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Panel of serum long non-coding RNAs as potential non-invasive biomarkers for gallbladder carcinoma

Sridhar Mishra [a], Pallavi Srivastava [a], Anshuman Pandey [b], Akash Agarwal [c], Saumya Shukla [a], Nuzhat Husain [a,*]

[a] Department of Pathology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, Uttar Pradesh, 226010, India
[b] Gastrosurgery, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, Uttar Pradesh, 226010, India
[c] Surgical Oncology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, Uttar Pradesh, 226010, India

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ABSTRACT

Gallbladder carcinoma (GBC) is a common malignancy and is usually diagnosed in the late stages of the disease. The identification of new effective early diagnostic biomarkers could represent an effective approach in reducing mortality in GBC. Altered expression of long non-coding RNAs (lncRNAs) is believed to be associated with the emergence and development of GBC. Our study aims to identify the expression of a range of circulating lncRNAs, including HOTAIR, ANRIL, H19, CCAT1 and MEG3, in matched serum and tissues of GBC for diagnosis and its association with clinicopathological features. The case and control study included matched serum and tissues from 63 GBC, 19 cholecystitis (CC), and 46 normal controls (NC). RNA extraction and cDNA synthesis from serum and fresh tissue match were performed using commercially available kits. Relative expression was assessed using SYBR Green real-time quantitative polymerase chain reaction. Circulating lncRNA levels including HOTAIR, ANRIL and H19 were upregulated in serum samples, while MEG3 and CCAT1 were downregulated in GBC compared to controls. The trend towards upregulation and downregulation was comparable in the tissue. HOTAIR and MEG3 levels were significantly different between serum CC and early-stage GBC ($p = 0.0373$, 0.0020), while H19 was significantly upregulated comparing early-stage GBC to advanced-stage GBC ($p = 0.018$). The expression of ANRIL was significant with M stage ($p = 0.0488$), H19 with stage ($p = 0.009$), M stage ($p=<0.0001$) & stage (0.009) and CCAT1 with M stage (0.044). When distinguishing GBC and NC, AUC for HOTAIR was 0.75, ANRIL 0.78, H19 0.74, CCAT1 0.80 and 0.96 for MEG3. The combination sensitivity for lncRNAs ranged from 84.13% (CI: 72.74–92.12%) to 100.0% (CI: 94.31–100.0%). Significant diagnostic value in discriminating pathologic stage was observed for ANRIL and MEG3 ($p = 0.022$, $p = 0.0005$). LncRNA show a significant change in expression in GBC and in discrimination of early stage from late-stage disease. The detection of 2 lncRNAs in panels, in coordination with radiology, could represent a potential serum-based biomarker for early-stage GBC diagnosis.

1. Introduction

Gallbladder carcinoma (GBC) is leading cause of cancer-related death and the most common form of bile duct cancer [1]. GBC is usually asymptomatic and diagnosis occurs late in the disease when resection is not possible. Early-stage GBC patients have a relatively higher survival rate with definitive treatment, but most patients are not diagnosed until advanced stages [2]. The 5-year survival rate of GBC patients is still 10% due to insufficient early detection methods. With the advancement of imaging modalities, it is quite difficult to detect gallbladder lesions and to differentiate GBC from adenomas and non-neoplastic gallbladder diseases such as xanthogranulomatous cholecystitis, chronic cholecystitis and cholesterol polyps, which are formed by a thickening of the gallbladder wall or a protruding gallbladder lesion [3,4]. For the definitive diagnosis, tissue histopathology is used after imaging methods with high diagnostic accuracy. The preoperative differentiation between early-stage cancer and cholecystitis can be challenging in radiological imaging. Bile cytology with endoscopic transpapillary gallbladder drainage (ETGD) was performed to differentiate between benign and malignant GB lesions [5]. The use of

* Corresponding author. Department of Pathology, Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow, 226010, Uttar Pradesh, India.
E-mail address: drnuzhathusian@hotmail.com (N. Husain).

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tumor markers such as carbohydrate antigen 19-9 (CA-19-9) and carcinoembryonic antigen (CEA) has shown limited diagnostic utility [6]. Therefore, a diagnostic biomarker for the early diagnosis and follow-up of GBC patients is urgently needed.

Early detection and treatment are among the most effective ways to enable curative treatment and reduce mortality in GBC. Non-invasive and easily accessible early detection markers are required. Liquid biopsy is a potential diagnostic modality and provides proteomic and transcriptomic information on tumor spatial and temporal heterogeneity [7,8]. Most of the human genome is transcribed into non-coding RNAs [9]. Non-coding RNA including long non-coding RNAs (lncRNAs) play an important role in physiological and pathological processes. LncRNAs have been shown to affect important cancer related signaling pathways in GBC such as WNT/-catenin, PI3K/Akt, EGFR, NOTCH, mTOR and TP53 signaling and contribute to every step of GBC carcinogenesis and tumor progression [10]. Furthermore, lncRNAs are highly stable and can be measured in various body fluid, supporting their use as a biomarker for early diagnosis and prognosis.

Altered expression of several lncRNAs has been reported in various solid tumors and could serve as an early diagnostic biomarker [10,11]. Previous studies have found the dysregulation of lncRNAs in GBC tissue, most of them showing an up regulation in GBC compared to normal gallbladder tissue (HOTAIR, ANRIL, H19, GCASPC and MALAT1) and other showing down regulation in neoplastic tissue compared to normal gallbladder tissue (CCAT1, MEG3). In GBC tissue, up/down regulation of these lncRNAs is well established and promote the cell proliferation, invasion and metastasis [10,11]. However, there are no studies on the diagnostic value of these lncRNAs in GBC. The lncRNA HOTAIR & H19 is overexpressed, promotes proliferation and migration in GBC tissue and has prognostic value [12,13]. In our previous study, we analyzed the panel of 5 microRNAs in GBC serum and found high diagnostic potential in distinguishing early-stage from late-stage GBC [14].

In the present study, we examined the expression of HOTAIR, ANRIL, H19, CCAT1 and MEG3 in matched serum and tissue from GBC and compared the levels with cholecystitis as disease control and with normal controls. Furthermore, we attempted to analyze these changes with a view to developing a non-invasive serum lncRNAs-based biomarker that could serve as a diagnostic biomarker for the early detection of GBC and correlate expression changes with clinicopathological features.

2. Material and methods

2.1 Patients sample and study design: The participants were recruited from the Department of Gastrosurgery & Surgical Oncology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India. A total of 128 participants were enrolled, including 63 primary GBC and 65 controls including cholecystitis as disease control and normal control. The Institutional Ethics committee of Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India, review and approve the study procedure (IEC 03/20). All the participants signed a written informed consent.

2.1. Sample collection

2.2.1 Blood Collection from cases and control: Pre-operative peripheral blood (5.0 ml) was collected from GBC (n = 63) and controls including cholecystitis as disease control (n = 19) and normal control (n = 21) in a silica gel vial (BD Vacutainer, USA) and processed to separate serum by centrifugation (1900g for 10 min and 16000 g for 10 min at room temperature). Serum samples were aliquot and stored at −80 °C till total RNA isolation.

2.2.2 Tissue collection from cases and control: After confirmation of the tumor in frozen section, tissue samples from the tumor area were stored in RNAlater solution (Thermo Fisher Scientific, USA) for total RNA extraction from GBC. For disease control, fresh tissue was collected from the gallbladder wall which were further confirmed on histopathologic examination as chronic cholecystitis/Xanthogranulomatous cholecystitis. Normal tissue with no or minimal inflammation and fibrosis was resected from healed cholecystitis/cases with/without stone, i.e., with normal morphology reviewed and confirmed by a histopathologist and served as a tissue reference control.

2.2. Long non-coding RNA profiling in paired samples of GBC and control

Matched serum and tissue samples were collected from 34 GBC. The procedure followed were radical cholecystectomy in 29/34 (85.30%), peritoneal nodule biopsies in 03/34(8.82%) and lymph nodes in 2/34 (5.88) were included. Matched serum and tissue samples from cholecystitis (n = 19) as disease control and 25 gallbladder with near normal histology and without marked inflammation & fibrosis were obtained from patients who underwent cholecystectomy due to gallstone disease and were used in this study as normal tissue reference.

2.3. Diagnostic evaluation of long non-coding RNA changes

For diagnostic evaluation, serum only samples were obtained from 29 non-resectable GBC cases diagnosed by radiology and cytology [22/29 (75.9%)] and radiology alone [07/29(24.13%)]. Cytology included fine-needle aspiration from the gallbladder mass, metastatic liver space occupying lesions and lymph nodes. Serum from 19 normal controls was used as normal serum reference. Blood samples were collected prior to the start of any treatment. Demographic and clinicopathological characteristics were obtained from all the participants.

2.4. Exclusion and inclusion criteria for cases and control selection

The cases were included on predefined inclusion and exclusion criteria. The histopathological confirmation as primary carcinoma gallbladder or unresectable advanced GBC with histologically confirmed peritoneal nodule/metastatic lymph node and radiologically/cytologically confirmed cases were included. Cases were excluded if presented with immunodeficiency disorder/other cancer, which may affect the long non-coding RNA analysis. Cases were also excluded if prior operated, on chemotherapy and/radiotherapy and subjects not giving consent to participate in the study.

2.5. Gold standard for diagnosis

GBC diagnosis was made based on histopathology of GBC according to WHO classification or radiological evidence of tumor with positive cytology. Histological parameters were assessed on hematoxylin and eosin-stained GBC slides by an experienced pathologist (NH, PS). The College of American Pathologists (CAP) protocol was used to assess tumor size, tumor site, TNM stage, nodal metastasis, and histomorphological features including mitosis, necrosis, nuclear grade, perineural invasion (PNI), and lymphovascular invasion (LVI) [15,16].

2.6. Total RNA isolation and quantitate real-time PCR

Total RNA from serum samples was extracted using the TRIzolLS reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. For qRT-PCR analysis, 500 ng of total RNA was reverse transcribed into complementary DNA using the Revert Aid H Minus First Strand cDNA Synthesis Kit (cat #K1632, Thermo Fisher Scientific, USA) according to the manufacturer's protocol and stored at −20 °C further use.

Real-time quantitative PCR was performed using SYBR Green PCR mix (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions using the primer sequences and annealing temperatures listed in Supplementary Table 1 on the CFX96 real-time PCR system (Bio-Rad, USA). The data was normalized using GAPDH

housekeeping gene expression. ΔΔCT method was applied for analysis of real time RT-PCR results. For this purpose, cycle threshold (CT) values for the reference gene and each of the lncRNAs was generated using the quantitative real time PCR in a 20 μl reaction volume including 10 μl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1.0 μl forward primer, and reverse primer, 02 μl of cDNA and 07 μl nuclease-free water. All samples were processed on Bio-Rad thermal cycler (USA) using the following thermal cycling conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30sec, annealing) as listed in Supplementary Table 1) 30 s, and elongation at 72 °C for 30 s. Melt curve analysis was performed on the same instrument from 65 °C to 95 °C with increments of 0.1 °C. Threshold (CT value) is well-defined as the number of PCR cycles in which the fluorescent signal crosses the threshold. The difference of CT values of individual lncRNAs and the GAPDH reference gene was reported as ΔCT. The ΔΔCT was calculated by subtracting the average ΔCt value of the controls from the ΔCt value of each case. The change in gene expression was calculated using equation $2^{-\Delta\Delta Ct}$ and was presented as relative fold change. The $2^{-\Delta\Delta CT}$ which characterizes the exponential value of ΔCT is measured as the fold change difference in expression of lncRNAs between GBC and controls in paired samples. For defining the up regulation and down regulation of studied lncRNAs, the mean relative fold change was compared with the mean relative fold change of controls. The upregulation for lncRNAs was defined as the high mean relative fold change in cases as compared controls. The down regulation for lncRNAs was defined as the lower mean relative fold change in cases as compared controls.

3. Statistical analysis

Categorical variables were represented as numbers and percentages (%) and continuous variables as mean SE. Association of mean of relative expression of lncRNAs with demographic and clinicopathological parameters was assessed using Mann-Whitney *U* test, one-way analysis of variance (ANOVA), and the Kruskal-Walli's test (for more than two groups as needed). For overall diagnostic value calculation receiver operator characteristic curve (ROC) analysis was performed. The MedCalc calculated the sensitivity, specificity and positive and negative predictive values. Statistical significance was defined as a p-value <0.05. All statistical analyzes were performed with GraphPad Prism version 9 (San Diego, USA).

4. Results

Relative expression levels of 5 selected serum circulating lncRNAs including HOTAIR, H19, ANRIL, CCAT1 and MEG3 were measured in matched samples (serum + tissue) and mismatched samples (serum only) using qRT-PCR as shown in Supplementary Table 2. The relative expression of these lncRNAs were then compared between groups including comparison of GBC with cholecystitis (CC) and GBC with normal control (NC). The comparison of GBC with controls (NC + CC) was also performed. The age and gender distribution of the cases and controls is shown in Supplementary Table 3. Age and sex of the cases and controls did not differ significantly; therefore, the groups were comparable.

4.1. LncRNA analysis in paired samples

LncRNA analysis, including HOTAIR, H19, ANRIL, CCAT1 and MEG3, was performed in 34 paired samples from GBC, and controls including matched cholecystitis (n = 19), as well as in 21 normal serum samples and 25 normal tissue samples. The mean relative expression difference between the matched samples was calculated for studied lncRNAs. The mean relative difference for HOTAIR was −6.621 (p = 0.334), H19 was 58.18 (p = 0.186), ANRIL was −15.85 (p = 0.584), CCAT1 was 1.04 (p = 0.159) and MEG3 was 1.96 (p = 0.005) respectively. In paired samples, the mean relative fold change of HOTAIR, ANRIL and H19 was higher GBC compared to normal control and termed as up regulated. The mean relative fold change of CCAT1 and MEG3 was lower compared to normal control as was termed as downregulated. The percent of cases upregulated in the serum and tissue was calculated by dividing the number of cases (relative fold change above the mean relative fold change of the normal control) to the total number of cases. The percentage of downregulated cases in the serum and tissue was calculated by dividing the number of cases (relative fold change below the mean relative fold change of the normal control) to the total number of cases. The change related to down-regulation and up-regulation in matched samples (serum + tissue) is presented in Fig. 1.

4.2. LncRNA value in serum samples

In addition, lncRNAs were additionally analyzed in 63 serum samples, including serum from 34 matched samples and 29 unmatched serum samples. The lncRNA value for the serum (n = 63) and tissue samples (n = 34) is shown in Table 1 and Fig. 2. Relative expression of CCAT1 and MEG3 was significantly down-regulated in GBC compared to normal control in serum samples, and the down-regulation trend was also confirmed in tissue samples. Furthermore, mean relative expression of HOTIAR, ANRIL, and H19 was significantly upregulated in serum of GBC compared to normal controls, and the trend of upregulation was consistent with tissue samples.

We also compared relative expression of these lncRNAs between GBC and cholecystitis to more accurately discriminate cases of cholecystitis. In serum samples, the mean of relative expression of HOTAIR (p=<0.0001) and ANRIL (p = 0.0002) showed an overall upregulation in GBC compared to CC. Furthermore, mean of relative expression of expression of CCAT1, MEG3 and H19 followed the trend of down-regulation in both serum and tissue samples, however the difference

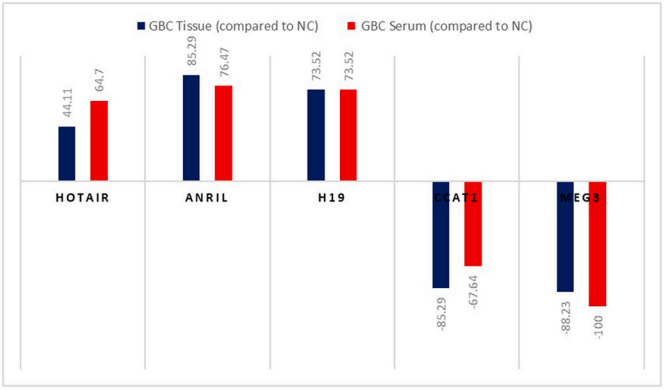

Fig. 1. Comparison of relative mean expression of lncRNAs in paired tissue and serum samples (n = 34). For this purpose, the cycle threshold (CT) for the reference gene (GAPDH) and each of the lncRNAs was generated using Syber Green-based real-time quantitative PCR. The difference in CT values of lncRNAs and the reference gene was presented as ΔCT. The ΔΔCt value was then calculated by subtracting the ΔCt value of the normal control from the ΔCt value of the GBC. The change in gene expression was calculated using equation $2^{-\Delta\Delta Ct}$ and was presented as relative fold change. The mean relative fold change of HOTAIR, ANRIL and H19 was higher GBC compared to normal control and termed as up regulated. The mean relative fold change of CCAT1 and MEG3 was lower compared to normal control and was termed as down-regulated. The up- and down-regulation of individual lncRNAs is presented for both tissue and serum samples. The percent of cases upregulated in the serum and tissue was calculated by dividing the number of cases (where relative fold change was above the mean of relative fold change of the normal control) to the total number of cases. The percentage of downregulated cases in the serum and tissue was calculated by dividing the number of cases (where relative fold change was below the mean of relative fold change of the normal control) to the total number of cases.

Table 1
Relative expression of LncRNA in cases and controls.

	GBC vs. NC			GBC vs. CC			GBC vs. Controls*		
	GBC Mean ± SE	NC Mean ± SE	p value	GBC Mean ± SE	CC Mean ± SE	p Value	GBC Mean ± SE	Controls Mean ± SE	P value
Serum HOTAIR	7.74 ± 1.36	1.53 ± 0.25	0.0006	45.76 ± 8.09	5.12 ± 1.47	<0.0001	18.0 ± 3.18	2.83 ± 0.42	<0.0001
Tissue HOTAIR	2.96 ± 1.08	1.32 ± 0.18	0.201	51.20 ± 18.6	19.80 ± 5.88	0.213	10.14 ± 3.6	4.26 ± 0.61	0.983
Serum ANRIL	5.97 ± 1.61	2.88 ± 0.45	0.783	57.09 ± 15.40	4.61 ± 1.27	0.0002	17.45 ± 4.70	5.10 ± 0.91	0.012
Tissue ANRIL	16.41 ± 11.31	5.12 ± 2.07	0.584	397.0 ± 273.7	54.12 ± 28.4	0.096	66.67 ± 45.94	15.52 ± 5.19	0.521
Serum H19	14.52 ± 8.39	1.76 ± 0.52	0.0006	32.46 ± 18.76	3.10 ± 1.00	0.653	21.28 ± 12.30	2.32 ± 0.50	0.012
Tissue H19	12.54 ± 8.39	1.34 ± 0.25	0.008	54.48 ± 36.48	22.50 ± 21.2	0.181	23.64 ± 15.83	5.66 ± 3.96	0.314
Serum CCAT1	0.57 ± 0.11	1.14 ± 0.12	<0.0001	2.48 ± 0.48	4.21 ± 1.11	0.298	1.15 ± 0.22	2.06 ± 0.26	0.0004
Tissue CCAT1	1.42 ± 0.29	3.25 ± 1.62	0.544	7.60 ± 1.55	4.91 ± 1.61	0.220	2.93 ± 0.60	4.62 ± 1.93	0.800
Serum MEG3	0.13 ± 0.01	1.41 ± 0.26	<0.0001	1.18 ± 0.15	3.86 ± 1.13	0.091	0.38 ± 0.05	2.83 ± 0.47	<0.0001
Tissue MEG3	0.81 ± 0.25	1.40 ± 0.17	0.0002	8.57 ± 2.68	3.73 ± 1.01	0.521	2.25 ± 0.70	2.72 ± 0.35	0.020

*GBC: Gallbladder carcinoma, CC: Cholecystitis, NC Normal control, *Controls: CC + NC*

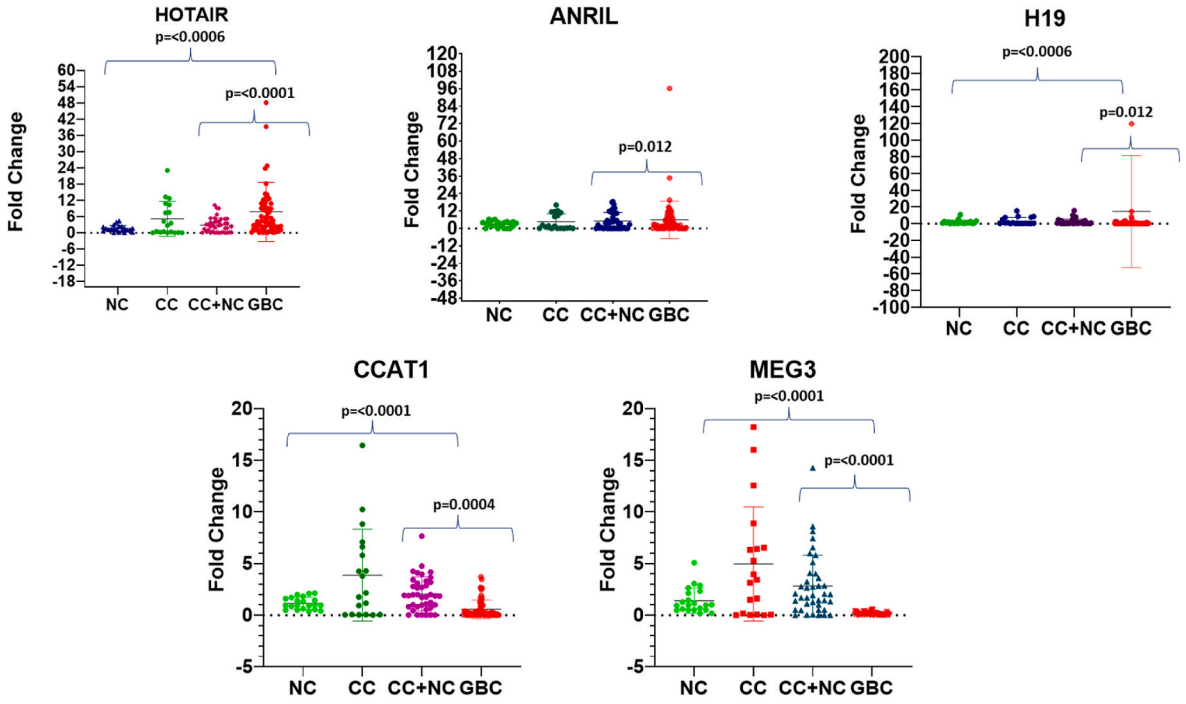

Fig. 2. Scatter dot plot of the serum level of five lncRNA in serum sample of GBC (n = 63) and controls including cholecystitis n = 19) & normal control (n = 21). The HOTAIR, ANRIL, H19 level were upregulated while CCAT1 and MEG3 weredown regulated in GBC compared to normal control and controls (NC + CC). The comparative threshold cycle method normalized data using GAPDH housekeeping gene expression. The Ct values were averaged; the relative expression of lncRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method. The scatter dot plot is presented in mean with SD. (p value was calculated using Man Whitney test).

was not significant.

In addition, the relative expression of serum lncRNAs were also analyzed with GBC stage as shown in Table 2. The mean of relative expression of HOTAIR (p = 0.037) and MEG3 (p = 0.002) differed significantly between cholecystitis and early stage of GBC. Furthermore, the mean of relative expression of H19 was significantly upregulated (p = 0.0188) when comparing early-stage GBC with advanced stage (Table 2). These findings suggest a probable role for HOTAIR and MEG3 for early GBC detection.

4.3. Serum lncRNAs diagnostic value

Studied serum lncRNAs including HOTAIR, ANRIL, H19, CCAT1 and MEG3 showed a significant diagnostic in discrimination of GBC form controls. The diagnosis of lncRNAs was determined in the serum of GBC (n = 63) by receiver operating characteristic curve (ROC) analysis and the area under the curve, sensitivity, specificity, positive and negative predictive value (Table 3). Among the HOTAIR, ANRIL, H19 CCAT1 and

Table 2
Comparison of relative expression of lncRNA in serum sample of cholecystitis (CC) and stage of GBC.

	Relative lncRNA expression in serum Mean ± SE			p value comparing	
	CC (n = 19)	GBC stage I + II (n = 16)	GBC stage III + IV (n = 47)	CC with GBC stage I + II	stage I + II with III + IV
HOTAIR	5.12 ± 1.47	26.35 ± 10.54	15.32 ± 2.42	0.034	0.131
ANRIL	4.61 ± 1.27	3.91 ± 1.53	22.05 ± 6.26	0.722	0.092
H19	3.10 ± 1.00	71.37 ± 46.83	4.67 ± 3.71	0.771	0.016
CCAT1	3.87 ± 1.02	1.56 ± 0.58	1.03 ± 0.23	0.070	0.315
MEG3	4.95 ± 1.27	0.29 ± 0.12	0.40 ± 0.05	0.002	0.342

Table 3
Diagnostic value of lncRNA in differentiation of cases and controls.

Marker	Cut off value	AUC (95% CI)	p-value	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95%CI)
HOTAIR								
GBC vs. NC	≥1.94	0.75 (0.65–0.85)	0.0006	73.02 (60.35–83.43)	71.43 (47.82–8872)	88.46 (79.32–99.88)	46.87 (35.13–5897)	72.62 (61.80–81.79)
GBC vs. CC	≥7.74	0.85 (0.77–0.94)	<0.0001	77.78 (65.54–87.28)	73.68 (48.80–90.85)	90.74 (82.03–95.46)	50.00 (36.95–63.05)	76.83 (66.20–85.44)
GBC vs. controls (CC + NC)	≥1.94	0.75 (0.72–0.88)	<0.0001	82.54 (70.90–90.95)	45.00 (29.26–61.51)	70.27 (63.59–76.18)	62.07 (46.40–75.57)	67.96 (58.04–76.82)
ANRIL								
GBC vs. NC	≥13.47	0.78 (0.69–0.88)	0.0001	78.73 (45.62–70.99)	42.86 (21.82–65.98)	75.51 (66.86–82.50)	25.71 (16.30–38.09)	54.76 (43.52–65.66)
GBC vs. CC	≥1.92	0.52 (0.39–0.65)	0.771	76.19 (63.79–86.02)	63.16 (38.36–83.71)	87.27 (78.93–92.62)	44.44 (31.37–58.33)	73.17 (62.24–82.36)
GBC vs. controls (CC + NC)	≥4.12	0.64 (0.54–0.75)	0.012	69.84 (56.98–80.77)	57.50 (40.89–72.96)	72.13 (63.54–79.35)	54.76 (43.30–65.74)	65.05 (55.02–74.18)
H19								
GBC vs. NC	≥0.38	0.74 (0.63–0.85)	0.0008	66.67 (53.66–78.05)	90.48 (69.62–98.83)	95.45 (84.74–98.76)	47.50 (38.32–56.85)	72.62 (61.80–81.79)
GBC vs. CC	≥0.81	0.53 (0.38–0.68)	0.642	65.08 (52.03–76.66)	47.37 (24.45–71.14)	80.39 (72.06–86.70)	29.03 (18.61–42.26)	60.98 (49.57–71.56)
GBC vs. controls (CC + NC)	≥0.54	0.64 (0.53–0.75)	0.013	65.08 (52.03–76.66)	70.00 (53.47–83.44)	77.36 (67.30–85.01)	56.00 (46.20–65.35)	66.99 (57.03–75.94)
CCAT1								
GBC vs. NC	≤0.36	0.81 (0.72–0.89)	<0.0001	69.84 (56.98–80.77)	100.00 (83.89–100.00)	100.00	52.50 (43.15–61.68)	77.38 (66.95–85.80)
GBC vs. CC	≤1.69	0.58 (0.40–0.76)	0.291	70.31 (57.58–81.09)	57.89 (33.50–29.75)	84.91 (76.43–90.70)	36.67 (25.27–49.78)	67.47 (56.30–77.35)
GBC vs. controls (CC + NC)	≤0.72	0.70 (0.59–0.81)	0.0005	69.84 (56.98–80.77)	80.00 (64.35–90.95)	84.62 (74.35–91.26)	62.75 (52.87–71.66)	73.79 (64.20–81.96)
MEG3								
GBC vs. NC	≤0.46	0.96 (0.93–1.00)	<0.0001	95.24 (86.71–99.01)	90.48 (69.62–98.83)	96.77 (88.91–99.12)	86.36 (67.54–95.07)	**94.05 (86.65 98.04)**
GBC vs. CC	≤1.47	0.63 (0.43–0.82)	0.091	71.43 (58.65–82.11)	68.42 (43.45–87.42)	88.24 (79.16–93.67)	41.94 (35.55–54.25)	**70.73 (59.65 80.26)**
GBC vs. controls (CC + NC)	≤0.49	0.82 (0.72–0.92)	<0.0001	73.02 (60.35–83.43)	82.50 (67.22–92.66)	86.79 (76.73–90.90)	66.00 (55.79–74.91)	**76.70 (67.34 84.46)**

GBC: Gallbladder carcinoma, CC: Cholecystitis, NC: Normal control, AUC: Area Under Curve, CI: Confidence Interval, PPV: Positive Predictive Value, NPV: Negative Predictive Value

MEG3 lncRNAs examined, only HOTAIR could significantly distinguish GBC from CC. Furthermore, among these lncRNAs, the highest sensitivity was for MEG3 (95.24%) and the specificity of CCAT1 was highest (100.0%) in distinguishing GBC from the normal control.

4.3.1. Diagnostic value of serum lncRNA HOTAIR

At a cutoff value of ≥1.94, the AUC for HOTAIR was 0.75 (p = 0.0006) in distinguishing GBC from normal control. The sensitivity, specificity PPV, NPV and diagnostic accuracy was 73.02%, 71.43% 88.46%, 46.87 and 72.62%, respectively. At a cutoff value of ≥7.74, the AUC was 0.85 (p=<0.0001), to distinguishing CC from GBC with sensitivity, specificity PPV, NPV and diagnostic accuracy of 77.78%, 73.68%, 90.74%, 50.0% and 76.83%, respectively. The AUC was 0.75, when distinguishing cases from controls (CC + NC), the sensitivity increased to 82.54% with PPV of 70.27%; however, the specificity dropped to 45.00% with NPV of 62.07% with a diagnostic accuracy of 67.96%.

4.3.2. Diagnostic value of serum lncRNA ANRIL

With a cutoff value of ≥13.47, the AUC for ANRIL was 0.78 (p = 0.0001) to distinguish GBC from normal control. The sensitivity, specificity PPV, NPV and diagnostic accuracy was 78.73%, 42.86% 75.51%, 25.71% and 54.76%, respectively. In distinguishing CC from GBC, the AUC was 0.52 (p = 0.771), with sensitivity, specificity PPV, NPV and diagnostic accuracy of 76.19%, 63.16%, 87.27%, 44.44% and 73.17%, respectively. When distinguishing cases from controls (CC + NC), the sensitivity decreased to 69.84% with PPV of 72.13%; and the specificity was 57.50% with NPV of 54.76% with a diagnostic accuracy of 65.05%.

4.3.3. Diagnostic value of serum lncRNA H19

With a cutoff value of ≥0.38, the AUC for H19 was 0.74 (p = 0.0001) to distinguish GBC from normal control. The sensitivity, specificity PPV, NPV and diagnostic accuracy was 66.67%, 90.48% 95.45%, 47.50% and 72.62%, respectively. At a cutoff value of ≥1.92, the AUC was 0.53 (p = 0.00.642) to distinguishing CC from GBC with sensitivity, specificity PPV, NPV and diagnostic accuracy of 65.08%, 47.37%, 80.39%, 29.03% and 60.98%, respectively. In distinguishing cases from controls (CC + NC), the AUC was 0.64, with sensitivity, specificity, PPV, NPV and diagnostic accuracy of 65.08%, 70.00%, 77.36%, 56.00% and 66.99% respectively.

4.3.4. Serum lncRNA CCAT1 and MEG3 diagnostic value

The down-regulated lncRNA, including CCAT1 and MEG3, showed higher AUC in distinguishing GBC from normal controls compared to up-regulated lncRNAs, as shown in Table 5. At a cutoff point of ≤0.36, the AUC for CCAT1 was 0.81 (p = 0.0001) in discrimination of cases from the control with a sensitivity, specificity, PPV, NPV and diagnostic accuracy of 69.84%, 100.0%, 100.0%, 52.505 and 77.38% respectively. At a cutoff value of ≤0.72, the AUC to distinguish GBC from controls (CC + NC) was 0.70 with a specificity of 80.0% and PPV, NPV of 84.62% & 62.75% respectively. Furthermore, at a cutoff value of ≤0.46, down-regulated MEG3 showed the highest sensitivity (95.24%) with an AUC of 0.96 (p=<0.0001) in distinguishing GBC from the normal control. In addition, the diagnostic accuracy for MEG3 (94.05%) was highest among the lncRNAs examined. In addition, the sensitivity and specificity of MEG3 was 73.02% & 82.50% in discrimination of GBC from controls with diagnostic accuracy of 76.70%.

Table 4
Diagnostics of HOTAIR, ANRIL, H19, CCAT1 and MEG3 in discrimination of stage in GBC.

GBC Parameter	Cut off value	AUC (95% CI)	p-value	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (%)
Stage I&II vs. III & IV								
HOTIAR	≥5.12	0.54 (0.35–0.73)	0.592	74.47 (59.65–86.06)	50.00 (24.65–75.35)	81.40 (72.27–88.01)	40.00 (25.03–57.11)	68.25 (55.31–79.42)
ANRIL	≥0.47	0.79 (0.67–0.91)	0.0005	87.23 (74.26–95.17)	56.25 (29.88–80.25)	87.42 (76.88–91.16)	60.00 (38.75–78.05)	79.37 (67.30–88.53)
H19	≤0.46	0.60 (0.42–0.77)	0.224	57.78 (42.15–72.34)	50.00 (24.65–75.35)	76.47 (65.22–84.92)	29.63 (18.81–43.35)	55.74 (42.45–68.45)
CCAT1	≥0.11	0.57 (0.36–0.76)	0.413	68.09 (52.88–80.91)	56.25 (29.88–80.25)	82.05 (71.72–89.18)	37.50 (24.75–52.25)	65.08 (52.30–76.66)
MEG3	≥0.03	0.70 (0.51–0.86)	0.022	91.49 (79.62–97.63)	50.00 (24.65–75.35)	84.31 (76.57–89.84)	66.67 (40.99–85.21)	80.95 (69.09–89.75)

4.4. Combination diagnostic of studied lncRNAs

In addition, combination diagnostic of lncRNAs was also analyzed. The diagnostic values of combination of two, three, four and five lncRNAs are presented in Supplementary Table 4. The combined sensitivity for lncRNAs ranged from 84.13% (CI: 72.74–92.12%) to 100.0% (CI: 94.31–100.0%), suggesting their use as a screening biomarker. The combination of HOTAIR + MEG3 showed the highest diagnostic sensitivity of 100.0% (CI: 94.31–100.0%) with an NPV of 100.0% in discrimination GBC from normal control. The specificity of two marker combinations, CCAT1+MEG3, showed the highest specificity of 77.50% (CI: 61.55–89.16). The diagnostic accuracy of the two marker combinations was between 66.99% (CI: 57.03–75.94) and 86.41% (CI: 78.25–92.37). In addition, the sensitivity three-marker combination ranged from 66.99% (CI: 57.03–75.94) to 80.58% (CI: 71.62–87.72). The three marker combinations HOTAIR + ANRIL + MEG and HOTAIR + H19++MEG3 showed a sensitivity of 100.0% (CI: 94.31–100.0%) and an NPV of 100.0%, respectively. The 4-marker combination also showed a sensitivity of 100.0%; however, specificity was reduced.

4.5. Diagnostics of HOTAIR, ANRIL, H19 CCAT1 and MEG3 stage discrimination

In discrimination of early-stage disease (stage I & II) from late-stage disease (stage III & IV), the diagnostic sensitivity of ANRIL (73.68%, CI: 56.60–86.60) and HOTAIR (68.42%, CI: 51.35–82.50) was highest among the studied lncRNAs as presented in Table 4.

4.6. Serum lncRNAs association with clinicopathological features of GBC

The association of mean of relative expression of HOTAIR, ANRIL, H19, CCAT1 and MEG3 with histopathological features of GBC is depicted in Table 5. The mean of relative expression of serum ANRIL was significantly associated with the M stage (p = 0.048) and stage (p = 0.009). The mean of relative expression of serum H19 showed a significant association with the N stage (p=<0.0001), M stage (p=<0.0001), and with GBC stage (p = 0.009). Moreover, the serum level of MEG3 showed a significant association with the M stage (0.044).

5. Discussion

Laboratory detection of asymptomatic GBC is a major challenge in early diagnosis. To overcome such limitations, minimally invasive and efficient biomarkers are urgently needed. Liquid biopsy can overcome this limitation to some extent [17–19]. The identification of diagnostic markers for cancer is of interest for the early detection and timely management of gallbladder cancer. Recently, various studies suggest that ectopic expression of lncRNAs could be a potential marker for diagnosis of cancer patients [20–22]. Furthermore, studies have shown that lncRNAs can act as a tumor suppressor or oncogenic in several cancer types including GBC and can monitor treatment response and survival [23–27]. Recently, circulating lncRNAs have been identified in hepatocellular carcinoma, colon, prostate and gastric cancer as an early detection biomarker using body fluids such as blood plasma [28–30]. Therefore, measurements of circulating lncRNAs can be beneficial for early detection and successive monitoring of GBC.

In the current study, we found that serum lncRNAs including HOTAIR, ANRIL, H19, CCAT1 and MEG3 are potential circulating biomarkers for GBC diagnosis. The 2- and 3-serum lncRNA combination offered high diagnostic sensitivity for GBC patients. Imaging and biomarker detection are currently used to diagnose GBC in current medical detection methods. However, early diagnosis of GBC still poses a challenge. The diagnostic value of circulating lncRNAs in GBC has therefore not been analyzed; very few studies have analyzed the role of tissue lncRNAs in malignancy development, aggressive phenotype and GBC diagnosis [11,12,31]. In our study, the diagnostic sensitivity of lncRNAs ranged from 66.67% (CI: 53.66–78.05) to 95.24% (CI: 86.71–99.01) and the specificity between 42.86% (CI: 21.82–65.98) and 100% (CI: 83.89–100.0), indicating the ability of lncRNAs to detect GBC and could target non-GBC cases. Among the HOTAIR, ANRIL, H19 CCAT1 and MEG3 lncRNAs examined, only HOTAIR could significantly distinguish GBC from CC, as it is known that the lncRNAs play role in regulation of inflammation firstly by increasing the transcription of pro-inflammatory cytokines or other inflammatory target genes, secondly by enhancing the NF-kB signaling pathways and lastly by modulating the polarization of macrophages which further results in release of proinflammatory mediators [32], However combination of markers have shown (HOTAIR + MEG3) higher diagnostic sensitivity (100.0%, CI: 94.31–100.0), and combination of CCAT1+MEG3 showed diagnostic specificity of 77.50% (CI: 61.55–89.16).

In clinical practice, tumor protein markers such as carbohydrate antigen 15-3 (CA15-3), cancer antigen 125 (CA125), and carcinoembryonic antigen (CEA) are commonly used; however, the use of these protein-based biomarkers showed limited sensitivity and specificity [33,34]. The stable expression of lncRNAs in serum supports their evaluation as diagnostic markers in GBC. Previous studies have identified dysregulated lncRNAs in GBC tissues; however, they have only been analyzed in serum/plasma in very few studies. Our study is interesting due to reason: first, based on previous studies, we selected 5 GBC related lncRNAs and analyzed the expression of these lncRNAs in matched samples (serum + tissue) to validate a parallel tumor-dependent increase in serum versus increase in the tissue. Second, we included cholecystitis with a narrow difference in radiology, as a disease control group. Subgroup analysis revealed that these lncRNAs offer moderate diagnostic sensitivity for distinguishing GBC patients from cholecystitis and normal controls. Consistent with the previous results, we observed up-regulation of ANRIL, HOTAIR and H19 and down-regulation of MEG3 in GBC compared to controls (CC + NC), and a similar trend was observed in the tissue samples. The ROC analysis confirms that analysis of these LncRNAs can be an effective marker to distinguish GBC patients

Table 5
Association of mean relative expression of lncRNAs with demographic and clinicopathological characteristics.

Characteristics	N	HOTAIR	p value	ANRIL	p value	H19	p value	CCAT1	p value	MEG3	P value
		Mean ± SE		Mean ± SE		Mean ± SE		Mean ± SE		Mean ± SE	
Age (Yrs.):											
≤45	17	9.02 ± 2.33	0.121	9.08 ± 2.63	0.441	1.36 ± 0.75	0.127	1.95 ± 0.55	0.070	0.39 ± 0.11	0.822
>45	46	21.49		20.54 ± 6.4		29.09 ± 17.02		0.87 ± 0.22		0.37 ± 0.05	
Gender:											
Female	44	16.84 ± 3.95	0.123	13.97 ± 2.925.50 ± 14.43	0.343	1.49 ± 0.55	0.071	0.97 ± 0.22	0.546	0.38 ± 0.06	0.979
Male	19	21.10 ± 5.70				68.19 ± 40.09		1.60 ± 0.55		0.36 ± 0.08	
Histological Grade:											
WD	1015	33.15 ± 15.93	0.323	4.52 ± 2.33	0.993	112.0 ± 73.18	0.231	0.69 ± 0.29	0.284	0.30 ± 0.13	0.571
MD	02	13.33 ± 4.16		3.60 ± 1.06		14.26 ± 11.60		2.02 ± 0.65		0.32 ± 0.13	
PD	07	1.20 ± 1.02		4.74 ± 4.72		3.37 ± 1.87		2.62 ± 0.68		0.43 ± 0.28	
Unknown		5.12 ± 0.83		6.75 ± 2.19		0.36 ± 0.08		3.13 ± 0.92		0.18 ± 0.04	
LVI:											
Evident	12	19.71 ± 11.23	0.984	2.83 ± 1.08	0.573	2.29 ± 1.02	0.735	1.43 ± 0.47	0.801	0.37 ± 0.12	0.193
Not evident	15	20.01 ± 7.81		5.04 ± 1.69		87.54 ± 49.93		1.76 ± 0.62		0.20 ± 0.10	
Unknown	07	4.75 ± 0.87′		6.64 ± 2.20		0.31 ± 0.08		2.98 ± 0.93		0.36 ± 0.19	
PNI											
Evident	07	12.98 ± 8.15	0.365	3.91 ± 1.79	1.00	4.22 ± 2.90	0.593	1.99 ± 1.01	0.135	0.18 ± 0.09	0.574
Not evident	20	22.29 ± 8.31		4.11 ± 1.30		65.55 ± 38.12		1.48 ± 0.42		0.31 ± 0.10	
Unknown	07	4.75 ± 0.87		6.64 ± 2.20		0.31 ± 0.08		2.98 ± 0.93		0.36 ± 0.19	
TILS											
1+	13	22.48 ± 10.85	0.925	4.14 ± 1.33	0.563	57.89 ± 42.87	1.00	0.93 ± 0.34	0.203	0.19 ± 0.05	0.887
2+	14	17.46 ± 7.76		3.98 ± 1.66		42.0 ± 39.49		2.25 ± 0.67		0.35 ± 0.14	
Unknown	07	4.75 ± 0.87		6.46 ± 2.20		0.31 ± 0.08		2.98 ± 0.93		0.36 ± 0.19	
T stage:											
Tis + T1	06	12.78 ± 5.02	0.287	3.47 ± 1.63	0.188	93.39 ± 90.70	0.113	0.92 ± 0.47	0.109	0.33 ± 0.23	0.837
T2	13	29.53 ± 12.73		5.56 ± 2.18		45.04 ± 42.54		1.76 ± 0.70		0.36 ± 0.14	
T3+T4	38	17.13 ± 2.90		25.37 ± 7.65		5.62 ± 4.59		0.67 ± 0.18		0.42 ± 0.06	
Tx	06	5.02 ± 0.98		6.95 ± 2.58		0.34 ± 0.09		3.18 ± 1.08		0.17 ± 0.04	
N stage											
N0	20	27.67 ± 8.58	0.325	9.29 ± 3.33	0.295	65.94 ± 38.10	<0.0001	1.48 ± 0.48	0.057	0.34 ± 0.10	0.623
N1	25	16.14 ± 3.71		28.47 ± 11.37		1.48 ± 0.51		0.68 ± 0.16		0.39 ± 0.07	
N2	09	15.86 ± 3.39		17.00 ± 4.75		0.30 ± 0.10		0.15 ± 0.05		0.51 ± 00.15	
Unknown	09	4.66 ± 1.05		5.36 ± 1.93		0.32 ± 0.06		2.80 ± 0.87		0.27 ± 0.11	
M stage											
M0	40	18.94 ± 4.53	0.753	16.33 ± 7.09	0.043	33.55 ± 19.51	<0.0001	0.99 ± 0.26	0.043	0.30 ± 0.06	0.061
M1	23	16.70 ± 4.12		19.38 ± 4.61		0.84 ± 0.25		1.46 ± 0.42		0.50 ± 0.08	
Stage											
Stage 0 + 1	06	12.78 ± 5.02	0.438	3.47 ± 1.63	0.009	93.39 ± 90.70	0.009	0.92 ± 0.47	0.643	0.33 ± 0.23	0.602
Stage 2	10	26.35 ± 10.54		3.91 ± 1.53		71.37 ± 46.83		1.56 ± 0.58		0.29 ± 0.12	
Stage 3	18	14.68 ± 3.37		29.13 ± 15.28		11.06 ± 9.67		0.79 ± 0.23		0.33 ± 0.08	
Stage 4	29	15.72 ± 3.36		17.65 ± 3.84		0.70 ± 0.21		1.17 ± 0.34		0.45 ± 0.07	
LN Metastasis:	09	8.60 ± 3.59	0.572	4.28 ± 2.16	0.503	2.57 ± 1.33	0.752	1.42 ± 0.41	0.061	0.31 ± 0.14	0.932
Present	16	26.27 ± 10.55		4.91 ± 1.56		82.09 ± 47.02		1.34 ± 0.45		0.32 ± 0.12	
Absent Unknown	09	7.99 ± 2.40		4.32 ± 1.13		0.68 ± 0.39		3.35 ± 1.01		0.23 ± 0.11	

WD: Well differentiated, MD: Moderately differentiated, PD: poorly differentiated, LVI: Lymphovascular invasion, PNI: Perinuclear invasion, LN: Lymph node metastasis.

from normal controls. We also provided new insight into the clinical relevance of HOTAIR, H19, ANRIL, CCAT1 and MEG3 and found that it was significantly associated with M-stage, N-stage and stage of GBC patients, suggesting that these lncRNAs may be positively associated with GBC progression.

Study by Longyang Jin et al., 2018 showed that MEG3 downregulation in GBC tissue and cells act as negative predictor of GBC. MEG3 has been presented as an effective target for GBC therapy and could help advance lncRNA-based diagnostics and therapy in GBC patients [33]. Liu et al., 2016 found, downregulation of MEG3 (6.26-fold) in GBC tissue compared to normal control [24]. Similarly, in our study, MEG3 expression was downregulated 7.4-fold in GBC tissue and 3.2-fold in CC compared to normal control. A similar trend was also observed for the serum of GBC patients. MEG3 significantly differentiate GBC stage I + II from cholecystitis patients ($p = 0.0020$). A possible justification for MEG3 downregulation in cancer and GBC could be hypermethylation of MEG3 promoters [35].

The lncRNA CCAT1 firstly describes in colon cancer, activated by c-Myc, and is essential for the invasion and proliferation of gastric cancer cells. MZ Ma et al., 2015, reported the up regulation of CCAT1 in GBC tissue (1.5-fold) compared to adjacent normal tissue, and was significantly associated with lymph node and tumor status [14].

H19 has been shown to upregulate various cancers including colon cancer, gastric cancer, non-small cell lung cancer (NSCLC) and breast cancer [36,37]. Upregulation of H19 has been reported in GBC tissue and was significantly associated with lymph node metastasis, tumor size and shorter survival [26,27]. Similarly, in our study, the serum H19 level in GBC was significantly different and higher than controls (CC + NC) and was significant associated with stage (0.00931), N stage ($p=<0.0001$) and M stage ($p=<0.0001$). Furthermore, serum H19 expression could significantly differentiated early-stage and late-stage GBC ($p = 0.0188$), suggesting the oncogenic function of H19. Studies have shown that tissue lncRNAs have low specificity in detecting specific cancers. For example, H19 has shown a specificity of 58% and 56.67% in gastric cancer patients [38]. Interestingly, we have found high specificity of H19 (90.48% CI: 69.62–98.43) in detecting GBC from NC and a specificity of 70% (CI: 53.47–83.44).

LncRNA HOTAIR promotes malignant tumor characteristics and is overexpressed in various tumors including gallbladder and gastric cancer [23,39,40]. We found a significant upregulation (6.3-fold) of HOTIAR in serum of GBC compared to controls (CC + NC, $p=<0.0001$) and was comparable in tissue samples. A study by MZ Ma et al., 2014 reported a 3.0-fold overexpression of HOTAIR in gallbladder tissue compared to adjacent non-tumor tissue [41]. Studies have reported the association of HOTAIR expression with the T and N stages of GBC. Furthermore, our study could not find any clinical-pathological association with HOTAIR. Furthermore, in our study, the serum level of HOTAIR was able to significantly differentiate cholecystitis from early stages (I + II) ($p = 0.0373$) disease. In our study, HOTAIR demonstrated sensitivity and specificity of 73.02% (CI: 60.35–83.43) and 71.43% (CI: 47.82–88.72), respectively, in differentiating GBC from normal control. In addition, the sensitivity in differentiating GBC patients from controls (CC + NC) reached 82.54% (CI: 70.90). Similarly, HOTAIR expression has demonstrated high diagnostic efficacy (92.5% specificity) in identifying CRC patients. The HOTIAR expression level was used as a negative prognostic biomarker for CRC patients with a sensitivity and specificity of 92.5% and 67.0% with an AUC of 0.87 [42]. These results suggest the oncogenic potential of HOTAIR in GBC progression and its expression could serve as a diagnostic marker in serum in GBC patients.

ANRIL is overexpressed and promotes malignancy in a variety of malignancies. In GBC tissue, expression of ANRIL was found to be upregulated 2.4-fold compared to adjacent normal tissue and shown to increase proliferation and tumor size in the mouse model study. ANRIL expression in GBC tissues compared to normal tissues have been found associated with age, histological type, differentiation and TNM stage [24]. In our study, the serum ANRIL level was 2.0-fold higher in the GBC patients compared to normal control and was 6.3-fold higher as compared to controls (CC + NC) ($p=<0.0131$). Interestingly, we also found a significant upregulation of serum ANRIL in GBC (12-fold) compared to cholecystitis ($p = 0.0002$). A similar trend was also observed in the tissue sample. In addition, serum ANRIL expression was associated with M stage (0.0488) and GBC stage (0.0097). Other studies have also reported comparable results, where increased expression of ANRIL promotes metastasis and decreased apoptosis in lung cancer cells [43]. Studies have shown that ANRIL affects carcinogenesis by modulating the INK4b-ARF-INK4a signaling pathways, which inhibits the expression of the p15INK4b and p16INK4a tumor suppressor genes and triggers the RAS signaling pathway to stimulate tumorigenesis [44,45]. These results suggest that ANRIL acts as an oncogene in GBC.

Interestingly, the expression level of ANRIL and MEG3 showed a significant diagnostic differentiation for early-stage vs. late-stage disease (Pathological stage I + II vs. III + IV). The sensitivity and specificity for ANRIL reached 87.23% (CI: 74.26–95.17) & 56.25% (CI: 29.88–80.25) with PPV of 87.42% (CI: 76.88–91.16) and NPV of 60.0% (CI: 38.75–78.05) respectively.

The MEG3 showed much higher sensitivity (91.49%, CI: 79.62–97.63) compared to other studied lncRNAs in the differentiation of early-stage vs. late-stage disease with PPV of 84.31% (CI: 76.57–89.84).

We calculated the combination diagnostics to overcome the limited diagnostic sensitivity and specificity of studies using circulating lncRNAs and to increase their diagnostic power. We analyzed the combination of two, three, and four lncRNAs to improve diagnostic performance. The combined sensitivity of the two manufacturer combinations ranged from 84.13% (CI: 72.74–92.12) to 100.0% (CI: 94.31–100.0). Diagnostic sensitivity was highest for HOTAIR and MEG3 (100.0%) and diagnostic specificity was highest for a combination of CCAT1 and MEG3 (77.50). In our study, the three marker combinations showed higher diagnostic sensitivity ranging from 95.24% (CI: 86.71–99.01) to 100.0% (94.31–100.0); however, specificity was reduced. A number of studies have proposed combination analysis to improve diagnostic performance. Zhao W et al., 2015 tested the combined diagnostic performance of circulating HOTAIR and CCAT1 in CRC and found higher sensitivity and specificity (84.3% and 80.2%) compared to diagnosis of the above markers alone. In addition, the markers showed an efficacy of 85.0% in identifying early-stage colorectal cancer [46]. Similarly, we also found the significant diagnosis of ANRIL (AUC = 0.78) in differentiating T-stage 1 + 2 from 3 + 4 with a sensitivity and specificity of 73.68 (CI: 56.90–86, 60), 73.68% (CI: 48.80–86.60) found. 90.85) and high PPV (84.85% (CI: 72.05–92.41)).

A combination diagnostic with lncRNA in GBC serum has not been reported; however, this has been reported in other cancers. A recent study reported the serum exosome UCA1 as a diagnostic marker of colorectal cancer with diagnostic sensitivity & specificity of 100.0% & 43.0% and could be increased to 93.0% sensitivity and 64% specificity by combining with lncRNA TUG1 [47]. A favorable panel comprising three lncRNAs (LOC152578, XLOC_000303, and XLOC_0006844) up-regulated in CRC was identified and confirmed on an independent large plasma sample cohort (220 CRCs, 180 controls) (positive predictive value: 0.80, negative predictive value: 0.84, AUC = 0.975) [25]. Hu et al. analyzed the 3 lncRNAs, including SPRY4-IT1, ANRIL, and NEAT1 in NSCLC patients and showed a diagnostic sensitivity and specificity of 82.8% & 92.3%, respectively, with an AUC of 0.87 [48]. A study by Tong YS et al., 2015 reported the combination of POU3F3, HNF1AAS1, and SPRY4-IT1 LncRNAs in the diagnosis of esophageal squamous cell carcinoma with diagnostic sensitivity and specificity of 72.8% and 89.4% respectively with an AUC of 0.84 [49]. To improve the diagnostic performance of the studied lncRNAs in HCC, Yu J et al., 2016, analyzed the combination of PVT1 and reported the sensitivity and specificity of 60.5% and 90.6%, respectively [50]. Thus, the signature created by combining a number of lncRNAs purportedly offers improved diagnostic performance compared to most individual circulating lncRNAs studied.

In addition to using the dysregulated expression of lncRNAs in diagnosis and prognosis, several studies have focused on investigating the role of lncRNAs in chemoresistance and in predicting therapeutic response in colorectal cancer, which could represent a potential therapeutic target. A study by Han P et al., 2017 found an association between elevated MALAT1 levels and poor response to oxaliplatin (OXA)-based chemotherapy [51]. Similarly, CRNDE also contributes to oxaliplatin resistance in CRC. The study by Li P et al. investigated HOTAIR overexpression and poor response to 5FU treatment by suppressing miR-218 and activating NF-kB signaling in CRC [52].

Few studies have provided clarity on the molecular mechanism behind the secretion and transport of circulating long noncoding RNAs in the extracellular environment. There is not much research indicating that the secretion of lncRNA and miRNA is almost identical. To generate RNase and survive, extracellular RNAs are first enclosed in membrane vesicles such as exosomes and macrovesicles. Lipoprotein vesicles with a diameter of 50–100 nm, called exosomes and macrovesicles, form when multivesicular bodies (MVBs) sprout inward via the endocytosis pathway. Once MVBs were fused with the plasma membrane, exosomes were reliably secreted [53]. Originally, exosomes were thought to be membrane proteins that were discarded and had no biological function [54]. However, recent studies have shown that exosomes and macrovesicles carrying both RNA and proteins can act as messengers of information by modulating transcription and translation processes. Using deep sequencing, Huang et al. exosomal RNAs extracted from human plasma [55], found that 3.36% of the sequences of exosomal RNAs were lncRNAs. An investigation found no significant difference in the amounts of lncRNA in plasma and exosomes. According to these findings, lncRNAs are mainly located in exosomes, which also act as their main protective shield in plasma [56]. Second, extracellular RNAs are released from tumor cells and tissues. Ren et al. [57] found a significant increase in plasma lncRNA levels in the presence of subcutaneous xenograft cancer. In addition, plasma lncRNA expression was lower in people who had undergone surgery than in people who had not. This suggests that long noncoding RNAs (lncRNAs) may originate from tumor cells and spread throughout the circulatory system. Immune cells, other blood cells, normal cells around the cancer, as well as circulating and primary tumor cells are the sources of circulating lncRNAs [58]. Third, extracellular RNAs are contained in high-density lipoprotein (HDL) or apoptotic bodies or bound to protein complexes. The most common protein complexes are the miRNA complexes Argonaute (Ago) [59] and Nucleophosmin 1 (NPM1) [60].

From a clinical perspective, the dysregulated lncRNAs could represent a valuable approach to improving outcomes in GBC patients. The upregulated expression of HOTAIR, ANRIL and H19 and the downregulated expression of CCAT1 and MEG3 in GBC compared to cholecystitis and normal control could be used for diagnostic purposes. Furthermore, the tumor suppressive properties of lncRNAs could be exploited for therapeutic options by controlling their expression level. In addition to this higher expression of serum HOTAIR, H19 showed a significant difference between cholecystitis and stage I + II patients. Further downregulation of MEG3 showed a significant difference between stages I + II and III + IV. These findings extend the utility of lncRNAs in stage-specific detection of GBC.

6. Conclusion

The lncRNAs have been shown to contribute to GBC carcinogenesis and tumor progression by promoting cancer-related signaling pathways. Altered expression of lncRNAs can be found in the premalignant stage of GBC, and dysregulated lncRNAs are associated with clinicopathological parameters indicative of GBC progression. In addition, lncRNAs detection in serum facilitates their use as biomarkers for early detection. However, before implementing a non-invasive lncRNAs-based test, some technical aspects should be considered, including the pre-processing and sample preparation procedure, the selection of an appropriate quantification method, and the use of a universal endogenous control. In order to develop sensitive and GBC-specific clinically applicable diagnostic tests based on lncRNAs in combination, multi-center validation studies with a larger sample cohort would be desirable. Furthermore, given our results, specific lncRNA expression in serum samples could contribute to improved early detection in conjunction with radiological findings.

Statements and declarations

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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CRediT authorship contribution statement

Sridhar Mishra: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Pallavi Srivastava:** Methodology, Formal analysis. **Anshuman Pandey:** Validation, Conceptualization. **Akash Agarwal:** Methodology, Data curation. **Saumya Shukla:** Formal analysis, Data curation. **Nuzhat Husain:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] H.S. Shukla, B. Sirohi, A. Behari, A. Sharma, J. Majumdar, M. Ganguly, , et al. T. Singh, Indian Council of Medical Research consensus document for the management of gall bladder cancer, Indian J. Med. Paediatr. Oncol. 36 (2015) 79–84. https://doi:10.4103/0971-5851.158829.
[2] C. Chen, Z. Geng, H. Shen, H. Song, Y. Zhao, G. Zhang, et al., Long-term outcomes and prognostic factors in advanced gallbladder cancer: focus on the advanced T stage, PLoS One 11 (2016) e0166361. https://doi:10.1371/journal.pone.0166361.
[3] M. Nagino, Fifty-year history of biliary surgery, Ann Gastroenterol Surg 3 (2019) 598–605. https://doi:10.1002/ags3.12289.
[4] S.W. Lee, H.J. Kim, J.H. Park, D.I. Park, Y.K. Cho, C.I. Sohn, et al., Clinical usefulness of 18F-FDG PET-CT for patients with gallbladder cancer and cholangiocarcinoma, J. Gastroenterol. 45 (2010) 560–566. https://doi:10.1007/s00535-009-0188-6.
[5] H. Itsuki, M. Serikawa, T. Sasaki, Y. Ishii, K. Tsushima, Y. Furukawa, et al., Indication and usefulness of bile juice cytology for diagnosis of gallbladder cancer, Gastroenterol Res Pract (2018) 5410349. https://doi:10.1155/2018/5410349.
[6] X. Zhou, Meta-analysis of the diagnostic performance of serum carbohydrate antigen 19-9 for the detection of gallbladder cancer, Int. J. Biol. Markers 37 (2022) 81–89. https://doi:10.1177/17246008211068866.

[7] R. Palmirotta, D. Lovero, P. Cafforio, C. Felici, F. Mannavola, E. Pellè, et al., Liquid biopsy of cancer: a multimodal diagnostic tool in clinical oncology, Ther Adv Med Oncol 10 (2018) 1758835918794630. https://doi:10.1177/1758835918794630.

[8] Z. Ding, N. Wang, N. Ji, Z.S. Chen, Proteomics technologies for cancer liquid biopsies, Mol. Cancer 21 (2022) 53. https://doi:10.1186/s12943-022-01526-8.

[9] J. Li, C. Li, Coding or noncoding, the converging concepts of RNAs, Front. Genet. 10 (2019) 496. https://doi:10.3389/fgene.2019.00496.

[10] O. alamb, B.K. Barták, A. Kalmár, Z.B. Nagy, K.A. Szigeti, Z. Tulassay, et al., Diagnostic and prognostic potential of tissue and circulating long non-coding RNAs in colorectal tumors, World J. Gastroenterol. 5 (2019) 5026–5048. https://doi:10.3748/wjg.v25.i34.5026.

[11] P. Pérez-Moreno, I. Riquelme, P. Brebi, J.C. Roa, Role of lncRNAs in the development of an aggressive phenotype in gallbladder cancer, J. Clin. Med. 10 (2021) 4206. https://doi:10.3390/jcm10184206.

[12] L. Zhang, Z. Geng, X. Meng, F. Meng, L. Wang, Screening for key lncRNAs in the progression of gallbladder cancer using bioinformatics analyses, Mol. Med. Rep. 17 (2018) 6449–6455. https://doi:10.3892/mmr.2018.8655.

[13] M.Z. Ma, B.F. Chu, Y. Zhang, M.Z. Weng, Y.Y. Qin, W. Gong, et al., Long non-coding RNA CCAT1 promotes gallbladder cancer development via negative modulation of miRNA-218-5p, Cell Death Dis. 6 (2015) e1583. https://doi:10.1038/cddis.2014.541.

[14] P. Srivastava, S. Mishra, A. Agarwal, A. Pandey, N. Husain, Circulating microRNAs in gallbladder cancer: is serum assay of diagnostic value? Pathol. Res. Pract. 242 (2023) 154320. https://doi:10.1016/j.prp.2023.154320.

[15] D.E. Henson, J. Albores-Saavedra, C.C. Compton, Protocol for the examination of specimens from patients with carcinomas of the gallbladder, including those showing focal endocrine differentiation: a basis for checklists, Arch. Pathol. Lab Med. 124 (2000) 37–40, https://doi.org/10.5858/2000-124-0037-PFTEOS.

[16] A.M. Dahlin, M.L. Henriksson, B. Van Guelpen, R. Stenling, Å. Öberg, J. Rutegård, R. Palmqvist, Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor, Mod. Pathol. 24 (2011) 671–682, https://doi.org/10.1038/modpathol.2010.234.

[17] S. Kumari, S. Tewari, N. Husain, A. Agarwal, A. Pandey, A. Singhal, et al., Quantification of circulating free DNA as a diagnostic marker in gall bladder cancer, Pathol. Oncol. Res. 23 (2017) 91–97, https://doi.org/10.1007/s12253-016-0087-0.

[18] S. Kumari, N. Husain, A. Agarwal, A. Neyaz, S. Gupta, A. Chaturvedi, et al., Diagnostic value of circulating free DNA integrity and global methylation status in gall bladder carcinoma, Pathol. Oncol. Res. 25 (2019) 925–936, https://doi.org/10.1007/s12253-017-0380-6.

[19] N.P. Awasthi, S. Kumari, A. Neyaz, S. Gupta, A. Agarwal, A. Singhal, et al., EpCAM-based flow cytometric detection of circulating tumor cells in gallbladder carcinoma cases, Asian Pac. J. Cancer Prev. APJCP 18 (2017) 3429, https://doi.org/10.22034/APJCP.2017.18.12.3429.

[20] O. Beylerli, I. Gareev, A. Sufianov, T. Ilyasova, Y. Guang, Long noncoding RNAs as promising biomarkers in cancer, Noncoding RNA Res 7 (2022) 66–70. https://doi:10.1016/j.ncrna.2022.02.004.

[21] Y. Qian, L. Shi, Z. Luo, Long non-coding RNAs in cancer: implications for diagnosis, prognosis, and therapy, Front. Med. 7 (2010) 612393. https://doi:10.3389/fmed.2020.612393.

[22] B. Pardini, A.A. Sabo, G. Birolo, G.A. Calin, Noncoding RNAs in extracellular fluids as cancer biomarkers: the new frontier of liquid biopsies, Cancers 11 (2019) 1170. https://doi:10.3390/cancers11081170.

[23] X.H. Liu, M. Sun, F.Q. Nie, Y.B. Ge, E.B. Zhang, D.D. Yin, et al., Lnc RNA HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in gastric cancer, Mol. Cancer 13 (2014) 92. https://doi:10.1186/1476-4598-13-92.

[24] B. Liu, E.D. Shen, M.M. Liao, Y.B. Hu, K. Wu, P. Yang, L. Zhou, et al., Expression and mechanisms of long non-coding RNA genes MEG3 and ANRIL in gallbladder cancer, Tumour Biol 37 (2016) 9875–9886. https://doi:10.1007/s13277-016-4863-y.

[25] J. Shi, X. Li, F. Zhang, C. Zhang, Q. Guan, X. Cao, et al., Circulating lncRNAs associated with occurrence of colorectal cancer progression, Am. J. Cancer Res. 15 (2015) 2258–2265. PMID: 26328256.

[26] S.H. Wang, X.C. Wu, M.D. Zhang, M.Z. Weng, D. Zhou, Z.W. Quan, Long noncoding RNA H19 contributes to gallbladder cancer cell proliferation by modulated miR-194-5p targeting AKT2, Tumor Biol. 37 (2016) 9721–9730. https://doi:10.1007/s13277-016-4852-1.

[27] S.H. Wang, X.C. Wu, M.D. Zhang, M.Z. Weng, D. Zhou, Z.W. Quan, Upregulation of H19 indicates a poor prognosis in gallbladder carcinoma and promotes epithelial-mesenchymal transition, Am. J. Cancer Res. 6 (2015) 15–26.

[28] G.H. Leyten, D. Hessels, S.A. Jannink, F.P. Smit, H. de Jong, E.B. Cornel, Prospective multicenter evaluation of PCA3 and TMPRSS2-ERG gene fusions as diagnostic and prognostic urinary biomarkers for prostate cancer, Eur. Urol. 65 (2014) 534–542. https://doi:10.1016/j.eururo.2012.11.014.

[29] R. Kumarswamy, C. Bauters, I. Volkmann, F. Maury, J. Fetisch, A. Holzmann, et al., Thum, circulating long noncoding RNA, LIPCAR, predicts survival in patients with heart failure, Circ. Res. 114 (2014) 1569–1575. https://doi:10.1161/CIRCRESAHA.114.303915.

[30] X. Zhou, C. Yin, Y. Dang, F. Ye, G. Zhang, Identification of the long non-coding RNA H19 in plasma as a novel biomarker for diagnosis of gastric cancer, Sci. Rep. 5 (2015) 11516. https://doi:10.1038/srep11516.

[31] L. Jin, Q. Cai, S. Wang, S. Wang, J. Wang, Z. Quan, Long noncoding RNA PVT1 promoted gallbladder cancer proliferation by epigenetically suppressing miR-18b-5p via DNA methylation, Cell Death Dis. 11 (2020) 871. https://doi:10.1038/s41419-020-03080-x.

[32] N.W. Mathy, X.M. Chen, Long non-coding RNAs (lncRNAs) and their transcriptional control of inflammatory responses, J. Biol. Chem. 292 (30) (2017) 12375–12382. https://doi:10.1074/jbc.R116.760884.

[33] Y.F. Wang, F.L. Feng, X.H. Zhao, Z.X. Ye, H.P. Zeng, Z. Li, et al., Combined detection tumor markers for diagnosis and prognosis of gallbladder cancer, World J. Gastroenterol. 20 (2014) 4085–4092. https://doi:10.3748/wjg.v20.i14.4085.

[34] J.S. Kang, S.Y. Hong, Y. Han, H.J. Sohn, M. Lee, Y.H. Kang, et al., Limits of serum carcinoembryonic antigen and carbohydrate antigen 19-9 as the diagnosis of gallbladder cancer, Ann Surg Treat Res 101 (2021) 266–273. https://doi:10.4174/astr.2021.101.5.266.

[35] Y. Zhou, X. Zhang, A. Klibanski, MEG3 noncoding RNA: a tumor suppressor, J. Mol. Endocrinol. 48 (2012) R45–R53. https://doi:10.1530/JME-12-0008.

[36] D. Han, X. Gao, M. Wang, Y. Qiao, Y. Xu, J. Yang, et al., Long noncoding RNA H19 indicates a poor prognosis of colorectal cancer and promotes tumor growth by recruiting and binding to eIF4A3, Oncotarget 7 (2016) 22159–22173. https://doi:10.18632/oncotarget.8063.

[37] E.B. Zhang, L. Han, D.D. Yin, R. Kong, W. De, J. Chen, c-Myc-induced, long, noncoding H19 affects cell proliferation and predicts a poor prognosis in patients with gastric cancer, Med. Oncol. 31 (2014) 914. https://doi:10.1007/s12032-014-0914-7.

[38] T. Arita, D. Ichikawa, H. Konishi, S. Komatsu, A. Shiozaki, K. Shoda, T. Kawaguchi, et al., Circulating long non-coding RNAs in plasma of patients with gastric cancer, Anticancer Res. 33 (2013) 3185–3193. PMID: 23898077.

[39] H. Ono, N. Motoi, H. Nagano, E. Miyauchi, M. Ushijima, M. Matsuura, et al., Long noncoding RNA HOTAIR is relevant to cellular proliferation, invasiveness, and clinical relapse in small-cell lung cancer, Cancer Med. 3 (2014) 632–642. https://doi:10.1002/cam4.220.

[40] T.H. Yan, S.W. Lu, Y.Q. Huang, G.B. Que, J.H. Chen, Y.P. Chen, et al., Upregulation of the long noncoding RNA HOTAIR predicts recurrence in stage Ta/T1 bladder cancer, Tumour Biol 35 (2014) 10249–10257. https://doi:10.1007/s13277-014-2344-8.

[41] M.Z. Ma, C.X. Li, Y. Zhang, M.Z. Weng, M.D. Zhang, Y.Y. Qin, et al., Long non-coding RNA HOTAIR, a c-Myc activated driver of malignancy, negatively regulates miRNA-130a in gallbladder cancer, Mol. Cancer 13 (2014) 156. https://doi:10.1186/1476-4598-13-156.

[42] J. Cai, X. Zuo, Z. Chen, Y. Zhang, J. Wang, J. Wang, et al., Long noncoding RNAs serve as potential diagnostic biomarkers for colorectal cancer, J. Cancer 10 (2019) 611–619. https://doi:10.7150/jca.28780.

[43] L. Lin, Z.T. Gu, W.H. Chen, K.J. Cao, Increased expression of the long non-coding RNA ANRIL promotes lung cancer cell metastasis and correlates with poor prognosis, Diagn. Pathol. 10 (2015) 14. https://doi:10.1186/s13000-015-0247-7.

[44] L. Hua, C.Y. Wang, K.H. Yao, J.T. Chen, J.J. Zhang, W.L. Ma, High expression of long non-coding RNA ANRIL is associated with poor prognosis in hepatocellular carcinoma, Int. J. Clin. Exp. Pathol. 8 (2015) 3076–3082. PMID: 26045820.

[45] F. Aguilo, S. Di Cecilia, M.J. Walsh, Long non-coding RNA ANRIL and polycomb in human cancers and cardiovascular disease, Curr. Top. Microbiol. Immunol. 394 (2016) 29–39. https://doi:10.1007/82_2015_455.

[46] W. Zhao, M. Song, J. Zhang, M. Kuerban, H. Wang, Combined identification of long non-coding RNA CCAT1 and HOTAIR in serum as an effective screening for colorectal carcinoma, Int. J. Clin. Exp. Pathol. 8 (2015) 14131–14140. PMID: 26823726.

[47] C. Barbagallo, D. Brex, A. Caponnetto, M. Cirnigliaro, M. Scalia, A. Magnano, et al., LncRNA UCA1, upregulated in CRC biopsies and downregulated in serum exosomes, controls mRNA expression by RNA-RNA interactions, Mol. Ther. Nucleic Acids 12 (2018) 229–241. https://doi:10.1016/j.omShitn.2018.05.009.

[48] X. Hu, J. Bao, Z. Wang, Z. Zhang, P. Gu, F. Tao, et al., The plasma lncRNA acting as fingerprint in non-small-cell lung cancer, Tumour Biol 37 (2016) 3497–3504. https://doi:10.1007/s13277-015-4023-9.

[49] Y.S. Tong, X.W. Wang, X.L. Zhou, Z.H. Liu, T.X. Yang, W.H. Shi, et al., Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma, Mol. Cancer 14 (2015) 3. https://doi:10.1186/1476-4598-14-3.

[50] J. Yu, J. Han, J. Zhang, G. Li, H. Liu, X. Cui, et al., The long noncoding RNAs PVT1 and uc002mbe.2 in sera provide a new supplementary method for hepatocellular carcinoma diagnosis, Medicine (Baltim.) 95 (2016) e4436. https://doi:10.1097/MD.0000000000004436.

[51] P. Han, J.W. Li, B.M. Zhang, J.C. Lv, Y.M. Li, X.Y. Gu, et al., The lncRNA CRNDE promotes colorectal cancer cell proliferation and chemoresistance via miR-181a-5p-mediated regulation of Wnt/β-catenin signaling, Mol. Cancer 16 (2017) 9. https://doi:10.1186/s12943-017-0583-1.

[52] P. Li, X. Zhang, L. Wang, L. Du, Y. Yang, et al., lncRNA HOTAIR contributes to 5FU resistance through suppressing miR-218 and activating NF-κB/TS signaling in colorectal cancer, Mol. Ther. Nucleic Acids 8 (2017) 356–369. https://doi:10.1016/j.omtn.2017.07.007.

[53] K. Trajkovic, C. Hsu, S. Chiantia, et al., Ceramide triggers budding of exosome vesicles into multivesicular endosomes, Science 319 (2008) 1244–1247.

[54] R.M. Johnstone, Exosomes biological significance: a concise review, Blood Cell Mol. Dis. 36 (2) (2006) 315–321.

[55] X. Huang, T. Yuan, M. Tschannen, et al., Characterization of human plasma-derived exosomal RNAs by deep sequencing, BMC Genom. 14 (2013) 319.

[56] Q. Li, Y. Shao, X. Zhang, et al., Plasma long noncoding RNA protected by exosomes as a potential stable biomarker for gastric cancer, Tumor Biol. 36 (3) (2015) 2007–2012.

[57] S. Ren, F. Wang, J. Shen, et al., Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer, Eur. J. Cancer 49 (13) (2013) 2949–2959.

[58] T. Arita, D. Ichikawa, H. Konishi et al. Circulating long non-coding RNAs in plasma of patients with gastric cancer. Anticancer Res.; 33 (8): 3185–3194..
[59] J.D. Arroyo, J.R. Chevillet, E.M. Kroh, et al., Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma, Proc. Natl. Acad. Sci. U.S.A. 108 (12) (2011) 5003–5008.
[60] K. Wang, S. Zhang, J. Weber, et al., Export of microRNAs and microRNA-protective protein by mammalian cells, Nucleic Acids Res. 38 (20) (2010) 7248–7259.