

## Original Article

# Telomere length variation and expression analysis of shelterin complex genes during gallbladder carcinogenesis

### ABSTRACT

**Background:** Telomeres, which are bound with shelterin protein complex, play an important role in maintaining genomic stability and its dysfunction may lead to carcinogenesis. Here, we aimed to analyze whether shelterin complex gene expression and telomere length variation, play any role in gallbladder carcinogenesis.

**Methods:** Telomere length analysis was carried out by monochrome multiplex qPCR, whereas expression analysis of shelterin genes was carried out using RT-qPCR. Statistical analysis was carried out using SigmaPlot 11 software.

**Results:** We found significantly reduced telomere length in tumor tissues, and this reduction was seen in both, tumors with or without gallstones in comparison to adjacent non tumor and gallstone (chronic calculous cholecystitis: Inflamed) tissues. Inflamed tissues showed increased telomere length as compared to both adjacent non tumor and tumor tissues. Expression analysis of five shelterin genes showed significant downregulation of *TERF1*, *POT1*, and *TINF2* genes in inflamed tissues as compared to non tumor and tumor tissues. *POT1* was also found to be significantly upregulated in tumor tissues and specifically in tumor tissues with gallstones compared to inflamed tissues.

**Conclusion:** This study, thus, suggests that, gallstone does not affect telomere length and even after having increased telomere length, decreased expression of some shelterin genes in inflamed tissue might cause telomeres to cap improperly, possibly leading to telomere dysfunction and further, gallbladder carcinogenesis. Also, increased expression of *POT1* in tumor tissues with gallstones could act as a diagnostic marker in patients with gallstones.

**KEY WORDS:** Gallbladder cancer, gallstone, shelterin complex, telomere

### INTRODUCTION

Gallbladder cancer (GBC) is the most common cancer of the biliary tract with a very poor prognosis and low survival rates.<sup>[1]</sup> Although it is a relatively uncommon cancer, its incidence is very high in certain South American countries, as well as in North India.<sup>[2]</sup> An analysis of the last 5 years data on the incidence of various cancers in North Central India showed significant increase in GBC cases.<sup>[3]</sup> Gallstones are present in most of the GBC patients; hence, it is thought to be an important risk factor.<sup>[4]</sup> As compared to most other cancers, not much has been investigated on its pathogenesis. Due to inaccessibility and late diagnosis, discovery of an early and reliable molecular marker having better diagnostic or prognostic value is still a challenge.

Telomeres are nucleoprotein structures found at chromosome ends, made up of tandem repeats

of TTAGGG and bound to a specialized protein complex called the shelterin complex.<sup>[5]</sup> The shelterin complex, which is composed of six proteins; TRF1 (*TERF1*), TRF2 (*TERF2*), POT1 (*POT1*), RAP1 (*TERF2IP*), TIN2 (*TINF2*), and TPP1 (*ACD*) are known to regulate telomere length and thereby, maintain telomere integrity by protecting the telomeres from chromosomal abnormalities and DNA-damage responses and serve to protect chromosome ends.<sup>[6]</sup>

Telomere dysfunction due to loss of telomere length (end replication problem) or loss of these proteins leads to inhibition of DNA repair pathways,

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causing chromosome instability, leading to cancer initiation and progression.<sup>[7]</sup> Hence, the present study was aimed to look into the relationship between variation in telomere length and expression of the shelterin genes in gallbladder carcinogenesis with a view to elucidate possible role of shelterin proteins in the molecular mechanisms of GBC pathogenesis.

## METHODS

### Patients and samples

A total of 40 fresh surgical specimens were collected from 25 patients, including 15 GBC patients and 10 gallstone patients, undergoing cholecystectomy for gallbladder ailments. These 40 tissue samples included 30 gallbladder adenocarcinoma (AC) tissues and their paired adjacent non tumor (ANT) tissues (15 each) and 10 gallstone (inflamed) tissues with chronic calculous cholecystitis (CCC). The tissue samples were stored immediately after excision in an ice box containing frozen ice packs and transferred to the laboratory where DNA and RNA were isolated right away. The diagnosis of GBC and CCC was confirmed by histological examination in all cases. All samples were collected with prior informed consent of the patients. The methods employed in the study were already cleared by the Institutional Ethics Committee and were in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

### DNA isolation and assessment of telomere length

DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Relative telomere length was assessed as previously described,<sup>[8]</sup> using SYBR Green-based monochrome multiplex qPCR. Briefly, qPCR was performed with 20 ng sample DNA in a final volume of 15  $\mu$ l containing 7.5  $\mu$ l 2X QuantiTect SYBR Green PCR mix (Qiagen, Germany) and 500 nM and 900 nM of telomere primer pair (T) and human beta-globin primer pair (S), respectively. A reference human DNA sample was used to prepare a three-fold serial dilution of five concentrations across 150–1.85 ng and assayed in duplicate. For experimental samples, the PCR was set up in triplicate. The ratio of the T and S signals were calculated for each sample and averaged.

### RNA isolation and RT-qPCR for expression analysis of shelterin genes

Briefly, total RNA was extracted using RNeasy Universal Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. RNA integrity and concentration were determined by analyzing on the Agilent 2100 Bioanalyzer using the eukaryotic total RNA nano assay (Agilent, USA). 700 ng of the total RNA was used for cDNA preparation using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). The prepared cDNA was diluted to a 1:30 dilution before carrying out qPCR analysis. mRNA levels of five shelterin genes (*TERF1*, *TERF2*, *POT1*, *TERF2IP*, *TINF2*) were determined by RT-qPCR using QuantiTect SYBR Green PCR Kit (Qiagen, Germany), performed

on CFX96 Real Time PCR detection system. Data was analyzed using CFX Manager Software (Bio-Rad, USA). To increase the accuracy of the result, three reference genes (*GAPDH*, *ACTB*, and *RRN18S*) were used for normalization. The primers used for the five shelterin genes and the three reference genes were procured from the QuantiTect Primer Assay (Qiagen, Germany). The reaction was set up after thawing all the components and mixing each component thoroughly according to the manufacturer's protocol. In each experiment, PCR was carried out in duplicate with appropriate positive and negative controls included along with a no template control. The data were expressed in terms of normalized fold expression.

### Statistical analysis

Student's *t*-test was applied to compare differences in telomere length and in shelterin protein expression levels among tumor, non tumor, and stone samples. Linear regression analysis was carried out to study the correlation between telomere length and expression levels of shelterin genes.  $P < 0.05$  was considered to be statistically significant. All statistical tests were carried out using SigmaPlot Version 11 software (Systat Software Inc., San Jose, USA).

## RESULTS

### Telomere length is shortened in gallbladder cancer tissues

The relative telomere length (T/S) was observed to be significantly reduced in tumor tissues as compared to both ANT and inflamed tissue samples ( $P < 0.001$ ,  $P < 0.001$ ). The relative telomere length was also significantly reduced in tumors with or without gallstone in comparison to ANT ( $P = 0.005$ ,  $P = 0.026$ ) and gallstone tissues ( $P = 0.039$ ,  $P = 0.004$ ). Furthermore, telomere length was observed to be increased in inflamed tissues as compared to ANT tissues. However, the difference was not significant [Figure 1].

### Downregulation of *TERF1*, *POT1*, and *TINF2* mRNA levels in inflamed tissues

Comparison of expression of five shelterin complex genes in inflamed tissue and ANT tissues samples showed significantly decreased mRNA levels of *TERF1*, *POT1*, and *TINF2* in inflamed tissues (*TERF1*:  $P \leq 0.001$ ; *POT1*:  $P \leq 0.001$ ; *TINF2*:  $P \leq 0.001$ ). However, when mRNA levels were compared between inflamed and tumor tissues, again *TERF1* and *POT1* mRNA levels were found to be significantly decreased in inflamed tissues (*TERF1*:  $P = 0.009$ ; *POT1*:  $P \leq 0.001$ ). Further, comparison of tumor tissues with both types of non tumor tissues (ANT and CCC) revealed an increased mRNA levels of all the five genes in tumor tissues; however, the data were statistically insignificant. Further, to understand the role of gallstone in gallbladder carcinogenesis, inflamed tissues (CCC) were compared with tumor tissues with gallstones (AC + GS). The results showed only *POT1* to be significantly upregulated in tumor tissues with gallstones [Figure 2].

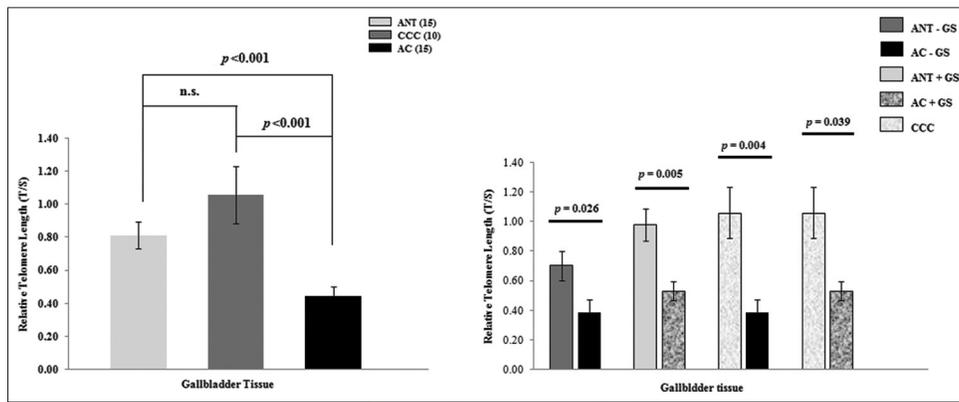
**POT1 expression positively correlates with telomere length in tumor tissues**

POT1 mRNA levels were found to be positively correlated with telomere length in tumor tissues ( $P = 0.0183$ ) and specifically in tumor tissues without gallstones ( $P = 0.0111$ ). Telomere length was independent of the mRNA levels of the other four shelterin genes in tumor tissues, with no statistically significant trend of telomere length-related variation in the mRNA levels of the five genes [Figure 3]. However, TERF2 mRNA levels showed positive correlation with telomere length in inflamed tissues ( $P = 0.033$ ). The other four genes did not show any correlation with telomere length in inflamed tissues.

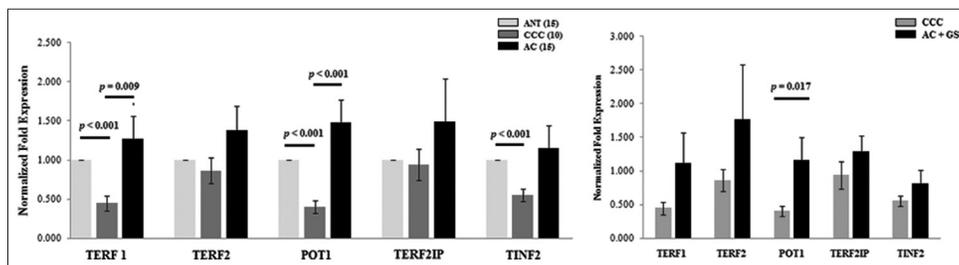
**DISCUSSION**

We investigated the possible association of telomere length variation and expression analysis of five shelterin

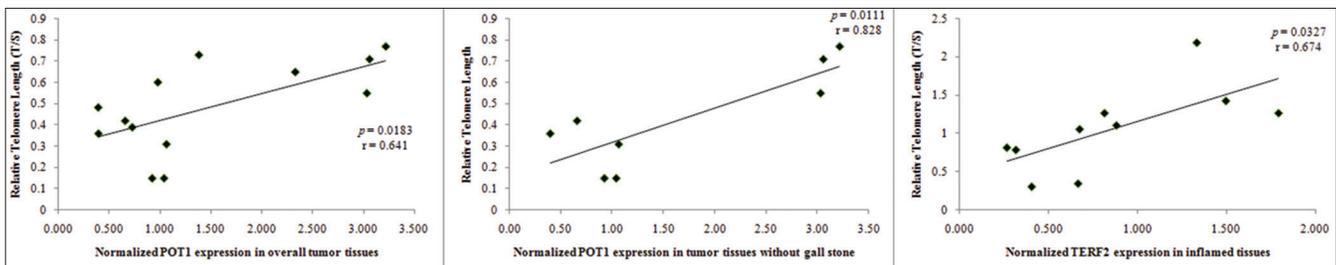
genes, which are essential for maintaining the integrity of telomeres, in GBC patients from North Central India. So far, to the best of our knowledge, this is the first study which reports the relationship between telomere length variation and expression of shelterin genes in gallbladder carcinogenesis, especially in gallstone disease, and provides insights into its possible molecular mechanism of being a risk factor to GBC. Our present analysis on telomere length variation among different tissue types of gallbladder (pathological and nonpathological) showed significantly decreased telomere length in tumor tissues and was similar in inflamed tissues, as compared to ANT tissues. Our results are also in corroboration to an earlier report in biliary tract cancer<sup>[9]</sup> and in many other cancers also.<sup>[10,11]</sup> This suggests the importance of telomere length in the process of neoplastic transformation, as telomere shortening may lead to genomic instability which is one of the hallmarks of



**Figure 1:** Comparison of telomere length in different tissue types of gallbladder. ANT = Adjacent non tumor, CCC = Chronic calculous cholecystitis (inflamed tissue), AC = Adenocarcinoma, -GS = Without gallstone; +GS = With gallstone



**Figure 2:** Normalized fold expression of five shelterin genes in different gallbladder tissue types. ANT = Adjacent non tumor, CCC = Chronic calculous cholecystitis, AC = Adenocarcinoma, -GS = Without gallstone; +GS = With gallstone



**Figure 3:** Correlation of POT1 gene expression with telomere length in gallbladder cancer (overall and in tumor without gallstones) and TERF2 gene expression in chronic calculous cholecystitis

cancer. However, it is also true that the cancer cells maintain the telomere length during multiple cell divisions and this is supported by our recent study, where we have shown telomere length to be shortened in early grade GBC, while it was increased in late grade cancer.<sup>[12]</sup> This also suggests the activation of telomere salvage pathway for maintaining telomere length during multiple cell divisions in late grade cancer. In the present study, the reduction of telomere length was also seen in both, tumors with, or without gallstone in comparison to ANT and gallstone tissues. This suggests that gallstone may not be having any effect on telomere length, since in both types of tumor tissues telomere length was found to be reduced.

We also analyzed mRNA expression of five shelterin genes in tumor, non tumor, and inflamed tissues. In the present study, we reported significant downregulation of *TERF1*, *POT1*, and *TINF2* mRNAs in inflamed tissues, as compared to ANT tissues. Furthermore, *TERF1* and *POT1* were found to be upregulated in tumor tissues in comparison to inflamed tissues; of these two, only *POT1* was found to be also upregulated in tumor tissues with gallstone. Previous reports on the expression of shelterin genes are somewhat confounding as in some studies, they were found to be upregulated, whereas, in some, they were observed to be downregulated. *TERF1* is downregulated in gastric cancer, breast cancer, and astroglial brain tumors,<sup>[13-15]</sup> whereas, it was found to be upregulated in hepatocarcinogenesis and adenocarcinoma of the lungs.<sup>[16,17]</sup> *TINF2* was found to be upregulated in hepatocarcinogenesis<sup>[13]</sup> and downregulated in gastric cancer.<sup>[16]</sup> Similarly, in one report, *POT1* expression was shown to be higher in tumor tissues,<sup>[18]</sup> whereas, in other, it was found to be downregulated.<sup>[19]</sup> These reports on *TERF1*, *POT1*, and *TINF2* may be on account of dissimilar type of tumors, which might be associated with the varied expression of these proteins. However, *TERF1* and *POT1* are directly binding proteins to the telomere, whereas *TINF2* binds with *TERF1* and *TERF2* and is the keystone that helps bond *POT1/ACD* complex to the rest of the shelterin complex.<sup>[6]</sup> Loading of shelterin proteins, mainly *POT1*, onto the telomeres, blocks the action of telomerase enzyme at the 3' overhang. Hence, when longer telomeres are present, they tend to harbor more shelterin proteins, masking the 3' overhang, and blocking the activity of telomerase. This inhibition is lost when telomeres are short with lesser shelterin proteins.<sup>[20]</sup> Stoichiometry analysis of shelterin proteins, carried out by Takai *et al.*, 2010, revealed the levels of *TRF1* and *TRF2* in the chromatin bound fraction of human cells, to be plentiful to cover the telomere length, however, the *POT1/ACD* heterodimer was found in more copies with respect to their available binding sites, suggesting they may have other binding sites as well. Also the heterodimers, *TRF2/TERF2IP* and *POT1/ACD* were found to be in a 1:1 stoichiometry.<sup>[21]</sup> This suggests the importance of shelterin proteins in maintaining telomere structure. In our present study, *POT1* was also found to positively correlate with telomere length in tumor tissues, specifically, tumor tissues without gallstone, suggesting that

*POT1* might play a crucial role in the process of carcinogenesis. Telomere length also positively correlated with the expression of *TERF2* in inflamed tissues. However, *TERF2* did not show any significant difference in the different tissue types studied. This may be a chance occurrence due to the small sample size that was analyzed.

Further, in our analysis, tumor tissues showed upregulation of all the five shelterin genes as compared to non tumor tissues, but the difference was statistically not significant. Despite this, it indicates that high levels of these proteins might be inhibiting telomerase and regulating telomere length. This is evident by the decreased telomere length observed in tumor tissues. These results further substantiate the earlier finding that expressions of shelterin proteins are known to regulate telomere length by inhibiting telomerase enzyme.<sup>[20]</sup>

The other important finding from our study is that despite the significant downregulation of *TERF1*, *POT1*, and *TINF2* in inflamed tissues, the telomere length in these samples was found to be longer as compared to both non tumor and tumor samples. This suggests that longer telomere length, but then, reduced expression of these primary proteins of the shelterin complex (*TERF1*, *POT1*, and *TINF2*) might lead to uniform telomere region but, with improper shelterin capping in inflamed tissues with gallstones. Overall, our results in inflamed tissues further strengthen the view that gallstone is an important risk factor and is likely to be the initial event in gallbladder carcinogenesis. Reduction in the expression of *TERF1*, *POT1*, and *TINF2*, although not in telomere length, in inflamed tissues, may likely be making telomeres vulnerable to degradation or resulting in telomere dysfunction, initiating gallbladder carcinogenesis. Lingering presence of gallstones may lead to immense distress along with constant irritation around the tissue where it is located. This prolonged exposure to gallstone results in chronic inflammation.<sup>[22]</sup> Recently, inflammation has been acknowledged as the seventh hallmark of cancer and has also been proposed to be the cause of genetic instability.<sup>[23]</sup> This seems to suggest that telomere dysfunction occurs by way of uncapping of some of the shelterin proteins during stone formation leading to genetic instability, which could be one of the reasons for gallbladder carcinogenesis.

## CONCLUSION

We suggest that gallstone does not affect telomere length, and during inflamed condition, telomere length remains unaltered. However, low level of its protective proteins (*TERF1*, *POT1*, and *TINF2*) may lead to telomere dysfunction and ultimately influence carcinogenesis. In addition, the presence of high levels of shelterin proteins in tumor tissues indicates that telomerase enzyme might be getting inhibited, which explains the short telomeres found in tumor tissues; however, it requires further clarifications by analysis in different grades of cancer. Further validation of *POT1*, for its utility to be used as a biomarker, in tumor tissues with gallstones, in a

larger pool of samples, is also required. Additionally, analysis of these shelterin genes at the protein level along with expression analysis of telomerase enzyme in different stages of tumorigenesis might throw some more light in understanding the pathogenesis of this highly aggressive gallbladder cancer.

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#### Conflicts of interest

There are no conflicts of interest.

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