Research Article

Biomarkers in Medicine

Clinical significance of miRNA-autophagy transcript expression in patients with hepatocellular carcinoma

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Aim: This study integrates autophagy transcripts miRNAs expression based on bioinformatic analysis followed by clinical validation. **Methodology:** Cellular jun proto-oncogene mRNA, LAMP2 mRNA, miR-16 and miR-146a level were investigated in the serum and tissue of patients with hepatocellular carcinoma (HCC), chronic hepatitis C and healthy volunteers by quantitative real-time PCR. The prognostic power of this serum RNA panel was explored. **Results:** The expression of serum cellular jun proto-oncogene mRNA, LAMP2 mRNA, miR-16 and miR-146a were positive in 85.1, 94, 97.1 and 84.2% HCC patients, respectively and they were correlated with tissue levels. Our results suggested that the chosen panel is an independent prognostic factor for survival in patients with HCC. **Conclusion:** The current work provides four RNA-based biomarker panel for HCC diagnosis and prognosis.

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Keywords: bioinformatics • biomarkers • C-JUN • hepatocellular carcinoma • LAMP2 • miRNA • prognosis

Hepatocellular carcinoma (HCC) ranks second and sixth most common cancer among men and women in Egypt due to high prevalence of viral hepatitis and its complications [1]. The alarming incidence of HCC worldwide and in Egypt provides strong support for the rapid implementation of novel biomarkers for the early detection of HCC in high-risk individuals to reduce morbidity and mortality [2].

Autophagy is a mechanism essential for many physiologic processes including development, tissue remodeling, cell survival or death, cellular homeostasis and innate immunity [3]. Recent studies discussed the role of autophagy in the regulation of tumor development, progression and response to chemotherapy. Interestingly, autophagy genes emerge as promising biomarkers in cancer diagnostics and therapeutics [4]. The autophagic pathway is subject to complex regulation including miRNA-mediated regulation of autophagy-related gene as a critical part of the molecular mechanisms in autophagy [5]. Recently, scientists sought that autophagy genes had dual role in HCC, in other words, either in tumorigenesis or tumor suppression with emerging evidence to promising role of autophagy biomarkers in HCC diagnosis [6].

Many studies have demonstrated dysregulated expression of specific miRNA in HCC patients [7]. Several hundred microRNAs (miRs) encoded in the genome act as key regulator for thousands of potential target of human mRNAs. Scientists hypothesize that miRNAs may downregulate protein expression by inhibiting target mRNA translation or by increasing mRNA degradation [8] or it may upregulate protein expression by upregulating transcription [9]. Both mechanisms would result in a quantifiable correlation between the miRNA and its target mRNA expression levels.

Future Medicine

Recent literatures addressed the interaction of miRNAs with autophagy and the application of miRNA-mediated autophagic networks as a potential tool in preclinical cancer models [10].

Given that autophagy plays an important role in HCC development and progression [11], we first retrieved HCC-associated autophagy genes (*LAMP2* as key player and *C-JUN* as regulator of autophagy) highly expressed in HCC based on two approaches; *in silico* data analysis and previous literature. *In silico* data analyses revealed that the chosen gene was highly correlated to HCC with high ranking score and tissue specificity from more than two public microarray databases. Both literature search and bioinformatics assumed possible association between the chosen autophagy transcript and HCC. Afterward, we have retrieved a set of two HCC-characteristic miRNAs (miR-16 and miR-146a) based on previous microarray studies which distinguished HCC from other diseases. The chosen miRNAs were chosen related to HCC and targeting the selected autophagy genes. Then, to confirm this panel, we screened serum C-JUN, LAMP2, miR-16, MiR-146a in HCC patients compared with chronic Hepatitis C virus (HCV) patients and healthy volunteers. Finally, the biological significance and the expression pattern of the selected RNAs in HCC tumor tissue have been also investigated.

Patients & methods

Patients & samples

The study was approved by the Ethical Committee of Ain Shams University faculty of Medicine and in accordance with the ethical standards of the Declaration of Helsinki. An informed consent was obtained from all the participants before enrollment in the study. This study was conducted on 140 Egyptian adults including 70 newly diagnosed HCC patients, 32 chronic HCV patients without HCC and 38 apparently healthy subjects as a control group, who were recruited from individuals seeking a routine medical check-up. Patients enrolled in this study were recruited from the Ain Shams University faculty of Medicine from May 2013 to August 2014. All adult patients more than 18 years or older of both sexes with proved HCC diagnosis according to the practice guidelines recommended by the American Association for the Study of Liver Diseases with no restriction to the number or the size of the tumor were enrolled in the study. HCC clinical stage was determined according to Barcelona Clinic Liver Cancer staging classification [12] and Child–Pugh classification [13]. Among the 70 HCC patients, 60 (85.7%), 6 (8.6%) and 4 (5.7%) were classified as stage A, B and C carcinomas, respectively. All patients and controls were subjected to full history taking, abdominal ultrasound and triphasic abdominal CT scan. Patients who received previous chemotherapy or radiotherapy or patients with liver tumors other than HCC and those with Barcelona Clinic Liver Cancer stage D were excluded from the study. Complete follow-up data of all participants were collected from medical records and personal interviews.

Blood samples were collected before any therapeutic intervention. All blood samples were centrifuged at 3000 rpm for 15 min at room temperature to obtain the serum and stored at -80°C till use. Fresh HCC and corresponding nontumor samples were collected from 20 out of 70 HCC patients, who underwent surgical resection. Tissue samples were stored in RNAlater™ (Qiagen) at -80°C immediately after resection for extraction of RNA. Clinicopathological features of all the participants are summarized in Table 1.

Laboratory investigations

Sera samples were investigated for alanine transaminase, aspartate transaminase, total and direct bilirubin (TB, DB), total protein and albumin on the automated BECKMAN COULTERAU680 auto analyzer (Beckman Coulter, Inc., CA, USA). For prothrombin time and International Normalized Ratio (INR), the collected blood was added in trisodium citrate, then centrifuged and the test was performed within 4 h of sample collection using coagulation analyzer.

Hepatitis markers, HCV antibodies and HBsAg were analyzed by ELISA (Diasorin, Saluggia, Italy) and confirmed by real-time PCR in HCV-positive antibody patients. Alpha fetoprotein (AFP) was quantitatively determined using a commercial ELISA (Cobas 411, Roche Diagnostic GmbH, Mannheim, Germany).

Bioinformatics-based selection of the miRNA-mediated autophagic networks

The construction of miRNA-mediated autophagic networks included three steps: autophagy genes LAMP2 (key player of autophagy) and c-JUN mRNA (potent regulator of autophagy) related to HCC were retrieved (Supplementary Figures 1 & 2). We have selected those autophagy genes which are highly correlated to hepatocellular carcinoma based on *in silico* data analysis adapted from more than two databases to decrease false discovery rate, namely; Genatlas at Paris Descartes University database available at [14]; and protein atlas database available at [15].

Table 1. Study population demographic and clinical characteristics (n = 140).

Parameter	Malignant (n = 70)	CHC (n = 32)	Normal (n = 38)	p-value	$\chi^{2(\dagger)}$ $F^{(\ddagger)}$
Age: – ≥57 years (n = 96) – <57 years (n = 44)	 54 (77.1%) 16 (21.9%)	 18 (56.2%) 14 (43.8%)	 24 (63.2%) 14 (36.8%)	0.076	$\chi^{2(\dagger)} = 5.157$
Sex: – Male (n = 110) – Female (n = 30)	 52 (74.3%) 18 (25.7%)	 26 (81.2%) 6 (18.8%)	 32 (84.2%) 6 (15.8%)	0.445	$\chi^{2(\dagger)} = 1.618$
Smoking: – Nonsmoker (n = 50) – Smoker (n = 90)	 24 (34.3%) 46 (65.7%)	 16 (50%) 16 (50%)	 10 (26.3%) 28 (73.7%)	0.113	$\chi^{2(\dagger)} = 4.379$
HCV-antibodies: – Positive (n = 92) – Negative (n = 48)	 60 (85.7%) 10 (14.3%)	 32 (100%) 0 (0%)	 0 (0%) 38 (100%)	<0.001§	$\chi^{2(\dagger)} = 101.9$
HBVsAg: – Positive (n = 4) – Negative (n = 136)	 4 (5.7%) 66 (94.3%)	 0 (0%) 32 (100%)	 0 (0%) 38 (100%)	0.128	$\chi^{2(\dagger)} =$ 4.118
Cirrhosis: – Cirrhotic (76) – Noncirrhotic (64)	 60 (85.7%) 10 (14.3%)	 16 (50%) 16 (50%)	 0 (0%) 38 (100%)	<0.001§	$\chi^{2(\dagger)} =$ 73.224
AST	71.7 ± 42.9	52.2 ± 11.66	24.26 ± 7.5	<0.001§	$F^{(\ddagger)} = 28.584$
ALT	56.5 ± 46.6	40.7 ± 19.9	21.26 ± 6.59	<0.001§	$F^{(\ddagger)} = 12.954$
Albumin	2.1 ± 0.55	2.4 ± 0.51	3.8 ± 0.257	<0.001§	$F^{(\ddagger)} = 72.29$
Total bilirubin	2.15 ± 1.9	1.8 ± 0.93	0.87 ± 0.21	<0.001§	$F^{(\ddagger)} = 9.7$
Direct bilirubin	1.1 ± 1.688	1 ± 0.55	0.12 ± 0.07	<0.001§	$F^{(\ddagger)} = 8.507$
INR	1.89 ± 0.37	1.69 ± 0.27	1.14 ± 0.05	<0.001§	$F^{(\ddagger)} = 34.04$
α-feto-protein	921 ± 349	18.5 ± 9.5	8.05 ± 7.04	0.005§	$F^{(\ddagger)} = 22.377$
Child score: – A5 – A6 – B7 – B8 – B9 – C10	 8 (11.4%) 20 (28.6%) 2 (2.9%) 12 (17.1%) 16 (22.9%) 12 (17.1%)	–	–	–	–
Mean size of the tumor: – ≥3 cm – <3 cm	 64 (91.4%) 6 (8.6 %)	–	–	–	–
BCLC stage: – A – B – C	 60 (85.7%) 6 (8.6%) 4 (5.7%)	–	–	–	–

†2 test.
‡One-way ANOVA test.
§Highly significant correlation was detected between investigated groups at $p < 0.01$.
ALT: Alanine transaminase; AST: Aspartate transaminase; BCLC: Barcelona clinic liver cancer; CHC: Chronic HCV infection; HBVsAg: Hepatitis B virus surface Antigen; HCV: Hepatitis C virus; INR: International normalized ratio.

Those genes which were up- or downregulated with fold change ≥2.0 and p-value < 0.05; miRNA regulating autophagy genes which are highly expressed in HCC were chosen from miRFocus database, DIANA microT and TargetScan databases; pathway enrichment analysis of miR-16 and miR-146a was performed using the DIANA-mirPath software [16] and the KEGG pathway. It revealed that the selected two miRNAs have got higher number of target genes related to tumorigenesis, for example, MAPK, lysosomal enzymes, focal adhesion, autophagy and apoptosis. Together, c-JUN mRNA was chosen with miR-146a as an epigenetic regulator and LAMP2 was also selected with miR-16.

Extraction of total RNA, including miRNA from sera & tissue samples

Total RNA was extracted from 200 µl serum using miREasy RNA isolation kit (Qiagen, Hilden, Germany) according to manufacturer's instructions for sera/tissue samples. Total RNA was eluted in 30µl of RNase-free water. The concentration and purity of RNA were determined by A260/A280 ratio using (Ultraspec 1000, UV/visible spectrophotometer, Amersham Pharmacia Biotech, Cambridge, UK) and 1.5% agarose gel electrophoresis. Pure

RNA had a ratio of from 1.8–2.1. The extracted total RNA was reverse transcribed into cDNA, following the manufacturer's protocol) of miScript II RT Kit (Qiagen/SABiosciences Corporation, MD, USA) using Hybaid thermal cycler (Thermo Electron, MA, USA).

Quantitative reverse transcriptase PCR

C-JUN mRNA and LAMP2 mRNA abundance in serum and tissue samples from HCC patients were assessed using (Hs_LOC100288387_QF_1 QuantiFast Probe Assay [QF00007504] and Hs_LAMP2_QF_1 QuantiFast Probe Assay, respectively), TaqMan Universal PCR Master Mix on Step One Plus™ System (Applied Biosystems, Inc., CA, USA; Accession: NM_002228 and NM_001122606, respectively) and β-actin (Accession: NM_001101) was used as an internal control.

MiR-16 and miR-146a level in serum and tissue samples were measured by mixing cDNAs with miScript SYBR Green PCR kit (Qiagen/SABiosciences Corporation) according to the manufacturer's suggested protocol; along with an miRNA-specific forward primer. RNU-6 was used as an internal control. All the PCR primers were purchased from Qiagen. Samples were analyzed in duplicate.

Relative quantification miRNAs and autophagy transcripts abundance were calculated using the $2^{-\Delta\Delta Ct}$ method [17]. The raw data of mRNAs and miRNAs were normalized to the housekeeping gene (β-actin) and to the serum control miRNA (RNU-6), respectively, and compared with a control sample.

Statistics

Statistical analyses were conducted using SPSS 22.0 software. The significance level is 0.05. Data were expressed as mean ± standard deviation for quantitative parametric measures in addition to mean rank for quantitative nonparametric measures and both number and percentage for categorical data. The receiver operating characteristic (ROC) was constructed to obtain the best cut-off value for the investigated biomarkers in diagnosing HCC. We conducted risk score analysis to assess the association between HCC and the miRs and the associated autophagy target genes levels followed by ROC curves to evaluate the diagnostic effectiveness of the combined four RNA-based biomarker panel. The prognostic power of the investigated parameters was analyzed with Kaplan–Meier method, and log-rank test (details in Supplementary Material).

Results

Clinical & laboratory characteristics of study population

As shown in Table 1, there was no significant difference in age, sex and smoking ratio among the study groups ($p > 0.05$). Details of the clinical data are shown in Table 1. Liver function tests revealed elevated level of aspartate transaminase, alanine transaminase, TB, DB, AFP and INR, and decreased albumin was noticed in HCC patients and HCV patients (all $p < 0.001$). Among the 70 HCC cases, 64 cases had a tumor size of more than 3 cm while others were less than 3 cm.

Expression of autophagy transcripts among the study groups

Based on relative quantity (RQ), the highest serum c-JUN mRNA level was detected in HCC group, (mean rank = 103.7), followed by chronic HCV group (mean rank = 46.7), meanwhile, the lowest serum level of c-JUN mRNA was detected in healthy control group (mean rank = 39.3). The highest serum level of LAMP2 mRNA was detected in healthy control group (mean rank = 105.5). While, the lowest serum LAMP2 mRNA level was presented in HCC group (mean rank = 40.5), followed by chronic HCV group (mean rank = 94.5). There were highly statistical significant differences in serum c-JUN mRNA, LAMP2 mRNA levels among the three studied groups taken together ($p < 0.001$; Tables 2 & 3; Figures 1 & 2).

On pairwise comparison; serum LAMP2 and c-JUN mRNA levels showed significant differences between (HCC and HCV) and (HCC and healthy control) but no statistical difference was detected between HCV and healthy control (Tables 2B & C).

Last, serum c-JUN mRNA, LAMP2 mRNA were positive in (63, 63 HCC cases, respectively; 5, 4 CHC cases, respectively) and (6, 0 healthy volunteers, respectively; Table 4), based on the cut-off values previously calculated according to the ROC curves (Supplementary Figures 3 & 4).

Table 2. Positivity rates of serum miRNAs and selected target genes among the study groups.

Sub-group	miR-16† cutoff ≥1.817		miR-146‡ cutoff ≤1.526		c-Jun§ cutoff ≥1.011		LAMP2# cutoff ≤0.745	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
HCC (n = 70)	68 (97.1%)	2 (2.1%)	64 (91.4%)	7 (10%)	63 (90%)	7 (10%)	63 (90%)	7 (10%)
CHC (n = 32)	2 (6.2%)	30 (93.8%)	6 (18.8%)	26 (81.2%)	5 (15.8%)	27 (84.2%)	4 (12.5%)	28 (87.5%)
Healthy control (n = 38)	0 (0%)	38 (100%)	6 (15.8%)	32 (84.2%)	6 (15.8%)	32 (84.2%)	0 (0%)	38 (100%)

Significant difference was detected between investigated groups at $p < 0.001$ using ^{2} test.
†($\chi^2 = 124.73$ at $p < 0.0001$).
‡($\chi^2 = 77.89$ at $p < 0.0001$).
§($\chi^2 = 77.5$ at $p < 0.0001$).
#($\chi^2 = 100.728$ at $p < 0.0001$).
CHC: Chronic HCV infection; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

Table 3. Significance of LAMP2, cellular jun proto-oncogene, miRNA 16-2 and miRNA 146a relative quantity differences among the different study groups, using Mann–Whitney test.

RNA marker	HCC	HCV	Healthy control	Significance	p-value
LAMP2 (RQ)	38.64	79.63	—-	†U = 220.0	†p = 0.000‡
Mean ranks	37.36	—-	86.08	§U = 130.0	§p = 0.000‡
	—-	31.38	38.97	#U = 476.0	#p = 0.119
c-jun (RQ)	63.03	26.28	—-	†U = 313.0	†p = 0.000‡
Mean ranks	70.41	—-	25.18	§U = 216.0	§p = 0.000‡
	—-	36.94	34.29	#U = 562.0	#p = 0.586
miRNA 16-2 (RQ)	65.94	19.91	—-	†U = 109.0	†p = 0.000‡
Mean ranks	72.89	—-	20.63	§U = 43.0	§p = 0.000‡
	—-	38.72	32.79	#U = 505.0	#p = 0.224
miRNA 146a (RQ)	37.96	81.13	—-	†U = 172.0	†p = 0.000‡
Mean ranks	37.39	—-	86.03	§U = 132.0	§p = 0.000‡
	—-	33.69	37.03	#U = 550.0	#p = 0.493

†HCC versus HCV·‡ HCC versus healthy control·§ HCV versus healthy control.
#Significant at ≤0.05 level.
c-Jun: Cellular jun proto-oncogene; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; RQ: Relative quantity.

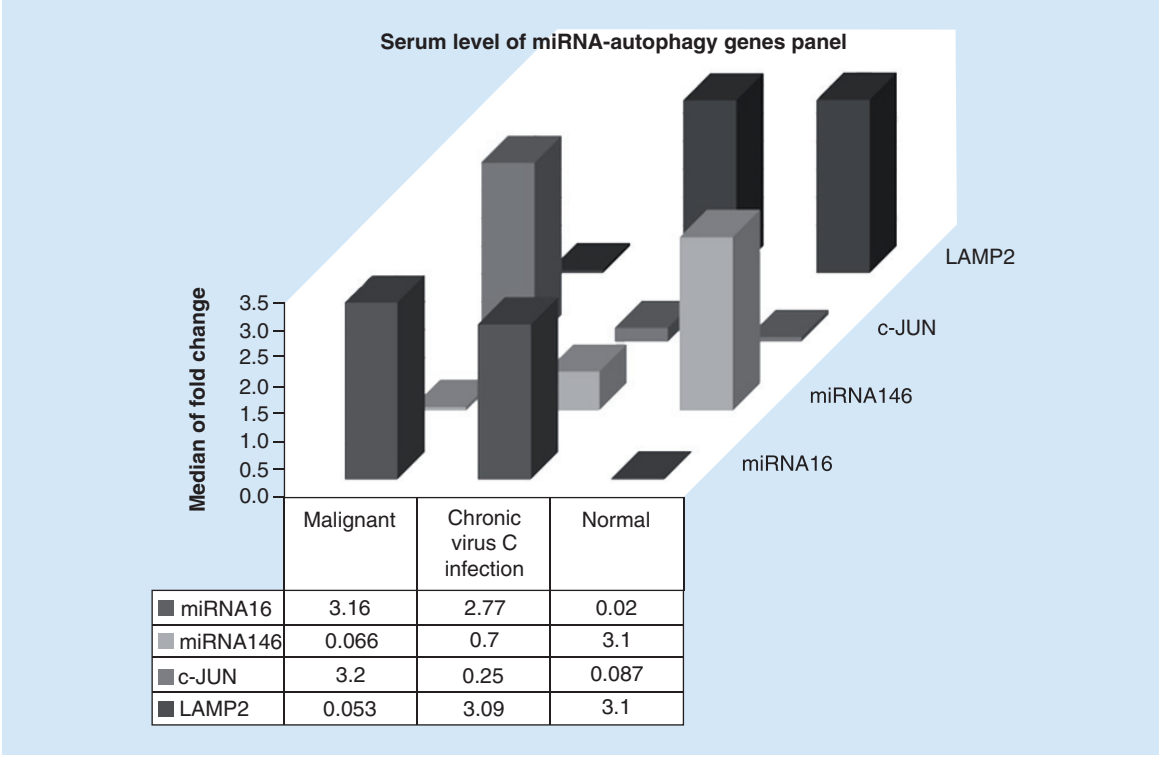
Table 4. Positivity rates of serum miRNAs and selected target genes among the study groups, using ^{2} test.

RNA marker	HCC		HCV		Healthy control		Pearson ^{2}	p-value
	Positive	Negative	Positive	Negative	Positive	Negative		
LAMP2 (RQ)	63	7	4	28	0	38	†$\chi^2 = 58.521$	†p = 0.000#
Cut off ≤0.745	(90%)	(10%)	(12.5%)	(87.5%)	(0%)	(100%)	‡$\chi^2 = 82.080$	‡p = 0.000#
							§$\chi^2 = 5.038$	§p = 0.025#
C-Jun (RQ)	63	7	5	27	6	32	†$\chi^2 = 49.185$	†p = 0.000#
Cut off ≥1.011	(90%)	(10%)	(15.8%)	(84.2%)	(15.8%)	(84.2%)	‡$\chi^2 = 53.245$	‡p = 0.000#
							§$\chi^2 = 0.000$	§p = 0.985
miRNA 16-2 (RQ)	68	2	2	30	0	38	†$\chi^2 = 84.267$	†p = 0.000#
Cut off ≥1.817	(97.1%)	(2.1%)	(6.2%)	(93.8%)	(0%)	(100%)	‡$\chi^2 = 99.669$	‡p = 0.000#
							§$\chi^2 = 2.445$	§p = 0.118
miRNA 146a (RQ) cut off ≤1.526	64 (91.4%)	7 (10%)	6 (18.8%)	26 (81.2%)	6 (15.8%)	32 (84.2%)	†$\chi^2 = 53.878$ ‡$\chi^2 = 61.790$ §$\chi^2 = 0.107$	†p = 0.000# ‡p = 0.000# §p = 0.743

†HCC versus HCV·‡HCC versus healthy control.
§ HCV versus healthy control.
#Significant at ≤0.05 level.
HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; RQ: Relative quantity.

Expression of miR-16 and miR-146a among the study groups

Based on relative quantity, the highest serum miR-16 level was detected in HCC group (mean rank = 103.7), followed by chronic HCV group (mean rank = 42.6), Meanwhile, the lowest serum level of miR-16 was detected in



	Malignant	Chronic virus C infection	Normal
miRNA16	3.16	2.77	0.02
miRNA146	0.066	0.7	3.1
c-JUN	3.2	0.25	0.087
LAMP2	0.053	3.09	3.1

Figure 1. Medians of the fold changes in serum levels of miRNAs and selected target genes in different study groups.

healthy control group (mean rank = 33.5). While, the lowest serum miR-146a level was presented in HCC group (mean rank = 39.8), followed by chronic HCV group (mean rank = 89.3). The highest serum level of miR-146a was detected in healthy control group (mean rank = 133.5). There were highly statistical significant differences in serum miR-16 and miR-146a levels among the three studied groups taken together ($p < 0.001$; Table 2).

On pairwise comparison; serum miR-16 and miR-146a levels showed significant differences between (HCC and HCV) and (HCC and healthy control) but not between HCV and healthy control (Supplementary Tables 2B & C).

Last, serum miR-16 and miR-146a were positive in (68, 64 HCC cases, respectively), (2, 6 CHC cases, respectively) and (0, 6 healthy volunteers, respectively) (Table 4), based on the cut-off values previously calculated according to ROC curves (Supplementary Figures 5 & 6).

Correlation of the serum level of miRNA-associated autophagy network with clinicopathological data of the malignant group

There was a significant association of serum c-JUN mRNA levels in the malignant group with smoking, Child–Pugh score, early stage, increase tumor size and cirrhosis; LAMP2 mRNA with HBsAg and tumor size; miR-16 level with Child–Pugh score and early stage; and miR-146a with Child–Pugh score and tumor size (Table 5). There were significant associations between miR-16 and TB or DB in the malignant group of the study ($p < 0.05$). Additionally, there was a significant positive correlation between c-JUN mRNA and TB, age, INR and AFP (Table 6B). Interestingly, regression analysis revealed that HBsAg, cirrhosis, serum AFP, c-JUN mRNA, LAMP2 mRNA, miR-16 and miR-146a levels were statistically significant independent predictors of HCC risk (all $p < 0.05$) (Supplementary Table 1)

Prediction of HCC cases by risk score analysis using serum miRNA-associated autophagy network

Risk score analysis of this four-RNA profiling system, was carried out on the patient's dataset. The risk score for prediction of HCC was formulated to take in consideration the chosen biomarkers with age, sex, smoking, HCV antibodies and AFP for each patient. According to the risk scores and a set of cut offs, samples could be divided into

Table 5. Positivity rate of serum miRNAs and selected target genes expression among the study groups in relation to different clinicopathological factors of the malignant group of the study (n = 70).

	MiR-16				MiRNA-146				c-Jun				LAMP2			
Clinicopathological factors	**Mean rank**	**Statistics**	**Number of cases ≥1.817 (%)**	**P $\chi^{2(\dagger)}$**	**Mean rank**	**Statistics**	**Number of cases ≤1.526 (%)**	**P $\chi^{2(\dagger)}$**	**Mean rank**	**Statistics**	**Number of cases ≥1.11 (%)**	**P $\chi^{2(\dagger)}$**	**Mean rank**	**Statistics**	**Number of cases ≤0.745 (%)**	**P $\chi^{2(\dagger)}$**
Mean age (years):																
≥57 (n = 54)	35.8	p = 0.437	52(76.5%)	p = 0.610	36.39	p = 0.166	48 (75%)	p = 0.163	33.9	p = 0.024#	51 (81%)	p = 0.023*	34.5	p = 0.187	50 (79.4%)	p = 0.18
<57 (n = 16)	34.5	$U^{(\ddagger)} = 416$	16(23.5%)	$\chi^{2(\S)} = 0.435$	32.5	$U^{(\ddagger)} = 384$	16 (25%)	$\chi^{2(\S)} = 1.944$	40.7	$U^{(\ddagger)} = 324$	12 (19%)	$\chi^{2(\S)} = 5.18$	38.5	$U^{(\ddagger)} = 383$	13 (20.6%)	$\chi^{2(\S)} = 1.7$
Sex:																
Male (n = 52)	35.8	p = 0.4	50 (73.5%)	p = 0.399	35.19	p = 0.657	49 (75%)	p = 0.655	36.7	p = 0.103	45 (71.4%)	p = 0.1	36.7	p = 0.103	45 (71.4%)	p = 0.1
Female (n = 18)	34.5	$U^{(\ddagger)} = 450$	18 (26.5%)	$\chi^{2(\S)} = 0.713$	36.39	$U^{(\ddagger)} = 452$	16 (25%)	$\chi^{2(\S)} = 0.199$	32	$U^{(\ddagger)} = 405$	18 (28.6%)	$\chi^{2(\S)} = 2.69$	32	$U^{(\ddagger)} = 405$	18 (28.6%)	$\chi^{2(\S)} = 2.69$
Smoking:																
Smoker (n = 24)	34.5	p = 0.303	24 (35.3%)	p = 0.3	32.5	p = 0.066	24 (37.5%)	p = 0.064	40.75	p = 0.003**	18 (28.6%)	p = 0.003**	36.38	p = 0.617	21 (33.3%)	p = 0.615
Nonsmoker (n = 46)	36.02	$U^{(\ddagger)} = 528$	44 (64.7%)	$\chi^{2(\S)} = 1.07$	37.07	$U^{(\ddagger)} = 480$	40 (62.5%)	$\chi^{2(\S)} = 3.4$	32.76	$U^{(\ddagger)} = 427$	45 (71.4%)	$\chi^{2(\S)} = 9.1$	35.03	$U^{(\ddagger)} = 531$	42 (66.7%)	$\chi^{2(\S)} = 0.254$
HCV-Abs:																
Positive (n = 60)	35.67	p = 0.56	58 (85.3%)	p = 0.343	34.8	p = 0.166	56 (85.5%)	p = 0.163	35.5	p = 1	54 (85.7%)	p = 1	36	p = 0.258	53 (84.1%)	p = 0.255
Negative (n = 10)	34.5	$U^{(\ddagger)} = 290$	10 (14.7%)	$\chi^{2(\S)} = 0.585$	39.5	$U^{(\ddagger)} = 260$	8 (12.5%)	$\chi^{2(\S)} = 1.94$	35.5	$U^{(\ddagger)} = 300$	9 (14.3%)	$\chi^{2(\S)} = 0$	32	$U^{(\ddagger)} = 265$	10 (15.9%)	$\chi^{2(\S)} = 1.29$
HBVsAg:																
Positive (n = 4)	34.5	p = 0.93	4 (5.9%)	p = 0.724	32.5	p = 0.7798	4 (6.2%)	p = 0.528	49.5	p = 0.16	2 (3.2%)	p = 0.006**	49.5	p = 0.16	2 (3.2%)	p = 0.006**
Negative (n = 66)	35.59	$U^{(\ddagger)} = 128$	64 (94.1%)	$\chi^{2(\S)} = 0.125$	35.6	$U^{(\ddagger)} = 120$	60 (93.8%)	$\chi^{2(\S)} = 0.390$	34.6	$U^{(\ddagger)} = 76$	61 (96.8%)	$\chi^{2(\S)} = 7.5$	34.6	$U^{(\ddagger)} = 76$	61 (96.8%)	$\chi^{2(\S)} = 7.5$
Child–Pugh score:																
A5 (n = 8)	38	p = 0.019*	8 (11.8%)	p = 0.077	44.7	p = 0.02*	4 (6.2%)	p = 0.001	32.7	p = 0.02*	8 (12.7%)	p = 0.352	49.5	p = 0.128	6 (9.5%)	p = 0.494
A6 (n = 20)	28.5	$\chi^{2(\S)} = 13.9$	20 (29.4%)	$\chi^{2(\S)} = 9.9$	29.6	$\chi^{2(\S)} = 13.2$	20 (31.2%)	$\chi^{2(\S)} = 23.6$	36	$\chi^{2(\S)} = 13.2$	18 (28.6%)	$\chi^{2(\S)} = 5.5$	35.15	$\chi^{2(\S)} = 8.5$	18 (28.6%)	$\chi^{2(\S)} = 4.3$
B6 (n = 2)	37		2 (2.9%)		24.5		2 (3.1%)		42.3		2 (3.2%)		52.5		2 (3.2%)	
B7 (n = 12)	25.5		10 (14.7%)		40.3		12 (18.8%)		46		12 (19%)		34.2		12 (19%)	
B8 (n = 16)	37		16 (23.5%)		26		16 (25%)		21.5		14 (22.2%)		26.3		15 (23.8%)	
C10 (n = 12)	52		12 (17.6)%		48.83		10 (15.6%)		42.3		9 (14.3%)		37.3		10 (15.9%)	
BCLC stage:																
A (n = 60)	33.4	p = 0.09	58 (85.3%)	p = 0.843	34.2	p = 0.423	56 (87.5%)	p = 0.34	36	p = 0.356	55 (87.5%)	p = 0.47	34.6	p = 0.608	55 (87.5%)	p = 0.47
B (n = 6)	45.17	$\chi^{2(\S)} = 4.7$	6 (8.8%)	$\chi^{2(\S)} = 0.343$	43.5	$\chi^{2(\S)} = 1.7$	5 (7.8%)	$\chi^{2(\S)} = 2.17$	25.3	$\chi^{2(\S)} = 2.06$	5 (7.9%)	$\chi^{2(\S)} = 1.4$	43.17	$\chi^{2(\S)} = 0.995$	5 (7.9%)	$\chi^{2(\S)} = 1.4$
C (n = 4)	52.5		4 (5.9%)		43		3 (4.6%)		42.8		3 (4.8%)		37.15		3 (4.8%)	

Non-significant correlation was detected between investigated groups at $p > 0.05$.† χ^2 test.

‡Mann–Whitney.

§Kruskal–Wallis test.

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

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

BCLC: Barcelona clinic liver cancer; c-Jun: Cellular jun proto-oncogene; HBVsAg: Hepatitis B virus surface Antigen; HCV: Hepatitis C virus.

Table 5. (cont.). Positivity rate of serum miRNAs and selected target genes expression among the study groups in relation to different clinicopathological factors of the malignant group of the study (n = 70).

	MiR-16				MiRNA-146				c-Jun				LAMP2			
Clinicopathological factors	**Mean rank**	**Statistics**	**Number of cases ≥1.817 (%)**	**P $\chi^{2(\dagger)}$**	**Mean rank**	**Statistics**	**Number of cases ≤1.526 (%)**	**P $\chi^{2(\dagger)}$**	**Mean rank**	**Statistics**	**Number of cases ≥1.11 (%)**	**P $\chi^{2(\dagger)}$**	**Mean rank**	**Statistics**	**Number of cases ≤0.745 (%)**	**P $\chi^{2(\dagger)}$**
Stage:																
Early (BCLC A, B) (n = 60)	32.7	p = 0.006**	58 (85.3%)	p = 0.55	33.6	p = 0.06	56 (87.5%)	p = 0.163	34.4	p = 0.3	56 (88.9%)	p = 0.023**	34.4	p = 0.687	55 (87.3%)	p = 0.25
Late (BCLC; C) (n = 10)	51.9	$U^{(\ddagger)} = 136$	10 (14.7%)	$\chi^{2(\S)} = 0.343$	46.7	$U^{(\ddagger)} = 188$	8 (12.5%)	$\chi^{2(\S)} = 1.9$	41.6	$U^{(\ddagger)} = 238$	7 (11.1%)	$\chi^{2(\S)} = 5.18$	41.6	$U^{(\ddagger)} = 276$	8 (12.7%)	$\chi^{2(\S)} = 1.29$
Tumor size:																
≥3 cm (n = 64)	45.5	p = 0.2	62 (91.2%)	p = 0.66	46.8	p = 0.165	60 (93.8%)	p = 0.023*	37	p = 0.04*	59 (93.7%)	p = 0.046*	43.8	p = 0.294	59 (93.7%)	p = 0.046*
<3 cm (n = 6)	34.5	$U^{(\ddagger)} = 132$	6 (8.8%)	$\chi^{2(\S)} = 0.19$	34.4	$U^{(\ddagger)} = 124$	4 (6.2%)	$\chi^{2(\S)} = 5.13$	19	$U^{(\ddagger)} = 96$	4 (6.3%)	$\chi^{2(\S)} = 3.9$	34.7	$U^{(\ddagger)} = 142$	4 (6.3%)	$\chi^{2(\S)} = 3.9$
Cirrhosis:																
Cirrhotic (n = 60)	35.5	p = 1	58 (85.3%)	p = 0.558	34	p = 0.4	54 (84.4%)	p = 0.29	38.7	p = 0.003**	56 (88.9%)	p = 0.02*	34.8	p = 0.46	55 (87.3%)	p = 0.259
Noncirrhotic (n = 10)	35.5	$U^{(\ddagger)} = 300$	10 (14.7%)	$\chi^{2(\S)} = 0.343$	39.7	$U^{(\ddagger)} = 256$	10+ (15.6%)	$\chi^{2(\S)} = 1.09$	17.7	$U^{(\ddagger)} = 122$	7 (11.1%)	$\chi^{2(\S)} = 5.17$	39	$U^{(\ddagger)} = 256$	8 (12.3%)	$\chi^{2(\S)} = 1.2$

Non-significant correlation was detected between investigated groups at $p > 0.05$. † χ^2 test.

‡Mann–Whitney.

§Kruskal–Wallis test.

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

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

BCLC: Barcelona clinic liver cancer; c-Jun: Cellular jun proto-oncogene; HBVsAg: Hepatitis B virus surface Antigen; HCV: Hepatitis C virus.

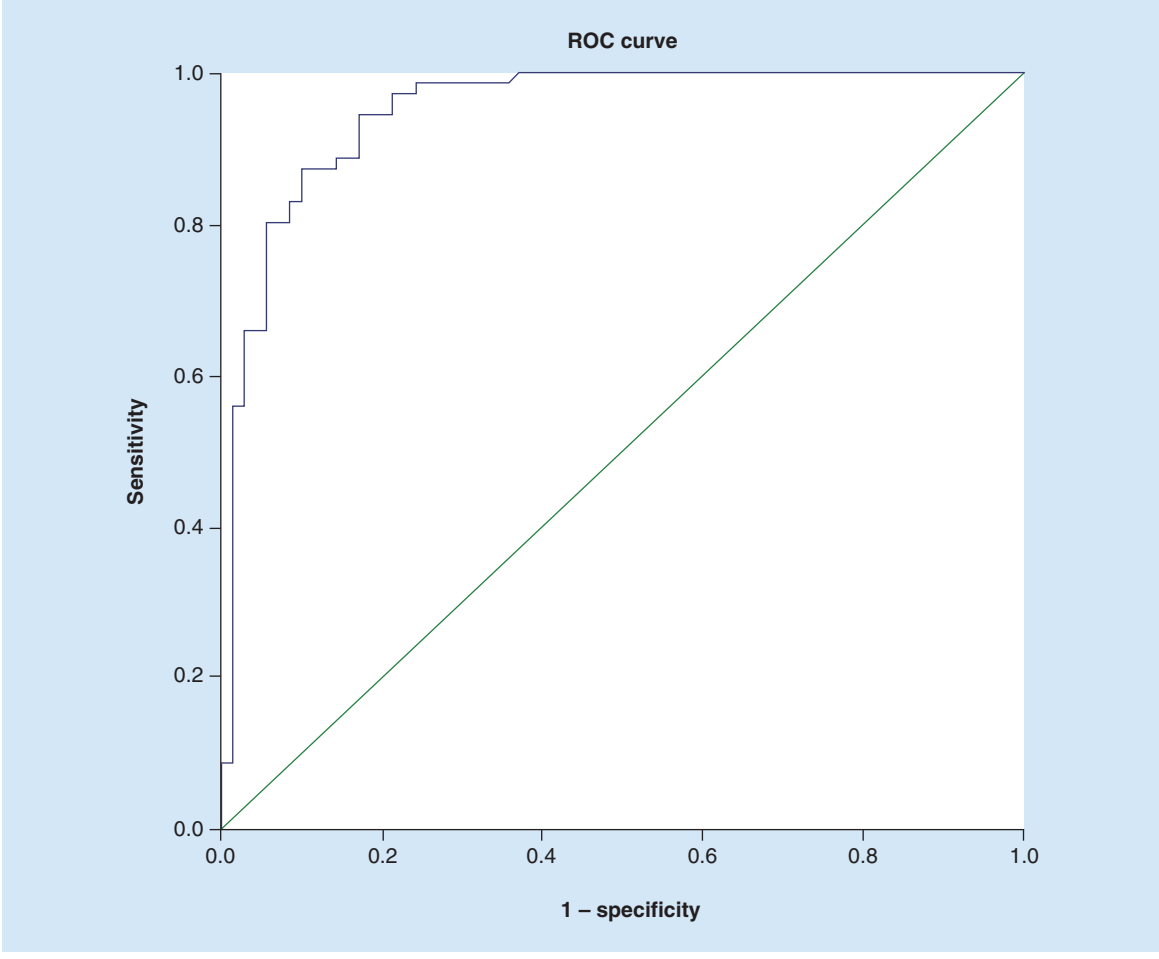


Figure 2. ROC curve of the risk score analysis of the four-RNA profiling system.

a high-risk group or a low risk group. At the optimal cut-off value (2.07), the diagnostic sensitivity and specificity values of the four-RNA based markers for HCC detection were 88.6 and 100%, respectively. None of the controls had a risk score >2.07, whereas 8 of the 70 HCC samples exhibited a risk score <2.07 (Table 4).

Abundance of miRNA-associated autophagy network in matched liver cancer tissues & adjacent cancer-free tissues

We examined miRNA-associated autophagy network level in 20 paired HCC tissues and adjacent nontumor tissues out of 70 HCC patients by quantitative reverse-transcriptase PCR. In tumor tissues, c-JUN mRNA and miR-16 levels were at a higher level than that of nontumor tissues, with the median of 464.5, 2.04 and 221, 0.36, respectively; however, tumor tissue showed lower level of LAMP2, miR-146a compared with normal tissue with a median of 4, 76.6; and 8.6, 105.6, respectively. These data indicated that aberrant expression of miRNA-associated autophagy network may be related to HCC pathogenesis (Figures 3A & B & Figure 4). Of note, there was a strong significant correlation between serum and tissue level of C-JUN mRNA, LAMP2 MRNA, miR-16 and miR-146a ($r = 0.59$, 0.583, 0.602 and 0.533, respectively at $p < 0.05$) (Table 5).

Serum level of miRNA-associated autophagy network as a potential diagnostic marker for HCC

The diagnostic value of the four selected RNAs (c-JUN mRNA, LAMP2 mRNA, miR-16 and miR-146a) were evaluated by ROC curve analysis and the area under the curve values through all the HCC patients. ROC curves were shown in Supplementary Figures 3–6.

We found that serum levels of c-JUN mRNA, LAMP2 MRNA, miR-16 and miR-146a discriminated HCC patients from healthy controls, with an area under the curves of 0.892, 0.929, 0.975 and 0.938, respectively. At

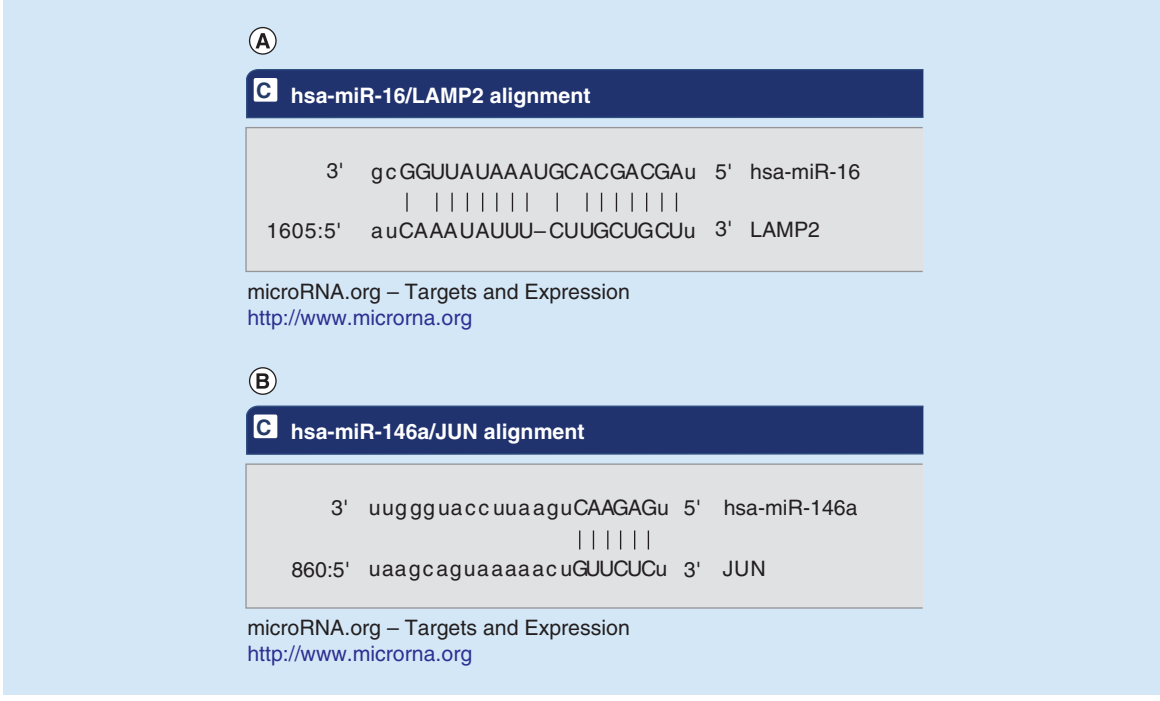
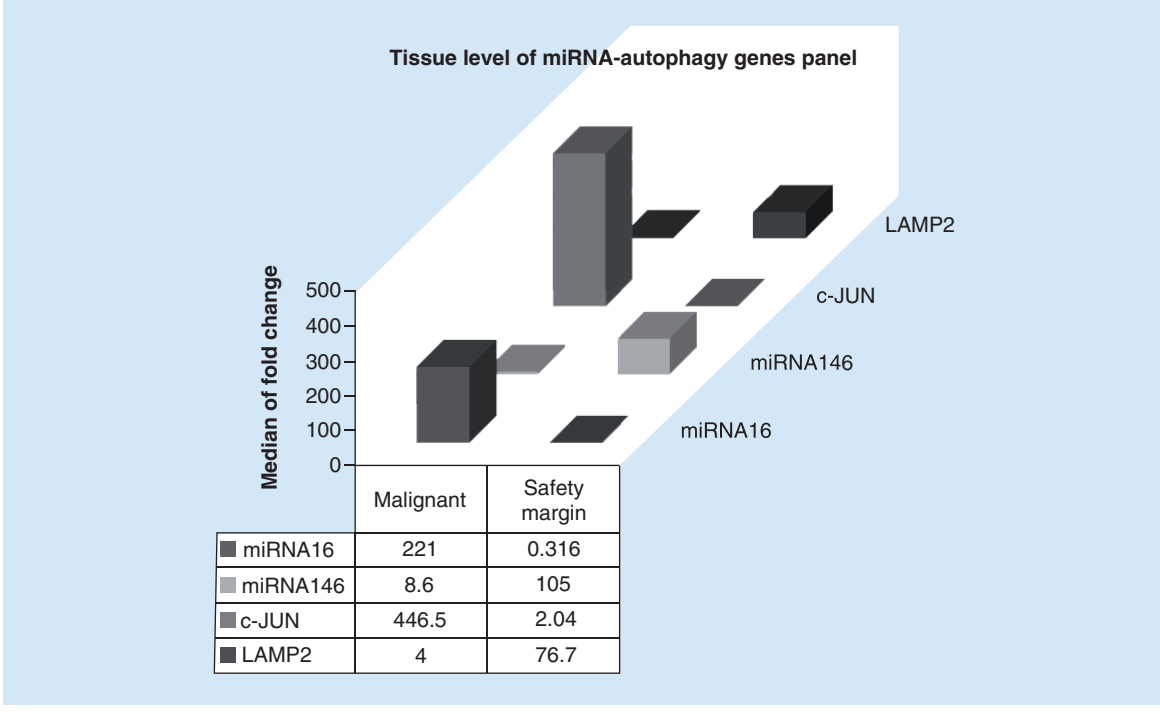


Figure 3. The sequence of miRNA interacting with its target genes. (A) The sequence of miR-16 interacting with its target gene *LAMP2*. **(B)** The sequence of miR-146 interacting with its target gene c-Jun.



	Malignant	Safety margin
miRNA16	221	0.316
miRNA146	8.6	105
c-JUN	446.5	2.04
LAMP2	4	76.7

Figure 4. Medians of the differential expression of tissue miRNAs and selected target genes in paired malignant liver tissue and safety margin.

Table 6. Significance of LAMP2, cellular jun proto-oncogene, miRNA 16-2 and miRNA 146a relative quantity differences among the different study groups, using ANOVA and post-hoc tests.

RNA marker	Groups		Mean difference	Standard error	Sig.	95% CI	
						Lower bound	Upper bound
LAMP2 (RQ)	HCC	HCV	-2.6	1.47	0.076	-5.528	0.278
		Healthy control	-10.7†	1.39	0.000†	-13.407	-7.924
	HCV	HCC	2.6	1.47	0.076	-0.278	5.528
		Healthy control	-8.0†	1.65	0.000†	-11.305	-4.777
	Healthy control	HCC	10.7†	1.39	0.000†	7.924	13.407
		HCV	8.0†	1.65	0.000†	4.777	11.305
c-jun (RQ)	HCC	HCV	51.4†	17.26	0.003†	17.293	85.569
		Healthy control	52.0†	16.30	0.002†	19.760	84.231
	HCV	HCC	-51.4†	17.26	0.003	-85.569	-17.293
		Healthy control	0.6	19.41	0.977	-37.819	38.947
	Healthy control	HCC	-52.0†	16.30	0.002†	-84.231	-19.760
		HCV	-0.6	19.41	0.977	-38.947	37.819
miRNA 16-2 (RQ)	HCC	HCV	31.6	18.10	0.083	-4.158	67.430
		Healthy control	32.0	17.09	0.063	-1.770	65.827
	HCV	HCC	-31.6	18.10	0.083	-67.430	4.158
		Healthy control	0.4	20.35	0.985	-39.853	40.637
	Healthy control	HCC	-32.0	17.09	0.063	-65.827	1.770
		HCV	-0.4	20.35	0.985	-40.637	39.853
miRNA 146a (RQ)	HCC	HCV	-3.3†	0.53	0.000†	-4.349	-2.271
		Healthy control	-3.9†	0.50	0.000†	-4.919	-2.958
	HCV	HCC	3.3†	0.53	0.000†	2.271	4.349
		Healthy control	-0.6	0.59	0.289	-1.796	0.539
	Healthy control	HCC	3.9†	0.50	0.000†	2.958	4.919
		HCV	0.6	0.59	0.289	-0.539	1.796

†Significant at ≤0.05 level.
HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; RQ: Relative quantity; Sig.: Significance.

the cut-off value of 1.011, 0.745, 1.817 and 1.526, the sensitivities were 90, 90, 97.1 and 91.4% respectively; and the specificities were 87.1, 94.3, 97 and 82.9%, respectively.

In order to determine whether miRNA-associated autophagy network aberrant expression was HCC specific, we compared HCC with CHC patients, the cut-off values of 0.943, 0.745, 1.45 and 0.857 for c-JUN mRNA, LAMP2 MRNA, miR-16 and miR-146a, respectively, could be used to discriminate the two groups. By applying these cut-off values we noticed that C-JUN mRNA and LAMP2 mRNA had the same sensitivity (90%) but the specificities decreased to 84.4 and 91.6%, respectively (90%). However, the sensitivity and specificity for miR-146a decreased to (88.6 and 94.7%). Moreover, miR-16 had increased sensitivity and specificity to 97.1 and 97.7%. Last, LAMP2 mRNA had a higher sensitivity than others, as regard to its predicting probability of detecting HCV from normal group.

Moreover, our data revealed that AFP has relatively limited sensitivity and specificity (only 87.7 and 75.9%), whereas miR-16 and LAMP2 mRNA were superior to AFP in specificity (97.1 and 97.1%) and sensitivity (94 and 90.4%), respectively. The selected miRNA-associated autophagy genes panel reduces false-negative results detected by AFP from 13.3 to 0% of 70 HCC cases (Table 7).

Prognostic efficacy of miRNA-associated autophagy network in HCC

A prognostic efficacy of miRNA-associated autophagy network provides information on the likely outcome of the HCC disease in patients to identify who are at high risk of relapse and therefore potential candidates for adjuvant systemic treatment. By following-up all the study cases, the recurrence rate was 28.6% (26/70) of the HCC patients. In univariate analysis, HCC patients with negative C-JUN mRNA, LAMP2 mRNA, miR-16 and miR-146a had relatively longer relapse-free survival (RFS) than patients with positive RNA. Kaplan–Meier analysis revealed significant decrease in RFS and increase in cumulative hazards among C-JUN mRNA- and LAMP2

Table 7. Performance characteristics of the investigated serum parameters.

Biomarker	Sensitivity	Specificity	PPV	NPV	Accuracy
All the study groups (n = 140)					
Alpha-fetoprotein	87.7%	75.9%	71.4%	90%	80.7%
MiRNA-16	97.1%	97.1%	97.1%	97.1%	97.1%
MiRNA-146	84.2%	90.6%	91.4%	82.9%	87.1%
c-JUN	85.1%	89.4%	90%	84.3%	87.1%
LAMP2	94%	90.4%	90%	94.3%	92.1%
Panel of miRNA and selected target genes	100%	85.7%	85.5%	100%	92.85%
Tumor size <3 cm (n = 6)					
Alpha-fetoprotein	66.7%	75.9%	36.4%	96.6%	88%
MiRNA-16	100%	97.1%	75%	100%	97.3%
MiRNA-146	66.7%	90.6%	25%	96.67%	81.57%
c-JUN	33.3%	89.4%	15.3%	93.65%	80.2%
LAMP2	66.7%	90.4%	50%	97%	92%
Panel of miRNA and selected target genes	100%	85.7%	37.5%	100%	86.8%

mRNA-positive HCC patients (Log-rank test: ^{2} 3.77; p = 0.05; 3.67, p = 0.05; respectively) (Supplementary Figures 7–10; and Supplementary Tables 2 & 3). The results of Cox multivariate analyses showed that C-JUN mRNA and LAMP2 mRNA were independent prognostic factors of RFS.

Discussion

Since the reliability of AFP and des-carboxyprothrombin is still limited, especially in benign liver disease and HCC tumor <3 cm [18], the quest for a novel optimal biomarker hence continues.

Due to its crucial importance for cellular survival, autophagy regulation has been involved in many human disorders, such as neurodegenerative disorders and cancer. Autophagy is regulated by the autophagy-related genes and several epigenetic factors including miRs [19]. Interestingly, the miRNAs so far seem to regulate autophagy genes predominantly targeting early stages of such pathway [20].

The current study hypothesizes getting lower number of false results than the simpler single-marker approach upon enrolling multiple HCC-specific and genetically related genes (miRNA-associated autophagy transcripts).

Several transcription factors were also found to regulate autophagy. These include oncogenes as well as tumor suppressors. C-JUN is an oncogene, a well-characterized JNK substrate that positively regulates the cell cycle but can also modulate apoptosis, especially in cells exposed to genotoxic stress [21]. C-JUN regulates the expression of several important factors controlling proliferation, such as cyclin D and cyclin A. Jun proteins are regulated by JNK but are also induced by DNA damages [22]. There is a significant regulatory role for the JNK1 signaling pathway in autophagy [23]. C-JUN is a potent inhibitor of autophagy whose expression is regulated by autophagy-inducing stimuli like starvation [24]. C-JUN NH2-terminal kinase activation may result in overexpression of LC3 during autophagy in human nasopharyngeal carcinoma cells [25]. However, Li *et al.* found that C-JUN and JNK activation could induce autophagy through beclin 1 overexpression during anticancer agent-induced autophagy [26]. Recent findings indicated that C-JUN has an essential role in hepatocyte proliferation and dysplasia [27]. Trierweiler *et al.* noticed that targeting C-JUN may be a potential strategy to prevent hepatitis-associated HCC [28].

LAMP1 and LAMP2 are N-glycosylated proteins, present in the lysosomal membranes. Interestingly, LAMP-2, are important regulators in successful maturation of both autophagosomes and phagosomes [29]. An accumulation of autophagic vacuoles was detected in multiple LAMP-2 deficient tissues, including heart and liver [30]. Earlier reports identified LAMP2 gene to be deregulated in metastatic colon cancer, pancreatic carcinoma and HCC cell lines [31–33]. Altogether, the current work presents autophagy as an opponent to the process of early carcinogenesis in hepatic tissues which seek for rapid growth rather than the quiescent autophagy state. The potential for high cellular growth rate in early cancer is usually accompanied by multitude of growth factors which are upstream activators for c-Jun; a negative regulator of autophagy. It is no surprise then that the final outcome would be an inhibition of autophagy by suppressing LAMP2 which is responsible for substrate translocation into the lysosomes.

Our study agreed with previous data confirming the role of miR-16 and miR-146a in HCC. MiR-16 plays a significant role in induction of apoptosis by targeting the BCL-2 gene and cell-cycle regulation [34]. Although, Ge *et al.*, Qu *et al.* and El-Abd *et al.* reported that serum miR-16 was down regulated in HCC patients compared with chronic liver disease or healthy control [35–37]; in accordance with the current results, it was found to be overexpressed by Varnholt *et al.* in HCC [38]. The differences in the results may be due to variation in sample size, ethnic origin or technical variation. Furthermore, the fact that miR-16 can act as an oncogene was reported in various types of cancer. Chen *et al.*, declared that miR-16 acts as an oncogene and is upregulated in renal cell carcinoma [39]. Moreover, Cimmino *et al.* found that miR-16 is upregulated in chronic lymphocytic leukemia and targeting BCL-2 [40].

MiR-146a is located in a region that is often deleted in human tumors [41], and has been reported to be aberrantly expressed in several cancers. MiR-146a acts as a potential tumor suppressor in pancreatic cancer [42], breast [43] and prostate cancer [44]. MiR-146a is involved in HCC cell development and progression [45] through negative correlation with VEGF and NF-κB [46].

In the present study, there was negative correlation between miR-16 and LAMP2 mRNA or miR-146 and c-JUN mRNA without statistical significance. It seemed that deregulated miR-16 and miR-146 are linked to overexpression of c-JUN mRNA and simultaneous downregulation of LAMP2 mRNA suggesting their synergistic effect in HCC pathogenesis. That was in agreement with *in silico* data analysis.

The strong positive correlation between the level of serum and tissue miRNA-associated autophagy network, identified in this study, was previously identified in the same type of tumor suggesting that these serum miRNA-associated autophagy network could be derived from tumor cells and provide additional evidence supporting the use of serum RNAs as potential diagnostic biomarkers

HCC patients with positive c-JUN mRNA and LAMP2 mRNA had an increased likelihood of distant recurrence in the period of 1–2 years after diagnosis compared with those with negative c-JUN mRNA and LAMP2 mRNA.

Of note, the study limitations include relatively small sample size as a pilot study with further *in vitro* functional analysis which is needed to elucidate the biological mechanisms of miRNA-associated autophagy network in HCC and larger multicenter studies are also strongly recommended.

Conclusion

Our integrated data analysis can assist in revealing important findings through combining the differential LAMP2 mRNA and c-JUN mRNA gene expression with the selected epigenetic regulators (miR-16 and miR-146a). This approach has been shown to generate potential serum-based biomarker panel (c-JUN mRNA, LAMP2 mRNA, miR-16 and miR-146a) for HCC diagnosis and prognosis. These findings expand the existing knowledge of miRNA-associated autophagy network characteristics and offer potential new targets for HCC therapy. Besides, c-JUN mRNA and LAMP2 mRNA levels are useful prognostic markers for RFS in HCC.

Financial & competing interests disclosure

This work was supported by the Egyptian Science and Technology Development Fund, RSTDG 12597. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study was approved by the Ethical Committee of Ain Shams University and in accordance with the ethical standards of the Declaration of Helsinki. An informed consent was obtained from all the participants before enrollment in the study. This study was conducted on 140 Egyptian adults including; 70 newly diagnosed HCC patients, 32 chronic HCV patients without HCC, and 38 apparently healthy subjects as a control group, who were recruited from individuals seeking a routine medical check-up. Patients enrolled in this study were recruited from the Ain shams University hospitals from May 2013 to August 2014. All adult patients more than 18 years or older of both sexes with proved HCC diagnosis according to the practice guidelines recommended by the American Association for the Study of Liver Diseases (AASLD with no restriction to the number or the size of the tumor were enrolled in the study. HCC clinical stage was determined according to Barcelona Clinic Liver Cancer (BCLC) staging classification [12] and Child-Pugh classification [13]. Among the 70 HCC patients, 60(85.7%), 6(8.6%) and 4(5.7%) were classified as stage A, B and C carcinomas, respectively. All patients and controls were subjected to full history taking, abdominal ultrasound and triphasic abdominal CT scan. Patients who received previous chemotherapy or radiotherapy or patients with liver tumors other than HCC

and those with Barcelona Clinic Liver Cancer (BCLC) stage D were excluded from the study. Complete follow-up data of all participants were collected from medical records and personal interviews.

Summary points

- α-fetoprotein has been called-off for surveillance or diagnosis of hepatocellular carcinoma (HCC) and new biomarkers are required.
- Autophagic pathway plays a role in tumor development and is subject to complex regulation by miRNA.
- Dysregulated autophagy genes and their corresponding miRNA should be screened as useful HCC biomarkers.
- C-JUN is a negative regulator of autophagy and its expression was found as upregulated in the present study.
- LAMP2; an important protein in autophagy was found downregulated in HCC patients.
- Deregulated miR-16 and miR-146 were found linked to overexpression of c-JUN mRNA and lower expression of LAMP2 mRNA.
- There was a strong positive correlation between the level of serum and tissue miRNA-associated autophagy network.
- C-JUN mRNA and LAMP2 mRNA levels are useful prognostic markers for relapse-free survival in HCC.

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