ORIGINAL ARTICLE

Dysfunction of subtelomeric methylation and telomere length in gallstone disease and gallbladder cancer patients of North Central India

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Abstract

Background Telomeres play an important role in cancer progression. Recently it has been shown that subtelomeric methylation negatively regulates telomere length in various diseases, including cancers. Here, we evaluated the influence of subtelomeric methylation in telomere dysfunction in gallbladder cancer (GBC), and whether this dysfunction is affected by the presence of gallstones.

Methods Relative telomere length and subtelomeric methylation levels were assessed using monochrome multiplex quantitative polymerase chain reaction and bisulfite sequencing, respectively, in different gallbladder tissue types including different grades of GBC, gallstones and adjacent non-tumor.

Results We found telomere length to shorten significantly in overall GBC, but specifically in early grade cancer. We also found D4Z4 and DNF92 subtelomeric sequences to be hypermethylated and hypomethylated, respectively, in GBC; however, their methylation levels differed significantly, only in early grade cancer. We could not find any specific correlation between subtelomeric methylation and telomere length in GBC. Interestingly, telomere length and subtelomeric methylation differed significantly in GBC without gallstones but not in GBC with gallstones.

Conclusions This study, thus, suggests that telomere dysfunction and changes in methylation levels may occur earlier in the progression of GBC, while the presence of gallstones may have no influence on telomere length as well as on methylation levels.

Keywords Gallbladder cancer · Gallstones · Methylation · Subtelomere · Telomere

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Introduction

Gallbladder cancer (GBC) is relatively a rare cancer [1] with very poor overall prognosis. The mortality rate of GBC is also very high owing to the late stage of diagnosis, with most of them being inoperable. The 5-year survival rate based on the American Cancer Society National Cancer Database is 8% at Stage IIIA to as low as 2% at Stage IVB [2]. Apart from geographic variation and ethnicity, the most important risk factor is gallstones [3], which is present in approximately 85% of patients with GBC [4]. The highest incidence rates are found in Chile, Bolivia, North India and Mexico [5–7]. Our data from North Central India report an age standardized incidence rate of 7.5/100,000 [8]. So far very little information is available that focuses on exploring the diagnostic and prognostic issues of this highly aggressive cancer.

Telomeres are made up of tandem repeats of TTAGGG in vertebrates [9], which functions to protect the ends of the chromosome by capping them with the shelterin proteins [10], and preventing from being recognized as double stranded breaks (DSBs). Hence, telomere length is critical in deciding whether a cell survives or undergoes replicative senescence and apoptosis. Telomere dysfunction has been known to cause genomic instability, which ultimately could be one of the causes for carcinogenesis.

Both telomere and subtelomeric regions are characterized by constitutive heterochromatin as seen in pericentomeric regions [11–13]. Telomeric repeats cannot be

methylated because of the absence of CpG sites, but the adjacent subtelomeric regions are heavily methylated. Mammalian telomeres are able to silence the subtelomeric genes by telomere position effect (TPE), which is further influenced by telomere length [14, 15]. Recent studies have identified that a change in subtelomeric methylation levels acts as a negative regulator of telomere length in human cancer cell lines [16].

The present study was carried out to analyze the subtelomeric methylation and telomere length in different grades of GBC and gallstone disease with a view to decipher molecular changes happening at the telomere and subtelomere regions. Also, since gallstones are an important risk factor, whether or not it influences these changes to help progression of GBC, was the other important issue investigated in this study.

Materials and methods

Patients and samples

Seventy-four patients including 37 GBC and 37 gallstone cases were recruited in the present study. Samples from tumor tissue (AC) and their corresponding adjacent non-tumor tissue (ANT) along with tissue inflamed due to gallstones (CCC) were obtained from resected gallbladder from Cancer Hospital and Research Centre, Gwalior, India. A gross analysis of the sample was performed by an expert pathologist at the hospital and on the basis of their appearance, tissues were selected as non-tumor, tumor or inflamed (chronic cholecystitis). Table 1 shows the clinicopathological profiles of the participating patients, indicating their age and gender. No preoperative treatment was given to the patients who were recruited for the study. All samples were collected with the informed consent of the donors, after clearance from the Institutional Ethics Committee on research using human subjects at Jiwaji University (Gwalior, India) and were adhering to the tenets of the Declaration of Helsinki.

Bisulfite sequencing of D4Z4 and DNF92 subtelomeric repeats

Genomic DNA from gallbladder tissue was extracted using QIAamp DNA mini kit (Qiagen, Duesseldorf, Germany). For methylation analysis of the subtelomeric repeat sequences D4Z4 and DNF92, 2 µg of genomic DNA was processed for bisulfite modification using EpiTect Bisulfite Kit (Qiagen). Bisulfite genomic sequencing of 6–11 clones was carried out as described elsewhere [17]. Briefly, semi-nested PCR was carried out in a volume of 20 µl for both D4Z4 and DNF92 repeat sequences, using 2 µl of bisulfite modified DNA (~50 ng) for the first amplification. The primers used for the first amplification were 5′-GTATATTTTTAGGTTGAGTTTTGTAA (F1-D4Z4) and 5′-AATATACCAAACCCTCTCTC (R1-D4Z4) or 5′-GTGGAGGTAGTATTAGGTAGA (F1-DNF92) and 5′-CCCTACTTTCTCAAAATACTCCTA (R1-DNF92). For the second amplification, 5 µl of a 1/300 dilution (~15 ng/µl) of the first amplification product in a volume of 50 µl was used. The primers used for the second amplification were 5′-GTGTGGTTTTGTTTTTTGTAAGG (F2-D4Z4) and R1-D4Z4 or 5′-GGGGTTGAGGTATATTAGTATTAA (F2-DNF92) and RI-DNF92. The final amplification product was gel purified using GeneJET Gel Extraction Kit (Thermo Scientific, Pittsburgh, PA, USA) and cloned in pJET 1.2/blunt vector using CloneJET PCR Cloning kit (Thermo Scientific). The ligation mixture was used to transform competent *Escherichia coli* DH5α strains using TransformAid Bacterial Transformation kit (Thermo Scientific). Automated sequencing was carried out commercially (SciGenom Labs, Kochi, India) to obtain methylation status of CpG sites of D4Z4 and DNF92 subtelomeric repeats. Percentage methylation was calculated by dividing the total number of methylated sites by the total number of CpG sites and converted to percentage terms for each tissue type. The presence of a TpA dinucleotide at a CpG site after bisulfite modification, presumably due to spontaneous deamination of methylated

Table 1 Clinicopathological profiles of gallbladder cancer and gall stone patients

Tissue type	Sample size (n)	Age (years) Mean ± SD (range)[a]	Gender Male: Female
Gallbladder carcinoma	37	47 ± 10 (30–65)	1: 2.7
Adenocarcinoma grade I	03	59 ± 6 (53–65)	1: 0.5
Adenocarcinoma grade II	14	44 ± 11 (30–65)	1: 2.5
Adenocarcinoma grade III	20	47 ± 9 (32–65)	1: 5.7
Gallbladder carcinoma + stone	15	46 ± 8 (30–64)	1: 6.5
Gallbladder carcinoma - stone	22	48 ± 12 (32–65)	1: 1.75
Chronic calculus cholecystitis	37	49 ± 14 (10–80)	1: 4.3

[a] Mean age has been rounded off to the nearest integer

CpGs on the opposite strand [18] was excluded from the methylation analysis.

qPCR analysis for relative telomere length measurement

The relative telomere length was measured by monochrome multiplex quantitative polymerase chain reaction (MMqPCR) in tumor-normal pair and gallstone/inflamed tissues [19]. Briefly, telomeric DNA (T) and a single copy gene, human beta globin (S), were amplified and quantified in a single well against a standard curve of five concentrations of human reference DNA sample, across an 81 fold range (150 ng, 50 ng, 16.7 ng, 5.55 ng and 1.85 ng per well). The reference DNA samples were analyzed in duplicate in every plate. All experimental DNA samples were assayed in triplicate and, hence, three T/S values were obtained for each sample. For further analysis, average T/S value of the three was taken in each case. qPCR was performed on CFX96 Real Time PCR Detection System and the data was analyzed using CFX Manager software (BioRad, Hercules, CA, USA).

Statistical analysis

The data was statistically analyzed using SigmaPlot Version 11, Systat Software, San Jose, CA, USA. χ^2 test was applied to compare D4Z4 or DNF92 DNA methylation in ANT, different grades of AC and CCC tissues. Mann–Whitney Rank Sum test was applied to compare telomere length in different gallbladder tissue types. To test the correlation between telomere length and subtelomeric methylation, Pearson's correlation test was applied. $P < 0.05$ was considered to be statistically significant.

Results

Gallbladder cancer tissue exhibit shorter telomeres than adjacent non-tumor tissue and chronic calculus cholecystitis tissue
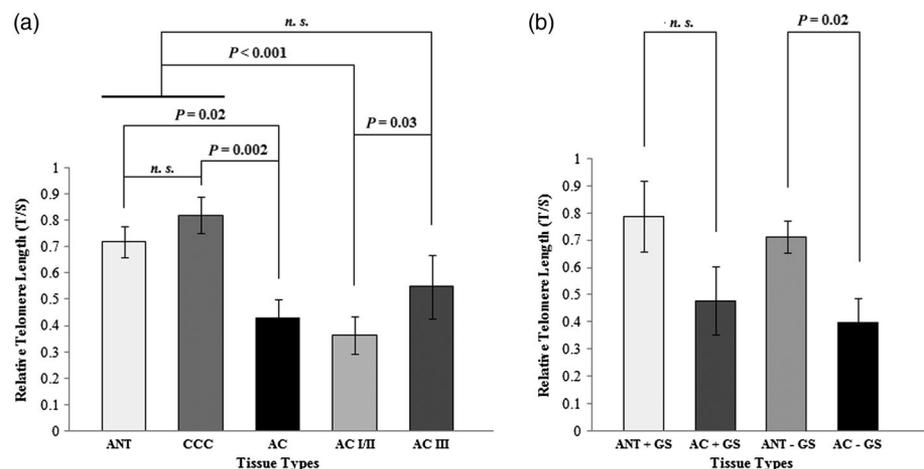
Telomere length comparison (Fig. 1a) revealed significant reduction in the mean telomere length in tumor tissues (AC) as compared to both adjacent non-tumor (ANT) and gallstone (CCC) tissues ($P = 0.02$ and $P = 0.002$, respectively). Further telomere length was found to be increased in CCC tissues, when compared to ANT tissues; however, this difference was statistically not significant.

Telomere attrition occurs in early grade adenocarcinoma and not thereafter

We also tried to look whether telomere length is in any way, responsible for the progression of cancer (Fig. 1a). We found a significant reduction in telomere length in Grade I/II tumor tissues ($P < 0.001$), when compared to ANT or CCC tissues. Telomere length was also found to be reduced in Grade III tumor when compared with ANT; however, this was not significant ($P = 0.634$). Interestingly, telomere length was found to increase from Grade I/II to Grade III tumor tissues ($P = 0.031$).

Telomere length is unaffected in the presence of gallstones

Also, when tumor samples were categorized on the basis of presence or absence of gallstones and compared with their respective adjacent non-tumor samples, we could

Fig. 1 Comparison of relative telomere length in different gallbladder tissue types. (**a**) Distribution of relative telomere length among adjacent non-tumor (ANT), chronic calculus cholecystitis (CCC), adenocarcinoma, in general (AC), early grade adenocarcinoma (AC I/II) and late grade adenocarcinoma (AC III) tissues. (**b**) Distribution of relative telomere length in adenocarcinoma with or without gallstones. +*GS* with gallstones, −*GS* without gallstones, *n.s.* non significant. Numbers in parenthesis indicate number of samples analyzed

find reduction of telomere length in both categories (Fig. 1b); however, it was statistically significant only in tumor tissues without gallstone ($P = 0.02$). No significant difference in telomere length between tumor with or without gallstone was found.

D4Z4 subtelomeric sequence display hypermethylation in gallbladder cancer and hypomethylation in gallstone tissues

To understand the role of subtelomeric methylation in GBC, the methylation level of subtelomeric repeats, D4Z4 and DNF92, were assessed in different grades of AC, CCC and ANT tissues (Fig. 2a,b). We could find hypermethylation of D4Z4 sequences in tumor tissues as compared to ANT and more specifically in tumor tissues without gallstone ($P = 0.01$ and $P = 0.005$, respectively) (Fig. 3a). On the other hand, hypomethylation of D4Z4 sequences was seen in CCC in comparison to ANT ($P = 1 \times 10^{-6}$) and tumor tissues with or without gallstones ($P < 0.0001$). D4Z4 methylation did not differ significantly between ANT and tumor tissues with gallstones.

DNF92 subtelomeric sequences display hypomethylation in gallbladder cancer and gallstone tissues

In contrast to D4Z4 methylation level, DNF92 sequences showed hypomethylation in ACs ($P = 0.01$), specifically in tumor tissues without gallstones ($P = 0.009$), as well as in gallstone tissues ($P = 0.006$), in comparison to ANTs (Fig. 3b). As in D4Z4, DNF92 methylation did not differ significantly between ANT and tumor tissues with gallstones.

Early grade adenocarcinoma without gallstones revealed significant changes in methylation pattern of subtelomeric sequences

Methylation levels were further categorized grade-wise to study whether there is any definite pattern of methylation during GBC progression (Fig. 3a,b). Comparison of D4Z4 methylation levels in different grades of cancer without gallstones and adjacent non-tumor revealed significant hypermethylation in Grade I/II tumor tissues ($P = 4 \times 10^{-6}$), but, was not significant in Grade III tumor tissues. Comparison of DNF92 methylation level in different grades revealed significant hypomethylation in Grade I/II tumor tissues ($P = 0.01$), but, it was also not significant in Grade III tumor tissues.

Absence of correlation between subtelomeric DNA methylation and telomere length in gallbladder cancer

Linear regression analysis was used to test the association between telomere length and subtelomeric DNA methylation. However, we could not find any correlation between methylation levels of D4Z4 and DNF92 sequences with telomere length in tumor tissues with or without gallstone.
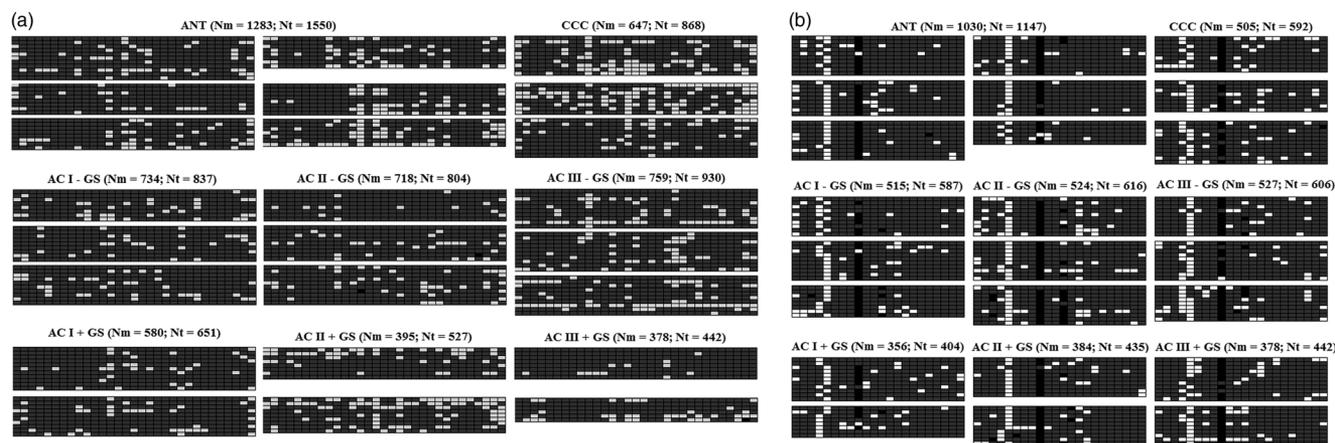
Fig. 2 Sequencing of PCR amplified (**a**) D4Z4 and (**b**) DNF92 fragments from bisulfite treated genomic DNA. Each line corresponds to the CpG residues of an individual clone of subtelomeric DNA. For each tissue type, 6–11 representative sequences are shown. Unmethylated cytosines are represented by white boxes, whereas methylated cytosines are represented by gray boxes. A black box indicates a TpA dinucleotide in the bisulfite-treated DNA, presumably due to spontaneous deamination of methylated CpGs on the opposite DNA strand and were excluded from the analysis. The total number of CpG analyzed for the subtelomeric DNA sequence is given for each tissue type under 'N′' and the number of methylated sites under 'Nm'. *ANT* adjacent non-tumor, *CCC* chronic calculus cholecystitis, *AC I* adenocarcinoma grade I, *AC II* adenocarcinoma grade II, *AC III* adenocarcinoma grade III, +*GS* with gallstones, −*GS* without gallstones

(a)

(b)
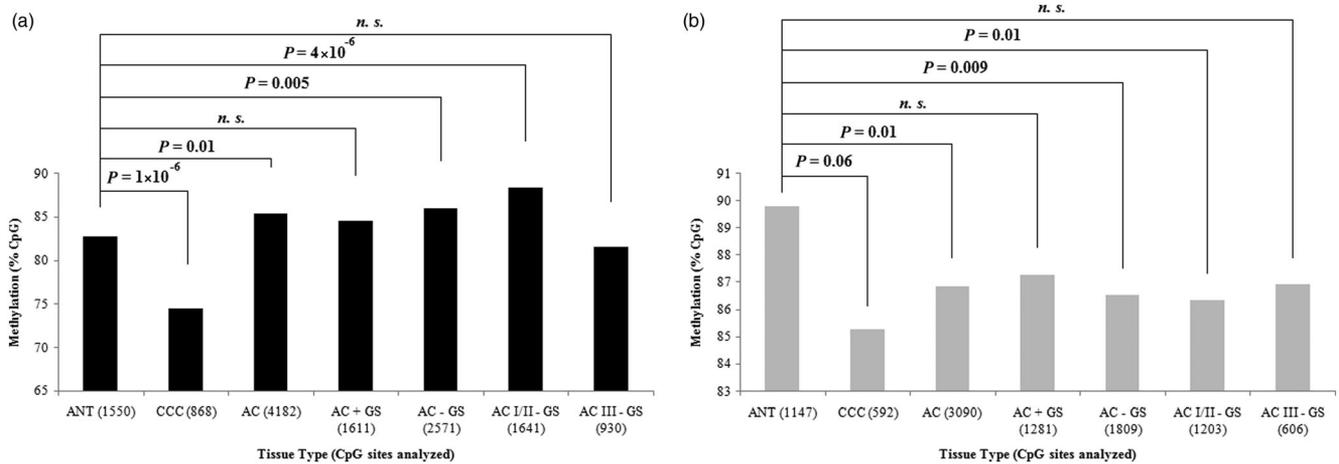
Fig. 3 Quantification of (**a**) D4Z4 (black bars) and (**b**) DNF92 (grey bars) methylation levels calculated from Figure 2. χ^2 test of association was applied to compare D4Z4 or DNF92 DNA methylation in different gallbladder tissue types. *ANT* adjacent non-tumor, *CCC* chronic calculus cholecystitis, *AC* adenocarcinoma, *+GS* with gallstones, *−GS* without gallstones, *n. s* non significant

Discussion

There has not been widespread investigation on the molecular mechanisms of GBC, as it has been in other cancers, resulting in lack of diagnostic and prognostic markers. This is also because of the fact that gallbladder cancer is a relatively uncommon cancer. However, due to late stage diagnosis and high mortality rate, there is a serious concern for this aggressive cancer. Although there are studies in GBC, which include detection of allelic loss of multiple chromosomal regions [20, 21], mutations of dominant oncogenes and tumor suppressor genes, such as *KRAS*, *TP53*, *FHIT* [22, 23], mutation in genes of cell cycle regulators, such as *p27*, *p21*, *RB*, *cyclin D1* [24–27], and mutations in DNA repair enzyme genes, like *MGMT*, *MLH1* and *MSH2* [28], but, so far no study has been carried out on the role of subtelomeric methylation in telomere length regulation and whether gallstone plays any role in it.

The present study showed that there is a significant decrease in telomere length in AC tissues as compared to both ANT and CCC tissues. Our data corroborates with an earlier study where it was shown that telomere shortening occurs in tumor tissues [29]. However, when we studied telomere length in different grades of cancer as compared to ANT, we could find that telomere attrition was confined only to the Grade I/II tumor samples and not in Grade III, suggesting that telomere length dysfunction occurs in tumor samples and, more specifically, in the initial stages of cancer progression, but not thereafter. Also, an increase in the telomere length was seen from Grade I/II to Grade III, suggesting the possible activation of telomere salvage pathways for telomere maintenance [30]. This is also substantiated by few earlier reports showing the activation of telomerase in late grade tumors in GBC [31–33]. However, no study has been carried out so far, correlating telomere length and activation of telomerase

in different grades of cancer. Hence, our study provides an insight into the possible molecular mechanism involving telomerase dependent telomere length regulation in GBC, which could be further validated by exploring the effect of telomerase on telomere length dysfunction.

As for the methylation analysis, no study has yet been conducted to understand the role of subtelomeric methylation in telomere attrition in gallbladder cancer. We studied two subtelomeric repeat sequence, D4Z4 (located at 4q35.2 and 10q26.3) and DNF92 (located at 1pter, 5qter, 6qter, 8pter, 17qter and at a lower frequency at 8 other chromosome ends) [17], of which, D4Z4 is a widely studied subtelomeric repeat in different diseases [34, 16]. We found significant hypermethylation of D4Z4 repeat sequences, whereas, DNF92 repeat sequences were significantly hypomethylated, in AC tissues as compared to ANT. These results do not corroborate with most of the studies where subtelomeric sequences are reported to be hypomethylated in various disease conditions when compared to normal [16, 17, 34, 35]. However, we could find similar hypomethylation only in gallstone cases and not in adenocarcinoma cases, suggesting that gallstone disease alone and gallstone leading to cancer may follow altogether different pathways. There are also reports consistent with our results, demonstrating methylation patterns of different subtelomeric regions to be varying, and thus, substantiating that the methylation patterns of subtelomeric sequences do not follow a particular pattern and varies from cancer to cancer [36, 37]. Also, the methylation levels of both markers differed significantly only in tumor tissues without gallstones and specifically in early grade adenocarcinoma. The difference in methylation was not significant in tumor tissues with gallstones. This further confirms that dysfunction in methylation level occurs only in early grade tumors, and hence, may have a role in the development of cancer. It also validates

our observation on telomere length variation, where telomere dysfunction is seen only in early grade cancer.

Further, in an earlier study, an association between subtelomeric hypomethylation and telomere loss in a panel of human cancer cell lines was reported, where telomere length was found to negatively correlate with methylation levels of two subtelomeric regions, D4Z4 and SRH [16]. Similar correlations have also been demonstrated in certain disease conditions, like Sarcoidosis, Parkinson's disease [38, 39] and Immunodeficiency, Centromere instability and Facial anomalies (ICF) syndrome [35]. Interestingly, in the present study, we did not find any correlation between the telomere length and methylation in the subtelomeric sequences, D4Z4 and DNF92, in GBC. This deviation can be attributed to the fact that the above studies were from cancer cell lines [16] and there could be significant differences in conditions prevailing *in vitro* and *in vivo*, while other studies [35, 38, 39] are related to ageing diseases and not particularly to cancer. Overall, we came across a single study in human hepatocarcinogenesis that showed negative correlation of telomere length and subtelomeric methylation levels in cancer tissues, where subtelomeric methylation was shown to be related to telomere lengthening or shortening, but only at certain subtelomeric regions [40]. These inconsistent methylation patterns in subtelomeric regions further complicate the role of subtelomeric methylation in disease pathogenesis, especially in GBC.

Moreover, the dysfunction in telomere length and subtelomeric methylation seen in our results appeared unaffected by the presence of gallstone, as we did not find significant difference in telomere length and subtelomeric methylation levels between ANT and tumor tissue with gallstone. In contrast, a significant difference was observed between ANT and tumor tissue without gallstone. This suggests that gallstone is not responsible for dysfunction in telomere length and subtelomeric methylation. This is further substantiated by the report that only 1–3% of patients with gallstones develop GBC [41], even if gallstones are present in most of the GBC cases.

In conclusion, this is the first ever study showing telomere dysfunction and changes in subtelomeric methylation levels occurring earlier in the progression of GBC. Also, the presence of gallstones in cancer may have no effect on telomere length as well as on subtelomeric methylation levels, suggesting that cancer due to gallstones and cancer without gallstones may have different etiologies. However, other mechanisms, like dysfunction of telomeric proteins may be responsible for telomere dysfunction in gallbladder tissues having gallstones. This can be explored in future studies to confirm the role of gallstone in gallbladder cancer along with validations of subtelomeric methylation by analyzing more number of subtelomeric markers. This will further add to our current

understanding as to how subtelomeric methylation and telomeric protein expression plays a role in telomere length regulation and cancer progression. Finally, even if telomere length and subtelomeric methylation levels may not be suitable to predict GBC progression from gallstone at this juncture, telomere length may be more convincingly used as an early diagnostic marker for GBC in future.

Conflict of interest   None declared.

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