

Alteration in transforming growth factor- β receptor expression in gallbladder disease: implications of chronic cholelithiasis and chronic *Salmonella typhi* infection

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Abstract

Gallbladder cancer prevalence is ever increasing with *Salmonella typhi* chronic infection being one of the predisposing factors. Altered ratios or expression of transforming growth factor- β (TGF- β) receptors and changes in its function are associated with loss in anti-proliferative effects of TGF- β and cancer progression. Using reverse transcriptase polymerase chain reaction we monitor any changes in TGF- β receptor gene expression. We simultaneously screen for *S. typhi* within the samples. From 73 patients undergoing cholecystectomy 39-50% had significant expression ($P < 0.05$) of TGF- β receptor (T β R)-I and T β R-II during chronic cholelithiasis as compared to the remaining 19-23% with acute chronic cholelithiasis. There was no significant increase in T β R-III receptor expression. Patient's positive for *S. typhi* (7/73) did not show any significant changes in expression of these receptors, thus indicating no direct relation in regulating the host TGF β -signaling pathway. Further analysis on expression of downstream Smad components revealed that patients with up-regulated TGF β receptor expression show >2-fold increase in the R-Smads and Co-Smads with a >2-fold decrease in I-Smads. Thus gain of T β R-I and II expression in epithelial cells of the gallbladder was associated with chronic inflammatory stages of the gallbladder disease.

Introduction

Transforming growth factor- β (TGF- β) plays a complex role in adult tissue homeostasis and embryonic development.^{1,2} TGF- β is also shown to have tumor suppressor and malignant activities and plays a complex role during carcinogenesis.³ The TGF- β ligands bind to the type TGF- β receptors which are a group of ser-

ine/threonine kinases that induce the downstream Smad and other signaling pathways. The binding of the ligand to type II receptor recruits the type I receptor to form a ternary complex, indicating the importance of both receptors in signal transduction. The receptor-regulated Smads (R-Smads) are phosphorylated by type I receptors and oligomerize with Smad4 (Co-Smad) which then translocate to the nucleus to regulate a variety of gene expressions. On a contrary, the inhibitory Smads block R-Smad phosphorylation and induce receptor dephosphorylation.^{2,4,5} The TGF- β signal transduction pathways play a major role in human diseases.⁶ Altered ratios or expression of TGF- β type I and II receptors and changes in its function are associated with loss in anti-proliferative effects of TGF- β and cancer progression.⁷⁻⁹ Various factors affect the TGF- β signaling pathway that includes disease condition, infection, genetic pre-disposition, etc. thus making it a strong marker of prognostic significance.¹²

Chronic cholelithiasis is a frequently encountered gallbladder disease being highly prevalent in Northern region of India.¹⁰⁻¹² The very fact that presence of such gallstones bring about alterations in molecular and histopathological characters in the gallbladder tissue, makes it a strong predisposing factor for development of gallbladder cancer.¹³ Also existence of *S. typhi* within gallbladder is considered to be a pre-disposing factor for gallbladder cancer.^{14,15} Thus in our study we try to reveal if the presence of *S. typhi* within gallbladder of patients could possible bring about a significant change in expression of TGF- β signaling pathway, making it a possible factor for progression towards gallbladder cancer.

Materials and Methods

Clinical specimen and sample collection

Human gallbladder tissue, bile and gallstones were obtained following laparoscopic cholecystectomy of patients suffering from chronic cholelithiasis at RG stone hospital and SMRC Hospital (Goa, India). For normal gallbladder specimens, tissue was obtained from dead bodies at Department of Forensic Science-Goa Medical College and Hospital (Goa, India). The study was approved by the institutional ethics committee. A small portion (~100 mg) of the tissue was cut and submerged in RNAlater® (Life technologies, USA) solution, and remaining of the freshly excised gallbladder tissue was immediately transported to the laboratory in sterile DMEM medium at 4°C for epithelial cell isolation.

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Histopathological classification of gallbladder tissue

A section of the tissue sample collected in RNAlater® was used for histological analysis as RNAlater® preserves the tissue histology. Transverse sections of the wall including mucosa were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) using routine histological procedures.

Detection of *S. typhi* DNA using PCR assay

All the clinical specimens (gallbladder, bile and gallstone) obtained were subjected to PCR assay for detection of *S. Typhi* specific gene fragment as described in our earlier work.¹⁶ The DNA was extracted from 25-30 mg of sample using the NucleoSpin® Tissue (Macherey-Nagel-GmbH & Co. KG, Duren, Germany) Kit as per manufacturer's instructions. The template DNA was subjected to PCR amplification using primers specific to H-1d region of flagellin gene fragment of *S. typhi* (Table 1). The expected 181bp fragment was visualized using

1.5% agarose gel. The fragment was eluted using QiaQuick Gel Extraction Kit (Qiagen), cloned in pGEM-T Easy vector system (Promega) and sequenced to confirm desired gene amplification.

Isolation of gallbladder epithelial cells

The isolation of gallbladder epithelial cells was performed as described by Miquel and colleagues with few modifications.¹⁷ Briefly, the isolation was carried out within 30-45 minutes post-surgery. The mucosa was rinsed carefully with DMEM to remove any adherent mucous and bile. The tunica mucosa was placed in 0.125% collagenase solution prepared in DMEM for 15 minutes at 37°C. The mucosa was abraded every 5 minutes using a scalpel and flushed with DMEM. The resulting cell suspension was immediately subjected to centrifugation at 1000 rpm for five minutes. An aliquot from the cells obtained was used for characterization using immunostaining by cytokeratin 19 antibodies, and remaining epithelial cells were used for further expression analysis.

RNA isolation and reverse transcriptase PCR

Total RNA was extracted with RiboZol™ (Amresco, USA) and quantified using NanoDrop Lite spectrophotometer (Thermo Scientific, India). Reverse-Transcriptase PCR was carried out using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, India) and oligo(dT)¹⁸ primer as per the manufacturer's protocol. For every reaction of cDNA synthesis 1µg of purified RNA was used followed by PCR amplification using specific primers to candidate genes listed in Table 1 within the linear range of amplification (35 cycles). A 10 µL aliquot of the amplified product was loaded on 1.6% agarose gel and bands obtained were analyzed using ImageJ 1.46r software. The normalized gene expression with a >1.5 fold increase and a P<0.01 was considered to be significant.

Statistical analysis

All the values obtained are representative of at least three independent replicates with the standard mean, standard deviation, and standard error values. The statistical significance for independent variables was determined using the chi-square test with P<0.05 considered to be significant.

Results

Classification of gallbladder tissue samples and isolating gallbladder epithelial cells: Human gallbladders were obtained from

patients with gallstone disease after cholecystectomy, followed by a routine H&E staining that showed varying degree of chronic cholelithiasis (Figure 1c) with cholecystitis depending on the stage in which the patients were operated. In order to understand TGF-β receptor expression specifically within the gallbladder epithelium, gallbladder epithelial cells were isolated from freshly excised human gallbladder tissue (Figure 1a) and immunos-

tained using CK19 antibody (Figure 1b) which demonstrated an isolation efficiency of 85-90%. The cells obtained were used for harvesting mRNA so as to determine changes in expression of desired components.

Alteration in TGF-β receptor expression during chronic cholelithiasis: From 73 patients undergoing cholecystectomy, 39/50 showed significant over-expression (P<0.05) of TGF-β receptor (TβR)-I and TβR-II during chronic

Table 1. Primer pairs used for this study.

Gene	Primer pairs	Product size (bp)	
<i>H-1d</i>	LP	TCGTTTGAGGATAAAAAACGGTA	181
	RP	CAGTTTGAGCAACGCCAGTA	
<i>TβR I</i>	LP	GATGGGCTCTGCTTTGTCTC	214
	RP	CAAGGCCAGGTGATGACTTT	
<i>TβR II</i>	LP	GGGAAACAATACTGGCTGA	198
	RP	GAGCTCTTGAGGTCCCTGTG	
<i>TβR III</i>	LP	CCAAGATGAATGGCACACAC	151
	RP	CCATCTGGCCAACCACTACT	
<i>Smad 2</i>	LP	CGAAATGCCACGGTAGAAAT	223
	RP	CCAGAAGAGCAGCAAATTC	
<i>Smad 3</i>	LP	CCCCAGAGCAATATTCCAGA	173
	RP	GGCTCGCAGTAGGTAACGG	
<i>Smad 4</i>	LP	TCACAATGAGCTTGCAATCC	247
	RP	ACCTTTGCCATATGTGCAACC	
<i>Smad 7</i>	LP	GGGTCTAGCTCTGGCTGTG	358
	RP	CAGGCTCTTACAGCCCTTTG	

TβR, transforming growth factor-β receptor.

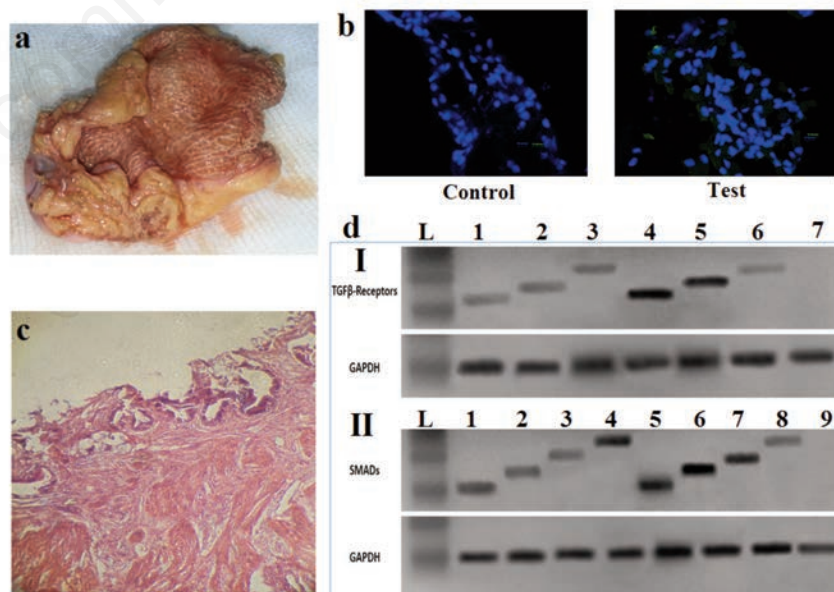


Figure 1. Expression of transforming growth factor-β (TGF-β)/Smad's in gallbladder epithelium. a) Gallbladder obtained following cholecystectomy. **b)** gallbladder epithelial cells isolated from gallbladder tissue *in vitro*. **c)** Histopathological characterization of gallbladder tissue obtained from patients. **d)** Reverse transcriptase polymerase chain reaction assay for detection of changes in candidate gene expression. L: ladder; 1d (I): Lane 1, 2, 3 and Lane 4, 5, 6 represent TGF-β receptor I, II, III respectively for d (I); 1d (II): Lane 1, 2, 3, 4 and Lane 5, 6, 7, 8 represent SMAD 2, 3, 4 and 7 respectively.

cholelithiasis as compared to 19/23 with acute chronic cholelithiasis (Figure 2a, b, c). Overall 79.45% of the patients had up-regulated (>1.5 fold increase) TGF- β receptor I and II expression in their gallbladder epithelium. There was no significant increase in T β R-III receptor expression observed in epithelium of any patients.

Effect of chronic *S. Typhi* persistence on TGF- β receptor signaling in gallbladder epithelium: Upon molecular screening of clinical samples (gallbladder tissue, bile and gallstones) 9.59% (7/73) of the patients showed positive results for the presence of *S. Typhi* DNA within the gallbladder of patients suffering from gallbladder disease. Interestingly, none of these patients showed any significant effect on the TGF- β receptor gene expression with similar fold changes as seen in patients without any chronic *S. Typhi* infection.

Regulation of the downstream-Smad signaling axis: It was observed that patients with up-regulated levels of TGF β receptors I and II had significantly altered expression levels of downstream Smad components. This includes the R-Smad's (Smad-2 and 3), Co-Smad (Smad 4) and the I-Smad (Smad 7). The R-and Co-Smads had lower expression levels in normal samples with higher levels of the I-Smads. In case of patients suffering from gallbladder disease, we observe that there was a nearly 2-fold increase in the expression of TGF- β receptor I and II with a ~2-fold decrease in the TGF- β receptor III (Figure 2d). Thus during chronic

cholelithiasis the gallbladder epithelium shows activation of the Smad-dependent TGF- β signalling axis.

Discussion

The TGF- β growth factor regulates growth and differentiation in various cell types and tissues, and plays a major role in human diseases. They are shown to play a dual role of tumor suppression and cancer metastasis.^{2,3} These primarily function via the TGF- β receptors and thus any alterations in the TGF- β receptors, may it be epigenetic changes or genetic modification, alters the TFG- β signaling axis.^{7,8} Thus monitoring expression of these receptors may be of prognostic significance during progression towards cancer.^{18,19} In this study we try to analyze the expression levels of these receptors in early stages of gallbladder disease and even reveal the alteration in expression of the downstream Smad signaling components. We also see if the present of chronically persisting *typhi* within the gallbladder could significantly bring about any changes in these components of the signal transduction pathway. As observed, *S. S. typhi* did not significantly alter the receptor expression or the Smad components indicating that the fold changes were due to the diseased state of the gallbladder. Though, *S. typhi* is shown to persist within the gallbladder even in earlier

literature,¹⁴⁻¹⁶ its role in significantly regulating the host to progress towards cancer needs to be understood. Thus gain of TGF- β receptor I and II expression in epithelial cells of the gallbladder was associated with chronic inflammatory stages of the gallbladder disease.

Conclusions

Both chronic cholelithiasis and *S. typhi* are considered to be pre-disposing factors for gallbladder cancers. We conclude from our study that chronic cholelithiasis is majorly involved in bringing about the changes in expression of the TGF- β /Smad signaling components in the host epithelium. Though *S. typhi* chronically persists within the gallbladder, their low numbers or Viable but non culturable state may not significantly alter the host mechanisms to cause cancer. This needs further investigation and analysis.

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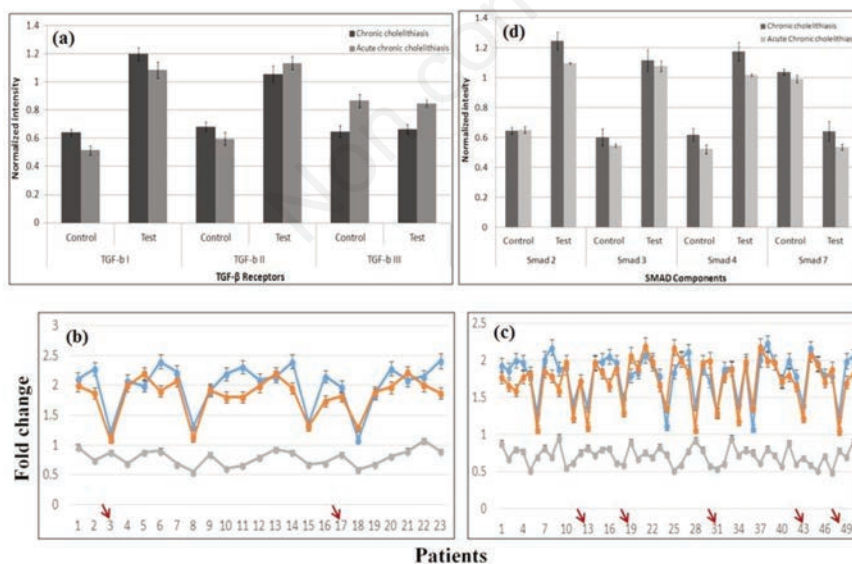


Figure 2. Changes in expression of transforming growth factor- β receptor (T β R) in given set of population. a) Changes in gene expression of T β R I, II, III in gallbladder epithelium of patients suffering from chronic cholelithiasis. Data is representative of means of at least three biological replicates with *P<0.05. b) and c) T β R expression in patients undergoing cholecystectomy. Red arrow indicates patients detected with *Salmonella Typhi* in their gallbladder. d) Changes in gene expression of Smad components.

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