β-catenin was found to be downregulated on HBx induction in the tumor cells of the nude mice injected with CCL13-HBx cells (fig. 3c). These results were consistent with those of our in vitro experiments [11]. Thus, it was concluded that HBx suppressed tumor growth via apoptosis in the nude mice injected with CCL13-HBx cells.

Results of 2D-PAGE and ESI-MS/MS

To identify possible molecules that are involved in the inhibition of tumorigenesis in the nude mice injected with CCL13-HBx and CCL13 cells, the tumor extracts were separated by 2D-PAGE. We compared the protein spots obtained in the 2 gels, identified the target spots,

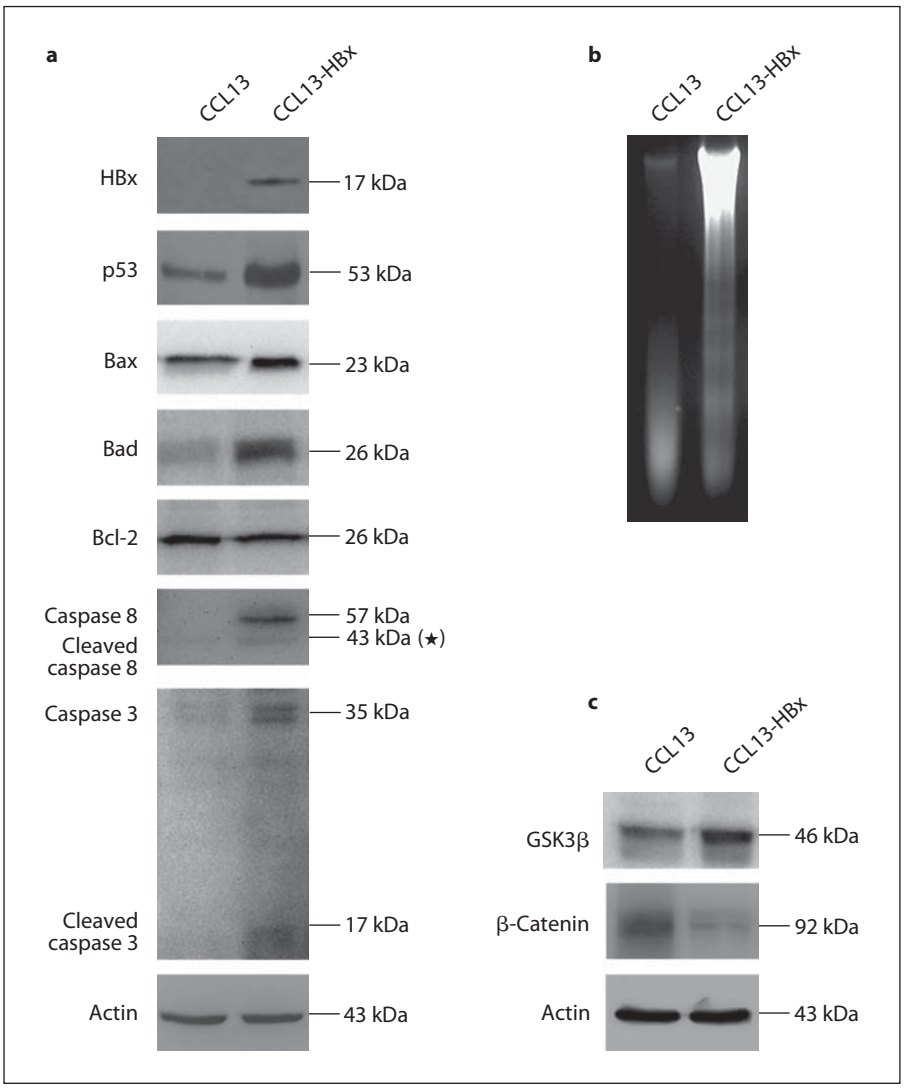

Fig. 3. HBx suppressed tumorigenesis via apoptosis in the nude mice injected with CCL13-HBx cells. **a** The expression of the proapoptotic proteins p53, Bax, Bad, Bcl-2, caspase-8, cleaved caspase-8 (★, 43 kDa), caspase-3, and cleaved caspase-3 (17 kDa) was detected by Western blotting. **b** DNA fragmentation was detected in the tumors induced by the CCL13-HBx cells but not in those induced by the CCL13 cells. **c** GSK3β expression was upregulated and β-catenin expression was downregulated in the tumors induced by the CCL13-HBx cells. Actin was used as the internal control.

and analyzed them by ESI-MS/MS (fig. 5a, arrow). Based on the findings, ^{32}EAFTVIDQNRDGIIDKEDLR^{51}, ^{60}LNVKNEELDAMMK^{72}, ^{91}LKGADPEDVITGAFK^{105}, and ^{155}NICYVITHGDAKDQE^{169} were identified as pMLCs (GenBank GI:7949078). The results obtained by Western blot analysis of MLCs revealed their upregulated expression in the tumor cells of the nude mice injected with CCL13-HBx cells (fig. 5b).

Discussion

HBV and hepatitis C virus (HCV) are the 2 major hepatitis viruses involved in the development of chronic hepatitis and HCC [33]. HBx plays a critical role in hepatocellular carcinogenesis [1, 3, 4, 34]. However, the intrinsic roles of HBx remain unclear and controversial. Conflicting results obtained with regard to the role of HBx may be attributed to the fact that different types of cells, immortalized or primary cells, and different experimental systems have been used in different studies [12]. Schuster et al. [35] demonstrated that low levels of HBx expression supported cellular transformation in immortalized cell lines. Thus, the obvious conclusion would be that HBx suppresses apoptosis to facilitate malignant transformation or the accumulation of transformation mutations in the early stages of HBV infection. However, it was found to promote apoptosis or sensitize cells to apoptosis, thereby facilitating the replication and dissemination of HBV [36], its escape from immunological responses [37, 38], or

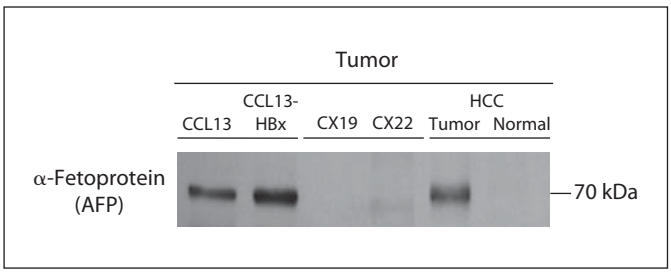

Fig. 4. Development of HCCs in the nude mice injected with CCL13 and CCL13-HBx cells. The results of Western blotting revealed AFP expression in the tumor cells of these mice and in the tumorous liver tissues of an HCC patient. However, AFP expression was not detected in the cervical tissues of patients with cervical cancer (CX19 and CX22) or in the normal liver tissue of the HCC patient.

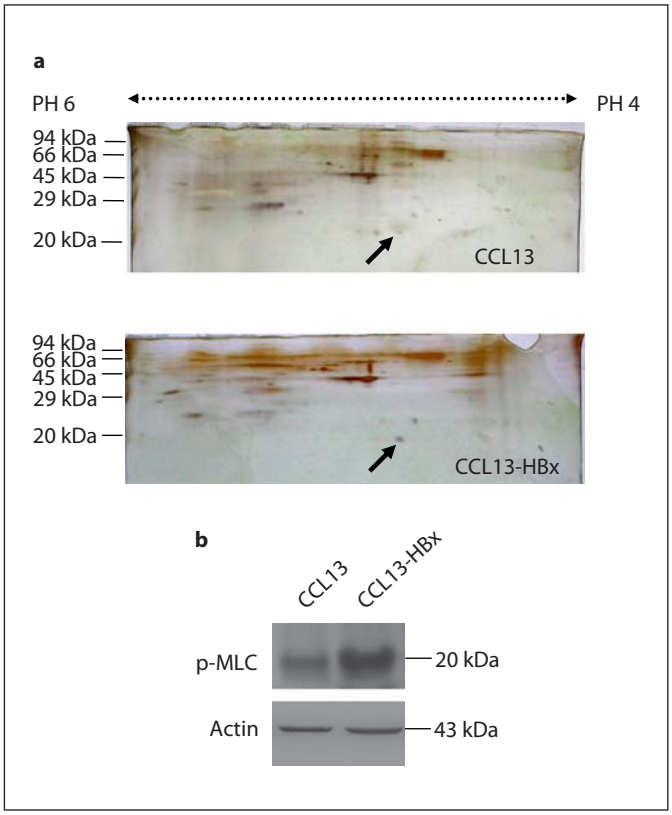

Fig. 5. Two-dimensional proteomics analysis. **a** 2D-PAGE of tumor extracts and silver nitrate staining. The molecular weights are shown on the left and the pH values on the top. Arrows indicate the candidate protein at 20 kDa, which was identified by ESI-MS/MS. **b** The candidate protein, namely, p-MLC, was confirmed by Western blotting. Actin was used as the internal control.

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malignant transformation in the advanced stages of HBV infection [39]. In addition, the different expression levels of HBx may contribute to the controversial results regarding its effects in HBV infection [12]. HBx is expressed at low levels in the early stage of HBV infection, and this contributes to transcriptional activation and virus replication via HBx-triggered apoptosis [40, 41]. Higher HBx expression was detected in chronic HBV infection [41–43].

In our previous studies, we reported that HBx induction in the CCL13-HBx stable cell line downregulated Wnt-3/β-catenin expression and suppressed cell growth by repressing cell proliferation or triggering apoptosis [11]. Moreover, we demonstrated that HBx negatively regulated the proliferation of CCL13-HBx stable cells via the GSK3β/β-catenin cascade [44]. Wang et al. [24] also reported that HBx suppressed tumorigenesis in the nude mice injected with CCL13-HBx stable cells. The experimental system for the subcutaneous injection of cells into nude mice has been well established in many other studies [45–49]. Therefore, we used the same experimental system to determine the in vivo effects of HBx on tumorigenesis. The experimental results revealed that HBx inhibited tumorigenesis via apoptosis in the nude mice injected with CCL13-HBx cells, as determined based on the upregulated expression of the pro-apoptotic proteins p53, Bax, Bad, caspase-3, caspase-8, and the downregulated expression of the anti-apoptotic protein Bcl-2 on HBx induction in the mice (fig. 3a). Furthermore, the phenomenon of apoptosis was detected by the DNA ladder assay only in the nude mice injected with CCL13-HBx cells and not in the nude mice injected with CCL13 cells (fig. 3b). By using 2D-PAGE, we analyzed the candidate molecule – pMLC – for its involvement in the inhibition of tumorigenesis on HBx induction in the nude mice injected with CCL13-HBx cells (fig. 5).

MLC is one of the major components of myosin in thick muscle filaments [50]. Smooth muscle activation depends on the balance between the phosphorylation and dephosphorylation of MLC, which are mediated by MLC kinase (MLCK) and MLC phosphatase, respectively [51, 52]. Sebbagh et al. [53] showed that MLC phosphorylation increases and membrane blebbing occurs after apoptosis is induced. Furthermore, MLCK is involved in tumor necrosis factor-α-induced apoptosis and caspase-3 activation [54, 55]. Su and Schneider [15] reported that HBx sensitizes cells to apoptosis by treatment with tumor necrosis factor-α. We also suggested that HBx sensitizes cells to apoptosis via MLC-depen-

dent pathway. However, we require more evidence to determine the possible mechanisms underlying the involvement of MLC in the HBx-induced inhibition of tumorigenesis in nude mice injected with CCL13-HBx cells.

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