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ORIGINAL ARTICLE

# Diagnostic Value of Circulating Free DNA Integrity and Global Methylation Status in Gall Bladder Carcinoma

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## Abstract

The current study investigates the role of circulating free DNA (cfDNA) as a liquid biopsy in diagnosis gall bladder carcinoma (GBC) utilizing levels of long DNA fragments (ALU247) derived from tumor necrosis, short apoptotic fragments (ALU115) denoting total cfDNA and cfDNA integrity denoting ratio of ALU247 and ALU115. The global methylation status of cfDNA was also estimated with the hypothesis that these parameters provide a diagnostic distinction between cancer and non-cancer subjects, with higher or altered values favoring presence of malignancy. Study group included 60 cases of GBC and 36 controls including diseased controls (cholecystitis) and healthy subjects. Median levels of ALU115, ALU247 and cfDNA integrity were significantly different in GBC at 1790.88, 673.75, 0.4718 vs. controls at 840.73, 165.03, 0.1989 ng/ml respectively. Global DNA methylation was not significantly different between GBC at 0.679% and controls at 0.695%. The sensitivity and specificity of ALU 247 in discriminating GBC from controls was highest with a sensitivity, specificity and diagnostic accuracy of 80.0%, 86.1% and 82.2% respectively. Global DNA methylation showed lowest sensitivity of 55.0% and specificity of 50.0%. Clinicopathological parameters showing significant association with cfDNA integrity, on ROC curve analysis, showed significant diagnostic discrimination of the tumor stage, lymphovascular invasion, disease stage and grade histology. This is a first time analysis of ALU115, ALU247 and cfDNA integrity in the diagnosis of GBC and confirms that the combination of ALU247 and cfDNA integrity provides good sensitivity, specificity and diagnostic accuracy in discriminating GBC from controls as well correlates with aggressive disease parameters.

**Keywords** Gall bladder carcinoma (GBC) · Circulating free DNA (cfDNA) · cfDNA integrity · Global DNA methylation · Liquid biopsy

## Introduction

Gall bladder carcinoma (GBC) is most frequent malignant neoplasm of the biliary tract in many developed and in developing countries including India. The highest incidence rates of GBC have been reported in women from India (21.5 / 100,000), Chile (18.1 /100,000), Pakistan (13.8 /100,000) and Ecuador (12.9 /100,000) [1]. Gall stone is a major risk factor associated with the disease and is present in 60– 90% of cases. Symptoms associated with the disease are usually non specific leading to the late stage disease diagnosis with overall survival of 5–10% only [2]. Incidental GBC discovered during cholecystectomy for benign disease, has a varying rate of 0.14% and 6.1%, depending on whether it is a high- or low-risk area [3]. The current diagnostic methods for GBC are mainly based on clinicopathological and radiological assessment such as Computed Tomographic (CT) scan, Magnetic

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Resonance Imaging (MRI) and Ultrasound (USG) and often fail to detect the malignancy at an early stage. Screening with established tumor markers such as carcinoembryonic antigen or CA19-9 has limited sensitivity. Consequently there is need for commonly applicable sensitive diagnostic biomarkers for malignant tumor [4, 5].

Liquid Biopsy has been emerged as a non invasive biomarker for early detection of the cancer, its progression and treatment response. Although circulating free DNA (cfDNA) has been investigated in the serum, plasma, sputum, bronchial lavage, milk, urine, and stool [6], the most frequently used sample is serum. Release of cfDNA occurs in circulation via apoptosis, necrosis, and from edge of the tumor & adjacent non-tumor cells; however the mechanism of its release into the circulation is not clear. Increased cfDNA levels have been reported in several malignancies. We have for the first time reported diagnostic value of cfDNA in GBC through the amplification of β-globin gene [7].

Highly repetitive sequences such as ALUs [8, 9] are distributed throughout the genome and have been suggested as good alternative for detection of amplification of short and long DNA fragments [8–10]. ALU repeats are the most abundant sequences in the human genome, with a copy number of about 1.4 million per genome [11, 12]. DNA fragments released from apoptotic cells are usually 185 to 200 base pair (bp) in length [13], this uniformly truncated DNA is produced by a programmed enzymatic cleavage process during apoptosis [14]. The main source of cfDNA in healthy controls is apoptotic cells. In contrast, malignant cells undergo pathologic cell death and necrosis and release DNA which varies in size [15]. Thus high levels of long DNA fragments may serve as a marker for presence of an underlying malignancy [16]. DNA integrity can be calculated as a ratio of longer to shorter DNA fragments, a higher ratio, therefore, favoring presence of malignancy. Epigenetic changes such as DNA methylation are the most frequent molecular alterations in human neoplasia [17, 18]. Methylation of tumor suppressor genes detected in cfDNA has been demonstrated to have prognostic potential [19, 20]. DNA methylation plays a crucial role in oncogenic point mutations, tumor suppressor gene silencing and initiation and activation of cancer process [21]. Data for detection of epigenetic changes in terms of global methylation of cfDNA in serum and plasma is minimal. We hypothesized that its estimation may be associated with the proportion of tumor and non tumor derived cfDNA.

In the current study we have assessed serum cfDNA integrity to distinguish cases of GBC from controls as well as in prediction of stage and regional lymph node metastasis in GBC. Global DNA hypomethylation in cases of GBC was also estimated and compared to controls. To the best of our knowledge this is a first time study evaluating diagnostic role of serum cfDNA integrity and global DNA methylation in GBC.

## Patients and Methods

**Patient Selection** The study group comprised of 60 cases of GBC and 36 controls which included 12 cases of cholecystitis and 9 cases of Xanthogranulomatous cholecystitis & 15 healthy individuals. The median (Range) age of the healthy and diseased controls ($n = 36$; 18 males and 18 females) was 38 years (24.00 – 72.00). The median age of the 15 normal controls (7 males and 8 females) was 30 years (24.00 – 48.00) while that of 12 cases of chronic cholecystitis (5 males and 7 females) was 47.5 years (29.00–65.00) and 9 xanthogranulomatous cholecystitis (6 males and 3 females) was 51.00 years (37.00 –72.00). The median age of the 60 GBC cases (17 males and 43 females) was 46.5 years (30.00–77.00). All cases that fulfilled the inclusion criteria were recruited for the study from the Department of Surgical Oncology, Dr. Ram Manohar Lohia Institute of Medical Sciences and Department of Surgical Oncology, King George's Medical University, Lucknow, India. Sample analysis was done in the molecular pathology lab of the department of Pathology. All participants signed an informed consent and ethical approval was obtained from Institutional Ethics Committee before recruiting patients. Diagnosis of all the cases was based on USG/CT/MRI/Endoscopy/or biopsy with definitive evidence of gall bladder carcinoma (if available). Cases who had undergone previous chemotherapy/radiotherapy as well as those with evidence of a significant clinical disorder or laboratory finding (which, in the opinion of the investigator would make it undesirable for the patient to participate in the study) and pregnant or breastfeeding women were excluded from the study. Detailed clinical and radiological assessment was done in all cases. Presenting symptoms, jaundice, stage of disease in terms of the TNM classification, T stage, nodal metastasis and presence of distant metastasis was assessed. In cases where histology of resection specimen or biopsy was done the histological parameters including grade, lympho-vascular invasion (LVI), perineural invasion were also assessed.

**Sample Collection and DNA Extraction** 3.5 ml of peripheral blood was collected from cases and controls in silica gel vials (B.D Vacutainer, UK) before surgery or treatment. Serum was separated by centrifugation and stored at −80 °C until further processing. Serum cfDNA was extracted by using ChargeSwitch® 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 lysate, 200 μl of purification buffer and 25 μl of ChargeSwitch magnetic beads were added, mixed gently by pipetting; tubes were placed in the magna Rack™for 3 min, followed by two time wash with 800 μl of wash buffer. 50 μl of Elution Buffer was added to the tube 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)** To assess the concentration and integrity of cfDNA, both short fragment (ALU115) and a long fragment (ALU247) of ALU repeats were amplified and quantified using a 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 S4 (10 ng–0.001 ng). The standard 1 ng (S2), 0.1 ng (S3), 0.01 ng (S4) and 0.001 ng (S5) were prepared by serial dilution of stock 10 ng (S1) in nuclease free water. Standard curves were created for both ALU115 and ALU247 primer sets.

The ALU115 primer set amplify both short (apoptotic) and long (non-apoptotic) DNA fragment and represent the total amount of cfDNA, whereas ALU247 primer set amplifies only long non apoptotic DNA fragments. cfDNA integrity was calculated as the ratio of ALU247-qPCR/ALU115-qPCR. The sequences of the ALU115 primers were forward: 5-CCTGAGGTCAGGAGTTCGAG-3 and reverse: 5-CCCGAGTAGCTGGGATTACA-3; ALU247 primers were forward: 5-GTGGCTCACGCCTGTAATC-3 and reverse: 5-CAGGCTG GAGTGCAGTGG-3. 20 μl reaction mixture for each of ALU-qPCR consisted of 2 μl of DNA, 0.5 μM each of forward primer and reverse primer (ALU115 or ALU247), 10 μl of SYBR Green Supermix (Applied Biosystems, USA), and the volume was adjusted by nuclease free water. qPCR was performed on CFX96 Real-Time PCR 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, 62 °C for 30s. To confirm the generation single 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. A negative control (without DNA) was set in each run.

**Determination of Global DNA Methylation** For global DNA methylation analysis, cfDNA concentration and purity was determined by taking the optical density measurement at $A_{260/280}$. Global DNA methylation was measured using Methyl Flash Methylated DNA Quantification Kit (Colorimetric) (Epigentek Group Inc., New York, NY, USA). The kit quantifies methyl cytosine content as a percent of total cytosine content. Briefly 200 ng of purified cfDNA was added to the ELISA plate. The methylated fraction of cfDNA was quantified by using 5-methylcytosine specific antibodies. The quantity of methylated DNA was proportional to the OD intensity at 450 nm. Relative quantification of DNA methylation was calculated using the formula: [(sample OD– M3OD)/S]/ [((M4OD– M3OD) ×2)/P] ×100; where OD is optical density; M3 is the negative control; S is the amount of input sample DNA in ng; M4 is the positive control; P is the amount of input positive control in ng. The amount of methylated DNA was expressed as percentage of total DNA.

## Statistical Analysis

Statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences) software package, version 16.0. The value of ALU115, ALU247, cfDNA integrity and global DNA methylation level in the groups categorized as GBC and controls including diseased controls with cholecystitis and healthy controls were compared by non parametric Kruskal-Wallis one way ANOVA and for analysis Mann-Whitney U-test between two group data with a quantitative response variable. The Receiver Operating Characteristics (ROC) curve analysis was performed to test the diagnostic potential of ALU115, ALU247, cfDNA integrity and global DNA methylation to discriminate GBC cases from control. A cut off value was defined to calculate sensitivity and specificity values defining the curve and the area under the curve (AUC). A $p$ value of less than 0.05 was considered significant.

## Results

The demographic characteristics of the groups (normal control, chronic cholecystitis, xanthogranulomatous cholecystitis and GBC) and clinico-pathological characteristics of cases of GBC are summarized in Table 2.

## Level of ALU115, ALU247 and cfDNA Integrity in Normal Controls, Chronic Cholecystitis, Xanthogranulomatous Cholecystitis and GBC Patients

**Controls** The median (IQR 25-75) concentration of ALU115, ALU247 was 840.73 (412.79–1263.39) & 165.03 (62.16–283.38) ng/ml and median cfDNA integrity was 0.19 (0.11–0.32) (Table 1).

**Normal Controls** The median concentration of ALU115, ALU247 was 817.51 (381.27– 1133.49), 73.53 (54.06 – 197.11) ng/ml and the median cfDNA integrity was 0.13 (0.11– 0.21) (Table 1).

**Chronic Cholecystitis & Xanthogranulomatous Cholecystitis** The median concentration of ALU115, ALU247 was 975.49 (738.50 –1295.51) & 224.47 (69.59 –328.66) ng/ml and the median cfDNA integrity was 0.22 (0.080–0.34) in chronic

**Table 1** Test values in cases and controls

| | GBC ($n = 60$) | | | | |
|---|---|---|---|---|---|
| | Median | Mean | S.D | Minimum | Maximum |
| ALU115 | 1790.88 | 2246.81 | 1767.86 | 61.19 | 6352.50 |
| ALU 247 | 673.75 | 1186.97 | 1230.07 | 55.16 | 5445.87 |
| cfDNA Integrity | 0.4718 | 0.5091 | 0.1975 | 0.137 | 0.936 |
| Global DNA methylation ($n = 40$) | 0.6790 | 0.9664 | 0.7659 | 0.0650 | 3.9380 |
| Control ($n = 36$) | | | | | |
| ALU115 | 840.73 | 947.48 | 607.03 | 216.39 | 2501.74 |
| ALU 247 | 165.03 | 195.92 | 159.01 | 2.31 | 529.26 |
| cfDNA Integrity | 0.1989 | 0.2194 | 0.1349 | 0.003 | 0.508 |
| Global DNA methylation ($n = 20$) | 0.6950 | 1.7478 | 2.4792 | 0.0270 | 8.8100 |
| Xanthogranulomatous Cholecystitis ($n = 09$) | | | | | |
| ALU115 | 643.93 | 828.64 | 576.43 | 296.47 | 1955.12 |
| ALU 247 | 194.84 | 215.45 | 113.56 | 56.97 | 402.18 |
| cfDNA Integrity | 0.3323 | 0.3210 | 0.1535 | 0.101 | 0.508 |
| Chronic Cholecystitis ($n = 12$) | | | | | |
| ALU115 | 975.49 | 1043.38 | 494.99 | 307.94 | 1881.25 |
| ALU 247 | 224.47 | 228.86 | 182.35 | 2.31 | 527.76 |
| cfDNA Integrity | 0.2288 | 0.2176 | 0.1454 | 0.003 | 0.422 |
| Global DNA methylation ($n = 10$) | 0.6950 | 1.4508 | 2.6148 | 0.0270 | 8.8100 |
| Normal Control ($n = 15$) | | | | | |
| ALU115 | 817.51 | 942.07 | 722.37 | 216.39 | 2501.74 |
| ALU 247 | 73.53 | 157.86 | 164.27 | 30.02 | 529.26 |
| cfDNA Integrity | 0.1388 | 0.1599 | 0.7051 | 0.057 | 0.288 |
| Global DNA methylation ($n = 10$) | 0.9620 | 2.0448 | 2.4378 | 0.2340 | 6.8440 |

*Done in 40 cases and 20 controls from the same set of samples

cholecystitis. The median concentration of ALU115, ALU247 was 643.93 (390.13– 1210.48) & 194.84 (166.68– 198.41) ng/ml and the median cfDNA integrity was 0.332 (0.19–0.45) in xanthogranulomatous cholecystitis (Table 1).

**GBC Cases** The median concentration of ALU115, ALU247 was 1790.88 (976.77–3154.33), 673.75 (488.93–1723.23) ng/ml and the median cfDNA integrity was 0.4718 (0.38 – 0.61).The level of ALU115, ALU247 and cfDNA integrity was significantly higher in GBC as compared to controls ($p = <0.001$) (Table 1 and Fig. 1a–c).

**Association of ALU115, ALU247 & cfDNA Integrity with clinico-pathological characteristics of cases with GBC** The association of ALU115, ALU247 and cfDNA integrity with clinico-pathological characteristics of GBC patients is summarized in Table 2. ALU115, ALU247 was higher cases with a poorly differentiated histology as compared to those with well differentiated adenocarcinoma. However these differences were not statistically significant ($p = 0.488$, 0.061). The cfDNA integrity values were significantly higher and associated with histological differentiation, lymphovascular invasion and lymph node metastasis ($p = 0.002$, 0.033, 0.015 respectively). CfDNA integrity was also significantly associated with T stage being highest in advanced stage ($p = 0.011$). Median level of ALU115 and 247 was higher in patients with lymph node metastasis though it was not statistically different ($p = 0.335$, 0.070). Metastasis was not significantly predicted by levels of ALU115, ALU247 & cfDNA integrity ($p = 0.551$, 0.781 & 0.893 respectively). Median level of ALU115 & ALU247 was higher in stage III & IV GBC as compared to stage I & II, though values were not statistically different ($p = 0.693$, 0.177). Median of cfDNA integrity was significantly associated with stage of patients ($p = 0.006$) Fig. 2.

## Diagnostic Utility of ALU115, ALU247 and cfDNA Integrity in GBC Patients

Levels of ALU115, ALU247 & cfDNA integrity were determined in the 60 GBC patients and 36 controls. ROC curves were drawn for distinguishing GBC patients from controls. Area under Curve (AUC) for ALU115 was 0.748. At a cut off point of >1128.429 ng/ml, ALU115 discriminated GBC from controls with sensitivity, specificity and diagnostic

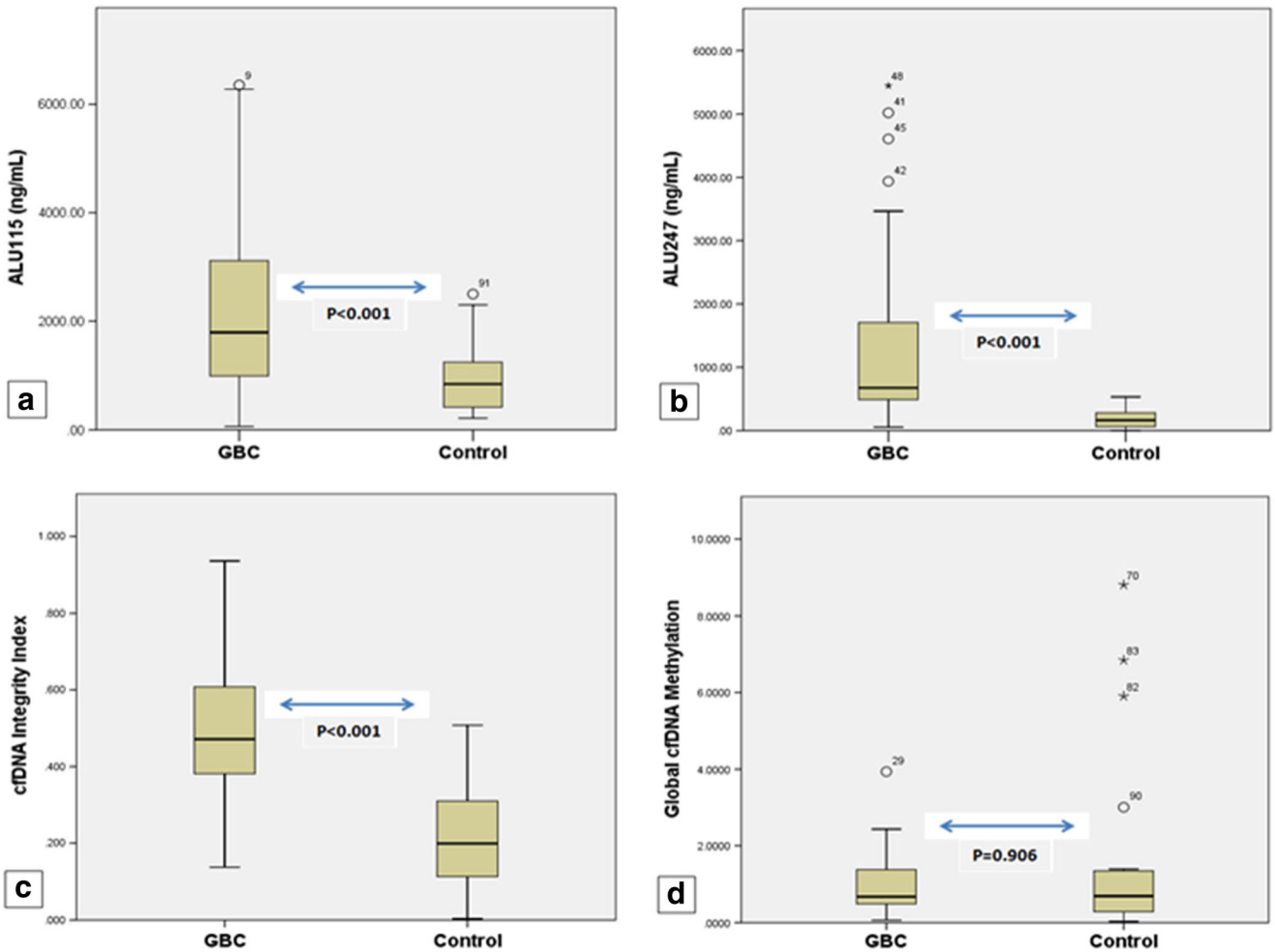


**Fig. 1** Box plot showing **a**) ALU115, **b**) ALU247, **c**) cfDNA integrity and **d**) Global DNA methylation levels of two groups. Groups were compared by Mann Whitney U test ($p < 0.05$ were considered as significant)

accuracy of 71.7%, 66.7% and 69.7% respectively. The AUC for ALU247 was 0.901. At a cut off point of >406.5825 ng/ml, ALU247 discriminated GBC from controls with sensitivity, specificity and diagnostic accuracy of 80.0%, 86.1% and 82.2% respectively. The AUC for cfDNA integrity was 0.895. At a cut off point of >0.356, cfDNA integrity distinguished GBC from controls with sensitivity, specificity and diagnostic accuracy of 78.3%, 80.6%, and 80.2% respectively (Table 3 and Fig. 3ia–c).

## Combined Diagnostics of ALU115, ALU247 and cfDNA Integrity in GBC Patients

Compared with individual diagnostics of ALU115, ALU247 and cfDNA integrity, combined diagnostics using one or more test positivity as a positive test, and all three tests negative as negative interpretation, the combination of ALU247 with cfDNA integrity gave best diagnostic efficiency in distinguishing GBC cases from controls (Table 3).

## Diagnostics of cfDNA Integrity in Discrimination of Stage, Lymph Node Metastasis, Lympho Vascular Invasion and T Stage in Cases of GBC

ROC curve analysis of cfDNA integrity showed significant diagnostic discrimination of the T stage I&II vs. III &IV with sensitivity, specificity and diagnostic accuracy of 90.70%, 57.14% and 82.4% respectively with an AUC of 0.748 and cut off value of >0.3422. cfDNA integrity had an AUC of 0.703 for discrimination of LN metastasis in patients vs. without LN metastasis with sensitivity, specificity and diagnostic accuracy of 83.87% & 55.0% and 72.5% respectively at a cut off value of >0.4049. cfDNA integrity had an AUC of 0.791 for discrimination of lymphovascular invasion

**Table 2** Association of ALU115, ALU247 & cfDNA integrity with clinico-pathological characteristics of cases with GBC

| Characteristics | *N* | ALU115 (ng/ml) Median (Q1-Q3) | *p* value | ALU247(ng/ml) Median (Q1-Q3) | *p* value | cfDNA Integrity Median (Q1-Q3) | *p* Value |
|---|---|---|---|---|---|---|---|
| Age (yrs): | | | | | | | |
| ≤ 45 | 25 | 1866.15 (143.05–3868.38) | 0.290 | 714.53 (546.08–2187.34) | 0.184 | 0.50 (0.40–0.64) | 0.311 |
| > 45 | 35 | 1657.78 (917.00– 2391.50) | | 613.86 (376.12–1016.06) | | 0.44 (0.37– .055) | |
| Sex: | | | | | | | |
| Female | 43 | 1866.15 (1059.79– 3550.65) | 0.346 | 840.35 (489.45– 2121.77) | 0.151 | 0.51 (0.38–0.64) | 0.215 |
| Male | 17 | 1170.54 (920.19– 2165.59) | | 546.08 (325.56– 790.25) | | 0.43 (0.31– 0.48) | |
| Jaundice: | | | | | | | |
| No | 40 | 1871.54 (1149.52– 4021.74) | 0.266 | 747.19 (496.11– 2170.95) | 0.279 | 0.49 (0.35– 0 .71) | 0.500 |
| Yes | 20 | 1280.39 (956.91– 2389.68) | | 573.93 (460.27– 886.27) | | 0.46 (0.39– 0.52) | |
| Histological Grade: | | | | | | | |
| WD | 14 | 1790.36 (766.44– 3078.81) | 0.488 | 467.50 (237.08– 1382.03) | 0.061 | 0.32 (0.26–0.45) | 0.002 |
| MD | 10 | 1574.49 (893.25– 4390.84) | | 828.83 (531.75– 2558.78) | | 0.60 (0.50– .74) | |
| PD | 11 | 1998.41 (1421.53– 5495.01) | | 1740.92 (579.41– 3936.91) | | 0.71 (0.47–0.91) | |
| LVI: | | | | | | | |
| Present | 6 | 1574.49 (935.00–4527.90) | 0.721 | 1023.52 (457.54–3047.98) | 0.454 | 0.607 (0.533 – 0.770) | 0.033 |
| Absent | 18 | 2026.86 (1221.07– 3358.92) | | 602.14 (337.61–2138.17) | | 0.358 (0.273– 0.583) | |
| T stage: | | | | | | | |
| T1 + T2 | 14 | 1790.36 (1076.95– 2316.42) | 0.868 | 584.91 (237.08–828.13) | 0.533 | 0.32 (0.27– 0.52) | 0.011 |
| T3 | 25 | 1866.15 (1101.42–3450.21) | | 714.53 (512.15– 1742.33) | | 0.42 (0 38– 0.55) | |
| T4 | 18 | 1785.49 (666.95– 3758.26) | | 866.26 (391.38– 2217.13) | | 0.57 (0.42– 0.79) | |
| LN Metastasis: | | | | | | | |
| Absent | 20 | 1721.15 (985.30– 2119.03) | 0.335 | 594.18 (368.59– 815.91) | 0.070 | 0.39 (0.28– 0.52) | 0.015 |
| Present | 31 | 1795.32 (1021.99– 4381.10) | | 918.54 (506.10– 2248.68) | | 0.47 (0.42– 0.72) | |
| Metastasis: | | | | | | | |
| M0 | 41 | 1866.15 977.35– 3525.74 | 0.551 | 684.66 (489.45– 1670.15) | 0.781 | 0.47 (0.38– 0.54) | 0.893 |
| M1 | 19 | 1784.53 (961.70– 2124.33) | | 598.44 (469.65– 1932.75) | | 0.44 (0.34– 0.65) | |
| Stage: | | | | | | | |
| I + II | 09 | 1922.95 (1150.10– 2165.22) | 0.693 | 579.41 (234.02– 713.63) | 0.177 | 0.28 (0.25– 0.44) | 0.006 |
| III | 18 | 1486.53 (960.20– 2764.13) | | 751.59 (464.89–1144.78) | | 0.47 (0.38– 0.55) | |
| IV | 33 | 1866.15 (958.51– 4406.53) | | 837.90 (489.10– 2256.94) | | 0.51 (0.42– 0.74) | |

present vs. absent with sensitivity, specificity and diagnostic accuracy of 83.0%, 80.0% and 80.7% respectively at a cut off value of >.5507. cfDNA integrity had an AUC of 0.813 and cut off of >0.3422 to discriminate the stage I&II vs. III &IV

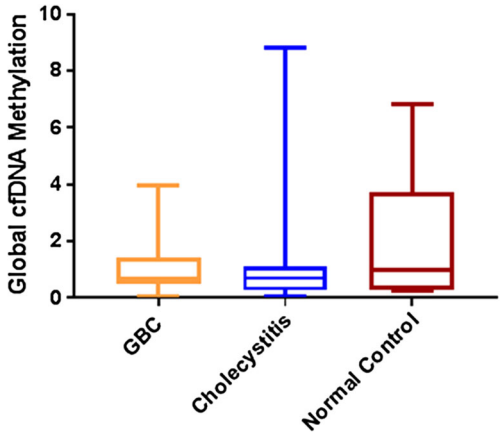


**Fig. 2** Box plot showing Global DNA methylation levels of three groups. Groups were compared by Kruskal-Wallis ANOVA

patients with sensitivity, specificity and diagnostic accuracy of 90.20%,77.78% and 85.0% respectively.(Table 3, Figs. 3ii–v and 4).

## Global DNA Methylation Level in Control and GBC cases

The median (IQR 25-75) of global DNA methylation levels in control and cancer patients were 0.69 (0.28–1.37) and 0.67 (0.48–1.40) respectively. Further on comparison of global DNA methylation level between controls and cancer, no statistical difference was observed ($p = 0.906$) (Table 1, Fig. 1d). Further comparison of global DNA methylation level of three groups, there was no statistical difference and values were overlapping (Fig. 2).

The global DNA methylation level of groups is summarized in Table 1.

**Association of Global DNA Methylation Level with Clinico-Pathological Characteristics of Cases with GBC** Global DNA methylation did not show any association with clinicopathological characteristics of GBC cases.

## Diagnostic Utility of Global DNA Methylation - ROC Curve analysis

ROC curve analysis was performed to evaluate diagnostic accuracy of global DNA methylation status to discriminate the cases from controls. The cutoff value of global DNA methylation at <0.7135, with an AUC of 0.509 showed a low sensitivity and specificity of 55.0% & 50.0% respectively (Table 3, Fig. 3i-D).

## Discussion

The current study is a novel use of cfDNA integrity to distinguish cases of gall bladder cancer from cholecystitis and normal controls. The DNA integrity was also applied to discriminate between low to high grade lesions, increasing size of tumor and stage, as well as presence of lymphovascular emboli and lymph node metastasis. The clinical value of using circulating free DNA as a relatively noninvasive biomarker in cancer has been actively explored. We have earlier quantified cfDNA in GBC and found it to be a promising biomarker for detection of GBC [7]. In the present study ALU sequences were chosen as they are most abundant and repeated DNA elements in the human genome and are typically 300 nucleotides in length representing more than 10% of the genome [22] and can be applied to measure length of circulating DNA fragments.

Analysis of genomic alterations in cell-free DNA is evolving as an approach to detect, monitor and genotype malignancies. Methods to separate the liquid from the cellular fraction of whole blood for circulating free DNA analyses have been largely unstudied although these may be a critical consideration for assay performance [23]. We used serum for studying cfDNA (ALU115, ALU247) levels. Although some studies have propagated the use of plasma for detection cfDNA in cancer [16], the possibility of contamination of plasma with leukocyte DNA after storage is a drawback with use of plasma. The analysis of cell-free DNA (cfDNA) as a sensitive biomarker for cancer diagnosis and monitoring has resulted in a need for efficient and standardized cfDNA isolation. Primary limitation may be the purification of cfDNA from serum/plasma that results in decreased DNA yield. This DNA loss may inversely depend on fragment size that would affect the DNA integrity. The methodology used to separate and quantify the cfDNA with magnetic bead separation using the specific ChargeSwitch® gDNA 1 ml Serum kit specific for cfDNA gave a high standardized yield in cases and controls in our study.

In the recent years potential of cfDNA and its integrity as a new tumor biomarker has been investigated. A number of research papers have reported elevated levels of cfDNA in serum/plasma of cases with cancer as compared to controls. Although evaluation of cfDNA integrity is not yet in clinical practice because the diagnostics of these methods have not been validated for clinical use, it appears to be a low cost and simple technique to evaluate the proportion of cfDNA coming from necrosis of cancer cells. For premalignant and early malignant lesions the diagnostic role of cfDNA integrity has not been ascertained. Utomo WK et al. in a study of serum and cyst fluid in cases of pancreatic cancer, cysts and normal controls observed that cfDNA integrity is not a useful marker to identify premalignant pancreatic lesions [24]. Agostini M et al. analyzed ALU247 and integrity values in pre and post

**Table 3** Diagnostic efficacy of ALU115, ALU247 and cfDNA integrity in GBC and cfDNA integrity in discrimination of stage, lymph node metastasis, tumor stage and lympho vascular invasion of GBC cases

| Diagnostic test | Cut off value (ng/ml) | AUC | *p*-value | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) | Diagnostic Accuracy (%) |
|---|---|---|---|---|---|---|---|---|
| Total groups | | | | | | | | |
| ALU115 | >1128.429 | 0.748 | 0.000 | 71.70 (58.56– 82.55) | 66.7 (49.03– 81.44) | 78.20 (68.73– 85.38) | 58.54 (47.02– 69.19) | 69.7 |
| ALU247 | >406.5825 | 0.901 | 0.000 | 80.00 (67.67– 89.22 | 86.11 (70.50– 95.33) | 90.60 (80.82– 95.63) | 72.10 (60.50– 81.33) | 82.2 |
| cfDNA Integrity | >0.35628 | 0.895 | 0.000 | 78.33 (65.80– 87.93) | 80.56 (63.98– 91.81) | 87.04 (77.31– 92.97) | 69.05 (57.33– 78.74) | 80.2 |
| Global Methylation (cfDNA) | <0.7135 | 0.509 | 0.906 | 55.0 (38.49– 70.74) | 50.0 (27.20 72.80) | 68.75 (56.67 –78.73) | 35.71 (24.16–9.21) | 51.6 |
| ALU115 + ALU247 | | | | 83.33 (71.48 – 91.71) | 66.67 (49.03– 81.44) | 80.65 (72.14 – 87.02) | 70.59 (56.57– 81.56 | 77.08 |
| ALU115+ Integrity index | | | | 96.67 (88.47– 99.59) | 52.78 (35.49– 69.59) | 77.33 (70.66– 82.86) | 90.48 (70.14– 97.46) | 80.20 |
| ALU247+ Integrity index | | | | 93.33 (83.80– 98.15) | 72.22 (54.81– 85.80) | 84.85 (76.70– 90.50) | 86.67 (71.17– 94.48) | 85.41 |
| ALU115 + ALU247 + Integrity index | | | | 96.67 (88.47– 99.59) | 52.78 (35.49– 69.59) | 77.33 (70.66– 82.86) | 90.48 (70.14– 97.46) | 80.20 |
| Subgroups (cfDNA Integrity) | | | | | | | | |
| T stage I&II vs. III&IV | >0.342 | 0.748 | 0.000 | 90.70 (77.86– 97.41) | 57.14 (28.86 –82.34) | 86.67 (77.89 –92.30) | 66.67 (41.47 – 84.95) | 82.4 |
| LVI Present vs. Absent | >0.550 | 0.792 | 0.000 | 83.33 (35.88 –99.58) | 80.00 (56.34 – 94.27) | 55.56 (32.66– 76.31) | 94.12 (72.51 –98.98) | 80.7 |
| Lymph node Metastasis Present vs. Absent | >0.404 | 0.703 | 0.000 | 83.87 (66.27 –94.55) | 55.00 (31.53 –76.94) | 74.29 (63.47 – 82.77) | 68.75 (47.33 –84.34) | 72.5 |
| Stage I&II vs. III & IV | >0.342 | 0.813 | 0.000 | 90.20 (78.59–96.74) | 77.78 (39.99 –97.19) | 95.83 (87.10–98.74) | 58.33 (36.21 –77.54) | 85.0 |

chemotherapy cases of colorectal cancer and reported plasma levels of the longer fragments (ALU247) of cfDNA and cfDNA integrity index to be promising markers to predict tumor response after preoperative chemotherapy for rectal cancer [25].

Till date there is no concurrence on the mechanism, for presence of cfDNA into circulation. Most common hypothesis for release of DNA into the circulation by damaged cells in both benign and malignant diseases. Shedding of lysed circulating tumor cells (CTC) may also be a factor for presence of DNA into the bloodstream; however low CTC count is unable to explain the observed amount of DNA in the plasma or serum [26]. Therefore apoptosis and or necrosis remains the likely hypothesis for increased level of cfDNA into the circulation by tumor. Differentiation between the apoptotic and necrotic DNA can be made by DNA fragments through amplification of short & long dimension of repetitive DNA elements. The finding of our study appears to validate this hypothesis. Due to rapid tumor growth the clearance of apoptotic and necrotic debris by infiltrating phagocytes is hampered resulting in accumulation of cellular debris and its secretion into the circulation [27]. In addition, the active secretion of apoptotic and necrotic DNA in circulation has also been indicated in the increase of cfDNA levels in blood [16].

In the present study, we observed significantly higher levels of absolute DNA (ALU115), longer DNA fragments (ALU247), as well as higher DNA integrity in GBC patients compared to controls. We also observed that the DNA integrity was indicative of tumor progression. Umetani et al. in a study on breast cancer also observed significant changes in cfDNA integrity associated with tumor progression [8]. In our study cfDNA integrity was found to significantly discriminate among histologic subtype, lymphovascular invasion, primary T stage, lymph node metastasis and TNM stage of GBC cases. In poorly differentiated adenocarcinoma of gall bladder level of ALU115 & ALU247 was higher as compared to moderately and well differentiated cases. Our results indicate that cfDNA integrity is an informative marker of aggressiveness of GBC with significant increase in level with higher stage, nodal metastasis, increasing T stage, LVI and grade. We have observed that the level of ALU247 was also higher in stage IV patients as compared to stage III & I + II. ALU247 levels were was also higher in patients with primary T4 tumor as compared to T3 & T2 lesions, possibly due to increasing tumor load and necrosis.

CfDNA and cfDNA integrity have been shown to be promising diagnostic biomarkers in many other cancers [8, 9, 28]. Diagnostic value of cfDNA for individual patients is limited

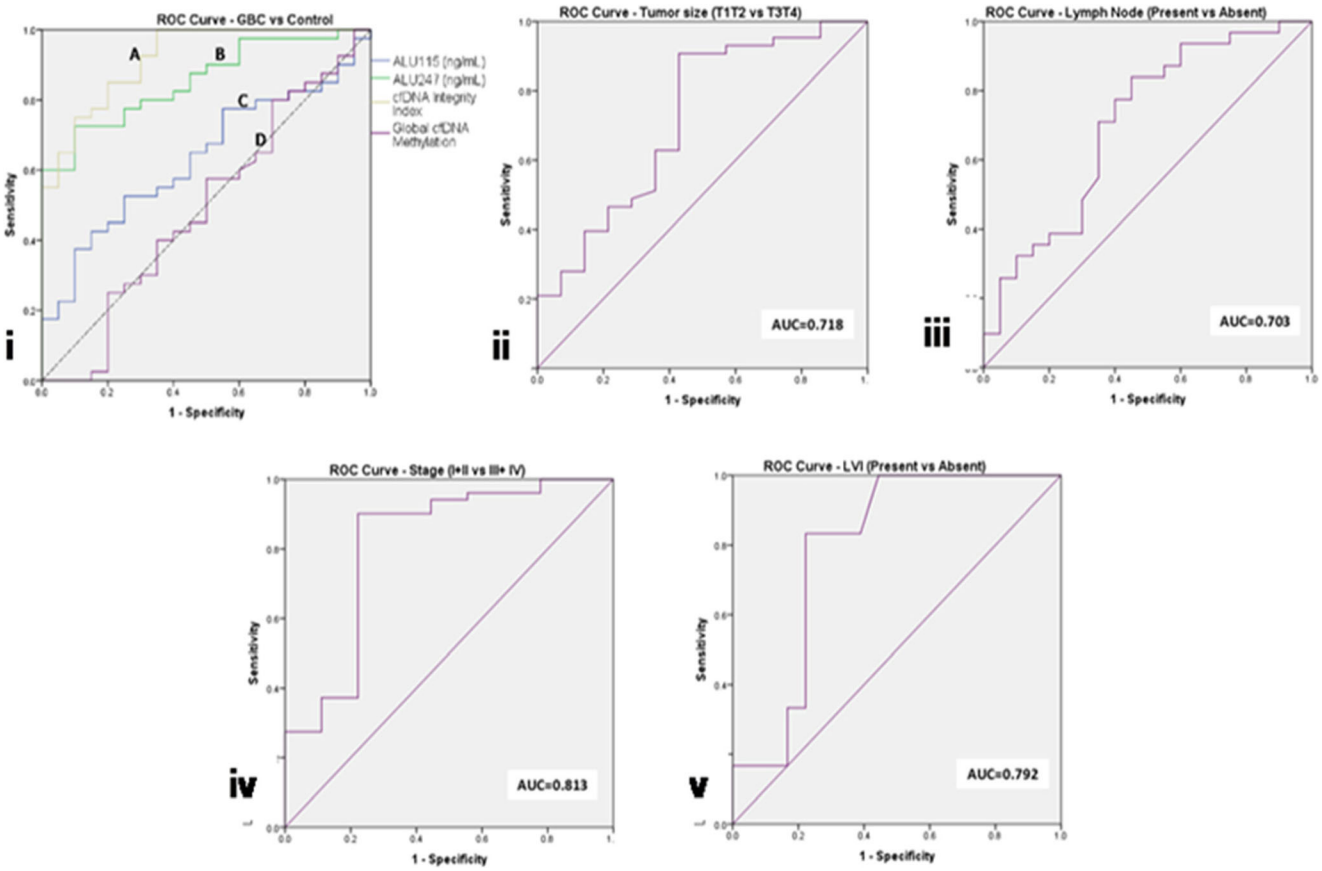


**Fig. 3** **i**) ROC curve analysis to determine diagnostic accuracy and cut offs for **A**) cfDNA integrity Index, **B**) ALU247, **C**) ALU115 and **D**) Global cfDNA methylation in discrimination of GBC cases from controls. **ii**) T stage I&II vs. III&IV, **iii**) Lymph node present vs. absent, **iv**) Stage I&II vs. III&IV and **v**) Lymphovascular invasion present vs. absent

by elevated level of cfDNA in benign pathologies that are important in differential diagnosis [29–31]. In our study the diseased controls including chronic cholecystic and xanthogranulomatous cholecystitis showed significanlty different values of ALU 115, ALU247 and cfDNA integrity. Development of malignancy is associated with higher cellular proliferation which initially is equilibrated by apoptosis and later by passive necrosis when tumor dedifferentiates and become invasive. Our study corroborates that elevated levels of longer DNA fragment (ALU247) and high cfDNA integrity may be potential non invasive surrogate biomarkers of malignancy and assist in differential diagnosis in the presence of imaging abnormalities. Level of ALU115 representing the total DNA amount, significantly discriminated GBC from controls but the specificity of the test was lower. ALU247 showed highest sensitivity and specificity (Table 3) in discrimination of GBC from normal controls with a diagnostic accuracy of 82.2%.

Our study analyses the diagnostics of the individual tests and the tests in combination. The combined sensitivity and specificty of ALU247 with cfDNA integrity showed a maximum sensitivity of 93.33% with a diagnostic accuracy of 85.41% to discriminate GBC from controls. Combined interpretation of ALU115 with cfDNA integrity and combination of all three tests including ALU115 + ALU247+ ALU247/115 improved the sensitivity but the specificity was considerably lowered to an unacceptable 52.78%.

ROC curve analysis cfDNA integrity to distinguish groups of tumor parameters fixed a cutoff point of >0.342 to discriminate T1&T2 patients from T3&T4 patients with sensitivity and specificity and overall diagnostic accuracy of 90.70, 57.14 & 82.4% respectively. Ohira et al. have reported that tumor volume determines the feasibility of cell-free DNA sequencing for mutation detection in non-small cell lung cancer. In their observation the yield of cfDNA did not differ among tumor stages but the detection of mutations was higher with higher T stages [32]. CfDNA integrity could also significantly discriminate stage I&II patients from stage III&IV with diagnostic accuracy of 85.0%. On the other hand cfDNA integrity showed higher sensitivity and specificity with diagnostic accuracy of 80.7% in discriminating patients with lymphovascular invasion from cases without lymphovascular invasion. Paradoxically presences of cfDNA integrity values were not significantly different in cases with or without metastasis. Agostini et al. in their study of fragments sizes and cfDNA integrity using ALU repeats in plasma of cases with

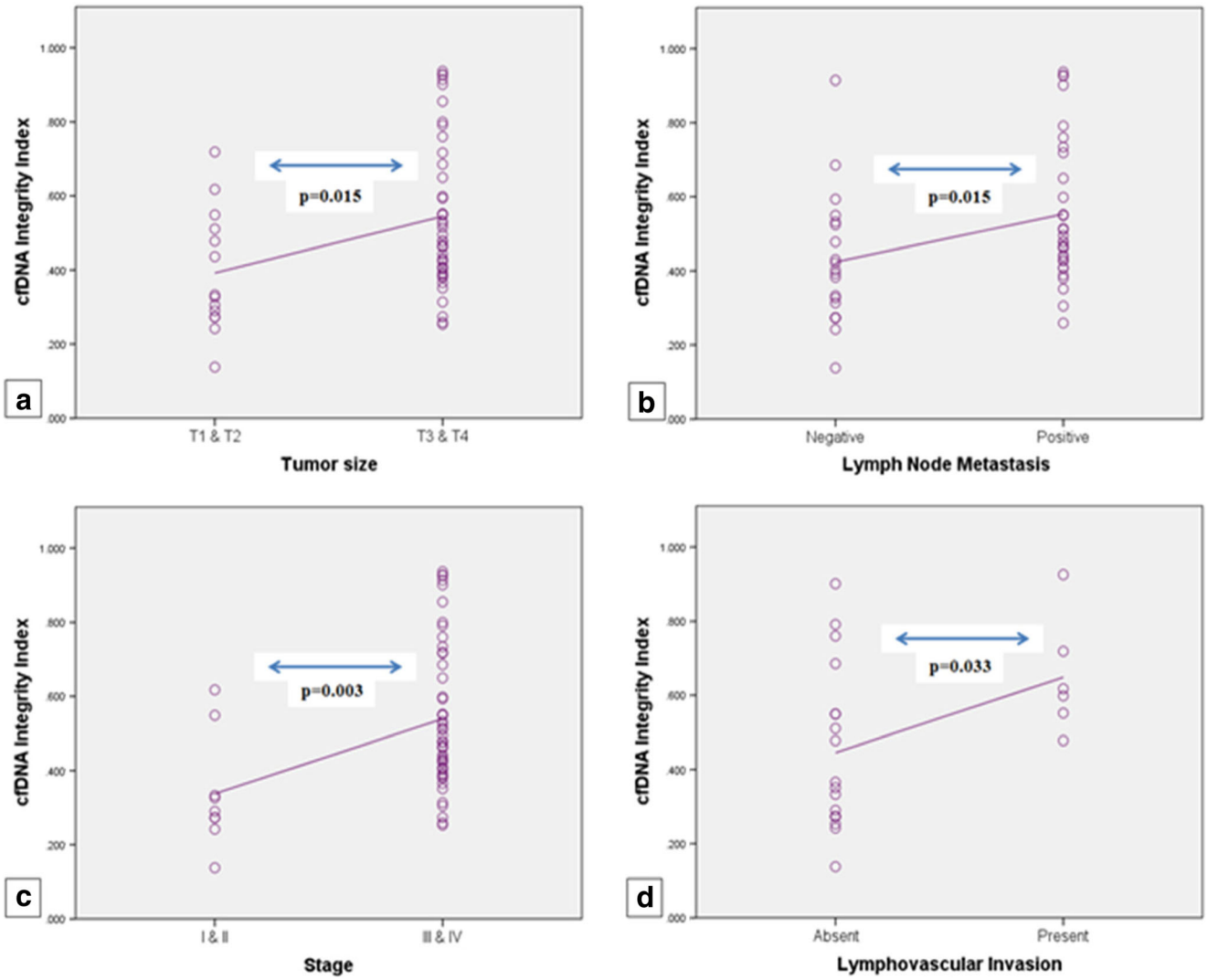


**Fig. 4** Scatter plot showing cfDNA integrity in GBC in relation to **a**) T stage of T1& T2 vs. T3&T4, **b**) Lymph node metastasis negative vs. positive, **c**) Stage I&II vs. III& IV and **d**) Lymphovascular invasion absent vs. present

breast cancer observed T stage and patients with regional LN metastasis positive cancers showed significantly higher cfDNA level of ALU247 [33].

Epigenetic events, such as DNA methylation in CpG islands, occur early in cancer development suggesting a potential role of DNA methylation as a biomarker for early diagnosis. From a theoretical point of view assessment of DNA methylation in circulating free DNA should provide a sensitive and specific distinction between cancer and non-cancer subjects. Based on this hypothesis we analysed the cfDNA in our cases and controls of gall bladder cancer for presence of significant global methylation changes. However in our study cfDNA methylation showed a very low diagnostic sensitivity and specificity. Studies have reported cfDNA methylation through bisulfite modification and methylation specific PCR of various genes. *CDKN2A (p16)* gene promoter is hypermethylated in a large number of diverse human cancer types and thereby inactivated. Sabine J et al. in an attempt to determine the source of non tumor and tumor DNA in cases of cancer reported the fraction of DNA with hypermethylated *CDKN2A*-promoter sequences varies from 90% to 10% in a variety of tumors [16]. Interestingly in their studies the total DNA levels did not correlate with the percent fraction of methylation in cases of cancer.

Methylation of SEPT9 is well studied gene and considered as clinically useful biomarker for screening and in detection of invasive colorectal cancer [34, 35]. Methylation of HPP1 and/or HLTF in serum cfDNA has been shown to be associated with poor outcome and a relative risk of mortality. Mutation of EGFR and KRAS gene has been reported in cfDNA. Point mutation of KRAS 2 gene in serum has been reported to effect the management of late stage colorectal cancer cases [36, 37]. Cancer-associated DNA hypomethylation is as prevalent as cancer-linked hypermethylation, but these two types of epigenetic abnormalities usually seem to affect different DNA sequences. Majority of the genomic sequence is subject to

hypomethylation rather than hypermethylation. Hypomethylation is seen in heterochromatic DNA repeats, dispersed retrotransposons, endogenous retroviral elements and transcription control sequences. Consequences incude increased karyotypic instability and activation of tumor-promoting genes by *cis* or *trans* effects. Multiple studies have investigated global DNA methylation profiles and gene-specific DNA methylation in blood-based DNA to develop powerful screening markers for cancer [38]. Global DNA methylation analysis in cfDNA could not provide any relevant information in discrimination of GBC cases from controls in the current study. Methylation of specific genes may be a direction to investigate diagnostic use of epigenetic changes in tumor derived DNA in serum or plasma [39–42].

## Conclusion

The present study investigated the use of cfDNA levels and its integrity to distinguish GBC cases from controls with chronic cholecystitis and xanthogranulomatous cholecystitis, a frequent pre operative and radiological differential diagnosis of GBC. This is a first time analysis of ALU 115, ALU247 and cfDNA integrity in the diagnosis of gall bladder carcinoma but is limited by its relatively small sample size. Further large-scale and prospective studies are needed to confirm the clinical utility of serum cfDNA integrity in the diagnosis of GBC as well as discriminate poor prognosis cases in terms of stage, histological grade and nodal metastasis. The current study confirmed that circulating free DNA and the ratio between the long and short cfDNA fragments (cfDNA integrity) have a potential to be used as diagnostic markers of GBC. The combination of ALU247 and cfDNA integrity provides good sensitivity, specificity and diagnostic accuracy for differentiating GBC from diseased (chronic cholecystitis) and normal controls.

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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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