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ORIGINAL RESEARCH ARTICLE

# Evaluation of Circulatory RNA-Based Biomarker Panel in Hepatocellular Carcinoma

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**Abstract**

*Background* The circulating transcriptome (coding and non-coding) plays a critical role in cancer. Novel accurate strategies for early detection of hepatocellular carcinoma (HCC) are strongly needed.

*Patients and Methods* We chose an HCC-specific RNA-based biomarker panel based on the integration of differential lysosomal-associated membrane protein 2 (*LAMP2*) gene expression with its selected epigenetic regulators using bioinformatic methods. This was followed by RT-qPCR validation in serum of 78 patients with HCC, 36 patients with chronic hepatitis C (CHC) infection and 44 healthy volunteers. We used risk-score analysis to evaluate the diagnostic efficacy of the serum profiling system. Moreover, in twenty of the 78 HCC cases involved in the study we examined the expression of RNA-based biomarker panel in both HCC and adjacent non-tumor tissues and assessed their correlation with the serum level of this panel.

*Results* The four ribonucleic acid (RNA)-based biomarker panel [long non-coding RNA-C terminal binding protein, androgen responsive (*lncRNA–CTBP*), microRNA-16-2 (*miR-16-2*), *microRNA-21-5-P* (*miR-21-5p*) and *LAMP2*], had high sensitivity and specificity for discriminating HCC from healthy controls and also from CHC patients. Among these four RNAs, serum miR-16-2 and miR-21-5p were independent prognostic factors.

*Conclusion* The circulatory RNA-based biomarker panel can serve as a potential biomarker for HCC diagnosis and prognosis.

**Key Points**

The circulating transcriptome has been revealed as a novel class of non-invasive disease biomarker with high specificity and stability for early detection of HCC.

We presented a novel approach that enables reliable integration of differential *LAMP2* gene expression with the selected epigenetic regulators.

This approach has generated an interesting biomarker panel (*lncRNA-CTBP, miR-16-2, miR-21-5p and LAMP2*) for HCC diagnosis and prognosis.

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## 1 Introduction

Hepatocellular carcinoma (HCC) ranks as the fifth most common malignant tumor worldwide and the leading cause of death among patients with cirrhosis globally [1]. HCC has become a real problem in Egypt as its incidence has been nearly doubled over the last decade [2] due to the high prevalence of hepatitis C virus infection (HCV) among the Egyptian population [3]. Most of the screening tools used for HCC, either ultrasound or serum alpha-fetoprotein (AFP), lack adequate sensitivity and specificity [4]. A great effort has been made towards the study of genomics and epigenomics in order to unravel the mechanisms of HCC and therefore identify novel therapeutic targets as well as early diagnostic and prognostic markers to improve the clinical outcome of patients [5].

There is accumulating evidence that highlights the critical role of autophagy in cancer [6–8]. Lysosomal-associated membrane protein 2 (*LAMP2*) is recognized as a major constituent of the lysosomal membrane and is needed for the fusion of lysosomes with autophagosomes in the late stage of the autophagy [9]. Of note, the content of poly-*N*-acetyllactosamines in *LAMP2* correlates with tumor differentiation and their metastatic potential by serving as a ligand for selectins [10]. Tsunedomi et al. [11], found *LAMP2* among 23 genes down-regulated in HCCs with portal vein invasion (PVI) in comparison with HCCs without PV1 by microarray [11]. Moreover, Li et al. [12] reported that *LAMP2* was significantly deregulated in HCC through gene expression profiling of liver tissue among 1820 genes with altered expression in HCC [12]. Another study that was conducted by He et al., who identified *LAMP2* deregulation in HCC derived exosomes using ribonucleic acid (RNA) deep sequencing [13].

Long noncoding RNAs (lncRNAs) have recently emerged as crucial players in regulating a number of diverse biological processes, e.g. differentiation, growth, apoptosis [14]; and about 18 % of the non-protein-coding genes that produce lncRNA are associated with cancers, whereas only 9 % of all human protein-coding genes are associated with oncogenesis and tumor metastasis [15]. Moreover, it has been reported that lncRNA can participate in competing endogenous RNAs (ceRNAs) regulation in order to communicate with other RNA transcripts at the post-transcriptional level [16]. RNA-RNA crosstalk, as part of a post-transcriptional regulatory loop, have been linked to human diseases [17]. lncRNA may interact with mRNA as the antisense strand to inhibit target translation. Recent studies [18, 19] show the lncRNA H19 is processed into microRNA fragments (miRNA-675), which target tumor suppressor retinoblastoma (Rb) mRNA and block its translation. Similarly, antisense lncRNA fragments are correlated with the grade of tumor differentiation in prostate cancer patients [20].

MicroRNAs (miRNAs), the most commonly studied subclass of small noncoding RNAs that regulate gene expression at the post-transcriptional level, act as key players in many diseases, including cancer [21]. Recently, circulating miRNAs has been considered as a stable potential, and non-invasive biomarkers for cancer diagnosis and prognosis [22]. Several research groups have reported that liver cancer exhibits an abnormal expression pattern of miRNAs [23, 24].

In the current study, we sought to identify novel and potential serum biomarkers that reliably define patients with HCC. We believe that an RNA-based biomarker panel derived from HCC could be used as a promising biomarker panel because lncRNA and miRNA are more informative than mRNA alone. We first identified HCC-associated autophagy genes and their epigenetic regulators via *in silico* data analysis. Then, to confirm this panel, we assessed whether lncRNA-C terminal binding protein–androgen responsive (*lncRNA–CTBP*), *microRNA-16-2(miR-16-2), MiR-21-5p* and *LAMP2* are altered in sera of HCC patients compared with chronic HCV patients and healthy volunteers. Finally, we verified whether the chosen RNA-based biomarker panel expression reflect tumor dynamics in paired tissue and sera samples

We have selected *LAMP2* as an autophagy gene highly correlated to hepatocellular carcinoma based on two approaches. Firstly, bioinformatics analysis was used to confirm the expression of *LAMP2* in HCC, and to reduce the false discovery rate we used three databases, Protein Atlas, Gene Atlas and Exocarta. Taken together, the three databases confirmed the correlation between deregulated *LAMP2* in HCC. The second approach was a literature review [11–13] of the limited data available. Both bioinformatics analysis and literature review suggested a possible role of *LAMP2* in HCC.

## 2 Patients and Methods

### 2.1 Patients and Samples

Seventy-eight HCC patients were enrolled in the present study; they were diagnosed according to American Association for the Study of Liver Diseases (AASLD) practice guidelines. The clinical stages of the HCC patients were determined [25, 26]; they were classified as 68 (78.2 %) stage A, 6 (7.7 %) stage B and 4 (5.1 %) stage C carcinomas according to the Barcelona Clinic Liver Cancer (BCLC) classification. All venous blood samples were collected before any therapeutic procedures, including

surgery, chemotherapy, and radiotherapy. Complete follow-up data were available for each patient [27]. Hepatocellular carcinoma and corresponding non-tumor fresh specimens were obtained from 20 out of the 78 HCC patients who underwent surgical tumor resection, the specimens were snapped-frozen in liquid nitrogen and stored at −80 °C immediately after resection for the extraction of RNA. In addition, we analyzed 36 CHC serum samples collected at Clinic Tropical Medicine Department, Ain Shams University Hospital from January 2013 to May 2014. Forty-four healthy controls were recruited during their routine medical checkup.

Venous blood samples were collected from each participant and centrifuged to obtain the serum. All serum samples were stored at −80 °C for further processing. Written informed consent was obtained from all the participants of this study, which was performed in accordance with Declaration of Helsinki, and was approved by the Ethics Committee of Ain Shams Faculty of Medicine, Egypt (ethical approval number; 25231). Clinical and demographic characteristics of all the participants are summarized in Table 1.

### 2.2 Viral Markers and Serum AFP Detection

Serum hepatitis B surface antigen (HBs Ag) and anti-hepatitis C virus antibody were investigated by enzyme linked immunoassay using commercial kits (ELISA). AFP was quantitatively determined using a commercial ELISA (AbCam, Cambridge, MA, USA).

### 2.3 Bioinformatics-Based Selection of RNA-Based Biomarker Panel

The identification of the RNA-based biomarker panel included four steps: (i) we identified an autophagy target gene related to HCC namely *LAMP2*. The autophagy target gene was down-regulated in HCC compared to normal liver, fold change $\geq 2.0$ and $p$ value $<0.05$ according to the data from Genatlas at Paris Descartes University database (available at http://genatlas.medecine.univ-paris5.fr/), Protein Atlas database (available at http://www.proteinatlas.org/ENSG00000005893-LAMP2/cell/CAB005272), Gene atlas database (available at http://genatlas.medecine.univ-paris5.fr/) and Exocarta database (available at http://exocarta.org/gene_summary?gene_id=3920); then to enhance the data reliability, we verified the expression of the autophagy target gene in HCC by Integrative OncoGenomics (IntOGen) (available at http://v03.intogen.org/home;jsessionid=18bgd621ylpwq); (ii) then we retrieved data about miRNA regulation of LAMP2 from the autophagy regulatory network (http://arn.elte.hu/) and identified (miR-16-2 and miR-21-5p); (iii) a pathway enrichment analysis of miR-16-2 and miR-21-5p was performed using the DIANA-mirPath software [28] and the KEGG pathway [29]. This revealed that the two selected miRNAs have a high number of target genes related to carcinogenesis, e.g. MAPK, apoptosis, lysosomal enzymes, cytokine, focal adhesion and Wnt signaling; (iv) we identified a lncRNA that acts as an epigenetic regulator of the above miRNAs and *LAMP2* by accessing the database of lnc-RBA acting as competing endogenous RNA (inCeDB) (http://gyanxet-beta.com/lncedb/index.php).

### 2.4 Extraction of Total RNA, Including lncRNA and miRNA

Total RNA was extracted from sera and tissue samples using miREasy RNA isolation kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The extracted total RNA was reverse-transcribed into cDNA as soon as possible with a miScript II RT Kit (Qiagen/SABiosciences Corporation, Frederick, MD) following the manufacturer protocol for sera/tissue samples) using Hybaid thermal cycler (Thermo Electron, Waltham, MA, USA).

### 2.5 Real Time-PCR (qPCR) Quantification of RNA-Based Biomarker Panel

LncRNA-CTBP and LAMP2 expression in serum and tissue samples from HCC patients were assessed using (Hs_C4orf42_QF_1 QuantiFast Probe Assay and Hs_LAMP2_QF_1 QuantiFast Probe Assay, respectively) TaqMan Universal PCR Master Mix on Step One Plus™ System (Applied Biosystems Inc., Foster, CA, USA) (Accession; NR_033339 and NM_001122606, respectively).

MiR-16-2 and miR-21-5p expression in serum and tissue samples were investigated by mixing the total cDNAs with miScript SYBR Green PCR Kit (Qiagen/SABiosciences Corporation, Frederick, MD, USA) according to the manufacturer's suggested protocol; along with the manufacturer-provided miScript Universal primer and miRNA-specific forward primer (Hs_miR-16-2*_1 miScript Primer Assay and Hs_miR-21_2 miScript Primer Assay, respectively) (Accession: MIMAT0004518 and MIMAT0000076, respectively). RNU-6 was used as an internal control. All the PCR primers were purchased from Qiagen, MD. Relative quantification of RNA-based biomarker panel expression was calculated using the $2^{-\Delta\Delta Ct}$ method [30]. The raw data were normalized to beta actin as a housekeeping gene as the invariant control for the samples, and compared with a reference sample. The PCR program for TaqMan probe-based qPCR was as follows: firstly, denaturation at 95 °C for 5 min; followed by 45 cycles of denaturation for 10 s at 95 °C; then annealing for

**Table 1** Study population demographic and clinical characteristics ($N = 156$)

| | Malignant ($n = 78$) | CHC ($n = 36$) | Normal ($n = 42$) | $P$ | $\chi^{2a}$ $F^{b}$ |
|---|---|---|---|---|---|
| Age | | | | | |
| <57 years ($n = 110$) | 61 (78.2 %) | 21 (58.3 %) | 28 (66.7 %) | 0.079 | $\chi^{2a} = 5.08$ |
| ≥57 years ($n = 46$) | 17 (21.3 %) | 15 (41.7 %) | 14 (33.3 %) | | |
| Sex | | | | | |
| Male ($n = 122$) | 56 (71.8 %) | 30 (83.3 %) | 36 (85.3 %) | 0.148 | $\chi^{2a} = 3.825$ |
| Female ($n = 34$) | 22 (22.2 %) | 6 (16.7 %) | 6 (14.7 %) | | |
| Smoking | | | | | |
| Non-Smoker ($n = 55$) | 26 (33.3 %) | 18 (50 %) | 11 (26.2 %) | 0.079 | $\chi^{2a} = 5.06$ |
| Smoker ($n = 101$) | 52 (66.7 %) | 18 (50 %) | 31 (73.8 %) | | |
| HCV-antibodies | | | | | |
| pos.($n = 104$) | 68 (76.2 %) | 36 (100 %) | 0 (0 %) | <0.001** | $\chi^{2a} = 116.76$ |
| neg.($n = 52$) | 10 (12.8 %) | 0 (0 %) | 42 (100 %) | | |
| HBVsAg | | | | | |
| pos.($n = 4$) | 4 (5.1 %) | 0 (0 %) | 0 (0 %) | 0.128 | $\chi^{2a} = 4.1$ |
| neg.($n = 152$) | 74 (94.9 %) | 36 (100 %) | 44 (100 %) | | |
| Cirrhosis | | | | | |
| Cirrhotic (96) | 67 (85.9 %) | 17 (47.2 %) | 0 (0 %) | <0.001** | $\chi^{2a} = 81.87$ |
| Non-cirrhotic (64) | 11 (14.1 %) | 19 (52.8 %) | 42 (100 %) | | |
| AST | 72.8 ± 42.4 | 52.5 ± 11.8 | 24.7 ± 7.7 | <0.001** | $F^{b} = 33.156$ |
| ALT | 56.35 ± 44.72 | 41.4 ± 20.05 | 21.31 ± 6.5 | <0.001** | $F^{b} = 15.17$ |
| Albumin | 3.2 ± 0.55 | 2.4 ± 0.5 | 3.8 ± 0.25 | <0.001** | $F^{b} = 89.6$ |
| Total bilirubin | 2.09 ± 1.8 | 1.8 ± 0.95 | 0.856 ± 0.21 | <0.001** | $F^{b} = 10.93$ |
| Direct bilirubin | 1.06 ± 1.636 | 1.02 ± 0.75 | 0.12 ± 0.06 | <0.001** | $F^{b} = 9.56$ |
| INR | 1.28 ± 0.35 | 1.69 ± 0.25 | 1.14 ± 0.05 | <0.001** | $F^{b} = 39.7$ |
| α-feto-protein | 856.8 ± 11.65 | 8.3 ± 9 | 8.5 ± 7.7 | 0.082 | $F^{b} = 2.54$ |
| Child score | | | | | |
| A5 | 9 (11.5 %) | – | – | – | – |
| A6 | 22 (28.2 %) | | | | |
| B7 | 2 (2.6 %) | | | | |
| B8 | 13 (16.7 %) | | | | |
| B9 | 19 (24.4 %) | | | | |
| C10 | 13 (16.7 %) | | | | |
| Mean size of the tumor | | | | | |
| ≥3 cm | 6 (7.7 %) | – | – | – | – |
| <3 cm | 72 (92.3 %) | | | | |
| BCLC stage | | | | | |
| A | 68 (78.2 %) | – | – | – | – |
| B | 6 (7.7 %) | | | | |
| C | 4 (5.1 %) | | | | |

*HCV* hepatitis C virus, *CHC* chronic HCV infection, *HBVs Ag* hepatitis B virus surface antigen, *AST* aspartate transaminase, *ALT* alanine transaminase, *INR* international normalized ratio, *BCLC* Barcelona clinic liver cancer

* Significant correlation was detected between investigated groups at $P < 0.05$

** Highly significant correlation was detected between investigated groups at $P < 0.01$

[a] Chi-square test

[b] One way Anova test

30 s at 60 °C. For relative *lncRNA and LAMP2* quantification, beta actin (Accession NM_001101) was detected in all cases, with a stable expression (Ct mean ± standard deviation: 24.8 ± 3.2).

The PCR program for Syber green-based QPCR was as follow: firstly, denaturation at 95 °C for 15 min; followed by 40 cycles of denaturation for 10 s at 94 °C; then annealing for 30 s at 55 °C; lastly, extension for 34 s at 70 °C. Each reaction was carried out in triplicate.

The threshold cycle (Ct) value of each sample was calculated using StepOnePlus™ software v2.2.2 (Applied Biosystems). Any Ct value more than 36 was considered negative. The results were analyzed by the melting curve analysis software of Applied Biosystem. Amplification plots and Tm values were analyzed to confirm the specificities of the amplicons for SybrGreen-based PCR amplification. All the samples were analyzed in triplicate to confirm the results.

### 2.6 Statistics

All statistical analyses were performed using SPSS 20. Comparisons were performed using Kruskal-Wallis, one-way analysis of variance (ANOVA), and chi-square test, as appropriate. To explore the predictive value of selected RNA-based biomarker panel for HCC, we performed the receiver operating characteristic (ROC) curve. The associations between RNA-based biomarker panel expression and clinicopathological parameters were assessed with the Spearman rank correlation. The prognostic significance was analyzed with Kaplan–Meier method. Two-tailed $p$ value of 0.05 or less was considered statistically significant. Taking into account the association of each RNA with HCC risk, each patient was assigned a risk score function (RSF) according to a linear combination of RNA expression levels. The regression coefficient of each risk score was used as the weight to indicate the contribution of each RNA to the RSF. We then used ROC curves to evaluate the diagnostic effectiveness of the profile and find an appropriate cut-off.

The prognostic significance was analyzed with Kaplan–Meier method, and differences in survival rates were assessed with log-rank test. Relapse-free survival (RFS) was defined as the time of diagnosis to the development of the first evidence of metastatic disease. Multivariate Cox regression analysis was used to determine independent prognostic factors. Hazard ratios (HRs) and 95 % Cl were estimated for each variable.

## 3 Results

### 3.1 Description of Study Population

There was no significant difference in age, sex and smokers: non-smokers ratio among the three groups ($p > 0.05$), details of the clinical data are shown in Table 1.

### 3.2 Expression of Serum RNA Based Biomarker Panel Among the Study Groups

The RNA-based biomarker panel levels based on relative quantity (RQ) values in serum, are summarized in Table 2. The medians (RQ) were 0.99, 1.2 and 3.46 for serum *lncRNA-CTBP-AS*, 0.095, 0.1, 3.39 for *miR-16*, 0.778, 0.89, 7.29 for *miR-21-5p-5p* and 4.04, 3.09, 0.038 *for LAMP2* in healthy donors, benign and malignant group, respectively. Compared with the non-malignant groups, the malignant group had a higher expression of *lncRNA-CTBP-AS* and *miR-16-2, miR-21-5p* and lower expression of *LAMP2* ($p < 0.001$) in the serum. The positivity rate of the serum (*lncRNA-CTBP-AS, miR-16-2, miR-21-5p,* and *LAMP2*) was 91, 92.3, 93.6 and 92.3 %, respectively, in the malignant group. However, they were not detected in normal individuals (0 %) ($p < 0.\ 01$), as shown in Table 2.

### 3.3 Correlation of the Serum Level of RNA Based Biomarker Panel with Demographic and Clinical Factors

There was a significant association of serum *lncRNA-CTBP* expression levels in the malignant group with smoking; *miR-16-2* expression with BCLC stage'*miR-21-5p-5p* with age and BCLC stage and *LAMP2* with HBs Ag, Child-Pugh score and tumor size (Table 3). The most notable findings were the significant associations between either *miR-16, ormiR-21-5p* and total or direct bilirubin in the malignant group of the study ($p < 0.05$) (Table 4). Interestingly, there was a significantly negative correlation between *lncRNA-CTBP* and *LAMP2* expression ($r = -0.4$ and $p < 0.001$). There was a significantly positive correlation between *lncRNA-CTBP* and miR-21-5p. Regression analysis revealed that serum AFP, *lncRNA CTBP, miR-16-2, miR-21-5p* and *LAMP2* levels were statistically significant independent predictors of hepatocellular carcinoma risk (all $p < 0.01$) (Table 5).

**Table 2** Differential expression of serum RNA based biomarker panel among different groups in the study (N = 156)

| Group | lnc-RNA-CTBP | miRNA 16-2 | miRNA 21-2 | LAMP2 |
|---|---|---|---|---|
| HCC | | | | |
| Mean | 11.952 | 30.207 | 143.67 | 0.564 |
| Std. deviation | 25.038 | 113.59 | 268.98 | 2.267 |
| Median | 3.465 | 3.394 | 7.292 | 0.038 |
| Mean rank[a] | 110.47 | 113.4 | 114.12 | 44.7 |
| Number of cases > cutoff [b] | 71 (91 %) | 72 (92.3 %) | 73 (93.6 %) | 72 (92.3 %) |
| CHC | | | | |
| Mean | 1.912 | 0.971 | 1.518 | 3.384 |
| Std. deviation | 1.317 | 2.038 | 2.872 | 2.35 |
| Median | 1.253 | 0.100 | 0.890 | 3.092 |
| Mean rank[a] | 61.6 | 50.11 | 46.96 | 104.22 |
| Number of cases > cutoff [b] | 9 (25 %) | 4 (11.1 %) | 4 (11.1 %) | 7 (19.4 %) |
| Healthy control | | | | |
| Mean | 0.764 | 0.345 | 0.722 | 11.659 |
| Std. deviation | 0.576 | 0.490 | 0.356 | 12.672 |
| Median | 0.994 | 0.095 | 0.778 | 4.021 |
| Mean rank[a] | 33.5 | 38.02 | 39.3 | 119.12 |
| Number of cases > cutoff [b] | 0 (0 %) | 0 (0 %) | 0 (0 %) | 0 (0 %) |
| Statistics | | | | |
| $P(\chi^2)$[a] | <0.001** | <0.001** | <0.001** | <0.001** |
| | (39.2) | (94.5) | 97.619 | 89.208 |
| $P(\chi^2)$[b] | <0.001** | <0.001** | <0.001** | <0.001** |
| | (103.48) | (119) | 123.05 | 111.28 |

Statistical significance was detected between the 2 studied groups (CHC, and HCC) regarding the RNA based biomarker panel expression

Statistical significance was detected between the 2 studied groups (control, CHC) regarding lnc-RNA-CTBP and miR-16-2 expression

The cutoff was ≥1.97 for *lnc-RNA-CTBP*, ≥1.8 for *miR-16-2*, ≥1.78 for *miR-21-5p* and ≤0.58 for *LAMP2*

*CHC* chronic HCV infection, *LAMP2* lysosomal associated membrane protein 2, *CTBP-AS* C terminal binding protein-androgen responsive, *lncRNA* long non coding RNA, *miRNA* micro RNA, *HCV* hepatitis C virus

** Statistical significance was detected between the 3 studied groups (control, chronic HCV, and malignant) regarding of l RNA based biomarker panel expression at $P < 0.01$

[a] Kruskal Wallis test based on relative quantity (RQ)values of RNA based biomarker panel

[b] Significant difference was detected between investigated groups at $P < 0.05$ using Chi-square test

### 3.4 Prediction of HCC Cases by Risk Score Analysis Using Serum RNA-Based Biomarker Panel Expression

To evaluate the diagnostic value of this 4-RNA profiling system, we performed a risk-score analysis on the patient‘s data set then used it to predict HCC case and control status. According to the risk scores and at a set cut-off, samples could be divided into a high-risk group, representing the predicted HCC cases, or a low-risk group, representing the predicted controls. At the optimal cut-off value (1.485), the diagnostic sensitivity (NPV) and specificity (PPV) of the 4 RNA-based markers for HCC detection were 79.5 and 100 %, respectively. None of the controls had a risk score >1.485, whereas 16 of the 78 HCC samples exhibited a risk score <1.485 (Table 6; Fig. 1).

### 3.5 Expression of RNA-Based Biomarker Panel in Matched Liver Cancer Tissues and Adjacent Cancer-Free Tissues

We examined RNA-based biomarker panel expression level in 20 paired HCC tissues and adjacent non-tumor tissues by qRT-PCR. In tumor tissues, *lncRNA-CTBP, miR-16-2, miR-21-5p* expression were at a level higher than that

**Table 3** Positivity rate of serum RNA based biomarker panel expression among the study groups in relation to different clinicopathological factors in the malignant group of the study ($N = 78$)

| Clinicopathological factors | Lnc-RNA-CTAB | | | | MiR-16-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean rank | Statistics | No of cases >1.97 (%) | $P$ $\chi^{2c}$ | Mean rank | Statistics | No of cases >1.8 (%) | $P$ $\chi^{2c}$ |
| **Mean age (years)** | | | | | | | | |
| ≥57 | 38.9 | $P = 0.67$ | 55 (77.5 %) | $P = 0.6$ | 38 | $P = 0.26$ | 55 (76.4 %) | $P = 0.178$ |
| < 57 | 41.5 | $U^a = 448$ | 16 (22.5 %) | $\chi^{2b} = 0.254$ | 44.8 | $U^a = 427$ | 17 (23.6 %) | $\chi^{2b} = 1.8$ |
| **Sex** | | | | | | | | |
| Male | 38.2 | $P = 0.66$ | 49 (69 %) | $P = 0.08$ | 39.2 | $P = 0.86$ | 52 (72.2 %) | $P = 0.77$ |
| Female | 41.2 | $U^a = 577$ | 22 (31 %) | $\chi^{2b} = 3.02$ | 40.18 | $U^a = 601$ | 20 (27.8 %) | $\chi^{2b} = 0.084$ |
| **Smoking** | | | | | | | | |
| Smoker | 47.5 | $P = 0.02$* | 26 (36.6 %) | $P = 0.05$* | 39.7 | $P = 0.94$ | 24 (33.3 %) | $P = 0.99$ |
| Non-smoker | 35.4 | $U^a = 467$ | 45 (63.4 %) | $\chi^{2b} = 3.8$ | 39.3 | $U^a = 67$ | 48 (66.7 %) | $\chi^{2b} = 0$ |
| **HCV-abs** | | | | | | | | |
| Pos. | 38.9 | $P = 0.57$ | 61 (85.9 %) | $P = 0.2$ 8 | 38.15 | $P = 0.16$ | 62 (86.1 %) | $P = 0.32$ |
| Neg. | 43.3 | $U^a = 302$ | 10 (14.1 %) | $\chi^{2b} = 1.1$ | 48.7 | $U^a = 248$ | 10 (13.9 %) | $\chi 2^{b} = 0.956$ |
| **HBV-sAg** | | | | | | | | |
| Pos. | 39 | $P = 0.947$ | 4 (5.5 %) | $P = 0.59$ | 53.5 | $P = 0.218$ | 4 (5.6 %) | $P = 0.553$ |
| Neg. | 39.5 | $U^a = 146$ | 67 (94.5 %) | $\chi^{2b} = 0.289$ | 38.7 | $U^a = 92$ | 68 (94.4 %) | $\chi^{2b} = 0.351$ |
| **Child-Pugh score** | | | | | | | | |
| A5 | 32.2 | $P = 0.2$ | 9 (12.7 %) | $P = 0.3$ | 52.8 | $P = 0.086$ | 9 (12.5 %) | $P = 0.08$ |
| A6 | 41.4 | $\chi^{2b} = 7.02$ | 18 (25.4 %) | $\chi^{2b} = 5.6$ | 40.14 | $\chi^{2b} = 9.6$ | 22 (30.6 %) | $\chi^{2b} = 9.6$ |
| B7 | 33.15 | | 2 (2.8 %) | | 35.9 | | 2 (2.8 %) | |
| B8 | 41.4 | | 13 (18.3 %) | | 29.7 | | 11 (15.3 %) | |
| B9 | 39.3 | | 18 (25.4 %) | | 43.3 | | 15 (20.8 %) | |
| C10 | 74.5 | | 11 (15.5) % | | 63.5 | | 13 (18.1 %) | |
| **BCLC stage** | | | | | | | | |
| A | 38.14 | $P = 0.211$ | 62 (87.3 %) | $P = 0.39$ | 39.8 | $P = 0.726$ | 63 (87.5 %) | $P = 0.59$ |
| B | 55.17 | $\chi^{2b} = 3.11$ | 6 (8.5 %) | $\chi^{2b} = 1.8$ | 33 | $\chi^{2b} = 0.64$ | 5 (6.9 %) | $\chi^{2b} = 1.02$ |
| C | 39.13 | | 3 (4.2 %) | | 43.6 | | 4 (5.6 %) | |
| **Stage** | | | | | | | | |
| Early (BCLC;A, B) | 36 | $P = 0.615$ | 59 (83.1 %) | $P = 0.443$ | 36 | $P = 0.01$** | 58 (80.6 %) | $P = 0.233$ |
| Late (BCLC;C) | 52 | $U^a = 385$ | 12 (16.9 %) | $\chi^{2b} = 0.589$ | 45 | $U^a = 150$ | 14 (19.4 %) | $\chi^{2b} = 1.4$ |
| **Tumor size** | | | | | | | | |
| ≥3 cm | 39.5 | $P = 0.97$ | 65 (91.5 %) | $P = 0.423$ | 38.47 | $P = 0.165$ | 66 (91.7 %) | $P = 0.462$ |
| <3 cm | 39.17 | $U^a = 214$ | 6 (8.5 %) | $\chi^{2b} = 0.621$ | 51.38 | $U^a = 142$ | 6 (8.3 %) | $\chi^{2b} = 0.542$ |
| **Cirrhosis** | | | | | | | | |
| Cirrhotic | 38.99 | $P = 0.62$ | 62 (87.3 %) | $P = 0.249$ | 39.6 | $P = 0.89$ | 61 (84.7 %) | $P = 0.3$ |
| Non-cirrhotic | 44.67 | $U^a = 334$ | 9 (12.7 %) | $\chi^{2b} = 1.3$ | 38.6 | $U^a = 359$ | 11 (15.3 %) | $\chi^{2b} = 1.06$ |
| Clinicopathological factors | MiR-21-5p | | | | LAMP2 | | | |
| | Mean rank | Statistics | No of cases >1.78 (%) | $P$ $\chi^{2c}$ | Mean rank | Statistics | No of cases ≤0.58 (%) | $P$ $\chi^{2c}$ |
| **Mean age (years)** | | | | | | | | |
| ≥57 | 36 | $P = 0.01$** | 56 (76.7 %) | $P = 0.22$ | 38.4 | $P = 0.08$ | 58 (80.6 %) | $P = 0.08$ |
| < 57 | 52 | $U^a = 306$ | 17 (23.3 %) | $\chi^{2\ b} = 1.48$ | 42.3 | $U^a = 452$ | 14 (19.4 %) | $\chi^{2b} = 3.03$ |
| **Sex** | | | | | | | | |
| Male | 42.4 | $P = 0.06$ | 54 (74 %) | $P = 0.1$ | 40.68 | $P = 0.12$ | 50 (69.4 %) | $P = 0.11$ |
| Female | 32 | $U^a = 451$ | 19 (26 %) | $\chi^{2b} = 2.6$ | 36.5 | $U^a = 550$ | 22 (30.6 %) | $\chi^{2b} = 2.5$ |

**Table 3** continued

| Clinicopathological factors | MiR-21-5p | | | | LAMP2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean rank | Statistics | No of cases >1.78 (%) | *P* $\chi^{2c}$ | Mean rank | Statistics | No of cases ≤0.58 (%) | *P* $\chi^{2c}$ |
| **Smoking** | | | | | | | | |
| Smoker | 42 | $P = 0.49$ | 24 (32.9 %) | $P = 0.77$ | 39.5 | $P = 0.94$ | 24 (33.3 %) | $P = 0.99$ |
| Non-smoker | 38.2 | $U^a = 611$ | 49 (67.1 %) | $\chi^{2b} = 0.1$ | 39.5 | $U^a = 676$ | 48 (66.7 %) | $\chi^{2b} = 0$ |
| **HCV-abs** | | | | | | | | |
| Pos. | 38.7 | $P = 0.47$ | 63 (86.3 %) | $P = 0.375$ | 39.9 | $P = 0.33$ | 62 (86.1 %) | $P = 0.328$ |
| Neg. | 44.3 | $U^a = 292$ | 10 (13.7 %) | $\chi^{2b} = 0.78$ | 36.5 | $U^a = 310$ | 10 (13.9 %) | $\chi^{2b} = 0.956$ |
| **HBV-sAg:** | | | | | | | | |
| Pos. | 26 | $P = 0.236$ | 4 (5.5 %) | $P = 0.59$ | 56 | $P = 0.38$ | 2 (2.8 %) | $P = 0.001$** |
| Neg. | 40.2 | $U^a = 94$ | 69 (94.5 %) | $\chi^{2b} = 0.289$ | 38.1 | $U^a = 142$ | 70 (79.2 %) | $\chi^{2b} = 10.28$ |
| **Child-Pugh score:** | | | | | | | | |
| A5 | 36.5 | $P = 0.088$ | 7 (9.6 %) | $P = 0.3$ | 31.78 | $P = 0.001$** | 7 (9.6 %) | $P = 0.248$ |
| A6 | 36.5 | $\chi^{2b} = 9.5$ | 20 (27.4 %) | $\chi^{2b} = 5.9$ | 30.6 | $\chi^{2b} = 20.5$ | 20 (27.4 %) | $\chi^{2b} = 6.65$ |
| B7 | 42.5 | | 2 (2.7 %) | | 38.08 | | 2 (2.8 %) | |
| B8 | 44.7 | | 13 (17.8 %) | | 36.2 | | 13 (17.8 %) | |
| B9 | 36.5 | | 18 (24.7 %) | | 61.6 | | 19 (26.4 %) | |
| c2 | 36.5 | | 13 (17.8 %) | | 68.5 | | 11 (15.3 %) | |
| **BCLC stage** | | | | | | | | |
| A | 39.3 | $P = 0.6$ | 63 (86.3 %) | $P = 0.67$ | 38.3 | $P = 0.13$ | 63 (87.5 %) | $P = 0.33$ |
| B | 43 | $\chi^{2b} = 1.01$ | 6 (8.2 %) | $\chi^{2b} = 0.786$ | 38.17 | $\chi^{2b} = 4.02$ | 6 (8.3 %) | $\chi^{2b} = 2.19$ |
| C | 36.5 | | 4 (5.5 %) | | 61.6 | | 3 (4.2 %) | |
| **Stage** | | | | | | | | |
| Early (BCLC;A, B) | 37.5 | $P = 0.01$** | 59 (80.8 %) | $P = 0.28$ | 38 | $P = 0.257$ | 60 (83.3 %) | $P = 0.3$ |
| Late (BCLC;C) | 37.4 | $U^a = 128$ | 14 (19.2 %) | $\chi^{2b} = 1.16$ | 31 | $U^a = 383$ | 12 (16.7 %) | $\chi^{2b} = 1.04$ |
| **Tumor size** | | | | | | | | |
| ≥3 cm | 39 | $P = 0.584$ | 67 (91.8 %) | $P = 0.505$ | 38.67 | $P = 0.015$* | 68 (94.4 %) | $P = 0.014$* |
| <3 cm | 44.8 | $U^a = 184$ | 6 (8.2 %) | $\chi^{2b} = 0.445$ | 49.5 | $U^a = 156$ | 4 (5.6 %) | $\chi^{2b} = 6.19$ |
| **Cirrhosis** | | | | | | | | |
| Cirrhotic | 40.8 | $P = 0.19$ | 62 (84.9 %) | $P = 0.349$ | 38.8 | $P = 0.162$ | 63 (87.5 %) | $P = 0.159$ |
| Non-cirrhotic | 31.18 | $U^a = 277$ | 11 (15.1 %) | $\chi^{2b} = 0.877$ | 43.59 | $U^a = 323$ | 9 (12.5 %) | $\chi^{2b} = 1.9$ |

*RQ* relative quantity, *CHC* chronic HCV infection, *LAMP2* lysosomal associated membrane protein 2, *CTBP-AS* C terminal binding protein-androgen responsive, *lncRNA* long non coding RNA, *miRNA* micro RNA Hepatitis C virus, *HBVs Ag* hepatitis B virus surface antigen, *BCLC* Barcelona clinic liver cancer

* Significant correlation was detected between investigated groups at $P < 0.05$

** Highly significant correlation was detected between investigated groups at $P < 0.01$

[a] Mann–Whitney

[b] Kruskal-Wallis test

[c] Chi- square test, Non-significant correlation was detected between investigated groups at $P > 0.05$

of non-tumor tissues, with the median ratio of 421, 0.26; 12.7, 0.24; and 13.02, 0.28, respectively, compared with normal counterparts. However, tumor tissue showed lower expression of *LAMP2* with the median ratio of 3.05 and 109.13 compared with normal tissue. These data indicated that abnormal RNA-based biomarker panel expression may be related to HCC pathogenesis (Supplementary Table 1s). Of note, there was a strong significant correlation between serum and tissue expression of RNA based biomarker panel ($r = 0.811$, 0.553, 0.53 and 0.695 at $p < 0.0$ 1, 0.014, 0.19 and 0.001 respectively) (Supplementary Table 2s A, B, C, D).

**Table 4** Correlation between RQ serum RNA based biomarker panel and laboratory parameters in the HCC group of study

| Correlations | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CTBP (RQ 1) | miRNA 16-2 (RQ3) | miRNA 21-2 (RQ4) | LAMP2 (RQ2) | AST | ALT | Total Bilirubin | Direct Bilirubin | Albumin | INR | AFP |
| Spearman's rho | CTBP (RQ 1) | Correlation coefficient | 1.000 | 0.172 | 0.281* | −0.419**– | −0.082– | −0.017– | −0.009– | −0.018– | −0.151– | −0.071– | −0.039– |
| | | Sig. (2-tailed) | – | 0.59 | 0.013 | 0.000 | 0.477 | 0.882 | 0.939 | 0.878 | 0.188 | 0.539 | 0.737 |
| | miRNA 16-2(RQ3) | Correlation coefficient | 0.172 | 1.000 | 0.565** | −0.058 | −0.062– | −0.208– | 0.388** | 0.498** | −0.183– | 0.012 | −0.171– |
| | | Sig. (2-tailed) | 0.132 | – | 0.000 | 0.616 | 0.589 | 0.068 | 0.000 | 0.000 | 0.109 | 0.920 | 0.135 |
| | miRNA 21-5p (RQ4) | Correlation coefficient | 0.281* | 0.565** | 1.000 | −0.096- | −0.001– | −0.064– | 0.259* | 0.392** | −0.308–** | 0.084 | −0.169– |
| | | Sig. (2-tailed) | 0.013 | 0.000 | – | 0.401 | 0.993 | 0.579 | 0.022 | 0.000 | 0.006 | 0.464 | 0.139 |
| | LAMP2 (RQ2) | Correlation coefficient | −0.419–** | −0.058 | −0.096– | 1.000 | 0.046 | 0.044 | −0.144– | −0.119– | 0.205 | −0.242–* | 0.237* |
| | | Sig. (2-tailed) | 0.000 | 0.616 | 0.401 | – | 0.687 | 0.701 | 0.209 | 0.298 | 0.072 | 0.033 | 0.037 |

*HCV* hepatitis C virus, *HBVs Ag* hepatitis B virus surface antigen, *AST* aspartate transaminase, *ALT* alanine transaminase, *INR* international normalized ratio

* Significant correlation was detected between investigated groups using Spearman's correlation at $P < 0.05$

** Highly significant correlation was detected between investigated groups at $P < 0.01$

**Table 5** Predictive power of RNA based biomarker panel fold change for the development of HCC by logistic regression analysis

| | | Standardized coefficients | | | 95.0 % confidence interval | |
|---|---|---|---|---|---|---|
| | | Beta | *t* | Sig. | Lower bound | Upper bound |
| 1 | (Constant) | | −1.935– | 0.055 | −0.604– | 0.006 |
| | Mean age | 0.035 | 1.573 | 0.118 | −0.010– | 0.086 |
| | Smoking | 0.000 | −0.020– | 0.984 | −0.046– | 0.045 |
| | Cirrhosis | 0.045 | 1.318 | 0.189 | −0.022– | 0.112 |
| | HCV Abs | −0.066– | −2.449– | 0.016 | −0.126– | −0.013– |
| | HBV sAg | 0.012 | 0.486 | 0.627 | −0.113– | 0.186 |
| | CTBP after cutoff | 0.147 | 4.256 | 0.000 | 0.079 | 0.215 |
| | lamp2 after cutoff | 0.225 | 6.389 | 0.000 | 0.155 | 0.295 |
| | miR-16-2 after cutoff | 0.315 | 8.776 | 0.000 | 0.244 | 0.387 |
| | miR-21-5p after cutoff | 0.300 | 7.830 | 0.000 | 0.224 | 0.376 |
| | AFP after cutoff 19.25 | 0.138 | 5.317 | 0.000 | 0.088 | 0.193 |

**Table 6** Risk score analysis of HCC cases and cancer-free controls

| Score | ≥1.485 | <1.485 | PPV | NPV |
|---|---|---|---|---|
| HCC | 62 (79.5 %) | 16 (20.5 %) | 100 % | 79.5 % |
| CHC and healthy control | 0 (0 %) | 78 (100 %) | | |

*PPV* positive predictive value, *NPV* negative predictive value

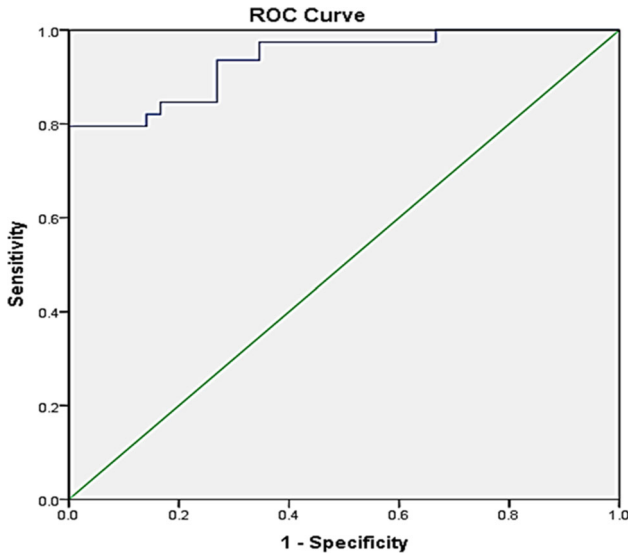


**Fig. 1** ROC curve analysis for discrimination between HCC cases, controls, and CHC cases by the 4-RNA-based biomarker panel using risk score analysis. Best cut-off point, 1.485, sensitivity = 79.5 % and specificity = 100 %. AUC [SE] = 0.938 [0.018], 95 % confidence limits range = 0.902–0.973, $p < 0.0001$

### 3.6 Accuracy of Serum Parameters for Predicting HCC by ROC Analysis

ROC curves analysis and the area under the curve (AUC) values were used to evaluate the diagnostic value of the four selected RNAs as illustrated in supplementary Figures 1s–4s).

When comparing HCC patients with healthy people, the best discriminating cut-off values of *lncRNA-CTBP, miR-16-2, miR-21-5p* and *LAMP2* were 1.9, 1.8, 1.78 and 0.58, respectively. Accordingly, the sensitivities were 91, 92.3, 93.6 and 92.3 % respectively, which indicated that these thresholds could be used to discriminate HCC patients from healthy subjects.

As regards HCC patients versus CHC patients, the cut-off values of 1.97, 1.49, 1.78 and 1.5 for the four RNAs *lncRNA-CTBP, miR-16-2, miR-21-5p* and *LAMP2,* respectively, could be used to discriminate the two groups. On applying these cut-off values we noticed no change in the sensitivity levels of *lncRNA-CTBP, miR-16-2* and *miR-21-5p*, while the specificities for each have decreased to 75, 88.9 and 88.9 %, respectively. On the other hand, a decrease in the sensitivity and specificity levels of *LAMP2* to 94.9 and 81.6 %, respectively, has been noticed (Supplementary Table 3s).

Lastly, the probability of detecting HCV patients from normal individuals, using *lncRNA-CTBP and miRNA-16* expression as discriminating markers has higher sensitivity and specificity than that of *miR-21-5p and LAMP2.*

### 3.7 Correlation Between RNA-Based Biomarker Panel Expression and Patients' Survival

Based on the follow-up for all the study cases (median follow-up was 22 months, the recurrence rate was 22.8 % (26/78) of the HCC patients. In univariate analysis, HCC

cancer patients with negative *lncRNA-CTBP, miR-16-2, miR-21-5p and LAMP2* in serum RNA had relatively longer RFS than patients with positive RNA (Supplementary Table 4s). Kaplan and Meier analysis revealed a significant decrease in RFS and increase in cumulative hazards among *lncRNA-CTBP, miR-16-2* and *miR-21-5p*-positive HCC patients (Log-Rank test: chi-square 3.6, $p = 0.05$; 9.99, $p \leq 0.002$; 4.1, $p = 0.041$ respectively) (Supplementary Figs 5–8). The results of Cox multivariate analyses showed that *miR-16-2* and *miR-21-5p* were independent prognostic factors of RFS (Supplementary Table 5s).

## 4 Discussion

Discovery of an efficient and reliable tool for early detection of HCC can play a pivotal role in improving the management of patients with HCC [4]. Recently, several studies reported that lncRNA-associated target gene activity profiles offered a means of exploring the complex interactions of RNAs that are potentially dysregulated in various types of cancer [31].

For the first time, we proposed a novel approach to study a panel of genetic and epigenetic biomarkers (*lncRNA-CTBP, miR-16-2, miR-21-5p and LAMP2*) in HCC using serum and tissue samples. For polygenic diseases such as HCC and a complex detection platform such as human serum, we recognized that a single gene biomarker approach will not suffice for the high performance requirement of HCC diagnosis. Therefore, by enlisting multiple genes that are mechanistically linked to each other and to HCC functional networks, we believe that the chance of success would be greater than the simpler conventional single-marker approach.

Moreover, highly metastatic colon cancer cell lines express higher levels of cell surface *Lamp-1 and Lamp-2* than low metastatic ones [32]. Künzli and his colleagues found that Lamp-1 affect local tumor progression in pancreatic carcinoma [33]. Previous reports identified *LAMP2* gene to be deregulated in HCV-related HCC samples [11, 34].

Our results expanded previous reports on the role of *miR-16-2 and miR-21-5p* in HCC. Several research groups have reported that miR-16-2 is implicated in the induction of apoptosis by targeting the BCL-2 gene, and was involved in cell-cycle regulation in several cancer cell lines [35, 36]. MiR-16 was found to be upregulated in lung, pancreas, ovaries, and HCC [37–39]. MiR-21-5p is an oncogenic miRNA with significant upregulation detected in many types of human cancer [40, 41]. miRNA-21 alone may not be a specific biomarker for the diagnosis of HCC, but it will improve the test performance when it is used in combination with other biomarkers [42–44]. miR-21-5p shares in the acquired resistance of sorafenib by inhibiting autophagy through the Akt/PTEN pathway [45].

C-terminal binding protein (CTBP) was found to directly inhibit the expression of many important tumor suppressor genes, and is involved in the epithelial to mesenchymal transition during the cancer cell metastasis [46, 47]. The anti-apoptotic transcriptional co-repressor *CCTBP2* was identified, and led to inhibition p53-independent apoptosis in colon cancer cells [48]. Remarkably, *CTBP* repressed *SIRT4* expression and thus contributes to the tumor growth. *LncRNA-CTBP1-AS* is located in the androgen responsive region of *CTBP1*, and is a co-repressor of the androgen receptor. *CTBP1-AS* is upregulated in prostate cancer by recruiting the RNA-binding transcriptional repressor PSF together with histone deacetylase [49]. To the best of our knowledge, it is the first investigation of LncRNA-CTBP1-AS expression in HCC patients.

There was a significantly positive correlation between *lncRNA-CTBP* and either *miR-16-2* or *miR-21-5p* ($p < 0.001$) with strong negative correlation between *lncRNA-CTBP* and *LAMP2*. We hypothesize that lncRNA-CTBP may be involved in epigenetic activation of miR-16 and miR-21 with subsequent inhibition of *LAMP2* with potential role in HCC pathogenesis. That was in agreement with the in silico data analysis.

The main drawback of alpha fetoprotein is its limited sensitivity and specificity (only 73.1 and 89.7 % in our study) in agreement with Abdel-Hamid et al., who calculated the AFP cut-off value for HCC diagnosis with sensitivity 80 % and specificity 85 % [50]. Also, Paul and his colleagues reported similar results with AFP 73 % sensitivity and 78 % specificity [51]. Whereas, *miR-16-2*, *miR-21-5p* and *LAMP2* were superior to the AFP in specificity (94.9, 94.9, 91 %, respectively). The selected RNA-based biomarker panel reduces false-negative results detected by AFP from 21 out of 78 to 0 cases.

Moreover, ROC curves were constructed for differentiating HCV and HCC from healthy controls. The results implied that *miR-16-2* and *miR-21-5p* are the most effective biomarkers when detecting HCC patients from healthy people. Collectively, we believe that the diagnostic accuracy for HCC detection would be improved by a concurrent measurement of serum (*lncRNA-CTBP, miR-16-2, miR-21-5p* and *LAMP2*) (approximately 100 % sensitivity and 91 % accuracy in the present study).

The strongly positive correlation between the expression of serum and tissue RNA-based biomarker panel identified in the same type of tumor suggests that this serum RNA-based biomarker panel could be derived from tumor cells.

Therefore, a serum based, noninvasive method could be superior to liver biopsies, which has drawbacks as regards convenience.

HCC patients with positive *lncRNA-CTBP, miR-16-2* and *miR-21-5p* had an increased likelihood of distant recurrence in the period of 1 to 2 years after diagnosis compared with those with negative *lncRNA-CTBP, miR-16-2* and *miR-21-5p*. In a Kaplan Meier survival analysis of our study, *lncRNA-CTBP, miR-16-2* and *miR-21-5p* prognostic significance for both RFS and hazard ratio of recurrences and this prognostic significance was validated in multivariate analysis.

The study limitations include the following: it was performed at a single center in Egypt with relatively limited sample size. Moreover, *in vitro* functional analysis is needed to elucidate the biological mechanisms of RNA-RNA crosstalk in HCC and to confirm the possible role of *LAMP2* in HCC suggested by both bioinformatics analysis and literature review. Further larger multicenter studies are strongly recommended.

## 5 Conclusion

We presented a novel approach that enables reliable integration of differential *LAMP2* gene expression with the selected epigenetic regulators. This approach has been shown to generate an interesting biomarker panel (*lncRNA-CTBP, miR-16-2, miR-21-5p* and *LAMP2*) for HCC diagnosis and prognosis. It is promising as a general strategy for future panel biomarker development in the serum of HCC patients and HCV patients. This can overcome the lower reliability of single-gene biomarker experiments while maintaining high accuracy by combining signals from multiple genetic levels. These findings expand the existing knowledge of RNA-RNA crosstalk characteristics and provide new tools to elucidate disease processes and offer new targets for HCC therapy. Besides, *miR-16-2* and *miR-21-5p* expression is a useful prognostic marker for RFS in HCC.

**Compliance with Ethical Standards**

**Conflict of interest** All the authors; El-Tawdi A, Matboli M, Shehata H, Tash F, Khazragy N, Ahmed EM Azazy and Abd El-Rahman O declared that they have no competing interest.

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## References

1. World Health Organization IAfRoC. GLOBOCAN 2012. Available from: http://globocan.iarc.fr/Default.aspx.
2. National Cancer Registry of Egypt. Magnitude of hepatocellular carcinoma in Egypt. 2010; [updated 2010 August 6].
3. Abdelgawad IA, Mossallam GI, Radwan NH, Elzawahry HM, Elhifnawy NM. Can Glypican3 be diagnostic for early hepatocellular carcinoma among Egyptian patients? Asian Pac J Cancer Prev. 2013;14(12):7345–9.
4. Stewart BW, Wild CP. In liver cancer, World Cancer Report 2014. IARC; 2014.
5. He Y, Meng XM, Huang C, Wu BM, Zhang L, Lv XW, et al. Long noncoding RNAs: novel insights into hepatocelluar carcinoma. Cancer Lett. 2014;344(1):20–7.
6. Papandreou I, Lim AL, Laderoute K, Denko NC. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. Cell Death Differ. 2008;15(10):1572–81.
7. Jin S, White E. Tumor suppression by autophagy through the management of metabolic stress. Autophagy. 2008;4:563–6.
8. Dalby KN, Tekedereli I, Lopez-Berestein G, Ozpolat B. Targeting prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy. 2010;6:322–9.
9. Chen JW, Murphy TL, Willingtham MC, Pastan I, August JT. Identification of two lysosomal membrane glycoproteins. J Cell Biol. 1985;101:85–95.
10. Lee N, Wang WC, Fukuda M. Granulocytic differentiation of HL-60 cells is associated with increase of poly-*N*-acetyllactosamine in Asn-linked oligosaccharides attached to human lysosomal membrane glycoproteins. J Biol Chem. 1990;265:20476–87.
11. Tsunedomi R, Iizuka N, Yamada-Okabe H, Tamesa T, Okada T, Sakamoto K, Takashima M, Hamaguchi T, Miyamoto T, Uchimura S, Hamamoto Y, Yamada M, Oka M. Identification of ID2 associated with invasion of hepatitis C virus-related hepatocellular carcinoma by gene expression profile. Int J Oncol. 2006;29(6):1445–51.
12. Li Y, Li Y, Tang R, Xu H, Qiu M, Chen Q, Chen J, Fu Z, Ying K, Xie Y, Mao Y. Discovery and analysis of hepatocellular carcinoma genes using cDNA microarrays. J Cancer Res ClinOncol. 2002;128(7):369–79.
13. He M, Qin H, Poon TC, Sze SC, Ding X, Co NN, Ngai SM, Chan TF, Wong N. Hepatocellular carcinoma-derived exosomes promote motility of immortalized hepatocyte through transfer of oncogenic proteins and RNAs. Carcinogenesis. 2015;36(9):1008–18.
14. Lei B, Xu SP, Liang XS, Li YW, Zhang JF, Zhang GQ, Pang D. Long non-coding RNA MVIH is associated with poor prognosis and malignant biological behavior in breast cancer. Tumour Biol. 2015. doi:10.1007/s13277-015-4360-8
15. Li JH, Liu S, Zhou H, Qu LH, Yang JH. StarBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res. 2014;42:D92–7.
16. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011;147:358–69.
17. Wang P, Ning S, Zhang Y, Li R, Ye J, Zhao Z, Zhi H, Wang T, Guo Z, Li X. Identification of lncRNA-associated competing triplets reveals global patterns and prognostic markers for cancer. Nucleic Acids Res. 2015;43(7):3478–89.
18. Li H, Yu B, Li J, Su L, Yan M, Zhu Z, et al. Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer. Oncotarget. 2014;5:2318–29.

19. Tsang WP, Ng EK, Ng SS, Jin H, Yu J, Sung JJ, et al. Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. Carcinogenesis. 2010;31:350–8.
20. Reis EM, Nakaya HI, Louro R, Canavez FC, Flatschart AV, Almeida GT, et al. Antisense intronic non-coding RNA levels correlate to the degree of tumor differentiation in prostate cancer. Oncogene. 2004;23:6684–92.
21. Farazi TA, Spitzer JI, Morozov P, Tuschl T. miRNAs in human cancer. J Pathol. 2011;223:102–15.
22. Ezzat H, Lotfy AM, Alalfy MN, El-Taher SM, Mokhtar A, Mohamed SA, El-Senosy FM. The significance of circulating MicroRNA-122 as a non invasive diagnostic marker of liver injury in Egyptian chronic hepatitis C virus infected and cirrhotic patients with and without hepatocellular carcinoma. Clin Med Diagnost. 2014;4(1):1–8.
23. Murakami Y, Yasuda T, Saigo K, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene. 2006;25:2537–45. doi:10.1038/sj.onc.1209283.
24. Zhao X, Zhen Y, Li G, Li D, Zhao Y, Wu Y, Robson S, He L, Xu Y, Miao R, Zhao HT. The role and clinical implications of microRNAs in hepatocellular carcinoma. Sci China Life Sci. 2012;55(10):906–19.
25. Child CG, Turcotte JG. Surgery and portal hypertension. Major Probl Clin Surg 1964;1:1–85 **[Medline]**.
26. Pugh RN, Murray-Lyon IM, Dawson JL, et al. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg. 1973;60:646–9.
27. Llovet JM, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis. 1999;19(3):329–38.
28. Paraskevopoulou MD, Georgakilas G, Kostoulas N, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 2013;41(Web Server issue):W169–73. Available at http://diana.cslab.ece.ntua.gr/pathways/.
29. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. 2012;40:D109–14.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta deltaC(T)) method. Methods. 2001;25:402–8.
31. Panzitt K, Tschernatsch MM, Guelly C, Moustafa T, Stradner M, Strohmaier HM, Buck C, Denk H, Schroeder R, Trauner M, et al. Characterization of HULC, a novel gene with striking up-regulation in hepatocellular carcinoma, as noncoding RNA. Gastroenterology. 2007;132:330–42.
32. Saitoh O, Wang WC, Lotan R, Fukuda M. Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. J Biol Chem. 1992;267:5700–11.
33. Künzli BM, Berberat PO, Zhu ZW, Martignoni M, Kleeff J, Tempia-Caliera AA, Fukuda M, Zimmermann A, Friess H, Büchler MW. Influences of the lysosomal associated membrane proteins (Lamp-1, Lamp-2) and Mac-2 binding protein (Mac-2-BP) on the prognosis of pancreatic carcinoma. Cancer. 2002;94(1):228–39.
34. Kaji H, Ocho M, Togayachi A, Kuno A, Sogabe M, Ohkura T, Nozaki H, Angata T, Chiba Y, Ozaki H, Hirabayashi J, Tanaka Y, Mizokami M, Ikehara Y, Narimatsu H. Glycoproteomic discovery of serological biomarker candidates for HCV/HBV infection-associated liver fibrosis and hepatocellular carcinoma. J Proteome Res. 2013;12(6):2630–40.
35. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. Cancer Res. 2007;67:6092–9.
36. Ceccarelli S, Panera N, Gnani D, Nobili V. Dual role of MicroRNAs in NAFLD. Int J Mol Sci. 2013;14:8437–55.
37. El-Abd NE, Fawzy NA, El-Sheikh SM, Soliman ME. Circulating miRNA-122, miRNA-199a, and miRNA-16 as biomarkers for early detection of hepatocellular carcinoma in egyptian patients with chronic hepatitis C virus infection. Mol Diagn Ther. 2015;19(4):213–20.
38. Ge W, Yu DC, Li QG, Chen X, Zhang CY, Ding YT. Expression of serum miR-16, let-7f, and miR-21 in patients with hepatocellular carcinoma and their clinical significances. Clin Lab. 2014;60(3):427–34.
39. Qu KZ, Zhang K, Li H, Afdhal NH, Albitar M. Circulating microRNAs as biomarkers for hepatocellular carcinoma. J Clin Gastroenterol. 2011;45(4):355–60.
40. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. MiR-21-mediated tumor growth. Oncogene. 2007;26:2799–803.
41. Schetter AJ, Leung SY, Sohn JJ, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA. 2008;299:425–36.
42. Tomimaru Y, Eguchi H, Nagano H, Wada H, Kobayashi S, et al. Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J Hepatol. 2012;56:167–75.
43. Xu J, Wu C, Che X, Wang L, Yu D, et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol Carcinog. 2011;50:136–42.
44. Fornari F, Ferracin M, Trerè D, Milazzo M, Marinelli S, Galassi M, Venerandi L, Pollutri D, Patrizi C, Borghi A, Foschi FG, Stefanini GF, Negrini M, Bolondi L, Gramantieri L. Circulating microRNAs, miR-939, miR-595, miR-519d and miR-494, Identify Cirrhotic Patients with HCC. PLoS One. 2015;10(10):e0141448. doi:10.1371/journal.pone.0141448 **(eCollection 2015)**.
45. He C, Dong X, Zhai B, Jiang X, Dong D, Li B, Jiang H, Xu S, Sun X. MiR-21 mediates sorafenib resistance of hepatocellular carcinoma cells by inhibiting autophagy via the PTEN/Akt pathway. Oncotarget. 2015;6(30):28867–81.
46. Yun JS, Gardner K. C-terminal binding protein: a molecular link between metabolic imbalance and epigenetic regulation in breast cancer. Int J Cell Biol. 2013;2013:647975.
47. Di LJ, Byun JS, Wong MM, Wakano C, Taylor T, Bilke S, et al. Genome-wide profiles of CtBP link metabolism with genome stability and epithelial reprogramming in breast cancer. Nat Commun. 2013;4:1449.
48. Paliwal S, Pande S, Kovi RC, Sharpless NE, Bardeesy N, Grossman SR. Targeting of C-terminal binding protein (CtBP) by ARF results in p53-independent apoptosis. Mol Cell Biol. 2006;26(6):2360–72.
49. Takayama K, Horie-Inoue K, Katayama S, Suzuki T, Tsutsumi S, Ikeda K, Urano T, Fujimura T, Takagi K, Takahashi S, Homma Y, Ouchi Y, Aburatani H, Hayashizaki Y, Inoue S. Androgen-responsive long noncoding RNA CTBP1-AS promotes prostate cancer. EMBO J. 2013;32(12):1665–80.
50. Abdel-Hamid M, Shaker OG, Ellakwa DE, Abdel-Maksoud EF. Detection of BCL2 polymorphism in patient with hepatocellular carcinoma. Am J Cancer Prevention. 2015;3(2):27–34. doi:10.12691/ajcp-3-2-2.
51. Paul SB, Gulati MS, Sreenivas V, Madan K, Gupta AK, Mukhopadhyay S, Acharya SK. Evaluating patients with cirrhosis for hepatocellular carcinoma: value of clinical symptomatology, imaging and alpha-fetoprotein. Oncology. 2007;72(Suppl 1):117–23.