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ORIGINAL ARTICLE

Quantification of Circulating Free DNA as a Diagnostic Marker in Gall Bladder Cancer

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Abstract Gall bladder Carcinoma (GBC) is the fifth most common cancer of the digestive tract and frequently diagnosed in late stage of disease. Estimation of circulating free DNA (cfDNA) in serum has been applied as a "liquid biopsy" in several deep seated malignancies. Its value in diagnosis of gall bladder carcinoma has not been studied. The present study was designed to assess the role of cfDNA in the diagnosis of GBC and correlate levels with the TNM stage. Serum was collected from 34 patients with GBC and 39 age and sex matched controls including 22 cholecystitis and 17 healthy individuals. Serum cfDNA levels were measured through quantitative polymerase chain reaction (qPCR) by amplification of β-globin gene. Performance of the assay was calculated through the receiver operating characteristic (ROC) curve. The cfDNA level was significantly lower in healthy controls and cholecystitis (89.32 ± 59.76 ng/ml, 174.21 ± 99.93 ng/ml) compared to GBC (1245.91 ± 892.46 ng/ml, p = <0.001). The cfDNA level was significantly associated with TNM stage, lymph node involvement and jaundice (0.002, 0.027, and 0.041, respectively). Area under curve of ROC analysis for cancer group versus healthy and cholecystitis group was 1.00 and 0.983 with sensitivity of 100 %, 88.24 % and specificity of 100 % respectively. Quantitative analysis of cfDNA may distinguish cholecystitis and gall bladder carcinoma and may serve as new diagnostic, noninvasive marker adjunct to imaging for the diagnosis of GBC.

Keywords Circulating free DNA · Gall bladder cancer · Real time PCR (qPCR) · DNA quantification

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Introduction

Gall bladder Carcinoma (GBC) is the most frequent malignant neoplasms of the biliary tract and is fifth most common gastrointestinal malignancy in India [1, 2]. It has very wide geographical variation worldwide, being least frequent in Europe and US to high incidence in Chile, Japan and India. The highest prevalence of GBC has been reported in India among females with age above 65 (21.5 out of 100,000) [3]. Gall stone is major risk factor associated with this cancer and is present in 60 to 90 % of patients of GBC [4]. Diagnosis usually occurs at an advanced stage of disease due to lack of early signs and symptoms, hence cases have a poor 5-year survival rate of 5–10 % [5, 6]. Early diagnosis and prediction of disease is important to improve the patient treatment and survival.

Circulating free DNA (cfDNA) has been reported to harbour information about disease diagnosis and its progression. It allows a noninvasive method to detect genetic material coming from diseased cells and tissues. CfDNA comprises of extracellular nucleic acids found in plasma/serum of human blood. Its levels may vary with the disease progression and thus have potential to be used as a diagnostic and prognostic biomarker. In a healthy individual, cfDNA is believed to enter circulation via apoptosis of lymphocytes and other nucleated cells, whereas in cancer patients it comes from lysis or active release of circulating cancer cells and tumor necrosis [7]. Hence, cfDNA levels

are useful to differentiate cancer patients from healthy individuals or those with various non-malignant diseases [8]. Compared to healthy controls increased levels of cfDNA have been reported in lung [9], colon [10], cervical [11], ovarian [12], breast [13], testis [14], bladder [15], prostate [16], gastric [17] and hepatocellular cancer [18]. However cfDNA levels have not been explored in GBC.

The present study uses real time PCR assay (qPCR) to quantify amount of cfDNA in GBC patients as compared to healthy controls and cholecystitis as disease control. CfDNA was isolated from serum of cases of GBC and controls. The amount was measured by quantifying the β-globin gene copies present in the sample and was correlated with clinico-pathological parameters. The sensitivity and specificity of the assay were also evaluated. To the best of our knowledge this is the first study evaluating serum cfDNA level in GBC.

Material and Methods

This case control study included 34 cases of GBC and 22 cases of cholecystitis and 17 healthy controls. All the patients who fulfilled the inclusion criteria were consecutively recruited for study from Department of Surgical Oncology & Gastrosurgery, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow. A written informed consent was taken from patients. Approval of the Institutional Ethical Committee was taken before commencement of the study. The patients were divided into the following groups:

Group I: 34 newly diagnosed patients with GBC (diagnosis of cases was based on Ultrasound Sonography (USG)/ Computed Tomography (CT)/Magnetic Resonance Imaging (MRI)/Endoscopy/biopsy).

Group II: 22 patients with cholecystitis of matched age and sex to group I.

Group III: 17 healthy controls of matched age and sex to group I.

Cases who have undergone previous chemotherapy/radiotherapy, those with evidence of significant clinical disorder or laboratory finding (which, in the opinion of the investigator, makes it undesirable for the patient to participate in the trial) and pregnant or breastfeeding women were excluded from the study.

Blood Sample Collection and DNA Extraction

Peripheral blood (3.5 ml) was collected from cases and controls before surgery or treatment in silica gel vials (B. D. Biosciences, USA). Samples were processed within 1 h of collection for serum isolation. Serum was separated from the cellular fraction by centrifugation at 1800×g for 10 min and stored at −80 °C until further processing.

DNA extraction from serum was performed using Charge Switch® gDNA 1 mL Serum Kit (Invitrogen, USA) as per the manufacturer's instructions. Briefly, 560 μl of lysis buffer and 30 μl Proteinase K was added to 800 μl of serum and incubated at room temperature for 20 min. To the mixture, 200 μl of purification buffer and 25 μl of charge Switch magnetic beads were added, mixed by pipetting gently, tubes were placed in the magna Rack™ for 3 min. The tubes were washed twice with 800 μl of wash buffer and 50 μl of Elution Buffer was added and left for 2 min at room temperature followed by 1 min incubation in magna Rack™. Eluate containing the purified cfDNA was transferred to a clean tube and frozen at -80 °C until further processing.

Quantitative Polymerase Chain Reaction (qPCR)

The amount of cfDNA extracted from the cases and controls was quantified by quantitative SYBR Green real-time PCR by amplification of *β-globin* gene in the samples compared to standard curve plotted by quantified DNA controls. Standard TaqMan Control Human Genomic DNA (Applied Biosystem, USA) with concentration of 10 ng/μL, was used to prepare DNA reference standards S1 to S5. The standard 1 ng (S3), 0.1 ng (S4) and 0.01 ng (S5) were prepared by serial dilution of stock 10 ng (S2) in nuclease free water, for 90 ng (S1) 9 μL of S2 was used.

Primers used for *β-globin* in the qPCR assay were forward primer-GTGCACCTGACTCCTGAGA and reverse primer-CCTTGATACCAACCTGCCCAG [19]. Each 20 μl qPCR reaction contained 10 μl of SYBR Green Supermix (Applied Biosystems, USA), 0.5 μM of each forward and reverse primers and DNA reference standard or 1 μl of sample cfDNA and the volume was adjusted by nuclease free water. qPCR was performed in CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with thermal cycling conditions of first denaturation at 95 °C for 9 min, followed by 35 cycles of 95 °C for 30s, 55 °C for 30s and 72 °C for 30s. To confirm the generation of specific PCR product melting curve analysis was performed from 65 °C to 95 °C (increment 0.5 °C every 30s) at the end of each reaction. All samples were made in duplicate and a negative control (without DNA) was set in each run. The machine generated Ct values and amplification plots were used to create standard curve, which was employed to quantify the DNA content in the samples.

Statistical Analysis

Statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences) software package, version 16.0. The data were expressed as mean ± SD. The age and sex of three groups were compared by one way analysis of variance (ANOVA) and chi-square ($\chi 2$) respectively. The levels of cfDNA in the groups categorized as GBC, cholecystitis and healthy controls were compared by non parametric

Kruskal-Wallis one way ANOVA followed by Z test. The non parametric Mann-Whitney U-test was used to compare cfDNA levels between two groups. The Receiver Operating Characteristics (ROC) curve analysis was performed to test the diagnostic potential of cfDNA to discriminate among the cases of two groups. A cut off cfDNA concentration value was defined to calculate sensitivity and specificity values defining the curve and the area under the curve (AUC). A p value less than 0.05 (two tailed) was considered significant.

Results

The demographic characteristics of the groups (healthy control, cholecystitis and cancer) and clinico-pathological characteristics of GBC patients are summarized in Table 1. The age of control, cholecystitis and cancer patients ranged from 36 to 70 yrs., 26–71 yrs. and 32–78 yrs. respectively, with mean (± SD) listed in Table 1. Male to female (M/F) ratio was 8/9 in control group, 8/14 in cholecystitis group and 11/ 23 in cancer group. The mean age of patients in all the three groups was statistically similar ($F = 0.82$, $p = 0.445$) and there was similar gender distribution ($\chi^2 = 1.06$, $p = 0.590$) among the study groups.

Most of the GBC patients were in stage IV (85.3 %) with tumor stage of T3/T4 (44.1 %). Regional lymph node involvement was present in 61.8 % cases of GBC, distant metastasis in 52.9 % and jaundice in 67.6 %.

Serum cfDNA Level

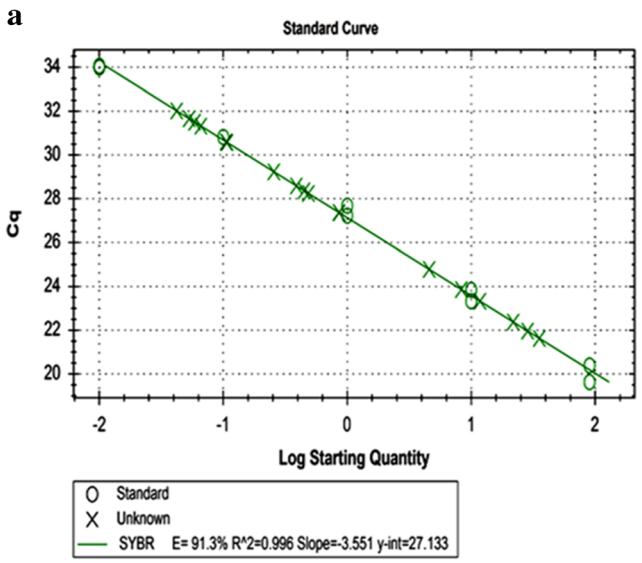
The standard curve plotted by β- globin gene amplification in DNA standards had R^2 value of 0.996 as shown in Fig.1a and b. Concentration of cfDNA in samples was calculated by plotting the Ct values on standard curve by the CFX96 software manager v3.0 (Bio-Rad Laboratories, Hercules, CA, USA). The mean (±SD) cfDNA levels in control, cholecystitis and cancer patients were 89.32 ng/ml(±59.76), 174.21 ng/ml(±99.93) and 1245.91 ng/ml(±892.46). Comparing the cfDNA levels of three groups, Kruskal-Wallis ANOVA revealed significantly different levels among the groups ($F = 54.06$, $p < 0.001$). Further Z test revealed significantly different and higher (92.8 %) cfDNA in cancer patients as compared to control group ($Z = 6.63$, $p < 0.001$) **(**Fig.1c**)**. Furthermore, the cfDNA in cancer patients was also found significantly higher (86.0 %) as compared to cholecystitis group ($Z = 5.37$, $p < 0.001$). However, cfDNA levels were not significantly different between control group and cholecystitis group, though it was 48.7 % higher in cholecystitis group compared to control ($Z = 1.55$, $p = 0.365$) **(**Fig.1c**)**. The cfDNA level of three groups is summarized in Table 2.

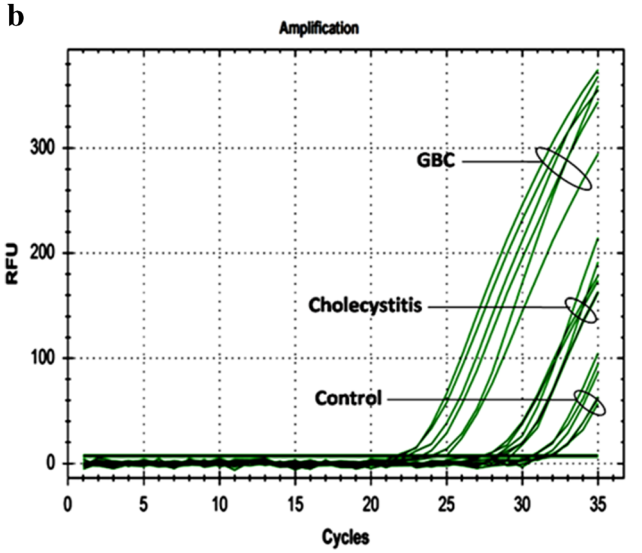
Association of cfDNA Level with Demographic and Clinico-Pathological Characteristics in GBC Patients

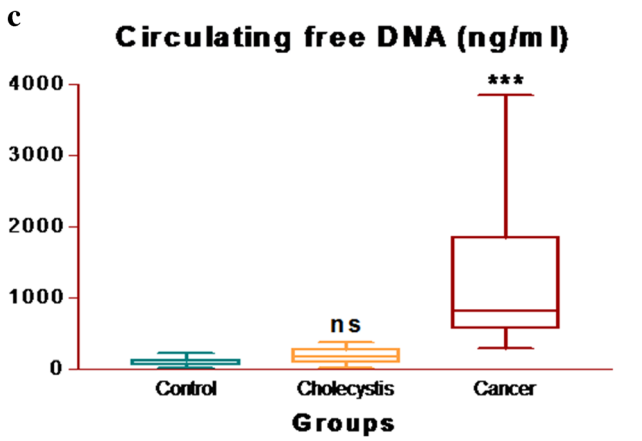
The association of cfDNA levels with demographic and clinico-pathological characteristics of GBC patients is summarized in Table 3. In GBC patients, the mean cfDNA level was lower among the age group of >45 yrs. than lower aged patients (≤45 yrs). CfDNA levels were higher in males than females. CfDNA levels were significantly elevated in patients with N1 and N2 lymph node status as compared to N0 and jaundice ($U = 7.21$, $p = 0.027$, $U = 71.00$, $p = 0.041$ respectively). Conversely the mean cfDNA level was higher, in patient with primary tumor of T4 than T3 & T2, and in patients with

Table 1 Demographic and Clinico-pathological characteristics of control groups and GBC patients

Characteristics		Control ($n = 17$) (%)	Cholecystitis ($n = 22$) (%)	Cancer ($n = 34$) (%)
Age (yrs) Mean (± SD)		47.12 ± 9.69	50.95 ± 14.34	51.53 ± 11.19
Sex Male/ Female		8 (47.1)/9 (52.9)	8 (36.4)/14 (63.6)	11 (32.4)/23 (67.6)
Stage	II			3 (8.8)
	III			2 (5.9)
	IV			29 (85.3)
Primary tumor	T2			4 (11.8)
	T3			15 (44.1)
	T4			15 (44.1)
Lymph node	N0			8 (23.5)
	N1			5 (14.7)
	N2			21 (61.8)
Metastasis	M0			16 (47.1)
	M1			18 (52.9)
Jaundice	No			11 (32.4)
	Yes			23 (67.6)







◀ **Fig. 1** Quantitative Real time PCR. **a)** A Standard curve for the β-globin assay, using fivefold serial dilution of genomic DNA (from 90 ng to 0.01 ng). The X- axis denotes the concentration of target (logarithmic scale) and the Y-axis denotes the threshold cycle (Cq). **b)** Amplification plots for sample. The X- axis denotes the threshold cycle and the Y-axis denotes the Relative Fluorescence Unit (RFU) for each sample per cycle. **c)** Box plot showing cfDNA levels of three groups. Groups were compared by Kruskal-Wallis ANOVA followed by Z test. ($^{ns}p > 0.05$ or $^{***}p < 0.001$- as compared to Control group). ns = not significant, *** = highly significant

metastasis vs. no metastasis; however the difference was not statistically significant. In addition, mean cfDNA levels were significantly higher in advanced stage patients (stage IV) than in early stage disease (stage II and III) (U = 10.00, $p = 0.002$).

Diagnostic Accuracy of cfDNA- ROC Curve Analysis

ROC curve analysis was performed to evaluate diagnostic accuracy of cfDNA levels to discriminate the cases of two groups (control and cholecystitis, control and cancer, and cholecystitis and cancer). The cutoff values of cfDNA with their sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Area Under Curve (AUC) are depicted in Fig.2 and Table 4. The ROC curve analysis found the significant diagnostic accuracy of cfDNA to discriminate the cases of two groups. The cfDNA value at >74.37 ng/ml discriminates healthy controls and cholecystitis significantly with 81.82 % sensitivity (95%CI = 59.7–94.7) and 64.71 % specificity (95 % CI = 38.4–85.7). Further, the cut off value of cfDNA at >218.55 ng/ml discriminates healthy controls and cancer patients significantly with 100.00 % sensitivity (95 % CI = 89.6–100.0) and 100.00 % specificity (95 % CI = 80.3–100.0). The cfDNA value at >372.92 ng/ml discriminates cases of cholecystitis and cancer patients significantly with 88.24 % sensitivity (95 % CI = 72.5–96.6) and 100.00 % specificity (95 % CI = 84.4–100.0).

Discussion

We have for the first time assessed cfDNA levels in GBC. It is the fifth most common gastrointestinal malignancy in India and usually diagnosed at advanced stage through USG, CT and/or MRI. In early stage GBC is usually asymptomatic or symptoms are very similar to those of benign conditions, like chronic cholecystitis. Clinical and radiological findings are often unable to detect early stage of disease. Ultrasound and CT are radiological diagnostic approaches and helpful in showing a thickened gallbladder wall (>5 mm), pericholecystic fluid or

Table 2 CfDNA levels (ng/ml) of three groups

Groups	n	Minimum	Maximum	Mean	SD	Median
Control	17	8.75	218.55	89.32	59.76	72
Cholecystitis	22	5.87	372.92	174.21	99.93	168
Cancer	34	283.94	3848.41	1245.91	892.46	802

Table 3 Association between demographic & clinicopathological characteristics of GBC patients with cfDNA level

Characteristics	N	CfDNA (ng/ml) Mean ± SD	U/F value	*p* value
Age (yrs):				
≤ 45	12	1329.53 ± 1054.74	131.0	0.971
> 45	22	1200.31 ± 814.07		
Sex:				
Female	23	1092.87 ± 746.11	94.00	0.232
Male	11	1565.92 ± 1112.27		
Stage:				
II + III	5	404.58 ± 135.77	10.00	0.002
IV	29	1390.97 ± 887.63		
Primary tumor:				
T2	4	763.39 ± 755.36	3.66	0.160
T3	15	1073.03 ± 816.87		
T4	15	1547.47 ± 944.40		
Lymph node:				
N0	8	709.24 ± 512.87	7.21	0.027
N1	5	1658.64 ± 564.60		
N2	21	1352.09 ± 994.35		
Metastasis:				
M0	16	987.24 ± 761.02	94.00	0.084
M1	18	1475.84 ± 957.15		
Jaundice:				
No	11	714.84 ± 427.73	71.00	0.041
Yes	23	1499.91 ± 950.31		

subserosal edema (without ascites), a sloughed mucosal membrane or intramural gas. However, there is no single specific tumor biomarker for diagnosis and prognosis of GBC. Tumor biomarkers such as cancer antigens (CA-125, 19.9, 242, 15.3) carcinoembryonic antigen (CEA) and malondialdehyde (MDA) have been extensively used for diagnosis of liver, gastric, colorectal and pancreatic cancer [20–23]. The individual biomarkers often show conflicting results and have low specificity. There is a need to find a method to diagnose GBC at early stage. In this novel study we have assessed the cfDNA levels in GBC and compared the value with healthy controls and cholecystitis as a disease control.

A promising cancer screening marker, CfDNA was first identified in 1948 by Mandel and Métais in human blood. Leon et al. [24] described increased levels of cfDNA in cancer in comparison to healthy controls in 1977. Our study suggests that the mean level of cfDNA in GBC is about 14-fold higher than healthy control group and about 7 fold of cholecystitis group. CfDNA quantification by real-time PCR is considered as the gold standard among various available DNA quantification methods [13]. The method we used was sensitive up to 10 picograms (around 2–3 copies) of human genome. Previous studies have analyzed the increased levels of cfDNA in other malignancies as compared to healthy controls and reported 18 times higher in lung

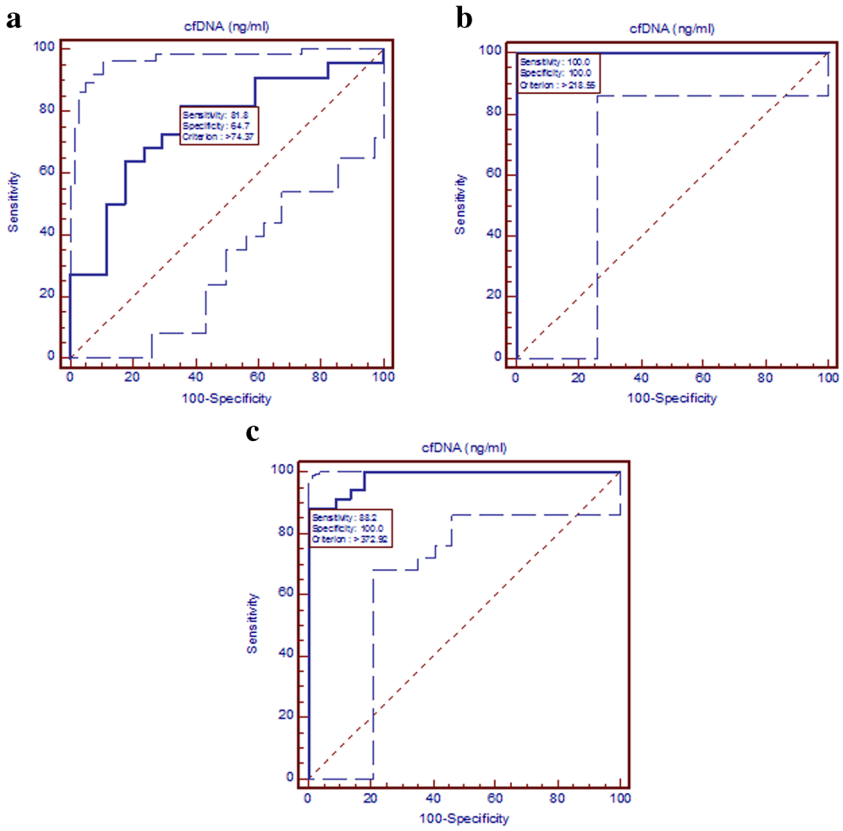


Fig. 2 ROC curve analysis to determine diagnostic accuracy of cfDNA to discriminate **a**. Control and Cholecystitis cases **b**. Control and Cancer cases **c**. Cholecystitis and Cancer cases

Table 4 Diagnostic accuracy of cfDNA to discriminate cases of three groups using ROC curve analysis

Diagnostic	Cutoff value (ng/ml)	Sensitivity (95 % CI)	Specificity (95 % CI)	PPV	NPV	AUC	*p* value
Control vs. Cholecystitis	>74.37	81.82 (59.7–94.7)	64.71 (38.4–85.7)	75.0	73.3	0.757	0.001
Control vs. Cancer	>218.55	100.00 (89.6–100.0)	100.00 (80.3–100.0)	100.0	100.0	1.000	<0.001
Cholecystitis vs. Cancer	>372.92	88.24 (72.5–96.6)	100.00 (84.4–100.0)	100.0	84.6	0.983	<0.001

[9], 5 times higher in colon [10], 3.5 times higher in breast [13], and 3 times higher in prostate [16] cancer.

Our results suggest cfDNA as a highly sensitive and specific marker to discriminate GBC patients from cholecystitis and healthy controls. Evaluation of cfDNA level achieved sensitivity and specificity of 100 % to differentiate cancer cases from healthy controls and with sensitivity and specificity of 88.24 % and 100 % to discriminate cholecystitis from cancer cases. Furthermore we found sensitivity and specificity of 81.82 % and 64.71 % to discriminate cholecystitis from healthy controls.

Serum has been used in our study as it is better source of cfDNA with six times more DNA compared to plasma [25] and has minimal leukocyte DNA contamination. It has been hypothesized that release of cfDNA occurs due to necrosis or apoptosis [7, 26, 27] or active release from cells [7, 28].

Our results indicate that cfDNA levels in patients do not relate with primary tumor size and metastasis; however patients with lymph node involvement and jaundice presented significantly higher level of cfDNA. Umetani et al. have observed that DNA integrity in cases with cancer positively correlated with the size of tumor and was also associated with lympho-vascular invasion and lymph node metastasis.

Due to the location and late appearance of symptoms, we got few cases in early stages. Most of the cases enrolled in the study had USG, CT and/ or MRI based staging. Twenty nine out of thirty four cases were in stage IV, two cases were in stage III and three were in stage II. Thus we can only emphasize the importance of high level cfDNA to screen the cases for GBC. To establish cfDNA levels as early diagnostic biomarker a study with larger sample size, having more number of early stage cases will be required. Our study suggests that patients with cholecystitis have higher cfDNA compared to normal, but have significantly low cfDNA when compared to GBC. A concomitant evaluation of cfDNA levels may assist in suspecting malignancy. It may be interesting to also observe changes in cfDNA level during the course of treatment.

We conclude that the cfDNA level increases significantly in serum of cases of GBC. These levels may serve as diagnostic marker to distinguish the GBC from chronic cholecystitis. Larger prospective studies in different stages of GBC may help to establish the use of cfDNA as a diagnostic marker in clinical practice.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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