Original Article

Medical Journal of Viral Hepatitis (MJVH)

Diagnostic value of Long Non-Coding RNA ZFAS1 as a Potential Biomarker for Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related deaths globally. Long non-coding RNAs (lncRNAs) may be considered as potential markers for HCC. The aim of this study is to evaluate the diagnostic and prognostic value of lncRNA ZFAS1 in HCC patients.

Materials and methods: The current study included 100 cirrhotic patients with HCC, in addition to 100 cirrhotic patients without HCC as control group. RNA extraction was performed for quantification of ZFAS1 expression by real-time quantitative polymerase chain reaction.

Results: ZFAS1 gene expression was significantly elevated in HCC group of patients compared to non-HCC group. The ability to distinguish HCC from cirrhotic controls by ZFAS1 was determined to be 1.00 (95% CI:1.00-1.00). Comparison between the diagnostic performance of ZFAS1 and AFP in differentiating HCC on top of cirrhosis from cirrhosis without HCC showed that, at cutoff values ≥ -4.65 ZFAS1 gene had 100 % specificity and 99% sensitivity while, AFP at cutoff value ≥ 9.4 ng/ml had 100 % specificity and 76% sensitivity. Comparison of AUC for the two parameters demonstrated a significantly higher AUC for ZFAS1 than for AFP (Difference = 0.168, P <0.001). No statistically significant correlation between ZFAS1gene and any HCC characteristic however, a statistically significant correlation between AFP level and sex , BCLC staging was found.

Conclusions: lncRNA ZFAS1 is a good diagnostic marker for HCC in cirrhotic patients with high sensitivity and specificity value, however ZFAS1has no prognostic importance in patients with HCC.s

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cause of cancer-related deaths globally [1]. Recent global cancer statistics indicate that primary liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer death [2]. The incidence of HCC has reportedly doubled in Egypt during the past decade, making it a particularly challenging health problem for this developing country [3]. Liver cirrhosis is the majour risk factors contributing to hepatocarcinogenesis via a series of complex interactions involving dysregulation of the cell cycle with increased apoptosis [4]. Although advancements in HCC diagnosis and therapy have progressed over time, the incidence of HCC has continued to rise [5], therefore necessitating the urgency for development of new diagnostic and therapeutic approaches to improve the clinical outcomes of this disease. Long non-coding RNAs (lncRNAs) are a class of RNAs lacking the ability to encode proteins. Earlier studies have proposed that, non-coding RNAs are “transcriptional noise” production during gene expression. Studies including sequencing technologies found that, lncRNAs have been closely concerned with cancer occurrence, progression, and prognosis [6, 7].

Most ncRNAs consist of long ncRNAs (lncRNAs) which contain over 200 nucleotides and participate substantially in processes of cell growth and differentiation and gene expression regulation [9, 10]. To date, the techniques of high-throughput sequencing and microarrays have identified upwards of 50,000 lncRNAs and counting [11], most of which are expressed in different malignancies including HCC [12], where they have been shown to regulate numerous cell processes including proliferation, epithelial-mesenchymal transition (EMT), angiogenesis, metastasis and autophagy [13]. The nature of circulating RNA has garnered widespread attention[14]. Long ncRNAs (lncRNAs) warrant consideration as potential markers for HCC based on findings that, they undergo cancer specific alterations when compared with protein-coding genes as well as their easy detectability as circulating lncRNAs in clinical samples of blood and urine of cancer patients [13-16].

Most lncRNAs maintain unspecified roles in HCC, but a small number of lncRNAs have been shown to take part in initiation and progression of this malignancy [17]. The expression of lncRNA HULC (highly upregulated in liver cancer) is increased in HCC [18]. Long lncRNA HULC has been shown to play a role in promotion of HCC growth, metastasis, and development of drug resistance [18-20]. Moreover, lncRNA DANCR has been shown to differentiate HCC patients from those without HCC more effectively when compared with AFP [21]. miR-150 inhibited HCC cell invasion by inhibiting ZEB1 and the matrix metalloproteinases MMP14 and MMP16. ZFAS1, a newly identified lncRNA, was shown to be dysregulated in many cancers and is involved in tumorigenesis and cancer

Keywords: Non-Coding RNA ZFAS1,hepatocellular Carcinoma, liver cirrhosis Received: 16-2-2022; Accepted: 26-3-2022

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progression. ZFAS1 functions as an oncogene in HCC progression by binding miR-150 and abrogating its tumor-suppressive function [22]. The aim of this study is to evaluate the diagnostic and prognostic value of lncRNA ZFAS1 in HCC patients.

Materials and methods

The current study included 100 patients with hepatocellular carcinoma on topof cirrhosis and 100 non-HCC cirrhotic patients as control group. All patients recruited from Mansoura University Hospitals from January 2018 till January 2019. Inclusion criteria for this study were adult patients (age above 18) with positive markers for hepatitis C virus (HCV), but negative markers for hepatitis B virus (HBV) and HIV. Patients were excluded if HCC was associated with other types of malignancy. The study was approved by the Mansoura Ethical Committee and written consent was obtained from each participant.

Diagnosis and staging of HCC

HCC was doubted by the recognition of a hepatic focal lesion by ultrasound or with elevated AFP level and confirmed by triphasic MSCT based on the characteristic arterial enhancement and early washout in delayed phase [23].

Laboratory investigation:

Ten milliliters of blood were obtained from each patients and divided into two sample; EDTA-free sample was used for determination of liver function tests (included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and albumin using spinreact kits and AFP) and blood sample in EDTA was subjected to RNA extraction for quantification of ZFAS1 expression by real-time quantitative polymerase chain reaction (qPCR).

RNA isolation and cDNA synthesis:

Isolation of total RNA was performed from 300µl plasma using blood total RNA isolation kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was measured by Nanodrop 2000 spectrophotometer (Thermo, CA, U.S.A.). The reverse-transcription reaction was performed using PrimeScript II cDNAsynthesis kit with gDNA Eraser (Takara, Dalian, China). The reaction conditions for cDNA synthesis involved incubation at 42°C for 2 min to remove the contaminated DNA, then 37°C for 15 min, and finally at 85°C for 5 sec.

Real-time PCR analysis

Real-time quantitative PCR assay was performed on Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) using SYBR-green I Premix Ex Taq following the instructions for users. The reaction protocol involved incubation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, 63.7°C for 30 sec, and 72°C for 30 sec. Primers for lncRNAsZFAS1 and 18S used as the endogenous control were listed the table below. Samples were analyzed in duplicate with no-template controls performedsimultaneously [24].

Results

Table1 show the primer sequence of for lncRNAsZFAS1. **Table 2** depicts the characteristics of HCC cases shows that, male sex predominated, with a mean age of 50.6 years, the majorty of patients had single lesion, twelve cases had two masses and eight cases had three masses, while all other cases had solitary lesion.

Table 3 shows statistically significant higher levels of AST, ALT, serum total bilirubin, AFP, and ZFAS1 gene in HCC group of patients compared with non-HCC patients.

The ROC curve analysis demonstrated that, a cut-off value of ≥ -4.65 for ZFAS1 gene and ≥ 9.4 mg/dL for AFP had optimum discriminative power (**Figure 1, Figure2**).

Table 4 shows that a cut-off values ≥ -4.65 ZFAS1 gene had 100 % specificity and 99% sensitivity versus 100 % specificity and 76% sensitivity for AFP at cutoff value ≥9.4 ng/ml. Comparison between the two AUCs showed that AUC for ZFAS1 gene was statistically significantly higher than that for AFP (Difference = 0.168, SE = 0.034, z statistic = 4.941, P < 0.001).

Table 5 shows no statistically significant correlation between ZFAS1gene and any HCC characteristic however, a statistically significant correlation between AFP level and sex, BCLC staging was found.

Table 1. The Primer sequence

	Primer sequence
ZFAS1	5-ACGTGCAGACATCTACAACCT--3 5-TACTTCCAACACCCGCAT-3
18S	5-CAGCCACCCGAGATTGAGCA-3 5-TAGTAGCGACGGGCGGTGTG-3

Table 2. Characteristics of hepatocellular carcinoma cases.

Characteristic	Statistic
Sex	N
Male	74
Female	26
Mean age (years) ± SD	50.6 ± 5.6
AST (IU/L)	60 (46 – 100)
ALT (IU/L)	47.5 (36 – 76)
Serum total bilirubin (mg/dl)	0.9 (0.7 – 1.5)
Serum direct bilirubin (mg/dl)	0.4 (0.3 – 0.6)
INR	1.1 (1.0 – 1.2)
Tumor Size (cm)	3 (1 – 3)
Number of masses	
One mass	80
Two masses	12
≥ 3 masses	8
BCLC stage	N
Stage 1	36
Stage 2	30
Stage 3	30
Stage 4	4

Table 3. Comparisons between hepatocellular carcinoma and control groups.

Parameters	Non-HCC group N = 100	HCC group N = 100	Test of significance	
			Z value	P value
AST (IU/L)	27 (25-32)	60 (46-100)	-9.553	<0.001
ALT (IU/L)	28.5 (25-33)	47.5 (36-76)	-8.020	<0.001
Total bilirubin (mg/dl)	0.8 (0.7-0.9)	0.9 (0.7-1.5)	-2.179	0.029
AFP (ng/ml)	6 (4-8)	51.7 (9.8-450)	-8.135	<0.001
ZFAS1 Gene	-5 (-5.2 to -4.9)	-4.15 (-4.4 to -4.0)	-12.248	<0.001

Notes: Data are Median (25th – 75th percentiles). Test of significance is Mann-Whitney U-test. AFP; alpha-fetoprotein.

Table 4. Level and area under ROC of ZFAS1 gene and AFP for detection hepatocellular carcinoma.

Biomarker	Cutoff value	AUC (95% CI)	P value	SP	SN	PPV	NPV
ZFAS1 gene	≥ -4.65	1.00 (1.00 – 1.00)	<0.001	100%	99%	100%	99%
AFP	≥ 9.4	0.83 (0.77 – 0.88)	<0.001	100%	76%	100%	80.6%

AUC, Area under the ROC curve; CI, confidence interval; SN, sensitivity; SP, specificity; PPV , positive predictive value; NPV, negative predictive value.

Table 5: Correlation between ZFAS1 gene and AFP with hepatocellular carcinoma characteristics.

Characteristic	ZFAS1 gene		AFP	
	Coefficient	P value	Coefficient	P value
Dichotomous	r_{pb}		r_{pb}	
Sex	0.039	0.699	0.197	**0.050**
Ordinal	r_s		r_s	
BCLC staging	-0.044	0.665	0.312	**0.002**
Number of masses	-0.075	0.461	0.010	0.920
Tumor size (cm)	0.101	0.322	0.012	0.903
Continuous	r_s		r_s	
Age (years)	-0.009	0.933	0.176	0.080
AST (IU/L)	-0.047	0.642	0.188	0.061
ALT (IU/L)	-0.178	0.077	0.159	0.115
Total bilirubin (mg/dl)	-0.001	0.996	0.121	0.232
direct bilirubin (mg/dl)	-0.124	0.29	0.021	0.837
INR	0.128	0.205	0.141	0.164

Notes: rpb = Point biserial correlation coefficient. rs = Spearman's correlation coefficient

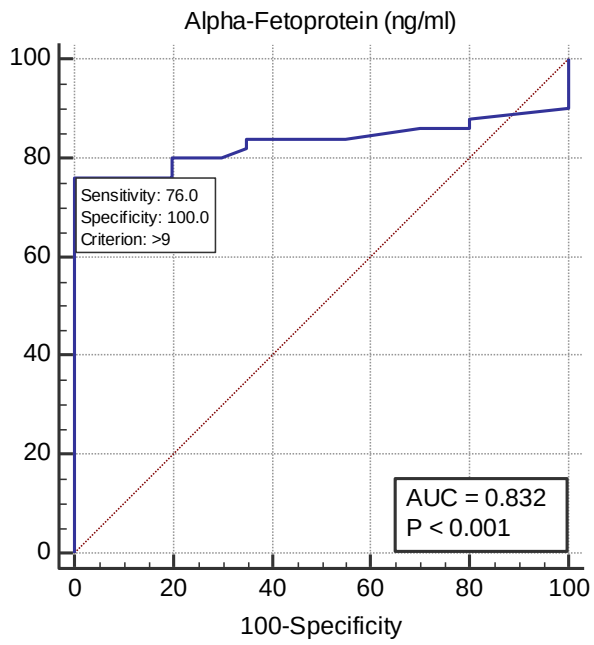

Figure (1): ROC curve for AFP

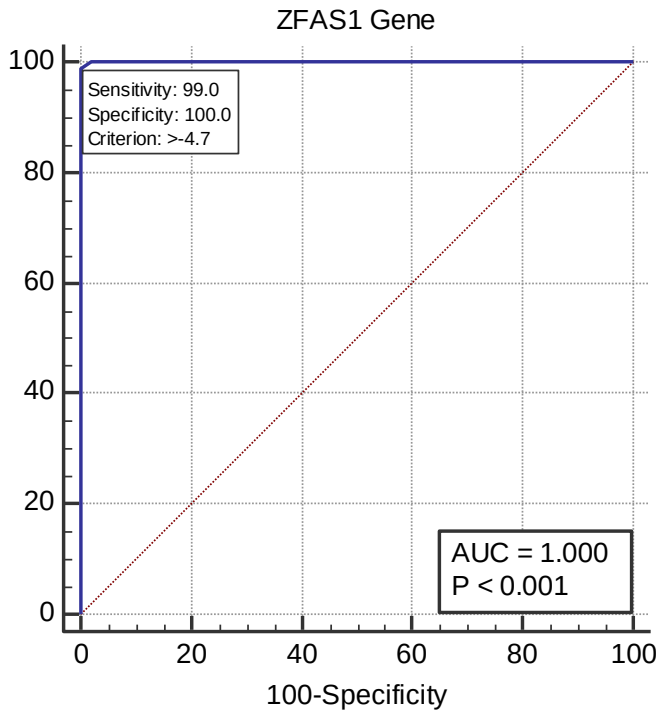

Figure (2): ROC curve for ZFAS1 gene

Discussion

Our study showed that ZFAS1 gene expression was significantly elevated in patients with hepatocellular carcinoma compared with the cirrhotic non-HCC group. In accordance with this result, Li et al. revealed an up-regulation of ZFAS1 in hepatocellular carcinoma patients and suggested that, lncRNA ZFAS1 could promote hepatocellular carcinoma progression by binding miR-150 that was correlated with worse prognosis [21]. It was suggested that, ZFAS1 acts as an endogenous sponge of miR-150, which abrogates the role of miR-150 in suppressing cancer metastasis. ZFAS1 increases zinc finger E-box-binding homeobox [1], matrix metallopeptidase [14], and matrix metallopeptidase[16] expression and promotes HCC metastasis by sponging miR-150 and inhibiting its function. in contrast, LncRNA ZFAS1, located at chromosome [20], is antisense to the 5´end of gene ZNFX1 promoter, which initially observed to be tumor suppressor in human cancer [21].

However, successive studies confirmed the oncogenic role of ZFAS1 gene which set the basis for this cancer-implicated ZFAS1 as promotive factor. For example, Thorenoor et al. suggest that ZFAS1 may function as oncogene in colorectal cancer by two main actions: (i) via destabilization of p53 and through (ii) interaction with CDK1/cyclin B1 complex leading to cell cycle progression and inhibition of apoptosis [25]. A study from Nie et al. displayed that ZFAS1 was overexpressed in gastric cancer and could repress KLF2 and NKD2 [26].

Our study found non statistically significant correlation between ZFAS1gene and any HCC characteristic including, tumor size, number and BCLC staging .These results indicating the non-prognostic value of ZFAS1 gene. In contrast, Li et al. found that, ZFAS1 gene is an unfavorable prognostic factor associated with a significantly higher ZFAS1 expression in portal vein tumor thrombus than primary HCC, demonstrating the correlation of ZFAS1 with HCC metastasis. Clinical data analysis showed that HCC patients with high ZFAS1 expression have higher recurrence rates and shorter overall survival than those with low expression of ZFAS1[21]. Also, there was a non-significant correlation between ZFAS1 expression and gender, age, tumor number and size, AFP level, or HCC stage [21].

Furthermore, recently, Lin et al found that, we sorafenib induced *ZFAS1* expression, specifically in sorafenib-resistant HCC cells via the PERK/ATF4-dependent pathway, which is associated with the sorafenib resistance in HCC cells [27].

In our study, comparison between the diagnostic performance of ZFAS1 and AFP in differentiating HCC on top of cirrhosis from cirrhosis without HCC showed that, at cutoff values ≥ -4.65 ZFAS1 gene had 100 % specificity and 99% sensitivity while, AFP at cutoff value

≥ 9.4 ng/ml had 100 % specificity and 76% sensitivity. Comparison of AUC for the two parameters demonstrated a significantly higher AUC for ZFAS1 than for AFP (Difference = 0.168, P <0.001). In accordance with our results, Luo et al showed that, the area under curve of ZFAS1 to distinguish HCC from healthy controls was 0.801 (95% CI: 0.724–0.875) and at the cut-off value of −4.006 the optical sensitivity and specificity was 55.7 % and 90.0%, respectively. However, the AUC of AFP was 0.798 (95% CI: 0.700–0.897) [27].

Conclusion

lncRNA ZFAS1 is a good diagnostic marker for HCC in cirrhotic patients with high sensitivity and specificity value, however ZFAS1has no prognostic importance in patients with HCC.

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