

RESEARCH ARTICLE

Prognostic Relevance of Human Telomerase Reverse Transcriptase (hTERT) Expression in Patients with Gall Bladder Disease and Carcinoma

Raj Kumari Deblakshmi¹, Manab Deka^{1,2}, Anjan Kumar Saikia³, Bir Kumar Sharma⁴, Nidhi Singh¹, NN Das⁵, Sujoy Bose^{1*}

Abstract

Background: Gallbladder carcinoma (GBC) has been stated as an Indian disease, with the highest number of cases being reported from certain districts of northeast India, which has an ethnically distinct population. Unfortunately there are no scientific reports on the underlying molecular mechanisms associated with the pathogenesis of the disease from this region. **Aim:** The present study evaluated the role of differential expression of human telomerase reverse transcriptase (hTERT) in the development of gall bladder anomalies. **Materials and Methods:** Blood and tissue samples were collected from patients undergoing routine surgical resection for clinically proven cases of gallbladder disease {cholelithiasis (CL, n=50), cholecystitis (CS, n=40) and GBC (n=30) along with adjacent histopathologically proved non-neoplastic controls (n=15)} with informed consent. Whole blood was also collected from age and sex matched healthy controls (n=25) for comparative analysis. Differential hTERT mRNA expression was evaluated by semi-quantitative rt-PCR and real-time PCR based analysis using β -actin as an internal control. Evaluation of differential hTERT protein expression was studied by Western blot analysis and immunofluorescence. Statistical analysis for differential expression and co-relation was performed by SPSSv13.0 software. **Results:** Gallbladder anomalies were mostly prevalent in females. The hTERT mRNA and protein expression increased gradiently from normal < CL < CS < GBC cases. Serum expression correlated statistically significantly with the tissue based mRNA expression pattern of hTERT, with highest expression observed in GBC cases and the lowest expression in normal gall bladder. **Conclusions:** Higher hTERT expression is associated with gallbladder disease susceptibility and severity; and may be a useful prognostic marker for gallbladder anomalies.

Keywords: Gall bladder carcinoma - cholelithiasis - cholecystitis - human telomerase reverse transcriptase - PCR

Asian Pac J Cancer Prev, 16 (7), 2923-2928

Introduction

Gall bladder carcinoma (GBC) is a rare neoplasm with poor prognostic rate (Randi et al., 2006). Incidences rate of the disease are observed in specific countries and in confined areas (Lazcano et al., 2001; Baez et al., 2010). GBC mortality remains high due to its aggressive and silent nature (Maurya et al., 2011). The highest Figureure for incidences of GBC is seen in India (Kapoor et al., 2003; Randi et al., 2006). As per 'the cancer atlas of India' the comparison of different population based cancer registries indicated that GBC is one of the commonest causes of cancer related mortality in women in northern and north-eastern states of India (Nandakumar et al., 2003). As per National Cancer Registry Program-ICMR (2011-2012) in India the highest number of gall bladder cancer incidences

is recorded in Kamrup district of Assam, followed by Imphal West, Manipur and Delhi. GBC shows marked ethnic distribution and endemic hotspots among female population worldwide. Gallstones (Cholelithiasis) and inflammation of the gallbladder (Cholecystitis) are the major risk factors for developing GBC and are present in between 60% and 90% of cases in different populations around the world (Tazuma and Kajiyama, 2001; Hsing, 2007), although the association between gallstones and GBC is strong, the casual relationship between them is not clear (Hamdani et al., 2013). Cancer is a multistep event, where genetic instability and deregulation of different signal transduction mechanism and apoptosis plays a predominant role, but importantly varies in cancers in different tissue etiologies. Therefore delineation of the underlying molecular mechanism(s) in specific

¹Department of Biotechnology, Gauhati University, ²Department of Biological Science, GUIST, ³Medicinal and Gastroenterology Unit, Central Hospital NF Railway, Maligaon, ⁴Department of HPB and Liver Transplant Surgery, Downtown Hospital, Khanapara, Guwahati, Assam, ⁵Department of General Surgery, Regional Institute of Medical Sciences, Imphal, Manipur, India *For correspondence: sujoybose1@gmail.com

tissues is critical in understanding the process of cancer development and plan therapeutic interventions based on the specificity and sensitivity of the molecular markers(s). Telomeres are protective nucleoprotein structures at the end of linear chromosomes (Greider, 1991). Telomerase is a cellular RNA-dependent DNA polymerase responsible for telomere maintenance and stabilization (Greider and Blackburn, 1985). The telomere-telomerase hypothesis of aging and cancer is based on the findings that the cells of most human tumors have telomerase activity while normal human somatic cells do not (Blasco and Hahn, 2003).

A high level of telomerase activity is detected in about 90% of human cancer specimens, whereas most somatic cells do not display telomerase activity or express it only at very low levels in a cell cycle-dependent manner (Kim, 2002; Cesare, 2010). The expression level of human telomerase reverse transcriptase (hTERT), a catalytic subunit bearing the enzymatic activity of telomerase, is the rate-limiting determinant of human telomerase function and activity, and has been implicated in cellular immortalization and transformation; whereas the other subunits are constitutively expressed both in normal and cancer cells (Nakayama et al., 1998; Kyo, 1999; Poole, 2001). Telomerase activation has been regarded as a crucial step in cellular carcinogenesis, and it is the broadest spectrum molecular marker of malignancies found (Shay, 1997). It has been reported that telomerase activity is significantly higher in about 80% of cancers and correlates well with the degree of malignancy (Cerni, 2000). hTERT mRNA in serum was detected in breast cancer but not in benign diseases, suggesting that hTERT is available for cancer diagnosis (Hiyama et al., 1996). It has been shown that hTERT mRNA is not only improved in both sensitivity and specificity but has closely correlation with tumor size and number and hTERT mRNA showed more sensitivity and specificity compared with AFP mRNA in HCC patients (Miura, 2005). Although, telomerase activity is reported in various tumor types, there is a limited literature regarding the significance of telomerase in GBC (Luzar et al., 2005), which needs critical evaluation. The present study evaluated the significance of differential expression of hTERT in the prognosis of gall bladder diseases and carcinoma.

Materials and Methods

Patient enrolment, sample collection and stratification

Whole blood and tissue specimens were obtained after routine surgical resection for gall bladder Carcinoma (n=30), cholelithiasis (n=50), cholecystitis (n=40) cases; and also from region of tumor free location situated at atleast 6cm distance from the tumor, and used as control(n=15) with informed consent. The samples were collected from Department of Surgery, RIMS, Lamphelpat, Central Hospital NF Railways, Maligaon and Downtown Hospital, Guwahati, Kamrup District, Assam with all clinical and histopathological details. The tissue samples were collected in liquid nitrogen and transferred to the Department of Biotechnology, Gauhati University for the experimental works. The study was approved by Institutional Ethics committee of the participating

institutions. Whole blood was also collected from few age and sex matched community based healthy control (n=25) blood donors for comparative mRNA based expression analysis for hTERT.

Total RNA was isolated by the standard trizol method. The quality and quantity of the total RNA of all the samples was checked using a nanodrop spectrophotometer, and subsequently 1µg of the RNA was converted to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). Initially, fifteen GBC cases in which both the tumour section and non-neoplastic control sections were evaluated for differential mRNA expression for hTERT by semi-quantitative RT-PCR analysis. β-actin was used as internal control. Next, the cases of all the cohorts were evaluated for fold change in mRNA expression of hTERT in the affected area compared to the non-neoplastic controls by real time PCR; β-actin expression being used as internal normalization control. The difference in mRNA expression in different cohorts was analyzed statistically using SPSS13.0 software. A Mann-Whitney non-parametric test was applied to calculate the p value; a two tailed p-value less than 0.05 was considered statistically significant.

Differential protein expression analysis

To evaluate the involvement of differential expression of telomerase protein (hTERT) in the gall bladder disease and carcinoma development, and its correlation with the mRNA expression status, the differential protein expression study was performed using western blotting method. Total protein was extracted using the Magnesium Lysis Buffer, and quantified using the BCA protein estimation kit. Proteins extracted from the disease (cholelithiasis and cholecystitis), carcinoma as well as non-neoplastic control cases (50µg each) were evaluated by western blotting analysis using antibody specific for telomerase (Y182 ab32020, Abcam). β-tubulin was used as loading control. Immunofluorescence study was done to evaluate the differential protein expression of gall bladder carcinoma and normal cases and validate the western blot analysis results, using antibody specific for telomerase (Y182 ab32020, Abcam) and goat anti-rabbit IgG HRP (Merck) secondary antibody.

hTERT mRNA expression in serum

Total RNA was extracted by standard trizol method using serum samples (250µl) collected from all the included cases and few age and sex matched community based healthy controls (n=25). The quality of the total RNA was checked by nano drop spectrophotometer and was converted to cDNA using the high capacity

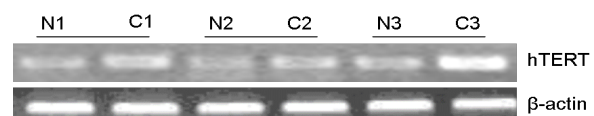


Figure 1. Representative Agarose Gel Electrophoresis Picture Panel Showing the Up-Regulation of the hTERT mRNA Expression in Affected areas(C1, C2 and C3) Compared to the Non-Neoplastic Control Areas(N1, N2 and N3)

cDNA reverse transcription kit (Applied Biosystems). The differential mRNA expression of hTERT in cases compared to the controls was studied by real time PCR; β -actin expression being used as internal normalization control. The difference in mRNA expression in different cohorts, and its correlation with tissue based expression was analyzed statistically using SPSS13.0 software.

Results

The present study included patients from two districts in India reported to have the highest distribution of gall bladder disease and carcinoma cases in India, i.e. Kamrup district of Assam, and Imphal, Manipur. The majority of the gall bladder disease (n=70, 87.5%) and GBC cases (n=25, 83.33%) were females. The average age of the GBC patients (44±13 years) was higher than the cholelithiasis (31±14 years) and cholecystitis cases (35±09 years). Cases having a history of alcoholism or liver disease were excluded from the study.

Semi-quantitative based analysis

Differential mRNA expression of hTERT initially was studied in GBC cases (n=15) between the affected area and the non-neoplastic control area, with β -actin used as internal control. The result showed a sharp up-regulation in the expression of hTERT in the affected area compared to the control area; indicating the role of hTERT re-activation in the susceptibility to GBC development (Figure 1).

Differential mRNA expression analysis

Since, the semi-quantitative RT-PCR based analysis was indicative of the deregulation of hTERT in the pathogenesis of GBC, and since both gall bladder diseases like cholelithiasis and cholecystitis has been established as a risk factor associated with the development of GBC; we evaluated the differential expression of hTERT by Real time PCR in the different cohorts compared to controls. The expression of hTERT was increased in cholelithiasis (3.0±2.08 folds) and cholecystitis (4.04±1.92 folds), the highest being in GBC (6.15±2.74 folds) compared to non-neoplastic controls (Figure 2).

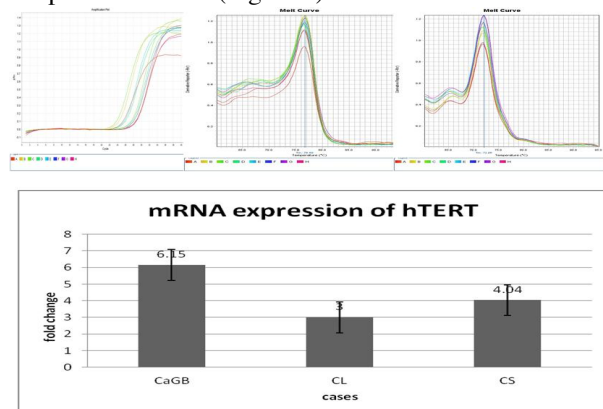


Figure 2. Representative figures in the upper panel showing (left to right) amplification graph of hTERT and β -actin; Melt curve of hTERT; and melt curve of β actin. Lower panel showing bar diagram representing the fold change in mRNA level expression of hTERT in gall bladder diseases and carcinoma cases normalized against β -actin

Differential protein expression Of hTERT

Differential expression of hTERT in the gall bladder disease cases and carcinoma cases was examined by western blotting with hTERT antibody (Abcam). Western blot data were similar to the mRNA expression pattern, and showed up-regulation of hTERT protein expression in cholecystitis and GBC cases, the highest being in GBC, whereas the expression pattern was comparative between normal and cholelithiasis cases in majority of the samples (Figure 3). The immunofluorescence based analysis using randomly selected gallbladder cancer and control cases was performed to validate the western blot analysis results. The immunofluorescence based data showed a marker up-regulation of the hTERT expression in the GBC cases compared to controls, thereby validating our western blot analysis data and indicating the importance of higher hTERT expression in the pathogenesis of gall bladder diseases and carcinoma (Figure 4).

Differential mRNA expression in serum (Figure 5)

Since the method for detecting tumor derived hTERT mRNA in serum was sensitive for the early detection of hepatocellular carcinoma in patients whose α -fetoprotein levels were low (Ito et.al 1998) and, lung cancer (Miura et

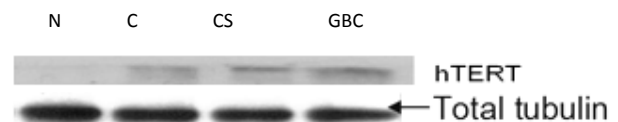


Figure 3. Representative Western Blot Analysis Showing Up-Regulation hTERT Expression in Gall Bladder Disease and Carcinoma in Comparison to Normal

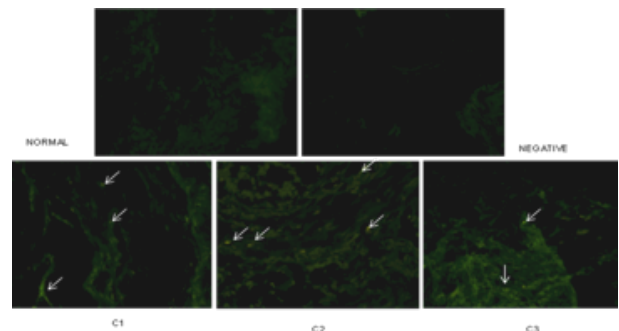


Figure 4. Representative Immunofluorescence Images for Normal, Negative and Carcinoma Cases. The hTERT expression was higher in carcinoma cases (C1, C2, C3) indicated by the arrows as compared to normal and no expression in negative slide

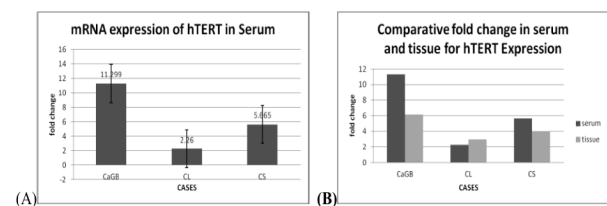


Figure 5. Representative bar diagram showing. A) mRNA expression of hTERT in Serum; B) comparative mRNA expression in serum and tissue in gall bladder diseases and carcinoma

Table 1. Showing Correlation of hTERT mRNA Expression in Serum and Tissue of different cohorts

Cases	Pearson correlation	P *	Spearman's rho correlation coefficient	P*
All cases	0.859	<0.001	0.868	<0.001
Cholelithiasis	0.726	0.003	0.745	0.002
Cholecystitis	0.685	0.01	0.665	0.013
GBC	0.814	<0.001	0.722	0.004

*Correlation is significant at the 0.01 level (2-tailed).

al., 2006), and since the serum based prognostic marker may be a better lesser invasive prognostic factor/marker; therefore the serum level differential mRNA expression of hTERT was evaluated by Real time PCR in the different cohorts compared to controls. The result uniform with data found in tissue mRNA expression of hTERT and co-related statistically significantly with the tissue level expression of individual cases of each cohorts (Figure 5). The expression of hTERT was increased in cholelithiasis (2.26±0.82) and cholecystitis (5.665±2.62), the highest being in GBC (11.299±5.28) compared to non-neoplastic controls (Figure 5).

Correlation analysis based on Pearson correlation and Spearman's rho correlation co-efficient was performed to correlate the levels of hTERT mRNA expression in serum and tissues of different cohorts. The correlation data showed significant positive correlation of hTERT mRNA expression in the individual cohorts i.e. cholelithiasis, cholecystitis, carcinoma as well as in a cumulative cohort considering all the cases taken together (Table 1).

Discussion

One of the fatal malignancies with poor prognosis, GBC, is one of the most commonly cancer encountered in northeastern region of India, particularly in Kamrup district of Assam and Imphal West, Manipur (Malik, 2004); with no studies elucidating the molecular mechanism underlying the development of the disease. Reports show a strong relation between gall stones and chronic cholecystitis with GBC (David, 1995; Zouet al., 2000; Nandakumar, 2005). Although global data have shown the association of deregulation in multiple signaling cascades in the development of GBC, the present data is still inconclusive. Telomerase or terminal transferase is a ribonucleoprotein complex that utilizes sequences of its own. The RNA component acts as a template for the de novo synthesis of telomeric DNA sequences. Its activity can be reconstituted *in vitro* by two essential components, the RNA subunit hTERC (which acts as the template for addition of new telomeric repeats) and the catalytic protein component hTERT. Telomerase activity is absent in most human somatic cells, but was detected in ~85% of 400 tumor tissue samples (Ryhu, 2005). Since hTERC is expressed at low level even in cells without telomerase activity (Koyanagi et al., 2000), hTERT expression has been more often used for detection of cancer cells in various samples (Hiyama and Hiyama, 2003). Telomerase activation has been regarded as a crucial step in cellular carcinogenesis, and it is the broadest spectrum molecular

marker of malignancies found (Shay, 1997). It has been reported that telomerase activity is significantly higher in about 80% of cancers and correlates well with the degree of malignancy (Cerni, 2000). Some studies have shown that hTERT gene expression is more specific and sensitive than telomerase activity in the diagnosis of malignant neoplasms, as the hTERT gene is overexpressed in about 90% of malignant tumors (Poole, 2001). In the present study, the role of differential hTERT expression in the development of gall bladder anomalies and progression to GBC was studied, and the prognostic significance of hTERT was evaluated.

The mRNA based expression analysis showed that the expression of hTERT correlates with the progression of the disease. The expression of hTERT was increased in cholelithiasis (3.0±2.08 folds) and cholecystitis (4.04±1.92 folds), the highest being in GBC (6.15±2.74 folds) compared to non-neoplastic controls. The protein level expression profile studied by western blot analysis also showed that there is a gradient increase in the hTERT expression starting from control, cholelithiasis, cholecystitis, and the highest being in GBC cases, which is similar to results reported by other groups (Shukla et al., 2006). The higher expression in cholecystitis cases compared to cholelithiasis case may be attributed to chronic inflammation linked to an increased telomere attrition rate and resulting malignant progression (O'Sullivan et al., 2002). The results of Immunofluorescence study also equivocal with the western data; there was higher expression of hTERT protein in carcinoma cases as compared to normal. Since reports suggest that telomerase is expressed almost exclusively in cancer cells, the detection of hTERT mRNA may become a powerful marker for detection of cancer cells also in blood (Hiyama and Hiyama, 2003). But contradictory reports from some groups have researchers have reported that hTERT expression is expressed both in normal and cancerous gastric specimens (Li et al., 2008; Cheng et al., 2014), which challenges the widespread concept that hTERT and telomerase are repressed in normal tissues. The contradictory data may be due to tissue or organ etiology, therefore requires specificity assessment based on tissue and cancer type. To confirm the finding shown in tissue samples, hTERT expression study was carried out in serum levels also and correlated with tissue level expression. The results showed similar up-regulation of hTERT mRNA expression at the serum levels in the gall bladder disease cases, highest being in GBC; and the serum level hTERT mRNA expression correlated significantly with the tissue level expressions, thereby highlighting the prognostic significance of hTERT expression in gall bladder anomalies. Similar to our results, RT-PCR based detection of the mRNA for hTERT has been used as prognostic diagnostic marker for cancer diagnosis in urogenital organs (Ito et al., 1998; Fukui et al., 2001) in breast cancer (Hiyama et al., 1996), cervical cancer (Takakura et al., 1998), HCC (Miura et al., 2005), thyroid neoplasm (Lerma et al., 2005), pancreatic juices and cancer (Ohuchida et al., 2005), biliary tract and pancreatic cancer (Kawahara et al., 2007), colorectal cancer (Baichoo et al., 2014) and limitedly in GBC (Uchida et al., 2003).

Recent studies have revealed that the levels of hTERT mRNA as well as telomerase activity were high in hepatobiliary neoplasm (Koyanagi et al., 2000) which can also be used as diagnostic marker. The clinical usefulness of hTERT mRNA, especially combined with EGFR mRNA (Miura et al., 2006), as a novel tumor marker in primary lung cancer, viewed in the light of early detection and diagnosis (Fujita et al., 2003).

To conclude, the present study shows significant association of higher hTERT expression in the susceptibility and severity of gall bladder diseases. Moreover, because the tissue level up-regulation correlates significantly with the serum level hTERT expression, it indicates the prognostic utility of hTERT as a biomarker in gall bladder disease predisposition, and as an important clinical target.

Acknowledgements

The corresponding author acknowledges the DST-SERB, Govt. of India for the financial support under the OYS/FastTrack scheme.

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