Original Article

Transcription factor 4 expression and correlation with tumor progression in gallbladder cancer

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ABSTRACT

Background: Dysregulation in Wnt/β-catenin signaling has been associated with the initiation and metastasis of cancer cells. Transcription factor 4 (TCF4) (also named as transcription factor 7-like 2) is a key transcriptional factor of the Wnt signaling pathway, which, when interact with β-catenin activates Wnt genes which plays an essential role in tumor development. The expression pattern and clinical significance of TCF4 in gallbladder cancer (GBC) are not yet established.

Aims: This study was performed to assess the expression pattern of TCF4 in GBC tissue and attempted to correlate its expression with different clinicopathological parameters.

Materials and Methods: The study was conducted on 33 surgically resected specimens of gallbladder carcinoma (GBC) and 12 cases of chronic cholecystitis (CC) as control, which had been confirmed from histology. The expression of TCF4 was performed by the reverse transcription polymerase chain reaction and immunohistochemistry.

Results: Relative mRNA expression levels of β-catenin and TCF4 in GBC tissues were significantly ($P < 0.05$) higher than in CC samples. TCF4 protein expression was observed in 81.82% (27/33) GBC cases. Specifically, among GBC samples, 21.21% (7/33) was graded as strongly positive, 60.61% (20/33) graded as moderately positive, whereas 18.18% (6/33) graded as negative. All 12 CC samples graded as negative. Overall, TCF4 expression in GBC tissues was statistically significant over CC samples ($P < 0.05$). Moreover, we observed that TCF4 expression was significantly higher ($P < 0.05$) in high tumor grades than low grade, higher ($P < 0.05$) in Stage 2 and Stage 3 than Stage 1.

Conclusion: The present study suggests that TCF4 may exert an oncogenic role in the progression of GBC and may serve as a new potential candidate biomarker for tumor progression, and it might be a potential therapeutic target against GBC.

KEY WORDS: Clinical significance, expression, gallbladder cancer, transcription factor 4, Wnt/β-catenin signaling, β-catenin

INTRODUCTION

Gallbladder cancer (GBC) is the most common malignancy of the biliary tract, ranked sixth among gastrointestinal cancers and is associated with a poor prognosis.[1-3] According to GLOBOCAN 2018 data, globally about 219,420 GBC new cases and 165,087 deaths were estimated to occur in 2018.[4] India is among the highest incidence area for GBC similar to the Latin American countries such as Chile, Bolivia, and Columbia.[2,5] In India, GBC is most prevalent in the north, east, northeast, and central part of India.[6-8] Other countries with high rates of GBC include Japan, Korea, China, Slovakia, Poland, Czech Republic, Israel, Pakistan, Thailand, Nepal, and Bangladesh. In contrast, GBC rates are low in most northern European countries, the United States, and Canada.[2,4,9-11] Cancer incidence and mortality are rapidly growing worldwide because of dietary patterns, lifestyle factors, exposed carcinogenic chemicals, and pathogenic microorganisms.[12,13]

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The development of GBC occurs over a span of 5–15 years, from chronic inflammation to tissue alterations, including metaplasia, dysplasia, carcinoma *in situ*, and invasive cancer.[1] The early diagnosis of GBC is not possible due to the lack of specific biomarkers.[2,5] The mean survival period for patients with advanced GBC is 6 months. However, if the disease is diagnosed in stage IV, the 5-year survival rate is less than 5%.[5,14,15] Some genetic and molecular changes such as K-Ras, epidermal growth factor receptor, HER2 (ERBB2), vascular endothelial growth factor A, TP53, cyclooxygenase-2, fragile histidine triad, retinoblastoma, MUC1, E-cadherins (CDH1), thrombospondin-1, Cyclin E, Cyclin D1, Cyclin-dependent kinase inhibitor 1B (p27Kip1), Caspases (CASP), Bcl2, Survivin, MYC, heat shock protein gp96, adenomatous polyposis coli, mammary serine protease inhibitor, p14, p16, cholecystokinin type-A, Phospho-mTOR, Methylene tetrahydrofolate reductase gene, Hedgehog pathway genes and Wnt signaling pathway genes had been studied in GBC.[16,17] There is certainly an unmet need to understand the pathophysiology of GBC and identify novel targets to manage both the early and advanced disease states.

Deregulation of Wnt/β-catenin signaling has been implicated in cancer initiation, cancer metastasis, and the development of cancer stem cells.[18-22] Transcription factor 4 (TCF4) (also named as transcription factor 7-like 2) is a key transcription factor of the Wnt/β-catenin signaling pathway which when interacts with nuclear β-catenin activates Wnt genes.[23,24] Wnt target genes are known to play an essential role in several aspects of tumor development, such as transformation, cell growth, proliferation, survival, migration, invasion, angiogenesis, and epithelial to mesenchymal transition.[25-36]

Aberrant Wnt/β-catenin signaling is an early progression event in 90% of colorectal cancers[37] and hepatocellular carcinomas.[38-40] Overactivation of β-catenin/TCF4 signaling by accumulated β-catenin in the nucleus has been shown to play a crucial role in the development of colorectal cancer[23,41-44] and hepatocellular carcinoma.[45-49] TCF4 expression is potentially involved in various tumors, including colorectal cancer and hepatocellular carcinoma, and in the progression of drug resistance.[47,50-53]

A great body of literature indicates that deregulation of Wnt/β-catenin signaling in GBC leads to increased nuclear localization of β-catenin.[54-59] As activation of β-catenin/TCF4 signaling primarily depends on nuclear β-catenin, we hypothesized that TCF4 may have a critical role in the progression of GBC. To the best of our knowledge, there have been no reports which described the expression pattern of TCF4 in GBC. The aim of this study was to investigate the expression patterns of TCF4 in GBC tissue samples by reverse transcription polymerase chain reaction (RTPCR) and immunohistochemistry (IHC) and attempted to correlate its expression with different clinicopathological parameters.

MATERIALS AND METHODS

Collection of gallbladder tissue specimens

The study was commenced after gaining the approval of the Institutional Ethical Committee of Institute of Science, Banaras Hindu University, Varanasi, India, and obtaining informed written consent from the patients. Newly diagnosed patients were included in the study. The study was conducted on 33 surgically resected specimens of gallbladder carcinoma (GBC) and 12 cases of chronic cholecystitis (CC) as control. The tissue specimens were collected between August 2017 to January 2019 undergoing cholecystectomy at the Department of Surgical Oncology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, a tertiary-level superspeciality hospital in northern India. All patients had a detailed clinical evaluation, blood investigations including tumor markers (Carcinoembryonic Antigen and Carbohydrate Antigen 19-9). Diagnostic imaging like ultrasonography and computed tomography (CT) were done. Positron emission tomography and computed tomography were done in selected patients. For histopathology and immunohistochemical analysis, tissue samples were fixed in 10% v/v buffered formalin. For mRNA expression, tissue samples were snap-frozen in liquid nitrogen immediately following excision prior to storing at −80°C until further processing.

Histology

Formalin fixed tissue samples were stored in 70% v/v ethanol and were embedded in paraffin in a routine manner. Five micrometer thick sections from formalin fixed paraffin embedded blocks were cut and stained with hematoxylin and eosin (H and E) for histopathological evaluations. After histological confirmation of all the samples, tumor grade, staging, and lymph node involvement were assessed as per the 8th edition of the American Joint Committee on Cancer (AJCC) in the gallbladder carcinoma group.[60] The tumor grades were also noted in each case as well differentiated (WD), moderately differentiated (MD), and poorly differentiated (PD) using previously described criteria.[61]

RNA isolation and reverse transcription polymerase chain reaction

The mRNA levels of TCF4 and β-catenin were quantified by RTPCR. Briefly, total RNA was extracted from the tissue samples using RNAiso Plus (Takara, Cat. #9108). The integrity of total RNA was checked electrophoretically and RNA concentration was quantified with a NanoDrop spectrophotometer. Two microgram of total RNA was used for the synthesis of complementary DNA (cDNA) using the cDNA Synthesis Kit (Thermo Scientific, #K1622) and amplified using the TopTaq DNA Polymerase (Qiagen, Cat. # 200201) according to the manufacturer's instruction. All primers were analyzed using Primer-Blast to ensure primer specificity for the gene of interest (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences for TCF4 forward 5'-GAATCGTCCCAGAGTGATGTCG-3' and reverse

5'-TGCACTCAGCTACGACCTTTGC-3'. The primer sequences for β-catenin forward 5'-CACAAGCAGAGTGCTGAAGGTG-3' and reverse 5'-GATTCCTGAGAGTCCAAAGACAG-3'. The primer sequences for reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) forward 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse 5'-ACCACCCTGTTGCTGTAGCCAA-3'. The PCR products were run on 1% w/v agarose gels, stained with ethidium bromide (0.5 μg/ml), and photographed by a BioRad Gel Doc™ EZ (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of bands was noted from Image Lab version 5.2.1 software of BioRad Gel Doc™ EZ.

Immunohistochemistry

The protein levels of TCF4 were quantified by IHC. Briefly, 5 μm formalin-fixed paraffin embedded sections were mounted on poly-L-lysine coated slides, deparaffinized in xylene, and rehydrated through graded ethanol solution. Antigen retrieval was done by heating in 10 mM sodium citrate buffer pH 6.0 for 20 min at 90°C. Endogenous peroxidase activity was neutralized with 3% v/v hydrogen peroxide in methanol for 15 min at room temperature. The sections were washed two times with 1% v/v goat serum in 10 mM phosphate-buffered saline with 0.4% v/v Triton X-100 (PBS-T) for 10 min. To block any nonspecific binding the tissue sections were incubated with 5% v/v goat serum in PBS-T for 30 min at room temperature. The sections were washed two times with 1% v/v goat serum in PBS-T for 10 min, and then samples were incubated with monoclonal anti-TCF-4 primary antibody, clone 6H5-3 (Upstate, Cat. # 05-511) used at 1: 200 dilution overnight at 4°C. After washing with 1% v/v goat serum in PBS-T for 10 min, samples were incubated with biotinylated secondary antibodies for 1 h at the room temperature followed by streptavidin peroxidase reagent for 1 h at room temperature. Chromogen solution 3,3-diaminobenzidine was used to visualize the binding and sections were counterstained with hematoxylin. Then sections were dehydrated in ascending concentrations of ethanol and cleared in xylene before cover-slipping. Microscopic images were captured with a BX41 light microscope (Olympus Optical Co., Tokyo, Japan) equipped with an Olympus DP70 digital camera and image analysis software (Analysis 3.2, Soft Imaging System, Hanover, Germany).

Immunohistochemical reactions were assessed using a previously described semiquantitative scoring system, in which the final immunoreactive score was expressed as the product of the percentage of positive cells and the staining intensity scores.[62] The percentage of positive cells was graded as follows: 0 = negative; 1 = <10% positive cells; 2 = 10%–50% positive cells; 3 = 51%–80% positive cells; 4 = >80% positive cells. The staining intensity was graded as follows: 0 = negative; 1 = weakly positive; 2 = moderately positive; and 3 = strongly positive. The product of the above two scores was given a numerical value from 0 to 12 as a final immunoreactive score and was graded as follows: 0–1 = negative, 2–3 = weakly positive, 4–8 = moderately positive, 9–12 = strongly positive. The percentage of positive cells were quantified from Color Deconvolution, a plugin of ImageJ software.[63,64] All staining results were independently evaluated by three different pathologists who were blinded to patient outcomes. TCF4 expression was compared between the gallbladder carcinoma and CC groups and was correlated with different clinicopathological parameters.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, Inc., USA). Results were compared between two groups using unpaired student's *t*-test (two-tailed) and among more than two groups by the one-way analysis of variance. Pearson correlation coefficients were determined between the mRNA and protein level of TCF4 in GBC tissue samples. A Chi-square test was applied

Table 1: Clinicopathologic characteristics of patients

	Number of cases, *n* (%)	
	GBC	CC
Age		
Age (years)		
21-30	-	2 (16.67)
31-40	-	6 (50)
41-50	9 (27.27)	4 (33.33)
51-60	17 (51.52)	0
>60	7 (21.21)	0
Sex distribution		
Gender		
Male	9 (27.27)	4 (33.33)
Female	24 (72.73)	8 (66.67)
Distribution of symptoms		
Symptoms		
Abdominal pain (right upper quandrant)	33 (100)	12 (100)
Dyspepsia, indigestion, bloating, loss of appetite	33 (100)	12 (100)
Vomiting	16 (48.48)	4 (33.33)
Weight loss	25 (75.76)	2 (16.67)
Fever	31 (93.94)	8 (66.67)
Abdominal lump	4 (12.12)	0
History of jaundice	26 (78.79)	8 (66.67)
History of gastrointestinal infections	24 (72.73)	6 (50)
Ultrasonography and computed tomography findings		
Findings		
GB mass	24 (72.73)	0
GB wall thickening	33 (100)	12 (100)
Gallstones present	26 (78.79)	12 (100)
Lymph nodes metastasis	7 (21.21)	0
Lymph node involvement	8 (24.24)	0
Histological grade of GBC patients		
Histological grade		
WD	4 (12.12)	
MD	19 (57.58)	
PD	10 (30.30)	
AJCC staging of GBC patients		
Stage		
I	6 (18.18)	
II	17 (51.52)	
III	10 (30.30)	
IV	-	

GBC=Gallbladder carcinoma, AJCC=American Joint Committee on Cancer, WD=Well differentiated, MD=Moderately differentiated, PD=Poorly differentiated, CC=Chronic cholecystitis, GB=Gallbladder

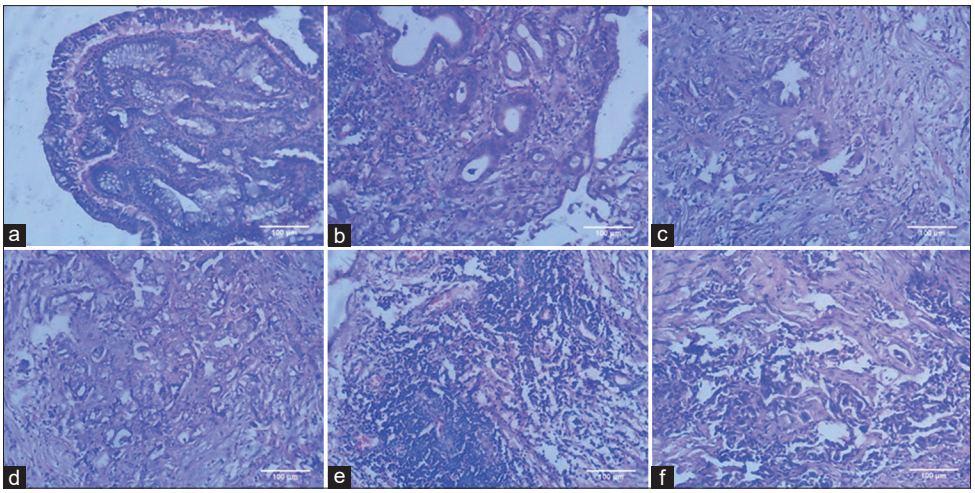

Figure 1: Histology images of chronic cholecystitis and gallbladder cancer patient tissue samples. Representative histology images stained with hematoxylin and eosin of (a and b) chronic cholecystitis tissue samples, (c and d) moderately differentiated gallbladder cancer tissue samples, (e and f) poorly differentiated gallbladder cancer tissue samples were shown. Magnification ×10 and scale bar 100 μm

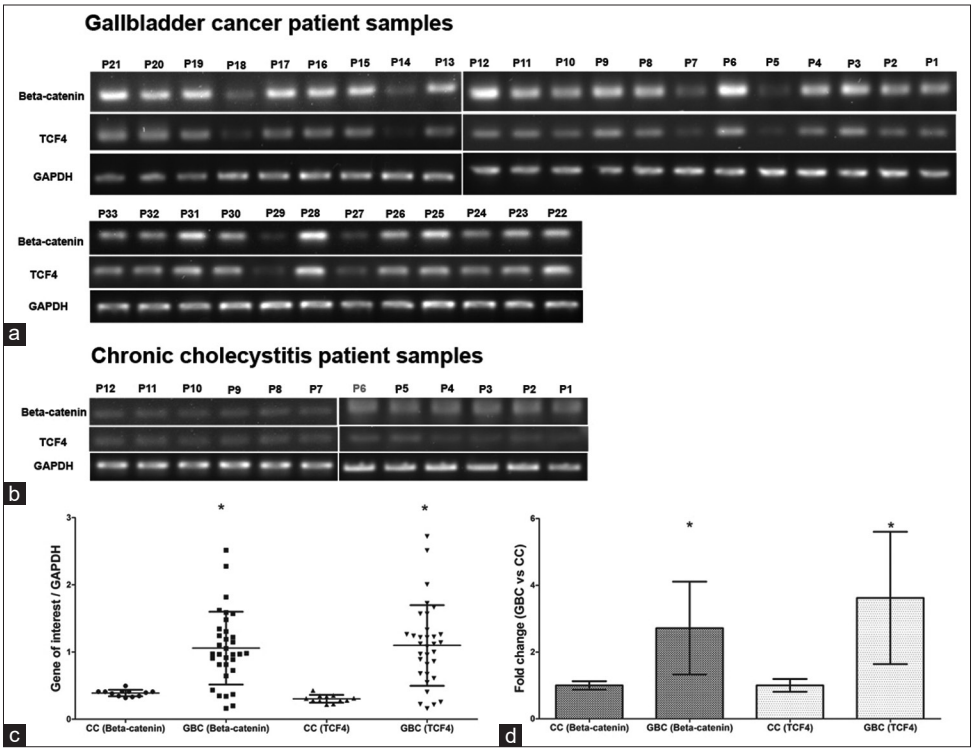

Figure 2: β-catenin and transcription factor 4 expression in chronic cholecystitis and gallbladder cancer tissue samples. Representative reverse transcription polymerase chain reaction images of (a) gallbladder cancer patient tissue samples, (b) chronic cholecystitis patient tissue samples, (c) β-catenin and transcription factor 4 mRNA expression levels in gallbladder cancer tissues ($n = 33$) were significantly higher than in chronic cholecystitis samples ($n = 12$), (d) Mean fold change in mRNA levels of β-catenin and transcription factor 4 in gallbladder cancer tissues compared to chronic cholecystitis samples. Data were presented as mean ± standard deviation. *$P < 0.05$ versus chronic cholecystitis (Beta-catenin), *$P < 0.05$ versus chronic cholecystitis (transcription factor 4)

to nonparametric variables. Statistical significance was considered at $P < 0.05$.

RESULTS

The purpose of this study was to investigate the expression patterns of TCF4 in GBC tissue samples and we attempted to correlate its expression with different clinicopathological parameters.

Patient characteristics

The study was conducted on 33 gallbladder carcinoma (GBC) patients and 12 chronic cholecystitis (CC) patients. All patients presented with right upper quadrant abdominal pain,

dyspepsia, indigestion, bloating, and loss of appetite. Most of the GBC patients had a history of jaundice (78.79%), history of gastrointestinal infections (72.73%), and gallstones (78.79%). Among the 33 cases of GBC, 4 were WD, 19 moderately and, 10 were PD. When categorized by AJCC staging, 6, 17, and 10 cases were in Stage I, Stage II, and Stage III, respectively. Nodal involvement was seen in eight cases and lymph nodes metastasis along the cystic duct, common bile duct, hepatic artery, and/or portal vein were present in seven cases. The clinicopathological characteristics of the patients are summarized in Table 1 and histology images of CC and GBC patient tissue samples are shown in Figure 1.

Transcription factor 4 expression

We first examined the expression levels of β-catenin and TCF4 mRNA in GBC ($n = 33$) and CC ($n = 12$) patients by RTPCR as shown in Figure 2. Relative mRNA expression levels of β-catenin and TCF4 in GBC tissues were significantly higher than in CC samples (the ratio of β-catenin: GAPDH and TCF4: GAPDH are 1.06 ± 0.54, 1.09 ± 0.60 respectively in GBC tissues, whereas 0.39 ± 0.05, 0.30 ± 0.06, respectively, in CC samples, the data were represented in mean ± standard deviation). The mean fold change in mRNA levels of β-catenin and TCF4 are 2.72, 3.62, respectively, in GBC tissues compared to CC samples, $P < 0.05$).

In the next study, we examined the protein level of TCF4 in the GBC and CC patient tissues to ascertain TCF4 expression by IHC, as shown in Figure 3. The IHC results revealed that the protein level of TCF4 was elevated in GBC tissues, which was consistent with the mRNA expression result. We found there was a statistically significant ($P < 0.05$) positive correlation between the mRNA and protein level of TCF4 in GBC tissue samples ($R^2 = 0.5544$), as shown in Figure 4a. In GBC, TCF4 expression was observed in 81.82% (27/33) cases. Specifically, among GBC samples, 21.21% (7/33) samples showed 51%–80% TCF4 positive cells with a final immunoreactive score from 9 to 12 and were graded as strongly positive, 60.61% (20/33) samples showed 10%–50% TCF4 positive cells with a final immunoreactive score from 4 to 8 and were graded as moderately positive, whereas 18.18% (6/33) samples showed <10% TCF4 positive cells with a final immunoreactive score from 0 to 1 and was graded as negative. All 12 CC samples showed <10% TCF4 positive cells with a final immunoreactive score from 0 to 1 and were graded as negative. Overall, TCF4 expression in GBC tissues was statistically significant over CC samples ($P < 0.05$).

Transcription factor 4 expression and clinicopathological correlation

We attempted to correlate TCF4 expression with different clinicopathological parameters as shown in Table 2 and Figure 4. We observed that: (1) Percentage of TCF4 positive cells and the final immunoreactive score increased significantly with tumor grades ($P < 0.05$ vs. WD); we found TCF4 was expressed in MD (89.47%, 17/19) and in PD (90%, 9/10) over WD (25%, 1/4) samples and it was statistically significant ($P < 0.05$, WD v/s MD + PD) (2) percentage of TCF4 positive cells and the final immunoreactive score increased significantly with tumor stage ($P < 0.05$ vs. stage 1); TCF4 was expressed in stage 1 (50%, 3/6), stage 2 (88.24%, 15/17), stage 3 (90%, 9/10), and it was statistically significant ($P < 0.05$, stage 1 v/s stage 2+ stage 3) (3) TCF4 expression was not correlated with the lymph node involvement ($P = 0.6321$, N0 v/s N1). Collectively, these results suggest that the upregulation of TCF4 may exert an oncogenic role in the progression of GBC.

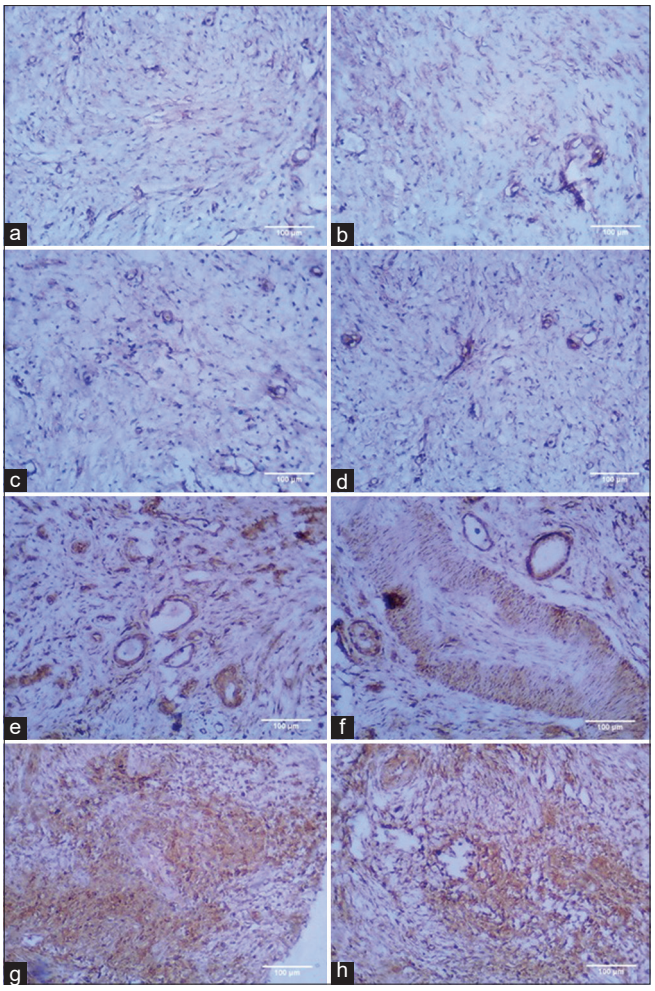

Figure 3: Expression of transcription factor 4 in chronic cholecystitis and gallbladder cancer tissue samples by immunohistochemistry. Representative immunohistochemistry images of (a and b) negatively graded chronic cholecystitis tissue samples, (c and d) negatively graded gallbladder cancer tissue samples, (e and f) moderately positive graded gallbladder cancer tissue samples, (g and h) strongly positive graded gallbladder cancer tissue samples were shown. Magnification ×10 and scale bar 100 μm

DISCUSSION

The Wnt canonical signaling pathway maintains cellular homeostasis by controlling fundamental cellular processes such as growth, proliferation, differentiation, migration,

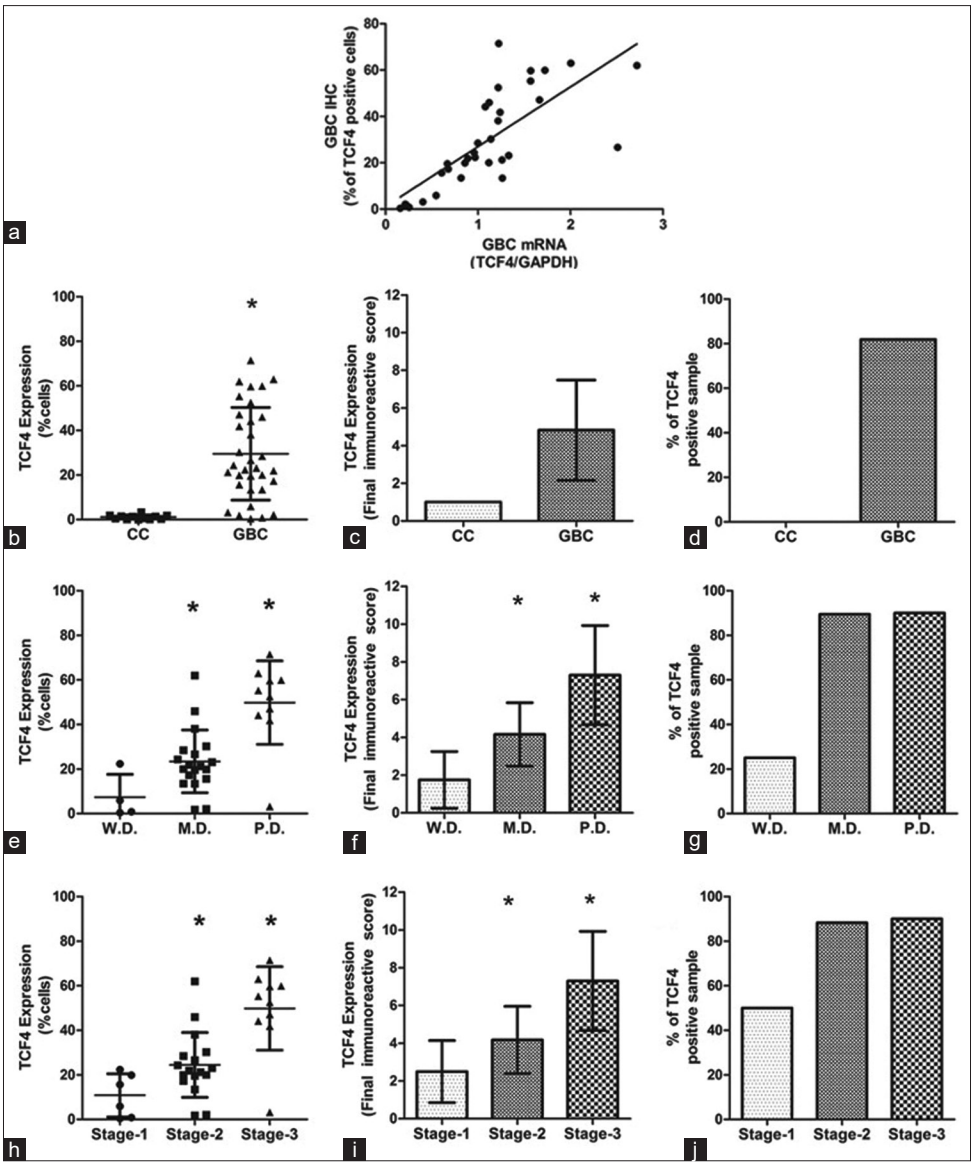

Figure 4: Correlation between the mRNA and protein level of transcription factor 4 in gallbladder cancer tissue samples; and analysis of transcription factor 4 protein expression from the percentage of transcription factor 4 positive cells, final immunoreactive score, and percentage of transcription factor 4 positive samples in chronic cholecystitis and gallbladder cancer tissue samples. (a) Representative graphical image shows a statistically significant ($P < 0.05$) positive correlation between the mRNA and protein level of transcription factor 4 in gallbladder cancer tissue samples. (b) The percentage of transcription factor 4 positive cells in gallbladder cancer tissues was statistically significant over chronic cholecystitis samples (*$P < 0.05$ vs. chronic cholecystitis). (c) representative graphical image of final immunoreactive score in gallbladder cancer tissues over chronic cholecystitis samples. (e, f) The percentage of transcription factor 4 positive cells and final immunoreactive score increased significantly with tumor grade (*$P < 0.05$ vs. well differentiated). (h, i) The percentage of transcription factor 4 positive cells and the final immunoreactive score increased significantly with tumor stage (*$P < 0.05$ vs. stage 1). The percentage of transcription factor 4 positive samples in (d) gallbladder cancer, among (g) different tumor grades and among (j) different tumor stages of gallbladder cancer tissue samples were shown. Data were presented as mean ± standard deviation

and apoptosis.[18-20] Dysregulation of Wnt/β-catenin signaling has been associated with cancer initiation, cancer metastasis, and development of cancer stem cells.[18-22] From our study, we found overexpression of β-catenin in GBC samples. Similar to our results, several clinical studies have reported overexpression of β-catenin in GBC samples,[54,57-59] indicating that deregulation of Wnt/β-catenin signaling in GBC patients.

TCF4 is a key transcriptional factor of the Wnt signaling pathway, which when interact with β-catenin activates Wnt genes which plays an essential role in tumor development such as transformation, cell growth, proliferation, survival, migration, invasion, angiogenesis, epithelial to mesenchymal transition, and development of cancer stem cells.[23,24] In our study, we have observed TCF4 expression was higher in GBC cases than that in CC cases. The evidence from this study

Table 2: Correlation of transcription factor 4 expression with different clinicopathological parameters

Characteristics	Number of cases	Strongly or moderately positive TCF4 expression, *n* (%)	Negative TCF4 expression, *n* (%)	*P*
Histological grade of GBC patients				
WD	4	1 (25)	3 (75)	0.0017 (WD vs. MD+PD)
MD	19	17 (89.47)	2 (10.53)	
PD	10	9 (90)	1 (10)	
AJCC staging of GBC patients				
I	6	3 (50)	3 (50)	0.0255 (I vs. II+III)
II	17	15 (88.24)	2 (11.76)	
III	10	9 (90)	1 (10)	
Lymph node involvement of GBC patients				
N0	25	20 (80)	5 (20)	0.6321 (N0 vs. N1)
N1	8	7 (87.5)	1 (12.5)	
Cases				
GBC	33	27 (81.82)	6 (18.18)	<0.05 (GBC vs. CC)
CC	12	0	12 (100)	

GBC=Gallbladder carcinoma, AJCC=American Joint Committee on Cancer, WD=Well differentiated, MD=Moderately differentiated, PD=Poorly differentiated, CC=Chronic cholecystitis, TCF4=Transcription factor 4

strongly supports the agreement that the expression of TCF4 is constitutively activated by β-catenin.[65] Several studies have reported that compared to β-catenin knockdown, TCF4 knockdown shows better efficacy to induce growth arrest and apoptosis in human colorectal cancer cells and suggests its significant role in controlling tumorigenesis.[44,52] In this study, we observed that TCF4 expression was (1) higher in high tumor grades (PD and MD) than low grade (WD), (2) higher in stage 2 and stage 3 than stage 1 and this showed that TCF4 overexpression was more likely to exhibit advanced disease. However, its expression was not correlated with lymph node involvement. Ishiguro *et al*. reported that TCF4 expression in esophageal squamous cell carcinoma was correlated with tumor grade, tumor stage, but did not correlate with lymph node status, similar to our finding.[66]

Our observations are consistent with the idea that TCF4 plays a tumor progression role and is an indicator of malignant potential or poor prognosis and we further suggest that TCF4 may play an important role in the GBC progression since higher TCF4 expression was associated with high tumor grades and higher stages.

A better understanding of the molecular pathophysiology and oncogenic mechanisms underlying the development and progression of GBC may help to establish more effective therapeutic agents and potential biomarkers. However, additional studies are required in a large sample size for further validation, which will give a better insight into the role of TCF4 in tumor progression, as a diagnostic and prognostic biomarker, and as a marker for chemotherapy resistance.

Taken together, these findings pointed toward the possibility that TCF4 may exert an oncogenic role in the progression of GBC and may serve as a new potential candidate biomarker for tumor progression. Moreover, we hypothesize that downregulation of Wnt/β-catenin signaling or selective β-catenin/TCF4 interaction inhibitors could be a potential therapeutic agent against GBC which might also be helpful in overcoming the prevalence of drug resistance.

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Conflicts of interest

There are no conflicts of interest.

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