

RESEARCH ARTICLE

Exosomal miR-1298 and lncRNA-RP11-583F2.2 Expression in Hepatocellular Carcinoma

Marwa Matboli^{1,*}, Mariam E. Labib¹, Hanaa El-Tayeb Nasser¹, Ahmed H.F. El-Tawdi², Eman K. Habib³ and Randa Ali-Labib¹

¹Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Ain Shams University, Abbassia, Cairo, 11381, Egypt; ²General and Plastic Surgery Department, Military Medical Academy, Cairo, Egypt; ³Anatomy Department, Faculty of Medicine, Ain Shams University, Abbassia, Cairo, Egypt

Abstract: Aim: The aim of this study was to explore the expression of exosomal non-coding RNAs (ncRNAs) in the sera of patients with HCC versus control.

Methods: Firstly, Bioinformatics analysis was conducted to retrieve ncRNAs specific to HCC (hsa-miRNA-1298 and lncRNA-RP11-583F2.2). Afterwards, extraction and characterization of exosomes were performed. We measured the expression of the chosen exosomal RNAs by reverse transcriptase quantitative real-time PCR in sera of 60 patients with HCC, 42 patients with chronic hepatitis C (CHC) infection and 18 healthy normal volunteers.

Results: The exosomal ncRNAs [hsa-miRNA-1298, lncRNA-RP11-583F2.2] had better sensitivity and specificity than alpha-fetoprotein (AFP) in HCC diagnosis.

Conclusion: The exosomal hsa-miRNA-1298, lncRNA-RP11-583F2.2 can be potential biomarkers for HCC diagnosis.

ARTICLE HISTORY

Received: October 28, 2019
Revised: November 16, 2019
Accepted: November 18, 2019

DOI:
10.2174/1389202920666191210111849

Keywords: Hepatocellular carcinoma, exosome, miRNA, lncRNA, diagnosis, chronic hepatitis C.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, with high morbidity and mortality [1]. According to the American Cancer Society, more than 700,000 people throughout the world are diagnosed with HCC each year and more than 600,000 deaths that occur each year are due to liver cancer [2].

In Egypt, cancer of the liver in males is the first most common cancer (33.63%) and is the second common cancer in females (13.5%). HCC represents the most common primary liver malignant tumor and it is the 2nd cause of mortality due to cancer in both sexes [3]. HCC occurs in a number of preexisting conditions, e.g. Hepatitis C virus (HCV) and hepatitis B virus (HBV), alcoholic and nonalcoholic cirrhosis. This is highly related to the HCV epidemic that involved about 10-15% of the Egyptian population through the last three decades, and was stated as the highest HCV prevalence in the world [4]. Egypt has the highest prevalence of HCV infection in the world with 13.8% of the population infected and seven million have chronic HCV liver disease [5].

Most of the screening tools used for HCC diagnosis, either serum alpha-fetoprotein (AFP) or ultrasound lack

adequate sensitivity and specificity [6]. Great efforts have been made towards the study of genomics and epigenomics to demonstrate the mechanisms of HCC and consequently identify novel therapeutic strategies and improve the clinical outcome of patients [7]. The development of cancer is a complex process in which exosomes have a major role. Exosomes are minute vesicles budded from the endosomal network in the form of vesicular bodies. The exosomes have a major role in the transport of important molecules between cells with concern in the targeted cells. The important molecules such as messenger RNAs (mRNAs), microRNAs (miRNAs), long noncoding RNAs (lncRNAs), ribosomal RNAs (rRNAs), small-nuclear RNAs (snRNAs) and transfer RNAs (tRNAs) [8, 9]. The exosomes derived from tumor liberated into the circulation and locally to react with the diversity of target cells, such as other cancer cells, immune and endothelial cells [10]. As the content of exosomes like that of the original cell, they can be considered as potential biomarkers for diagnosis and therapeutic strategies of cancer [11].

Sohn *et al.*, (2015) reported that exosomal microRNAs, such as miRNA-18a, miRNA-221, miRNA-222, and miRNA-224 are elevated in patients of hepatocellular carcinoma as compared to patients with chronic hepatitis B or cirrhosis of the liver. Meantime, the levels of serum exosomal miRNA-101, miRNA-106b, miRNA-122, and miRNA-195 were lower in patients of hepatocellular carcinoma as compared to

*Address correspondence to this author at the Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Ain Shams University, Abbassia, 11381, Cairo, Egypt; Tel: 01005824962; E-mail: marwasayed472@yahoo.com

chronic hepatitis B patients [12]. Moreover, Takahashi *et al.*, (2014) reported that the revelation of hepatocellular carcinoma cells to different agents of anticancer such as camptothecin, induce the lncRNA-VLDLR expression in transformed liver cells in addition to recruitment of these cells into exosomes derived from it. This result elucidates that this lncRNA-VLDLR could involve in chemo-resistance in HCC cells.

The same research group stated other lncRNA mediated in chemo-resistance of hepatocellular carcinoma is the regulator of reprogramming (ROR). LncRNA-ROR plays a role in inducing the maintenance of cancer stem cells and the advancement of HCC cells chemo-resistance, whereas knockdown of this lncRNA enhanced the chemo-sensitivity [13].

In the current study, ncRNAs specific to HCC were retrieved and the expression of the chosen ncRNAs in HCC was verified through *in silico* data analysis. Then the expression of serum exosomal ncRNAs was done to evaluate their usefulness as diagnostic biomarkers and the relationship between the selected RNA biomarkers and pathological changes of patients was explored.

2. PATIENTS AND METHODS

2.1. Patients and Samples

In the current study, 60 HCC patients were diagnosed based on the American Association for the Study of Liver Diseases (AASLD) practice guidelines. However, the clinical stage was determined by the Barcelona Clinic Liver Cancer (BCLC) classification, the clinical stages of HCC of patients classified as 54 (90%) early-stage (A and B) and 6 (C) (10%) late-stage HCC. All blood samples assembled before any surgical, chemotherapeutic or radiotherapeutic procedures. For each patient, complete follow-up data was available. Forty-two patients with chronic viral hepatitis C(CHC) were recruited at the tropical department Ain Shams University Hospital. As well as, blood samples were collected from 18 healthy normal volunteers during their routine medical checkup.

Venous blood samples from each participant were collected in plain collection tubes without clot activator and centrifuged at 1300xg at 4°C for 20 min. to obtain the serum. Then all sera samples were stored at -80°C until assayed. From all the participants of this study, written informed consent was obtained. The study was performed according to the Declaration of Helsinki, and approved by the Research Ethical Committee at the Faculty of Medicine, Ain Shams University, Egypt (ethical approval number; FWA 000017585). The clinical and demographic data of all the participants have been summarized (Table 1).

2.2. Viral Markers and Serum AFP Detection

Serum anti-HCV antibodies and hepatitis B surface antigen (HB_sAg) were investigated by enzyme-linked immunosorbent assay (ELISA) using commercial kits. Serum AFP was measured using ELISA commercial kits (AbCam, Cambridge, MA).

2.3. Bioinformatics Based Selection of RNA Based Biomarker Network

The identification of the RNA based biomarker network was done in the following steps:

- (i). We used scanning algorithms to search miRNA specific to HCC through several miR public databases, namely, miRWalk database, and miR2Disease. The 2 databases confirmed the correlation between selected hsa-miR-1298 dysregulation and HCC development depending on, fold change ≥ 2.0 , P-value < 0.05 , higher ranking score and novelty.
- (ii). Pathway enrichment analysis revealed that hsa-miR-1298 has a high number of target genes related to carcinogenesis, for example, Mitogen Activated Kinase-Like Protein, exosomes secretion, apoptosis and cell adhesion through Diana database.
- (iii). We chose LncRNA-RP11-583F2.2 targeting hsa-miR-1298 through the database of lncRBA acting as competing endogenous RNA (inCeDB) (<http://gyanxet-beta.com/lncedb/index.php>). LncRNA-RP11-583F2.2 has a higher number of target genes linked to HCC by bioinformatics tools, namely; Database of Cancer Gene Networks from Public Gene Expression Data(TCNG) (Available at <http://tcng.hgc.jp/index.html>) and lncRNA Expression database NRED database (Available at <http://nred.matticklab.com/cgi-bin/lncrnadb.pl/>) (Supplementary Table 1s).

2.4. Exosomal RNA Extraction and Characterization of Exosomes

Exosomal RNA was extracted from sera samples of all participants using exoRNeasy Serum/Plasma MidiKit (Cat. no. 77044, Qiagen, USA) according to manufacturer's instructions, the exoRNeasy Serum/Plasma Kits use a membrane-based affinity binding step to isolate exosomes. Purified exosomes suspensions were examined by A JEOL 1010 transmission electron microscopy (TEM) at various magnifications (Fig. 1). The extracted total RNA from exosomes was reverse transcribed into cDNA as soon as possible with a miScript II RT Kit (Cat. no. 218161, Qiagen, USA), following the manufacturer's protocol for sera samples using Thermo Hybaid PCR express (Thermo Scientific, USA).

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

Exosomal hsa-miR-1298 expression in sera samples was assessed by mixing the total cDNAs with miScript SYBR Green PCR Kit (Cat. no. 218076, Qiagen, Helman Germany), according to the manufacturer's protocol; along with the miScript Universal primer provided by the manufacturer and the forward primer specific to miRNA (Hs_miR-1298_1 miScript Primer Assay, MIMAT0005800, Cat. no. MS00014574).

Exosomal LncRNA-RP11-583F2.2 expression in sera samples was estimated using RT² SYBR Green ROX qPCR Master mix Kit (Cat. no. 330520, Qiagen, Germany) and RT² lncRNA qPCR Primer Assay for Human RP11-583F2.2 (ENST00000583416, Cat. no. LPH24879A).

Table 1. Shows the clinicopathological factors in different groups of the study.

-	Malignant (HCC) N (%)	CHC (HCV) N (%)	Healthy Control N (%)	P	χ^2 (a)
Age:					
≥ 57.8	35 (58.3%)	21 (50%)	9 (50%)	.657 NS	0.839
< 57.8	25 (41.7%)	21 (50%)	9 (50%)		
Sex:					
Male (88)	43 (71.7%)	33 (78.6%)	12 (66.7%)	.582 NS	1.084
Female (32)	17 (28.3%)	9 (21.4%)	6 (33.3%)		
Smoking:					
Smoker (53)	23 (38.3%)	21 (50%)	9 (50%)	.437 NS	1.656
Non-Smoker (67)	37 (61.7%)	21 (50%)	9 (50%)		
HCV-antibodies:					
Positive (94)	52 (86.7%)	42 (100%)	0 (0%)	.000**	79.149
Negative (26)	8 (13.3%)	0 (0%)	18 (100%)		
HBV-sAg:					
Positive(6)	6 (10%)	0 (0%)	0 (0%)	.043*	6.316
Negative(114)	54 (90%)	42 (100%)	18 (100%)		
Cirrhosis:					
Cirrhotic(70)	49(81.7%)	9(21.4%)	0(0%)	.000**	39.840
Non-cirrhotic(50)	11(18.3%)	33(78.6%)	18(100%)		

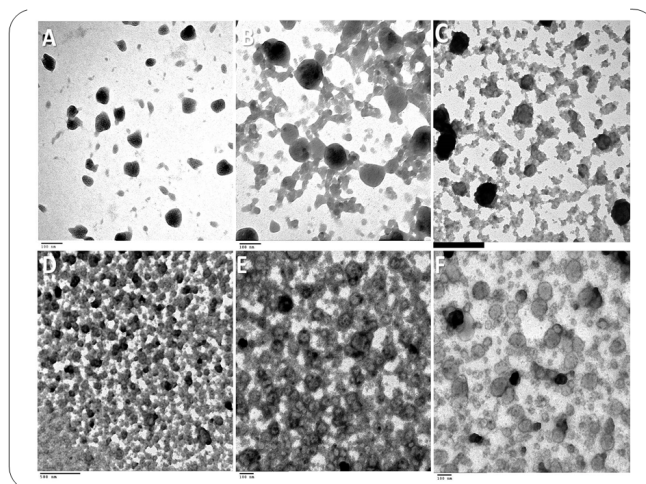


Fig. (1). Transmission electron microscopy image showing the morphology of Exosomes isolated from serum of control group (A&B), HCV serum-derived Exosomes (C) and HCC serum-derived Exosomes (D, E & F). The isolated Exosomes are intact small round shaped nanovesicles, morphologically homogeneous and of different sizes ranging from 30 to 100 nm in size, with a typical round or cup shape appearance (scale bar, 100 nm). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

All the PCR primers were purchased from Qiagen, (Hs_ACTB_1_SG QuantiTect Primer Assay, NM_001101, Cat. no. QT00095431) and both and small nucleolar RNA, C/D box 68 [SNORD-68]) were used as housekeeping genes to normalize our raw data for lncRNA and miRNA respectively. The PCR program for the Rotor gene 5Plex (Qiagen, Hilden, Germany) based qPCR is as follow: firstly, denaturation at 95°C for 15 min; followed by 45 cycles of denatura-

tion for 15 sec at 94°C; then annealing for 30 sec at 55°C. For relative lncRNA-RP11-583F2.2 quantification, the real-time cyler was programmed as follows: initial activation step for 10 min at 95°C to activate HotStarTaq DNA Polymerase. Forty cycles of PCR performed under the following conditions; 15 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C for denaturation, annealing and extension, respectively. Each reaction was carried out in duplicate.

Using the Leviak method, the relative quantification of RNA based biomarker panel expression was calculated, where the $RQ = 2^{-\Delta\Delta C_t}$ method [14]. We compared 2 snoRNAs to identify the best reference gene for relative miR quantification. SNORD-68 was detected in all cases, with higher and more stable expression than RNU-6. Using the Rotor-Gene 5Plex real-time PCR detection system, the threshold cycle (Ct) value of each sample was calculated. Any Ct value more than 36 is considered negative. The results were analyzed by the plot curve analysis of Rotor-Gene software.

2.6. Statistics

All statistical analyses were performed by Statistical Package for the Social Sciences (SPSS software version 20). Comparisons were performed using Krauskul-Wallis, one-way analysis of variance (ANOVA test), and chi-square test, as appropriate. The receiver operating characteristic (ROC) curve was generated to explore the predictive value of selected RNA based biomarker network for HCC. The association between expressions of RNAs and clinicopathological data assessed with the Spearman rank correlation. Two-tailed P value of ≤ 0.05 was considered statistically significant.

3. RESULTS

3.1. Description of the Study Population

There was no statistically significant difference as regard age, sex, smoking and HBV_sAg among the three study groups ($p > 0.05$), details of the clinical data are presented in Table 1.

3.2. TEM Canning of Serum Exosomes Among the Studied Groups

The isolated exosomes are intact small round shaped nanovesicles, morphologically homogeneous and of different sizes ranging from 30 to 100 nm in size, with a typical round or cup shape appearance (scale bar, 100 nm). TEM images in (Fig. 1A and B) shows a low level of abundance of exosomes in healthy control samples compared with more abundance exosomes noted chronic hepatitis C (HCV) patient, as shown in the image in (Fig. 1C). However, the highest abundance of exosomes was found in the HCC patients as per images when compared with healthy control and chronic HCV induction patients (Fig. 1D, E and F).

3.3. Expression of Serum Exosomal ncRNAs Among the Study Groups

The exosomal ncRNAs biomarker levels based on RQ values in serum have been summarized (Table 2). The median (RQ) were, 2.77, 2.3, 0.411, for miRNA-1298 and 0.96, 0.643 and 29.9 for lncRNA-RP11-583F2.2 in healthy control, CHC group and malignant group (HCC) respectively. Compared with the non-malignant groups, the malignant group (HCC) had a higher expression of lncRNA-RP11-583F2.2 and lower expression of miRNA-1298 ($p < 0.01$) in the serum. The positivity rate of the serum exosomal (miRNA-1298 and lncRNA-RP11-583F2.2) was 95% and 96.7% respectively in the malignant group. However, they were 0% in normal individuals ($p < 0.01$), as shown in Table 2, Figs. (2A, B and 3).

3.4. Correlation of the Serum Exosomal with Demographic and Clinical Factors

There was no statistically significant difference between fold change (RQ) value of exosomal miRNA-1298 and lncRNA-RP11-583F2.2 and the different clinicopathological factors within the malignant group (HCC) ($P > 0.05$) as shown in Table 3A. There was no statistically significant correlation between fold change (RQ) value of exosomal miRNA-1298 and lncRNA-RP11-583F2.2 and the different laboratory parameters within the malignant group (HCC) ($P > 0.05$) as shown in Table 3B. Interestingly, there was a highly significant negative correlation between exosomal miRNA-1298 and lncRNA-RP11-583F2.2 based on fold change (RQ) among the three study groups. While, there was no significant correlation between exosomal miRNA-1298 and lncRNA-RP11-583F2.2 based on fold change (RQ) in malignant group as shown in Table 4.

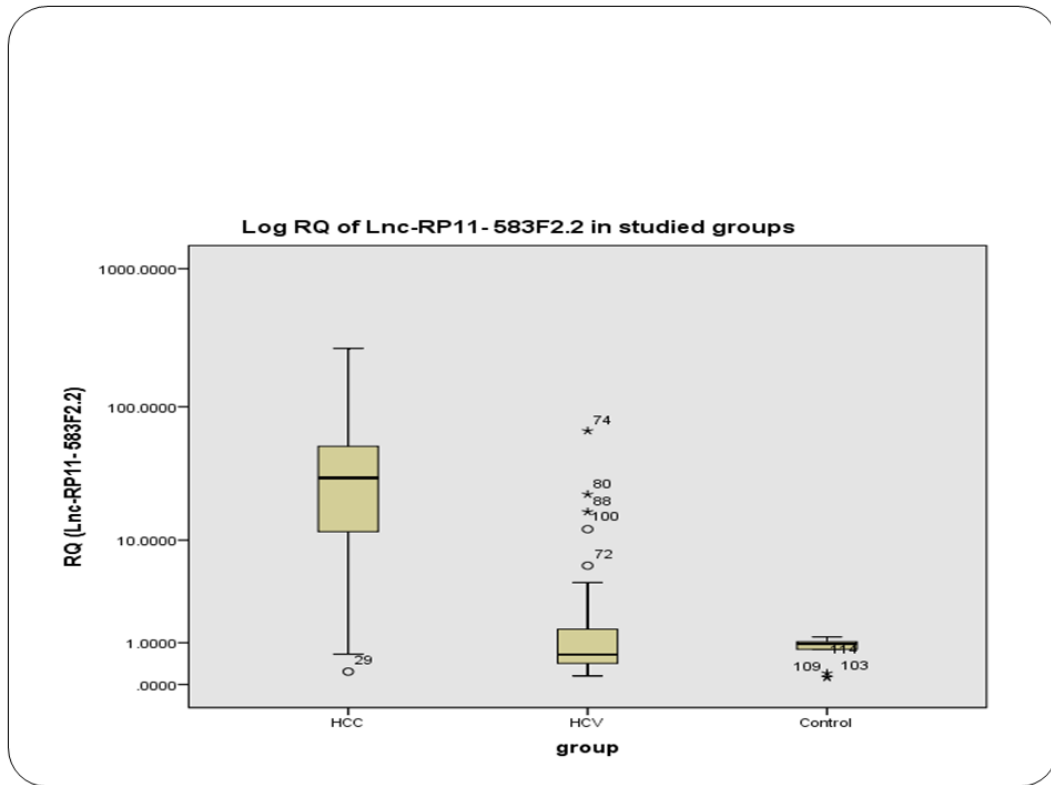
3.5. Accuracy of Serum Parameters for Predicting HCC by ROC Analysis

The use of ROC curves analysis and values of the area under the curve (AUC) evaluate the ncRNAs diagnostic value as shown in Supplementary Figs. (1s a-d).

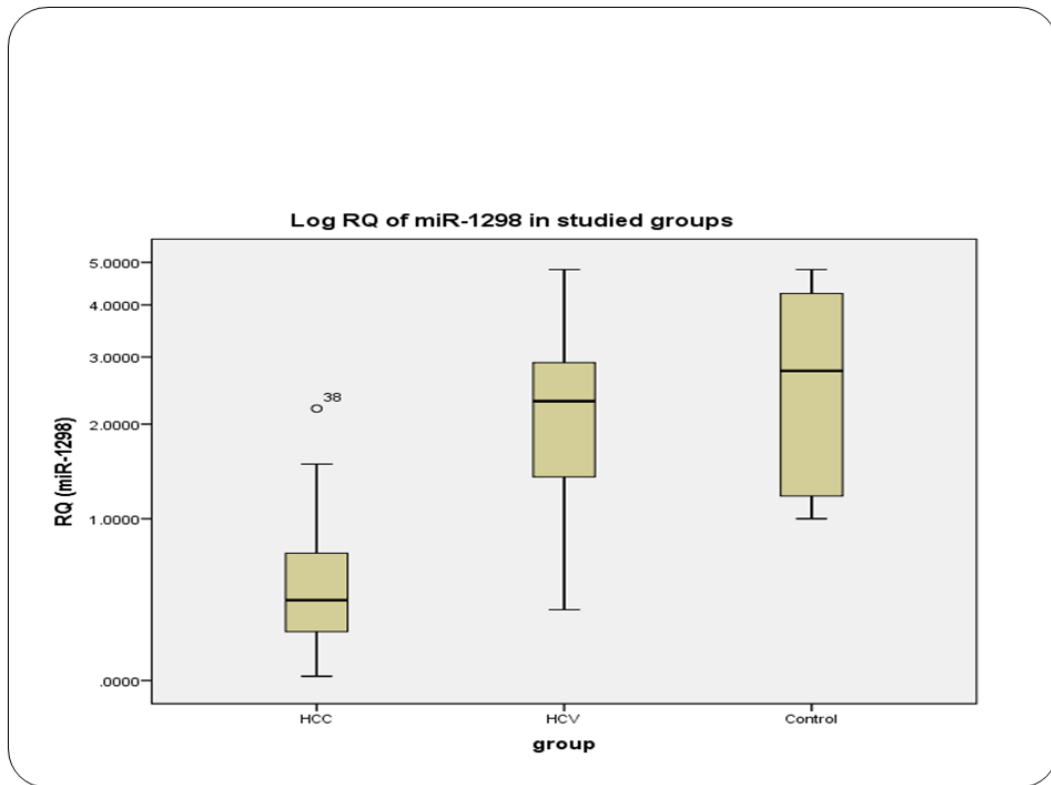
Regarding the HCC patients versus CHC patients and healthy control, the best discriminating cutoff values of miRNA-1298 and lncRNA-RP11-583F2.2 were ≤ 0.965 and ≥ 5.02 respectively. Accordingly, the sensitivities were 95% and 96.7% respectively and specificity were 98.3% and 91.7% respectively, which indicated that these threshold values

Table 2. Shows the median (RQ) and the positivity rate of serum exosomal RNA based biomarkers among the study groups.

-	Malignant (HCC)	CHC (HCV)	Healthy Control	P	$\chi^2(a)$
RQ of miRNA-1298	0.41	2.3	2.77	.000**	81.344
RQ of Lnc-RNA-RP11-583F2.2	29.9	0.643	.96	.000**	71.162
Positivity rate of miRNA-1298	57 (95%)	1 (2.4%)	0 (0%)	.000 **	104.678
Positivity rate of Lnc-RNA-RP11-583F2.2	58 (96.7%)	5 (11.9%)	0 (0%)	.000 **	86.022



(A)



(B)

Fig. (2). BOXPLOT: Serum exosomal lncRNA-RP11-583F2.2 and exosomal RAB11A mRNA as determined by qRT-PCR among the HCC, CHC, and healthy control groups. (A) LncRNA-RP11-513115.6; (B) Has-miR-1298; The data are presented as the median fold changes ($P < .05$). CHC, chronic hepatitis C virus; HCC, hepatocellular carcinoma; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Table 3A. Shows correlation of the serum level of RNA based biomarkers with different clinicopathological factors within the malignant group.

Clinicopathological Factors	RQ of miR-1298	RQ of Lnc-RNA-RP11-583F2.2
Mean age :		
≥57.8 years	.33	33.12
<57.8 years	.52	29.85
P, χ^2 (a)	133 NS, 2.256	.808 NS, .059
Sex :		
Male	.40	21.25
Female	.51	41.35
P, χ^2 (a)	.131 NS, 2.285	.489 NS, .478
Smoking :		
Smoker	.41	29.85
Non-smoker	.40	30.06
P, χ^2 (a)	.30 NS, 1.072	.257 NS, 1.286
HCV-Ab :		
Positive	.41	29.96
Negative	.46	26.82
P, χ^2 (a)	.296 NS, 1.093	.573 NS, .318
HBV-sAg :		
Positive	.39	41.09
Negative	.41	28.76
P, χ^2 (a)	.554 NS, .351	.632 NS, .230
Cirrhosis:		
Cirrhotic	.40	27.66
Non-cirrhotic	.42	35.01
P, χ^2 (a)	.491 NS, .475	.496 NS, .464
Child-Pugh score :		
A2	.236	5.61
A5	.524	11.31
A6	.408	44.94
B6	.459	25.63
B7	.476	49.40
B8	.414	29.85
C10	.407	19.40
P, χ^2 (a)	.575 NS, 4.762	.651NS, 4.19
BCLC stage :		
Early	.41	30.92
Late	.40	19.40
P, χ^2 (a)	.167 NS, 1.91	.632 NS, .230
Average Tumor size :		
< 3 cm	.408	28.76
≥ 3 cm	.903	57.81
P, χ^2 (a)	.741NS, .109	.789 NS, .071

Table 3B. Shows correlation of RQ of *miR-1298* and RQ of *Lnc-RNA RP11-583F2.2* with different laboratory parameters within the malignant group.

Spearman's rho								
-	-	AST	AST	Albumin	total bilirubin	direct bilirubin	INR	AFP
RQ (<i>miR-1298</i>)	Correlation Coefficient	-.017-	.175	-.127-	-.021-	.155	.107	.021
	Sig. (2-tailed)	.896	.181	.333	.871	.236	.417	.873
RQ (<i>Lnc-RNA RP11-583F2.2</i>)	Correlation Coefficient	-.117-	.186	-.005-	-.049-	-.115-	.088	-.244-
	Sig. (2-tailed)	.373	.154	.967	.710	.380	.503	.060

Table 4. Shows the correlation between RQ of *miR-1298* and RQ of *Lnc-RNA RP11-583F2.2* among all the study group and malignant group.

Group Type	-	RQ (<i>miR-1298</i>)	RQ (<i>Lnc-RNA RP11-583F2.2</i>)
All groups	RQ (<i>miR-1298</i>)	Correlation Coefficient	1.000
		Sig.	.000
	RQ (<i>Lnc-RNA RP11-583F2.2</i>)	Correlation Coefficient	-.602**
		Sig.	.000
Malignant	RQ (<i>miR-1298</i>)	Correlation Coefficient	.138
		Sig.	.293
	RQ (<i>Lnc-RNA RP11-583F2.2</i>)	Correlation Coefficient	.138
		Sig.	.293

could be used to distinguish malignant group (HCC) from non-malignant groups (CHC patients and healthy subjects) as shown in Supplementary Tables 2s and 3s.

4. DISCUSSION

The incidence of liver cancer has increased more than triple since 1980. Since 2000, liver cancer death rates have increased by almost 3% per year [2].

In Egypt, HCC represents an important public health problem, the estimated number of liver cancer cases in Egypt 2013 was 27,991 for both sexes and expected to be 85,471 for both sexes in 2050 due to population growth [15].

Therefore, the aim of bioinformatics in the cancer biomarker discovery is to give priority lists of marker candidates with the preferred sensitivity and specificity [16].

Currently, tumor-derived exosomes have shown potential in the field of cancer [17]. The contents of tumor-derived exosomes, such as miRNAs, lncRNAs and oncoproteins reflect pathophysiological status of their endosomal origin [18, 19]. Some studies reported that exchange of exosomal RNAs and proteins not only plays a major role in onset and progression of HCC but also it may be considered as potential noninvasive markers in addition to targets of therapy [20]. The cells of hepatocellular carcinoma excrete high amounts

of exosomes whose participation in pathogenesis is actually under investigation. Though the use of HCC derived exosomes as potential diagnostic and prognostic biomarkers is still at the primary stage, but there are supporting observations that exosomes released from HCC cells are different in RNA and protein content that released from untransformed cells [21]. Tumor-derived exosomes are protected against degrading enzymes (e.g., RNases), as they are enclosed in a lipid bilayer membrane [22]. Interestingly, these enriched mRNAs, miRNAs, lncRNAs and oncoproteins in tumor-derived exosomes are selected, suggesting that the exosomal content may provide novel serological biomarkers for various types of cancer [23-26].

In light of these findings, bioinformatics analysis was used to choose *hsa-miR-1298* and *lncRNA-RP11-583F2.2* as promising non-coding RNAs relevant to hepatocellular carcinoma based on previous microarray studies followed by clinical validation in HCC patients versus control.

Fornari *et al.*, (2015) stated the exosomal secretion of miRNA-21, miRNA-221a, miRNA-519d and miRNA-1228 from hepatocellular carcinoma patients and showed a relation between serum and tissue levels of miRNA-21, miRNA-494, and miRNA-519d [27]. Furthermore, Sugimachi *et al.* (2015) explored serum exosomal biomarkers that may predict HCC recurrence after surgery [28]. Kogure *et al.*, (2013)

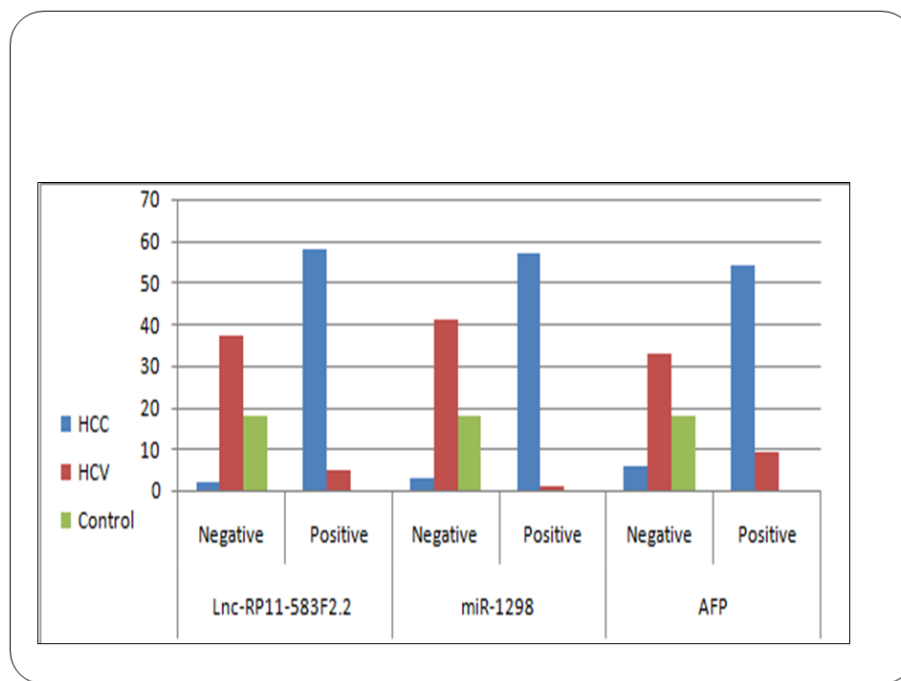


Fig. (3). Bar chart showing the positivity rates for serum exosomal lncRNA-RP11-583F2.2, and exosomal Hsa-miR-1298 expression among the HCC, the CHC, and healthy control groups. The data are presented as percentages, and * indicates $P < .05$ compared with the control group (chi-square test). CHC, chronic hepatitis C virus; HCC, hepatocellular carcinoma. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

reported that the certain miRNAs enriched in exosomes released from HCC cells *in vitro* e.g., miR-133b, miR142-5p, miR-215, miR-367, miR-376a, miR-378, miR-451, miR-517c, miR-518d, miR- 520f and miR-584 [29]. In addition, Liu *et al.*, (2015) stated that miR-10b and miR-21 were enriched in exosomes released from HCC cells *in vivo* [30]. Wei *et al.*, (2015) found miR-10b-5p and miR-486-5p were highly abundant in exosomes in the SMMC-7721 HCC cell line but less abundant in cellular RNAs. While, let-7b-5p, let-7d-5p, and let-7c-5p were highly abundant in cellular miRNAs, but occurred only in very low numbers in exosomal RNAs. These results revealed selective and specific enrichment of exosomes with specific miRNAs in HCC cells [31]. Zhou *et al.*, (2016) stated that miR-1298 inhibits mutant KRAS-driven tumor growth by repressing tyrosine kinase FAK and the laminin subunit LAMB3 [32].

Hsa-miR-1298 is a microRNA gene, and is affiliated with undefined RNA class, located on the X chromosome (Xq23), (114,715,233bp - 114,715,344bp) with the length of 112 bases. Fornari *et al.*, stated the excretion of circulating miRNA-21, miRNA-221a, miRNA-519d and miRNA-1228 from HCC patients [33]. The present study reports that exosomal *miR-1298* expression down-regulated in patients of hepatocellular carcinoma compared with patients of hepatitis C virus and normal good health control.

Increasing evidence also pointed out lncRNA's role as transmissible molecules in hepatocellular carcinoma, in addition to use exosomes to carry them. Kogure *et al.*, (2013) reported that the long noncoding RNA TUC339 was significantly up-regulated in HCC derived exosomes and this lncRNA TUC339 was mediated in the growth of the tumor, adhesion of cell and progression of cell cycle [28]. Liu *et al.*, (2017) stated

that lncRNA-RP11-62F24.2 was found significantly expressed in tissues of gastric cancer in comparison with normal gastric tissue ($p < 0.05$) and its expression level was significantly correlated with invasion and tumor size [34].

Meanwhile, Jingxu *et al.*, (2015) reported that lncRNA-RP11-119F7.4 expression was down-regulated in gastric cancer [35].

Abd El Gwad *et al.*, (2018) reported that the panel of 3 exosomal RNA-based biomarkers (*lncRNA-RP11-513I15.6*, *miR-1262*, and *RAB11A*) showed excellent sensitivity and specificity in discriminating HCC patients from CHC patients and healthy controls [36].

There was a statistically negative correlation between *miR-1298* and *lncRNA RP11-583F2.2*, mRNA statistical significance ($P < 0.01$). It seemed that *lncRNA RP11-583F2.2* may act as a sponge for *miR-1298* inhibiting its action suggesting the hypothesis of acting as competing endogenous RNA alongside possible participation in HCC pathogenesis [12]. These statistical associations agree with insilco data analysis for retrieval of ncRNAs specific to HCC. It is worth noting that the identification of the aforementioned statistical correlation where HCC cells can be targeted with knocking out lncRNA activity, to turn off specific target genes may have significant therapeutic potential in HCC.

To the best of our knowledge, the present study is the first to report that exosomal *lncRNA-RP11-583F2.2* expression up-regulated in the serum of hepatocellular carcinoma patients as compared with hepatitis C virus patients and normal good health control so exosomal *lncRNA-RP11-583F2.2* may be used as a potential novel biomarker in the diagnosis of HCC.

Our results indicated that *miR-1298* and *lncRNA-RP11-583F2.2* were superior to the AFP in sensitivity and specificity (95%, 96.7%) and (98.3%, 91.7%) respectively as compared to AFP(90%,85%). The selected *miR-1298* and *lncRNA-RP11-583F2.2* reduce false negative results as compared to AFP.

The findings of this study demonstrated that the estimation of serum exosomal *miR-1298* and *lncRNA-RP11-583F2.2* could have a potential diagnostic and therapeutic target in patients with HCC in the future especially when used in combination.

The study limitations include a relatively small sample size. Therefore, larger multicenter studies are needed for validation. Although, this study addressed the statistical association of the *lncRNA-RP11-583F2.2* by linking it to *miR-1298* but more mechanistic studies are needed.

CONCLUSION

In conclusion, the presented strategy enables us to identify a significant differential exosomal miRNA-1298 and *lncRNA-RP11-583F2.2* expression between HCC and control with high accuracy through *in silico* data analysis followed by clinical validation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of Faculty of Medicine, Ain Shams University, Egypt (ethical approval number; FWA 000017585).

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are the basis of this research. All human research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Written informed consent was taken from all the participants of this study.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

Ain Shams Faculty of Medicine, Grants Office and Grant No. 2016-36 supported this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

- [1] Torre, L.A.; Bray, F.; Siegel, R.L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A. Global cancer statistics, 2012. *CA Cancer J. Clin.*, **2015**, *65*(2), 87-108. <http://dx.doi.org/10.3322/caac.21262> PMID: 25651787
- [2] *Cancer facts and figures Atlanta*; American Cancer Society: Atlanta, GA, **2019**; 1-71.
- [3] National Cancer Registry Program of Egypt. *Magnitude of hepatocellular carcinoma in Egypt*, **2011**.
- [4] Elghazaly, H.; Gaballah, A.; Bahie Eldin, N. Clinic-pathological pattern of hepatocellular carcinoma (HCC) in Egypt. *Ann. Oncol.*, **2018**, *29*(5), v5-v6. <https://doi.org/10.1093/annonc/mdy151.018>
- [5] Amer, N.A.; Gemaay, M.A.; Mohamed, A.E.; Hussein, M.M.; Shehad, I. Prevalence of viral hepatitis in Egyptian patients with hepatocellular carcinoma. *Egyptian Liver J.*, **2013**, *3*(1), 6-9. <http://dx.doi.org/10.1097/01.ELX.0000424247.25858.79>
- [6] Papandreou, I.; Lim, A.L.; Laderoute, K.; Denko, N.C. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. *Cell Death Differ.*, **2008**, *15*(10), 1572-1581. <http://dx.doi.org/10.1038/cdd.2008.84> PMID: 18551130
- [7] Jin, S.; White, E. Tumor suppression by autophagy through the management of metabolic stress. *Autophagy*, **2008**, *4*, 563-566. <http://dx.doi.org/10.4161/auto.5830>
- [8] Christianson, H.C.; Svensson, K.J.; van Kuppevelt, T.H.; Li, J.P.; Belting, M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc. Natl. Acad. Sci. USA*, **2013**, *110*(43), 17380-17385. <http://dx.doi.org/10.1073/pnas.1304266110> PMID: 24101524]
- [9] Gajos-Michniewicz, A.; Duechler, M.; Czyz, M. MiRNA in melanoma-derived exosomes. *Cancer Lett.*, **2014**, *347*(1), 29-37. <http://dx.doi.org/10.1016/j.canlet.2014.02.004> PMID: 24513178
- [10] Stoorvogel, W.; Kleijmeer, M.J.; Geuze, H.J.; Raposo, G. The biogenesis and functions of exosomes. *Traffic*, **2002**, *3*, 321-30. <https://doi.org/10.1034/j.1600-0854.2002.30502.x>
- [11] Pant, S.; Hilton, H.; Burczynski, M.E. The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. *Biochem. Pharmacol.*, **2012**, *83*(11), 1484-1494. <http://dx.doi.org/10.1016/j.bcp.2011.12.037> PMID: 22230477
- [12] Sohn, W.; Kim, J.; Kang, S.H.; Yang, S.R.; Cho, J.-Y.; Cho, H.C.; Shim, S.G.; Paik, Y.-H. Serum exosomal microRNAs as novel biomarkers for hepatocellular carcinoma. *Exp. Mol. Med.*, **2015**, *47*, e184. <http://dx.doi.org/10.1038/emmm.2015.68> PMID: 26380927
- [13] Takahashi, K.; Yan, I.K.; Kogure, T.; Haga, H.; Patel, T. Extracellular vesicle-mediated transfer of long non-coding RNA ROR modulates chemosensitivity in human hepatocellular cancer. *FEBS Open Bio*, **2014**, *4*, 458-467. <http://dx.doi.org/10.1016/j.fob.2014.04.007> PMID: 24918061
- [14] Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, **2001**, *25*(4), 402-408. <http://dx.doi.org/10.1006/meth.2001.1262> PMID: 11846609
- [15] Amal, S.I.; Hussein, M.K.; Nabel, N.H.; Hoda, B.; Hossam, K. Cancer incidence in Egypt: results of the national population-based cancer registry program. *J. Cancer Epidemiol.*, **2014**, *43*7971, 18. <http://doi.org/10.1155/2014/437971>
- [16] Nair, M.; Sandhu, S.S.; Sharma, A.K. Prognostic and predictive biomarkers in cancer. *Curr. Cancer Drug Targets*, **2014**, *14*(5), 477-504. <http://dx.doi.org/10.2174/1568009614666140506111118> PMID: 24807144
- [17] Azmi, A.S.; Bao, B.; Sarkar, F.H. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer Metastasis Rev.*, **2013**, *32*(3-4), 623-642. <http://dx.doi.org/10.1007/s10555-013-9441-9> PMID: 23709120
- [18] Kogure, T.; Lin, W.-L.; Yan, I.K.; Braconi, C.; Patel, T. Intercellu-

- lar nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. *Hepatology*, **2011**, *54*(4), 1237-1248.
http://dx.doi.org/10.1002/hep.24504 PMID: 21721029
- [19] Ji, H.; Greening, D.W.; Barnes, T.W.; Lim, J.W.; Tauro, B.J.; Rai, A.; Xu, R.; Adda, C.; Mathivanan, S.; Zhao, W.; Xue, Y.; Xu, T.; Zhu, H.J.; Simpson, R.J. Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. *Proteomics*, **2013**, *13*(10-11), 1672-1686.
http://dx.doi.org/10.1002/pmhc.201200562 PMID: 23585443
- [20] Laura, S.; Cecilia, B.; Claudia, M.; Franca, C.; Raffaele, S.; Carla, C. Functional roles and therapeutic applications of exosomes in hepatocellular carcinoma. *BioMed Res. Int.*, **2017**, *2017*, 1-8.
https://doi.org/10.1155/2017/2931813
- [21] He, M.; Qin, H.; Poon, T.C.; Sze, S.-C.; Ding, X.; Co, N.N.; Ngai, S.-M.; Chan, T.-F.; Wong, N. Hepatocellular carcinoma-derived exosomes promote motility of immortalized hepatocyte through transfer of oncogenic proteins and RNAs. *Carcinogenesis*, **2015**, *36*(9), 1008-1018.
http://dx.doi.org/10.1093/carcin/bgv081 PMID: 26054723
- [22] Yuyama, K.; Sun, H.; Mitsutake, S.; Igarashi, Y. Sphingolipid-modulated exosome secretion promotes clearance of amyloid- β by microglia. *J. Biol. Chem.*, **2012**, *287*(14), 10977-10989.
http://dx.doi.org/10.1074/jbc.M111.324616 PMID: 22303002
- [23] Melo, S.A.; Luecke, L.B.; Kahlert, C.; Fernandez, A.F.; Gammon, S.T.; Kaye, J.; LeBlou, V.S.; Mittendorf, E.A.; Weitz, J.; Rahbari, N.; Reissfelder, C.; Pilarsky, C.; Fraga, M.F.; Piwnica-Worms, D.; Kalluri, R. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*, **2015**, *523*(7559), 177-182.
http://dx.doi.org/10.1038/nature14581 PMID: 26106858
- [24] Avgeris, M.; Panoutsopoulou, K.; Papadimitriou, M.A.; Scorilas, A. Circulating exosomal miRNAs: clinical significance in human cancers. *Expert Rev. Mol. Diagn.*, **2019**, *19*(11), 979-995.
http://dx.doi.org/10.1080/14737159.2019.1673732 PMID: 31594418
- [25] Alegre, E.; Zubiri, L.; Perez-Gracia, J.L.; González-Cao, M.; Soria, L.; Martín-Algarra, S.; González, A. Circulating melanoma exosomes as diagnostic and prognosis biomarkers. *Clin. Chim. Acta*, **2016**, *454*, 28-32.
http://dx.doi.org/10.1016/j.cca.2015.12.031 PMID: 26724367
- [26] Suchorska, W.M.; Lach, M.S. The role of exosomes in tumor progression and metastasis (Review). *Oncol. Rep.*, **2016**, *35*(3), 1237-1244.
http://dx.doi.org/10.3892/or.2015.4507 PMID: 26707854
- [27] Fornari, F.; Ferracin, M.; Trerè, D.; Milazzo, M.; Marinelli, S.; Galassi, M.; Venerandi, L.; Pollutri, D.; Patrizi, C.; Borghi, A.; Foschi, F.G.; Stefanini, G.F.; 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.
http://dx.doi.org/10.1371/journal.pone.0141448 PMID: 26509672
- [28] Sugimachi, K.; Matsumura, T.; Hirata, H.; Uchi, R.; Ueda, M.; Ueo, H.; Shinden, Y.; Iguchi, T.; Eguchi, H.; Shirabe, K.; Ochiya, T.; Maehara, Y.; Mimori, K. Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. *Br. J. Cancer*, **2015**, *112*(3), 532-538.
http://dx.doi.org/10.1038/bjc.2014.621 PMID: 25584485
- [29] Kogure, T.; Yan, I.K.; Lin, W.-L.; Patel, T. Extracellular vesicle-mediated transfer of a novel long noncoding RNA TUC339: a mechanism of intercellular signaling in human hepatocellular cancer. *Genes Cancer*, **2013**, *4*(7-8), 261-272.
http://dx.doi.org/10.1177/1947601913499020 PMID: 24167654
- [30] Liu, W.-H.; Ren, L.-N.; Wang, X.; Wang, T.; Zhang, N.; Gao, Y.; Luo, H.; Navarro-Alvarez, N.; Tang, L.J. Combination of exosomes and circulating microRNAs may serve as a promising tumor marker complementary to alpha-fetoprotein for early-stage hepatocellular carcinoma diagnosis in rats. *J. Cancer Res. Clin. Oncol.*, **2015**, *141*(10), 1767-1778.
http://dx.doi.org/10.1007/s00432-015-1943-0 PMID: 25724413
- [31] Wei, J.-X.; Lv, L.-H.; Wan, Y.L.; Cao, Y.; Li, G.L.; Lin, H.-M.; Zhou, R.; Shang, C.-Z.; Cao, J.; He, H.; Han, Q.-F.; Liu, P.-Q.; Zhou, G.; Min, J. Vps4A functions as a tumor suppressor by regulating the secretion and uptake of exosomal microRNAs in human hepatoma cells. *Hepatology*, **2015**, *61*(4), 1284-1294.
http://dx.doi.org/10.1002/hep.27660 PMID: 25503676
- [32] Zhou, Y.; Dang, J.; Chang, K.Y.; Yau, E.; Aza-Blanc, P.; Moscat, J.; Rana, T.M. miR-1298 inhibits mutant KRAS-driven tumor growth by repressing FAK and LAMB3. *Cancer Res.*, **2016**, *76*(19), 5777-5787.
http://dx.doi.org/10.1158/0008-5472.CAN-15-2936 PMID: 27698189
- [33] Fornari, F.; Pollutri, D.; Patrizi, C.; La Bella, T.; Marinelli, S.; Casadei Gardini, A.; Marisi, G.; Baron Toaldo, M.; Baglioni, M.; Salvatore, V.; Callegari, E.; Baldassarre, M.; Galassi, M.; Giovannini, C.; Cescon, M.; Ravaoli, M.; Negrini, M.; Bolondi, L.; Gramantieri, L. In hepatocellular carcinoma miR-221 modulates sorafenib resistance through inhibition of caspase-3-mediated apoptosis. *Clin. Cancer Res.*, **2017**, *23*(14), 3953-3965.
http://dx.doi.org/10.1158/1078-0432.CCR-16-1464 PMID: 28096271
- [34] Liu, C.; Cao, B.; Liu, N.; Zhou, Z.; Yang, G.; Zhou, P. Increased expression of long noncoding RNA RP11-62F24.2 in gastric cancer and its clinical significance. *Clin. Lab.*, **2017**, *63*(9), 1475-1479.
http://dx.doi.org/10.7754/Clin.Lab.2017.170334 PMID: 28879717
- [35] Sun, J.; Song, Y.; Chen, X.; Zhao, J.; Gao, P.; Huang, X.; Xu, H.; Wang, Z. Novel long non-coding RNA RP11-119F7.4 as a potential biomarker for the development and progression of gastric cancer. *Oncol. Lett.*, **2015**, *10*(1), 115-120.
http://dx.doi.org/10.3892/ol.2015.3186 PMID: 26170986
- [36] Abd, E.L.; Gwad, A.; Matboli, M.; El-Tawdi, A.; Habib, E.K.; Shehata, H.; Ibrahim, D.; Tash, F. Role of exosomal competing endogenous RNA in patients with hepatocellular carcinoma. *J. Cell. Biochem.*, **2018**, *119*(10), 8600-8610.
http://doi.org/10.1002/jcb.27109 PMID: 30015383