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• BASIC RESEARCH •

In situ detection of TGF betas, TGF beta receptor II mRNA and telomerase activity in rat cholangiocarcinogenesis

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Abstract

AIM: Initial report on the *in situ* examination of the mRNA expression of transforming growth factor betas (TGFβs), TGFβ type II receptor (TβRII) and telomerase activity in the experimental rat liver tissue during cholangiocarcinogenesis.

METHODS: Rat liver cholangiocarcinogenesis was induced by 3'-methyl 4-dimethylazobenzene (3'Me-DAB). *In situ* hybridization was used to examine the TGFβs) and TGFβ type II receptor (TβRII) mRNA, *in situ* TRAP was used to check the telomerase activity in the tissue samples.

RESULTS: There was no TGFβs, TβRII mRNA expression or telomerase activity in the control rat cholangiocytes. The expression of TGFβ1, TβRII was increased in regenerative, hyperplastic, dysplastic cholangiocytes and cholangiocarcinoma (CC) cells. The expression of TGFβ2 mRNA was observed in only a part of hyperplastic, dysplastic cholangiocytes. TGFβ3 expression was very weak, only in hyperplastic lesion. There was positive telomerase activity in the regenerative, hyperplastic, dysplastic cholangiocytes, and CC cells. Stroma fibroblasts of these lesions also showed positive TGFβs, TβRII mRNA expression and telomerase activity.

CONCLUSION: There were TGFβs, TβRII expression and telomerase activity in hyperplastic, dysplastic cholangiocytes, cholangiocarcinoma cells as well as in stroma fibroblasts during cholangiocarcinogenesis. Their expression or activity is important in cholangiocarcinogenesis andstroma formation.

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INTRODUCTION

Transforming growth factor beta (TGFβ)-TGFβ receptor (TβR) signaling system is important in growth regulating carcinogenesis and cancer progression[1,2]. Lacks of the expression of TGFβ and/or TβR, mutation of the related genes were reported in human and animal malignancies[3-5]. These abnormalities were considered to be the cause of interruption of the growth signal from the TGFβ to the cell nuclear, resulting in the uncontrolled growth of the involved cells. While there are reports on the efficacy of TGFβ which supported that TGFβ was involved in cancer invasion and metastasis[1, 2, 6].

Hepatocellular carcinoma (HC) and intrahepatic cholangiocarcinoma (CC) are two most common types of liver carcinoma[7, 8]. Though there are reports on their expression in HC[9, 10], few reports dealed with the expression of TGFβs and/or TβR in CC. Bile ducts had increased expression of TGFβ1 in inflammatory or obstructive lesions[11]. TGFβ1 was detected in small amount of cancer cells among 25 of 30 CC cases[12]. The expression and significance of TGFβs and their receptors during cholangiocarcino-genesis are poorly understood. Stroma fibrosis is one of the characteristics of CC[7, 8], but the mechanism of excessive stroma formation is not clear.

Telomerase is a key enzyme in the maintenance of the telomeric DNA length[13]. Telomerase is undetectable in most normal somatic tissues. Its activity was reported in most cancer cell lines as well as cancer tissues from human and experimental animals[13, 14]. mRNA of telomerase and telomerase-associated protein were detected in CC and its preneoplastic lesions[15]. It is not clear in which stage the telomerase is activated during cholangiocarcinogenesis.

Liver carcinogenesis was induced by feeding rats with 0.05 % 3'-methyl 4-dimethylazobenzene (3'Me-DAB) in maize flour. The model showed progressive changes from degeneration, necrosis, to cholangiocyte hyperplasia, dysplasia and CC[16]. We found obviously increased expression of TGFβ and TGFβ type II receptor (TβRII) mRNA and telomerase activity in the proliferative, dysplastic cholangiocytes, CC cells as well as stroma fibroblasts. Here we report these findings and discuss their significance.

MATERIALS AND METHODS

Animals and reagents

Male Wistar rats (*n*=100, weighing 65±10 g) and foods were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Science. All rats received humane care.

3' Me-DAB was purchased from the Tokyo Kasai Co. Ltd. (Tokyo, Japan Cat. 0207). DIG RNA labeling kit (Cat. No. 1175025), DIG nucleic acid detecting kit (Cat. No. 1175041), and Telomerase PCR ELISA kit (Cat. No. 1854666) were bought from Roche, Germany. Mouse anti-proliferating cell nuclear antigen (PCNA), goat anti-vimentin and biotinylated secondary antibodies were purchased from DAKO. ABC Kit was the product of Vector.

Alcian blue (8GS) was the product of Chroma. Sirius red (F3B) was from Sigma. All other reagents were of analytical or molecular biology research grade from Sigma, Merck or Shanghai Analytical Chemical Co.

Experimental design

The rats were divided into Experimental (*n*=60) and Control

(*n*=40) Groups randomly and fed with common compound food and tap water during the first week of adaptation. Maize flour containing 0.05 % 3' Me-DAB was prescribed to the Experimental Group rats for 12 weeks to induce liver cancer. The Control Group rats were fed with maize flour only for the 12-week-period. Common compound food was given to all rats after the period. The rats were sacrificed under anesthetization from 4-week to the end of 22-week since 3' Me-DAB feeding.

The liver tissues with macroscopic lesions were sampled. Samples from half of the lesions were fixed in 4 % buffered paraformaldehyde, embedded in paraffin for routine H.E, histochemical staining, immunohistochemistry and *in situ* hybridization. Samples from residual half of the lesions were embedded in OCT compounds, snap frozen, and cryostat section for histochemical staining and in situ TRAP reaction. H.E. alcian blue, PAS and sirius red staining were undertaken. The liver lesions were classified into not obvious, hyperplastic or cholangial proliferative, dysplastic proliferative foci and cancer[7, 8, 14]. The cholangial property of the cells in the lesion was confirmed by positive mucin staining with either serum albumin mRNA expression, or cytoplasmic glycogen.

In situ hybridization

Plasmids containing cDNAs of TGFβ1, 2, 3; TβRII and serum albumin (SA) were proliferated in *E. Coli*. The plasmids were extracted, purified and linearized with specified endonucleases (Table 1). Anti-sense and sense cRNAs were then made and labeled with digoxigenin *in vitro*[17].

Paraffin embedded tissue samples were sectioned (5 μm). The sections were deparaffinized in serial xylene and alcohol solvents, transferred into 100 mM PBS (pH7.4) and digested with proteinase K. The sections were pretreated with 4 % buffered paraformaldehyde, PBS, 200 mM HCl, 100 mM TEA-HCl buffer (pH8.0), 100 mM TEA· 0.25 % anhydrous acetate, PBS and further dehydrated with serial alcohol. Pre-warmed hybridization solution containing digoxigenin labeled probe was dropped on the pretreated sections, covered with parafilm and incubated in wet chamber for 15 hours at 50 ℃. After hybridization, the sections were washed in 5×SSC, 2×SSC with 50 % formamide and TNE solutions. Non-hybridized probe was digested with RNAse A. Digoxigenin labeled probe was detected with alkaline phosphatase labeled anti-digoxigenin antibody and visualized with NBT-BCIP substrate[17, 18]. Some of the sections were further counterstained with eosin, alcian blue, and /or hematoxylin.

Addition of SA anti-sense probe was used as positive control, sense probes were used as negative controls.

Table 1 Probes and plasmids

Probe	Vector	Endonuclease and promotor cRNA (-)		cRNA (+)		Length of cDNA
TβRII	pBluescript II KS(-)	EcoRI	T3	Hind III	T7	485bp
TGFβ1	pBluescript II KS(-)	XhoI	T3	Hind III	T7	400bp
TGFβ2	pGEM 3ZF(-)	HindIII	T7	EcoRI	SP6	500bp
TGFβ3	pGEM 3ZF(-)	BamHI	T7	EcoRI	SP6	280bp
SA	pBluescript II KS(-)	HindIII	T3	EcoRI	T7	620bp

In situ TRAP

Liver tissues embedded in O.C.T. compounds were sectioned (10 μm), air-dried shortly for further processing. The *in situ* TRAP was performed as reported[14, 19]. Briefly, the elongation and PCR mixture was dropped onto cryostat sections and incubated in wet chamber for 30 min at 30 ℃. Telomerase was inactivated at 94 ℃ for 5 min. The elongated telomere sequence was amplified within GeneAmp *in situ* PCR System 1000 (Perkin-Elmer Co. Foster City, CA 94404) for 30 cycles. Each cycle included: 94 ℃ for 30 sec, 50 ℃ for 30 sec, 72 ℃ for 20 sec. Last cycle was followed by 72 ℃ for 10 min. The sections were then washed with washing buffer and fixed with 4 % buffered paraformaldehyde.

The sections were further treated with digoxigenin labeled probes, peroxidase labeled anti-digoxigenin antibody and coloration substrate to show the products of amplification. The reaction products were directly photographed before the addition of stop solution. Negative controls included: elongation after inactivation of telomerase, no probe, no antibody or substrate only control.

Immunohistochemical and histochemical reactions

Paraffin sections were routinely deparaffinized and transferred to PBS. PCNA affinity to antibody was recovered by microwave oven treatment of the sections in 10mM TAE. Immunohistochemical detection of PCNA and vimentin was performed following routine procedure[20].

Alcian blue and sirius red staining were undertaken on paraffin sections. PAS staining was carried out on paraffin as well as frozen sections.

The experiment was undertaken on at least 6 rats from different period of carcinogenesis with lesions of regeneration, hyperplasia, dysplasia and carcinoma foci separately. The experiments on the same sample were duplicated to ensure the results.

RESULTS

The Control Group rats showed no obvious pathologic changes. There was no detectable expression of TGFβ1, 2, 3, TβRII mRNA in the cholangiocytes and bile duct cells from the control rat liver. There was a zonal expression of SA in hepatocytes, stronger at zone 1 and weaker at zone 3. Neither telomerase activity, nor PCNA reaction was detected in the cholangiocytes and bile duct cells. The stellate cells of the sinus were positive to vimentin.

There were successive histologial changes in the liver tissue samples in Experimental Group rats: from degeneration and necrosis, regeneration and proliferation, hyperplasia and dysplasia, to carcinoma.

At the early stage, there were massive degeneration and necrosis of the liver tissue samples. No TGFβ1, 2, 3, TβRII expression or telomerase were detected in the degenerative and necrotic liver tissue samples.

Later, regeneration and proliferation of cholangiocytes and hepatocytes were observed. Early in the regenerative and proliferative lesion, there were epithelial cells with edematous stroma. The epithelial cells were scattered in small clusters or forming cell cords, sometimes with lumen in the cords. When the cells differentiate toward cholangiocytes, the cytoplasm of the cells became basophilic without SA or glycogen. There was mucus accumulation in the cytoplasm or in some of the lumens. These cells showed positive TGFβ1 mRNA expression (Figure 1-2). Telomerase activity and PCNA positive nucleus appeared in these epithelial cells.

The proliferation of cholangiocytes continued but the edema of the stroma reduced with time, while vimentin positive fibroblast proliferation appeared in stroma followed by deposition of collagen. At this stage, the cholangiocytes and fibroblasts expressed high level of TGFβ1, 2 and TβRII mRNA (Figure 3-4). These cells were also positive to PCNA and telomerase reactions (Figure 7). TGFβ3 can also be detected transiently in some cholangiocytes. The lesion developed into cholangiocyte hyperplasia with stroma fibrosis (Figure 4, 7).

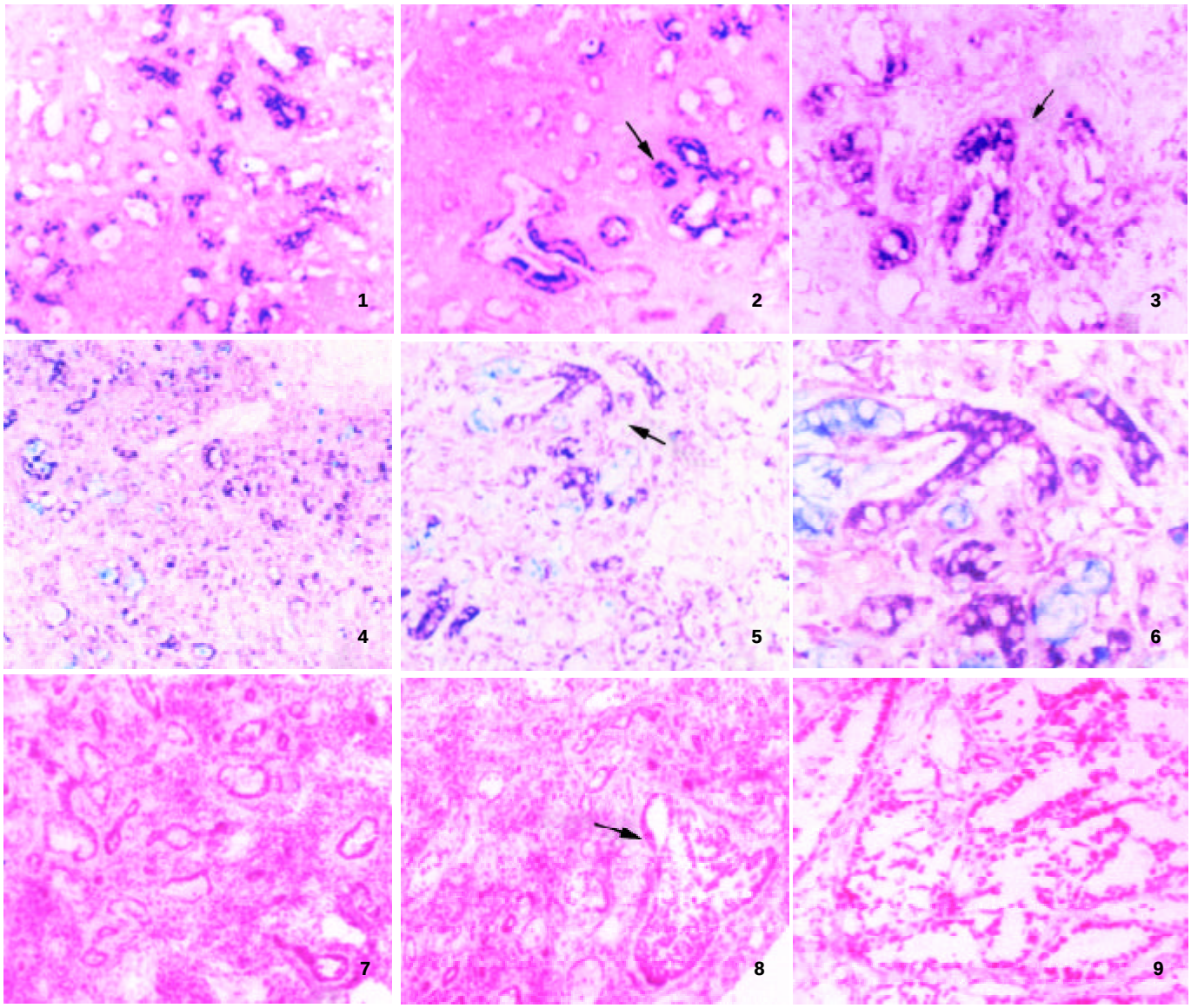

Figure 1-6 *In situ* hybridization showed the mRNA localization (NBT-BCIP purple colored) of TGFβ1 (Figure1, 2), TGFβ2 (Figure 3), TβR II (Figure 4-6). Figure 1-3 counterstained with eosin; Figure 4-6 counterstained with alcian blue and eosin.
Figure 7-9 *In situ* TRAP shows telomerase activity of the hyperplastic (Figure 7), dysplastic cholangiocytes (Figure 8) and cholangiocarcinoma (Figure 9) in rat liver tissue. Hyperplastic epithelial cells in the stroma form clusters, cords, and ducts (Figure 1, 4, 7). There was mucin formation in some of the ducts (Figure 4) indicating they were cholangiocytes. The hyperplastic cholangiocytes showed TGFβ1, TβR II mRNA expression. (Figure 1, 4), There was telomerase activity in their nuclei (Figure 7). Proliferative cholangiocytes forming duct structures. Some of them had structural and cellular atypia indicating cholangial dysplasia or cholangiocarcinoma in hyperplastic lesion (Figure 2, 3, 5, 6, 8). The expression of TGFβ1, 2, (Figure 2, 3), TβR II (Figure 4-6) and mucin formation in the hyperplastic and dysplastic ducts were uneven (Figure 2-3, 5-6). In Cholangial dysplasia foci and cholangiocarcinoma, there was telomerase activity in the nuclei of cancer cells and stroma fibroblasts (Figure 8-9). (Figure 1, 4, 7, 8×100; Figure 2, 3, 5, 9×200; Figure 6×400).

Later, the cholangiocytes in some areas disappeared with the maturation of fibroblasts to fibrocytes and increased deposition of collagen forming a "burnt-out" picture.

In other areas, the cholangiocytes kept growing with atypical cell morphology, forming irregular cellular clusters, and abortive tubular or glandular structures, indicating cholangiocyte dysplasia. Some of them may accompany with CC. The dysplasia was first found in the liver tissues after 12 weeks of 3' Me-DAB treatment. Small foci of CC appeared in the liver lesion at the 16th to 20th week of experiment.

There was mucin in the cytoplasm of the dysplastic cholangiocytes, CC cells or in the lumen formed in the cell clusters. The expressions of TGFβ1 and TβRII mRNA in the dysplastic cholangiocytes and CC cells differed greatly from negative to strong positive among different cells and different cell clusters (Figure 2, 5, 6). TGFβ2 mRNA expression was also uneven in the dysplastic lesions (Figure 3). TGFβ3 expression was undetectable. Most of the dysplastic cholangiocytes and cancer cells showed telomerase activity (Figure 8-9). Strong PCNA positive reaction was observed in the hyperplastic, dysplastic cholangiocytes and CC. The stroma was abundant with proliferative fibroblasts (PCNA and vimentin positive) and collagen deposition. The fibroblasts had positive TGFβ1, TbRII mRNA expression and telomerase activity.

DISCUSSION

The liver tissues from our carcinogenesis model had lesions from cholangiocyte hyperplasia, dysplasia to CC with positive mucin staining with neither albumin mRNA, nor glucagon in the cytoplasm.

TGFβ is well known for its effects on fibroblasts which can induce formation of stroma[1, 2, 21]. But there is no report on the expression of TβR during experimental cholangiocarcinogenesis. We observed the expression of TGFβ1 and TβR II expression in the fibroblasts of regenerative, dysplastic cholangiocyte lesions and in CC. There was increased fibrous stroma formation around the fibroblasts and fibrocytes. These results supported the function of TGFβ-TβR II system in the excessive stroma formation in these lesions.

Present experiment showed that there was no TGFβ1, 2, 3 and TβRII expression in normal bile duct cells. TGFβ1, 2, 3 and TβRII mRNA expression was detected in the repairing and proliferative cholangiocytes. In the dysplastic cholangiocytes and CC cells, their expression varied from negative to strong positive. TGFβ1 protein was also detected in experimental rat and human CC cells[21-23]. TGFβ1 can suppress the proliferation of epithelium, prevent epithelial carcinogenesis[1, 2]. On the other hand, there are reports that TGFβ can not inhibit the cancer growth or even accelerate the cancer invasion[2, 6]. TGFβ can suppress the growth of the normal bile duct cell but not the CC cells[12]. Transgenic mouse with TGFβ1 over expression accelerates hepatocarcinogenesis[24]. Dominant-negative TβR II mice had accelerated carcinogenesis[25]. Our results showed that TGFβ and TβRII expression accompanied with the cholangiocarcinogenesis procedure.

Cancer progress is related to the reaction between cancer cells and its stroma[26]. Treatment of Ras-transformed mammary epithelial cells with TGF-beta results in resistant to growth inhibition by TGF-beta. These cells start to secrete TGF-beta, leading to maintenance of the invasive phenotype. The action is dependent on epithelial-stromal interaction[27]. Our results showed that there was TGFβ1 and TβRII expression in the dysplastic cholangiocytes, CC cells and stroma fibroblasts. Thus, the paracrine and autocrine functions of TGFβ1 are important in supporting the process of cholangiocarcinogenesis.

The expression of TGFβ2 mRNA was only detected in part of hyperplastic, dysplastic cholangiocytes. TGFβ3 mRNA was only weakly positive in some hyperplastic cholangiocytes. There is few reports on the expression of TGFβ2, 3 mRNA in the process of cholangiocarcinogenesis. Their role may be transient.

Phase of telomerase activation during cholangiocarcinogenesis is not specified. Present experiment showed that normal bile duct cells were telomerase negative. There was telomerase activity in the regenerative, hyperplastic, and dysplastic cholangiocytes as well as CC cells. The activation of telomerase occurred in the early stage of cholangio-carcinogenesis. There were also reports on the positive hTR and TP1 mRNA expression in intrahepatic biliary dysplasia[28]. Increased telomerase activity was reported in dysplastic hepatocytes during hepatocellular carcinogenesis[29].

The expression of TERT can induce resistance to TGFβ growth inhibition[30]. This may be another reason for the hyperplastic, dysplastic cholangiocytes and CC cells escaping from TGFβ-TβR growth suppression in our cholangiocarcinogensis model.

The telomerase activity is a marker of immortalized or malignant cells[13,14, 31]. In present experiment, telomerase was positive in the proliferating cells no matter they were parenchyma or stroma cells. The phenomenon was observed in other liver proliferative lesions[14, 32]. So that telomerase activation was also a good marker of cell in proliferation.

In summary, this is the first report on the *in situ* detection of TGFβ1, 2, 3, TβRII mRNA and telomerase activity during rat cholangiocarcinogenesis. There is TGFβ1, 2, 3, TβRII mRNA and telomerase activity in the hyperplastic, dysplastic cholangiocytes, CC cells as well as stroma fibroblasts. There is gradual increase of the fibrous stroma (fibrosis) during the development of CC. It is considered that the expression of TGFβ1, 2, 3, TβRII and telomerase activation has important implication in cholangiocarcino-genesis and cancer stroma formation.

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