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Dermatopontin Is Expressed in Human Liver and Is Downregulated in Hepatocellular Carcinoma

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Abstract—Dermatopontin (DPT) was recently found as a downstream target of vitamin D receptor, which is a key molecule in the 1,25-dihydroxy-vitamin D_3 anti-hepatoma proliferation pathway. MCTx-1 from *Millepora*, a homolog of DPT, is identified as a cytotoxin towards leukemia cells. The aim of this study was to analyze DPT expression in hepatocellular carcinoma (HCC) based on the analysis for DPT gene in normal tissues in order to estimate its function in the progression of HCC. DPT mRNA expression was analyzed in normal tissues and HCC cell lines by RT-PCR, and in HCC tissue by RT-PCR and real-time PCR. Its protein was examined in HCC tissues by Western blot and immunohistochemistry assays. Meanwhile, transforming growth factor-β1 (TGF-β1) that is closely associated with HCC and DPT was observed by immunohistochemistry in HCC tissues. The results showed that DPT mRNA was strongly expressed in human fetal and adult liver, kidney, and spleen, weakly in ovary and heart, and absent in other tissues and HCC cell lines examined. Its mRNA was significantly downregulated in HCC tissues, while its protein was weakly expressed in tumor compared with non-tumor. DPT is located mainly in the cytoplasm of several cell types in the liver; it has been identified also in the extracellular matrix of the skin. TGF-β1 was observed in extensive tumor tissue of HCC. This fact suggests that DPT can play various roles in different tissues and might be a molecule related to carcinogenesis and the progression of HCC via possible interaction with TGF-β1 and other potential mechanisms.

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Key words: liver, hepatocellular carcinoma, dermatopontin, transforming growth factor-β1

Hepatocellular carcinoma (HCC) is the most common primary malignant cancer of the liver [1, 2]. Many biological substances contribute to hepatocarcinogenesis and its pathological process, such as transforming growth factor-β1 (TGF-β1), α3-integrin, and E-cadherin related to HCC invasion and migration [3, 4]. Dermatopontin (DPT) is a 22 kDa acidic extracellular matrix protein that has multiple functions [5]. Mesenchymal cells (fibroblasts and myofibroblasts) and macrophages are indicated as the source of DPT [6-8]. This molecule is critical for extracellular matrix assembly [9-11], cell adhesion [12], wound healing [8], and positive modification of the growth inhibition activity of TGF-β1 [13]. Its murine counterpart early quiescence-1 gene can induce quiescence and be induced by the quiescence of cells, suggesting a self-driven mechanism of anti-proliferation [14]. Recently, MCTx-1, a proteinaceous cytotoxin, was found in fire coral; it can kill mouse leukemia cells and was deduced to be a novel DPT from the corresponding cDNA sequence [15]. Additionally, DPT was identified as a new downstream target of the vitamin D receptor, participating in the regulation of the osteogenic differentiation of mesenchymal stem cells [16], while vitamin D receptor was clinically evidenced to mediate 1,25-dihydroxy-vitamin D_3 to exert anti-proliferative effect on hepatoma [17]. Furthermore, some hepatic cell populations including hepatocytes, sinusoidal endothelial cells (SECs), Kupffer cells (KCs), and hepatic stellate cells (HSCs) have been shown to clearly express both nuclear vitamin D receptor and are targets of the vitamin D endocrine/paracrine/intracrine system [18]. Early studies indicated a low mRNA level and no protein expression of

Abbreviations: DPT, dermatopontin; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; KCs, Kupffer cells; N, non-tumor; SECs, sinusoidal endothelial cells; T, tumor; TGF-β1, transforming growth factor-β1.

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DPT in the liver [6, 19, 20]. However, the small hepatic sinusoidal lining cell populations that contain HSCs, KCs, and SECs, known to produce DPT or its related protein in other tissues, might express this molecule [6-8]. In this context, we assumed that DPT might be present in the liver and be related to hepatocarcinogenesis. In the present study, we examined DPT gene expression in normal human tissues, HCC tissue, as well as 14 HCC cell lines, and further analyzed its protein expression in HCC tissue. Given that TGF-β1 is closely associated with the progression of HCC [3, 21-24] and can interact with DPT to affect the growth of epithelial cells and the extracellular matrix assembly [13, 25], we also investigated the expression of TGF-β1 in HCC with immunohistochemical assay in order to understand well the potential role of DPT in HCC.

MATERIALS AND METHODS

Tissues and cell lines. Normal tissues were obtained from 20 healthy adult donors who died as a result of accidents (12 males and eight females, 20-60-year-old). These tissues include human liver, kidney, spleen, heart, lung, ovary, smooth muscle, stomach, brain, breast, colon, and testes. Four-six-month-old fetal livers (nine males and 11 females) were donated by the West China Second University Hospital. HCC tissue samples were obtained from 25 randomly selected patients (14 males and 11 females, (48 ± 8)-year-old) with HCC undergoing hepatectomy at West China Hospital. The etiology of chronic liver disease associated with HCC includes chronic hepatitis B virus infection (16 cases), hepatitis C virus infection (five cases), alcoholic cirrhosis (three cases), and primary biliary cirrhosis (one case). According to the Edmondson–Steiner standard [26], grade I and II, namely well-differentiation and mature liver-cell types were noted in 11 cases, and grade III and IV, namely immature liver-cell and embryonal-cell types in 14; 12 patients had a complete amicula, and 13 had a tumor embolus in the portal vein. All cases were reviewed to confirm the diagnosis by pathologists. All tumor (T) samples were acquired from within the tumor, while corresponding non-tumor (N) samples were harvested from areas distant from the tumors. Access to these materials was in agreement with the requirements of the local Ethics Committee as well as the ethical standards formulated in the Helsinki Declaration. Informed consent was obtained from the patients. The tissues were immediately frozen in liquid nitrogen and immersed in formalin until required. All tissue sections were routinely analyzed after staining with hematoxylin–eosinsaffran and Sirius Red. Fourteen HCC cell lines (HA22T, Hep3B, Huh-1, Huh-4, Tong, PP5, SNU182, SNU449, SNU475, Huh-6, Huh-7, HepG2, Mahlavu, and Sk-Hep1) from the Cell Center of our department were cultured as previously described [4] and collected for RNA isolation when 80 to 90% confluence was attained.

RNA extraction and cDNA preparation. Total RNA was extracted using TRIzol® (Invitrogen, USA) reagent. First strand cDNA was synthesized from 2 μg of total RNA in a final volume of 20 μl using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Reverse transcription (RT) reactant (cDNA) was used for polymerase chain reaction (PCR) and real-time PCR assays.

PCR assay. PCR was performed in a total volume of 25 μl using PCR Master Mix (Fermentas) containing 0.1 μM of each DPT primer (forward, 5′-ATC CCA GGC AGC ATG GAC CTC AGT-3′; reverse, 5′-GCA TTG CAG TTA CCA GCT CA-3′) and 1 μl of cDNA. After an initial denaturation step at 95°C for 2 min, the PCR cycling conditions were 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 50 sec using a PCR Sprint Authorized Thermal Cycler (Thermo Hybaid, GB). PCR products were electrophoresed in agarose gels and photographed on a 280 nm UV light box after staining with ethidium bromide. β-Actin was used for endogenous reference (forward primer, 5′-GTG GGG CGC CCC AGG CAC CA-3′; reverse primer, 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′). Its PCR conditions were as described above.

Relative quantitative real-time PCR (RQ-PCR). The expression of the DPT gene in HCC tissue was further analyzed using SYBR® Premix Ex Taq™ (Perfect Real Time; TaKaRa, China) containing 2 μl of cDNA and 0.2 μM of each primer (forward, 5′-GGC TTC AGC TAC CAG TGT CC-3′; reverse, 5′-TCA TGT CCA TTT CCT CAC CA-3′) in 20 μl volumes in a 96-well plate (Applied Biosystems, USA). RQ-PCR reactions were carried out using the ABI PRISM 7300 Fast Real-Time PCR System (Applied Biosystems). Forty cycles of 95°C for 15 sec and 60°C for 1 min were used. β-Actin was used for a reference gene. To determine relative PCR efficiency, RT reactant of a calibrator that was a mean of one normal liver tissue in our assay was diluted in serial 10-fold ranges and the Ct value at each dilution for both DPT and β-actin was measured. The RQ-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification method [27]. The number of DPT copies in unknown samples was assessed relative to a calibrator (mean of one normal liver tissue in our assay) after normalization to β-actin copies.

Western blot analysis. Proteins were extracted from HCC tissue using the Whole Protein Extract kit (KeyGen, China), separated by 12% SDS-PAGE, and then blotted onto PVDF membrane (Millipore, USA). Membranes were incubated with rabbit polyclonal antibodies against human DPT (ProteinTech Group, USA) and mouse monoclonal antibody against human β-actin (ProteinTech Group), followed by horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse

antibodies. Proteins were visualized by the ECL system (KeyGen), followed by development against X-ray films. The data were analyzed by the Gray value ratios of DPT versus β-actin using BandScan v.5.0 software assay.

Immunohistochemical assay. HCC T and N tissue sections were probed with rabbit polyclonal antibodies against DPT, as described above, and rabbit polyclonal antibodies against TGF-β1 (Santa Cruz, USA), respectively. The assay was followed by development with the Rabbit Streptavidin-Peroxidase kit (Zymed, USA), and visualized with diaminobenzidine. Buffy staining of the cell membrane, cytoplasm, or nuclei was assessed as positive for each section. The expression of DPT and TGF-β1 was graded as negative (–) for ≤10% or positive (+) for >10% of the cells staining positively. Samples with light brownish-yellow cells indicated moderate immunoreactivity, while those with deep brownish-yellow cells denoted strong immunoreactivity [21].

Statistical analyses. The normalized data are presented as means ± SEM. Statistical analyses were performed using SPSS 11.1 for Windows software as follows: the *t*-test was used for paired samples; the χ^2-square test was used to determine differences between two groups. A *P* value less than 0.05 was considered as significant.

RESULTS

Expression of DPT gene in normal human tissues, HCC tissue, and HCC cell lines. RT-PCR assay indicated strong expression of DPT mRNA in fetal and adult liver, kidney, and spleen, weak expression in ovary and heart, and no expression in brain, breast, colon, lung, skeletal muscle, stomach, and testes samples (Fig. 1a). In 14 HCC cell lines, DPT mRNA was found to be absent in the RT-PCR assay (Fig. 1b). DPT mRNA could be observed in all N tissue obtained from HCC patients by obvious bands (100%), whereas strong bands in only four T samples were detected, faint bands in 11 corresponding T samples, and no bands in another 10 T samples (60%) (Fig. 1c). The findings suggested that DPT mRNA expression was significantly lower in T tissue than in N tissue ($P = 0.002$). This result was further confirmed by the RQ-PCR assay (Fig. 2, a-d). The log cDNA dilution versus ΔCt was plotted. The absolute value of the slope was 0.036 (<0.1) (Fig. 2e), reflecting the valid comparison of DPT and β-actin genes. The relative average $2^{-\Delta\Delta Ct}$ was as follows: 0.16 ± 0.03 in N tissue and 0.03 ± 0.02 in T tissue. The mRNA level of DPT in T tissue was significantly reduced (to 18.8%) compared with that in N tissue ($P = 0.003$) (Fig. 2f).

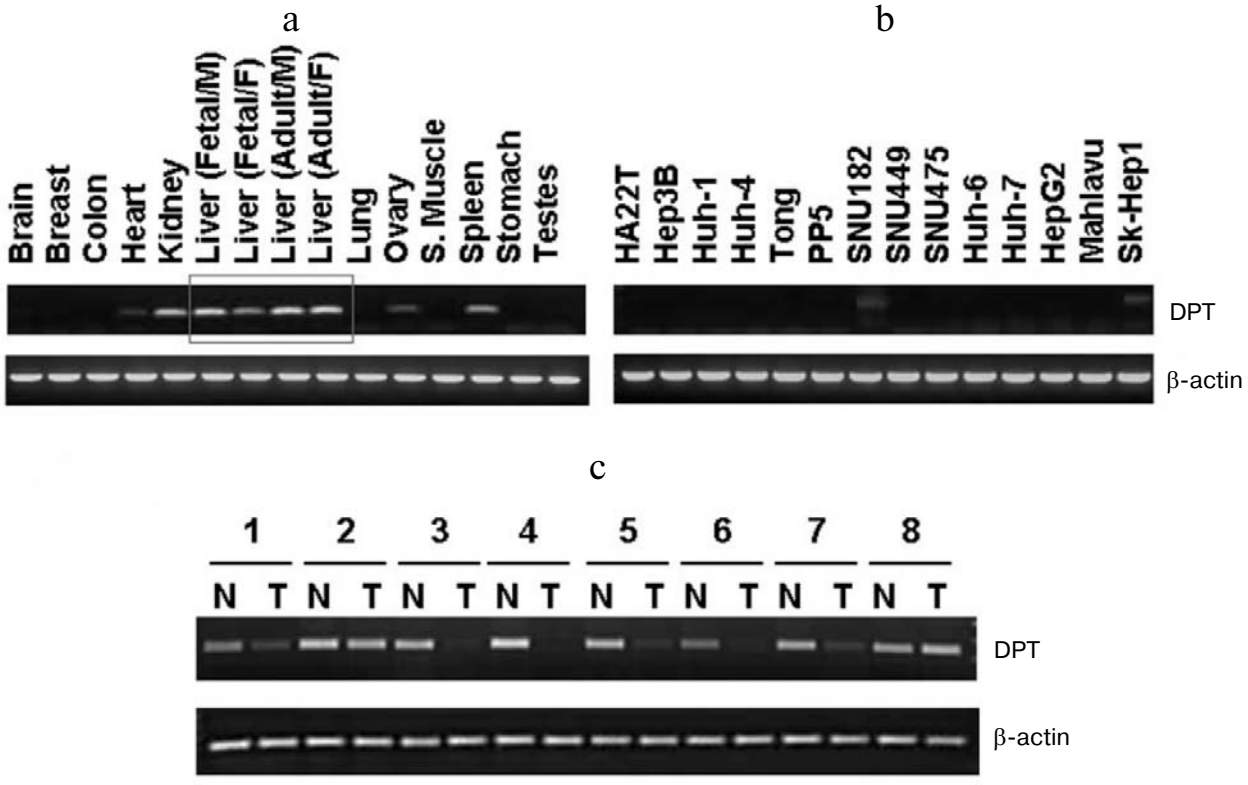

Fig. 1. DPT mRNA expression in representative normal tissues, HCC tissues, and its cell lines by RT-PCR. a) DPT gene was strongly expressed in fetal and adult liver, kidney, and spleen, weakly in ovary and heart, and was not expressed in brain, breast, colon, lung, skeletal muscle, stomach, and testes samples. b) Fourteen of the HCC cell lines tested appeared to lack DPT mRNA. c) DPT mRNA expression was obviously identified in non-tumor (N), but a drastic decrease in expression was detected in the corresponding tumor (T) ($P < 0.05$).

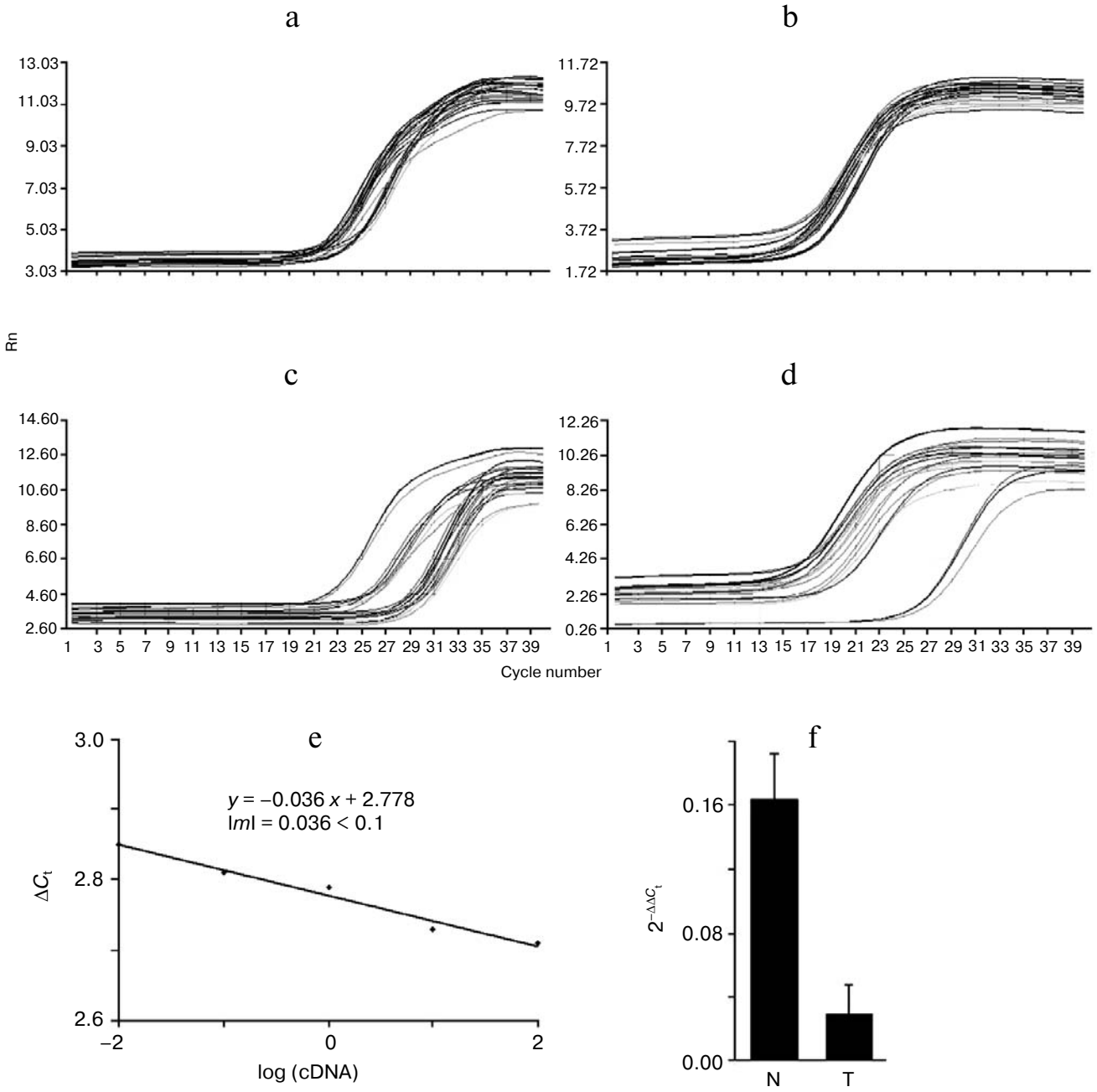

Fig. 2. DPT mRNA expression in representative HCC tissues by real-time PCR. Amplification plots of DPT and β-actin in non-tumor (N) tissues (a, b) and in tumor (T) tissues (c, d). e) Relative PCR efficiency of DPT. The log cDNA dilution versus ΔCt was plotted. The absolute value of this slope (*m*) was calculated as 0.036 (<0.1), reflecting the optimal relative efficiency. f) Relative DPT expression. The mRNA level of DPT in T was significantly decreased compared with that in N ($P < 0.05$).

Expression of DPT protein in HCC tissue. Western blot analysis showed that DPT-specific bands could be clearly visualized at 22 kDa in all N samples (100%), whereas only very weak bands were observed in 14 corresponding tumors and no signal was detected in another 11 tumors (56%) (Fig. 3a). The averages of the Gray value ratios (DPT/β-actin) were as follows: 0.2 ± 0.03 in N tissue and 0.05 ± 0.01 in T tissue ($P = 0.003$). DPT protein was downregulated to approximately 25% in T tissue (Fig. 3b). Furthermore, immunohistochemical assay revealed that moderate and strong immunoreactivity against DPT was detected in 92% (23 of 25) of N samples, two negative samples of which may be due to low level of DPT so that immunohistochemical assays could not detect. Forty eight percent (12 of 25) of T samples displayed positive staining, revealing significant differences in the DPT pro-

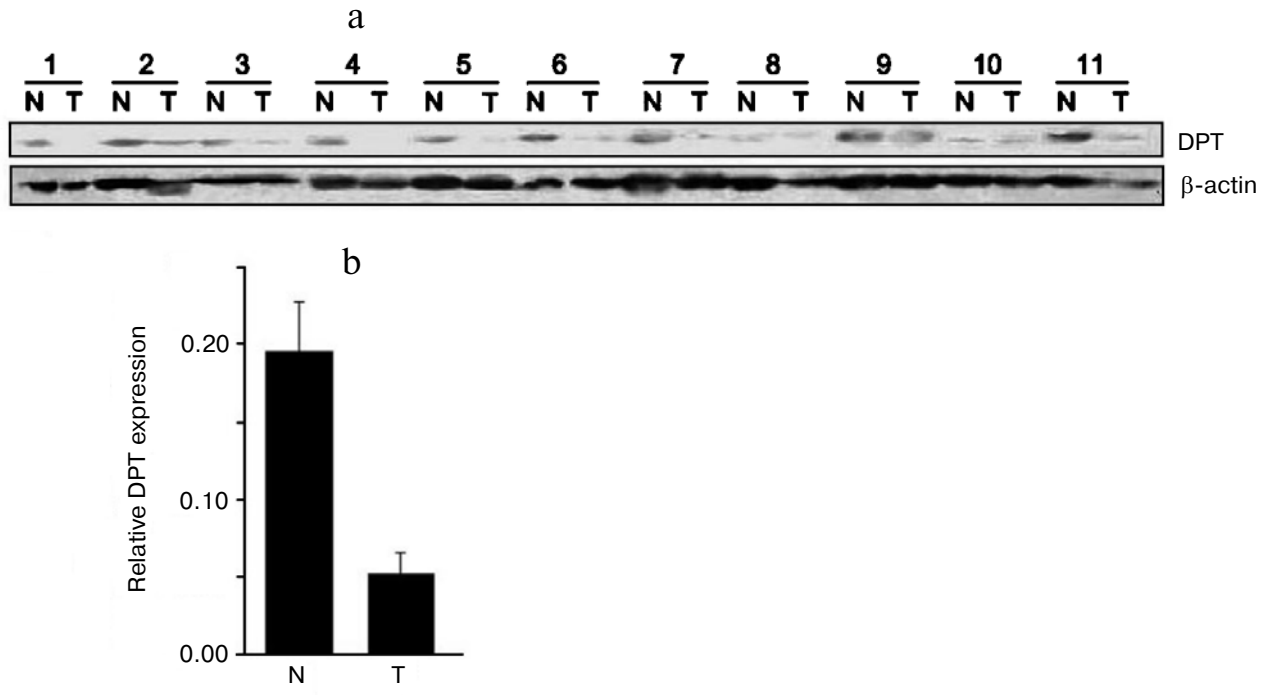

Fig. 3. DPT protein expression in matched non-tumor (N)/tumor (T) tissues from representative HCC patients by Western blotting. a) Extracted proteins were probed with antibodies specific for DPT and β-actin. b) BandScanning assay indicated that the DPT protein level was clearly downregulated in T compared with that in N ($P < 0.05$), normalized to β-actin.

tein expression between T and N tissue. DPT staining in T tissue was less intense than in N tissue (Fig. 4, a and b; see color insert). The staining appeared diffusely in the cytoplasm of several cell types, presenting morphology similar to parenchymal hepatocytes and Disse space cells including HSCs, KCs, and SECs in N tissue (Fig. 4a), as well as in many tumor cells (Fig. 4b). These findings suggested that DPT protein was weakly expressed in HCC tumor compared with non-tumor.

Expression of TGF-β1 protein in HCC tissues. The immunoreactivity of TGF-β1 showed a moderate and strong positive signal in 28% (7 of 25) of N samples and in 720% (18 of 25) of T samples, consistent with previous studies [3, 21]. TGF-β1 protein was detected in the cytoplasm and nuclei of some cells presenting morphology similar to hepatocytes and KCs. Its staining in the nuclear may be caused by nonspecific nuclear staining. This factor was also visualized in the cells similar to HSCs in N tissue, as well as in many tumor cells and in the extensive extracellular matrix of tumor (Fig. 4, c and d). Given the reported interaction between TGF-β1 and DPT [13, 25], TGF-β1 might be related with the possible roles of DPT in HCC.

DISCUSSION

In previous studies, it has been indicated that DPT is involved in anti-proliferation and cell differentiation [14-16]. To our knowledge, there is poor understanding of DPT expression in the liver and its relationship with HCC. In the present study, we first observed strong DPT mRNA expression in normal fetal and adult liver, kidney, and spleen, weak expression in ovary and heart, and no expression in brain, breast, colon, lung, ovary, skeletal muscle, and testes. Then our study indicated that DPT protein was present in the liver while it was weakly expressed in HCC T tissue. However, these results were not fully consistent with previous findings demonstrating low DPT mRNA level in human liver, strongest in human heart, and intermediate in lung and kidney as detected using the probe that was designed according to the bovine protein [20]. DPT could also not be detected by rabbit antiserum against tyrosine-rich acidic matrix protein (TRAMP) that is a porcine form of DPT in the porcine, mouse, and human liver in early study [6, 19]. The reason for these discrepancies is unclear but may be due to multiple factors, such as different individuals, ethnic groups, tissue areas used for specimen preparation, detection antibodies against different species DPT immunogens, as well as species variation. The role of DPT gene as a downstream target for the vitamin D receptor, which is an osteogenesis transcription factor in the differentiation of multipotent stromal cells from bone marrow to osteogenic lineage, was reported. The siRNA to DPT inhibited the differentiation of multipotent stromal cells from bone marrow to osteogenic lineage [16]. Over-expression of

vitamin D receptor in MG-63 osteosarcoma cells induced the expression of DPT [16]. Therefore, the strong expression of DPT gene in human fetal and adult liver might imply that it is related to liver development and play some potential role in the liver. Further study will be needed to determine whether the role of DPT is related to vitamin D receptor. The significant downregulation of DPT in HCC and its mRNA lacking in 14 HCC cell lines implicated its possible association with the progression of HCC. Since the Western blot and RT-PCR assays cannot differentiate the cell origin in liver tissue, it is possible that the higher level of DPT expression in N tissue is mainly from the cells of the Disse space, but not from hepatocytes. However, given the role of its murine counterpart in early quiescence-1 gene in inducing cell quiescence [14] and the important roles Disse space cells play in hepatic immune defense, relatively weak expression of DPT protein in T tissue may be a factor related to hepatocarcinogenesis and its pathological progression in the following ways: attenuating its possible anti-proliferation activity [14]; losing its potential cytotoxicity [15]; blocking its possible vitamin D receptor-associated pathway of anti-proliferation [16]. DPT staining appeared mainly in the cytoplasm of several cell types presenting morphology similar to a few parenchymal hepatocytes, HSCs, KCs, and SECs. However, it has been identified extensively in the periphery of collagen fibers and endothelial cells but absent in fibroblasts of the skin [6, 7]. This disaccord of DPT location in different tissues suggested that it might play various roles. It is worthwhile to further study whether the staining protein in hepatic cells is an acute immune response molecule in response to the microbial challenge, as reported previously in amphioxus [28], or has similar cytotoxic effect to MCTx-1 [15]. Additionally, the anti-proliferative effect of 1,25-dihydroxy-vitamin D_3 on hepatoma via vitamin D receptor may be related to DPT, a known downstream target for vitamin D receptor [16], because some hepatic cell populations including hepatocytes, SECs, KCs, and HSCs express nuclear vitamin D receptor and are targets of vitamin D endocrine/paracrine/intracrine system [18]. It is also important to further study whether DPT is related to the auto-regulation of hepatic cell proliferation as its murine counterpart early quiescence-1 gene inducing cell quiescence [14]. Taken together, DPT might be a key molecule in immune system defense against infection and tumor in the liver. TGF-β1 has been indicated to be closely associated with the invasion and migration of HCC cells [3, 4, 21, 22]. DPT can enhance the growth inhibition effects of TGF-β1 in epithelial cells and TGF-β1 can also increase DPT expression in skin fibroblasts [13, 25]. The present study suggested TGF-β1 expression in the extensive T tissue, which may affect the roles of DPT in HCC. Their interaction remains to be investigated.

The mechanism of DPT downexpression in HCC and its definite roles in the liver remain incomprehensible from our present study. Therefore, further study of the interaction mechanism of DPT with TGF-β1 and other potential molecules *in vitro* and in combination with clinical pathological features will be helpful to understand these unknown areas of DPT. In conclusion, the DPT gene was strongly expressed in human fetal and adult liver, while it was significantly downregulated in HCC tissues. Its protein was weakly expressed in HCC tumor compared with non-tumor. This disaccord of DPT location in different tissues suggested it might play various roles. DPT might be related to the liver development, carcinogenesis, and the progression of HCC via possible interaction with TGF-β1 and other possible mechanisms.

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