Egyptian Journal of Medical Human Genetics

RESEARCH Open Access

Long non-coding RNA HOTAIR and HOTTIP as potential biomarkers for hepatitis C virus genotype 4-induced hepatocellular carcinoma

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Abstract

Background: Long non-coding RNAs (lncRNAs) homeobox (Hox) transcript antisense intergenic RNA (HOTAIR) and HOXA transcript at the distal tip (HOTTIP) have been suggested to be implicated in liver cancer tumorigenesis and progression; however, little is known about the role of the plasma HOTAIR and HOTTIP in liver cancer diagnosis and prognosis. The current study aimed at measuring the plasma levels of long non-coding RNAs (HOTAIR and HOTTIP) expression in chronic liver disease (CLD) due to HCV genotype 4 infection with/without cirrhosis and HCC patients in an attempt to evaluate the potential benefits of these new circulating as non-invasive diagnostic biomarkers and a novel therapeutic strategy for liver cirrhosis and carcinogenesis of Egyptian patients. Hundred subjects were included in this study, divided into two groups; group I (50 patients) were classified into subgroup Ia (CLD without cirrhosis, $n = 25$) and subgroup Ib (CLD with cirrhosis, $n = 25$), group II (CLD patients with HCC, $n = 25$), and control (healthy volunteer, $n = 25$). The expression of lncRNAs (HOTAIR and HOTTIP) genes was analyzed by real-time PCR.

Results: LncRNAs (HOTAIR and HOTTIP) showed upregulation in all diseased groups, which was in consistent with the progression of the disease toward the HCC stage. In addition, HOTAIR and HOTTIP showed a diagnostic ability to discriminate between cases of cirrhosis and HCC compared with healthy control ($p < 0.001$), while HOTAIR and HOTTIP did not show a discrimination significant differences between cirrhotic cases and non-cirrhotic cases. By using receiver operating characteristic curve (ROC) analysis, it was found that LncRNAs (HOTAIR and HOTTIP) could diagnose liver cancer with 64.0% sensitivity and 86.0% specificity and 48.0% sensitivity and 88.0% specificity. Furthermore, both genes can be considered as the predictor and prognostic parameters for cirrhosis (OR = 1.111, $p = 0.05$) and (OR = 1.07, $p = 0.05$) respectively, and HCC (OR = 1.047, $p = 0.01$) and (OR = 1.05, $p = 0.003$). The increased HOTAIR and HOTTIP expression were associated with advanced tumor stages and higher grades.

Conclusion: These results strongly prompt us that HOTAIR and HOTTIP genes can be used as non-invasive prognostic biomarkers and new therapeutic targets for HCV genotype 4-induced HCC.

Keywords: Biomarker, HOTAIR, HOTTIP, HCV, Cirrhosis, HCC

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Background

Egypt has a very high prevalence of the hepatitis C virus (HCV) worldwide, about 22% of Egyptian blood donors suffer from HCV with 170,000 to 200,000 new cases yearly and 40,000 dying from the disease every year "(http://egyptianstreets.com/2015/05/04/egypt-has-highest-prevalence-of-hepatitis-c-in-the world-who)". HCV infection is a major cause of CLD, which can progress to liver fibrosis, cirrhosis and even HCC [1]. HCV increases the risk of HCC development by nearly 17-fold compared with healthy individuals [2]. Although the overall prevalence of chronic hepatitis C is declining, the complications of the disease are increasing because of the aging of the infected population and the progression of liver fibrosis [3].

LncRNAs account for a large portion of the non-coding transcripts. They comprise of in excess of 200 nucleotides and have no or restricted protein-coding potential. Previously, they were believed to be transcriptional noise [4]. But recently, expression analyses of lncRNAs in a wide range of tissues have revealed their importance for different homeostatic and physiological functions, such as gene imprinting, cell differentiation, and organogenesis [5, 6]. A solid affiliation has been found between deregulated lncRNA articulation and the improvement of maladies.

Through the modulation of oncogenic and tumor-suppressing pathways, it was noted that the aberrant expression of lncRNAs contributed to the development and progression of cancer [7–10]. LncRNA has attracted attention as a new participant in carcinogenesis and emerged as a new source of non-invasive cancer biomarkers [3, 11–13]. Most of these lncRNAs are upregulated in HCCs, but less expressed or undetectable in normal individuals [14].

The HOX family qualities are realized interpretation factors with a key job in embryogenesis and carcinogenesis [15, 16]. Their expression is deregulated in many cancers [17]. In humans, HOX genes are organized into 4 clusters (A, B, C, and D), which are situated on various chromosomes [18]. Interestingly, several lncRNAs associated with HOX genomic regions can partake in the regulation of HOX genes and work together in their functions [19]. HOTAIR lncRNA is transcribed from the antisense strand of the HoxC gene, which is located on chromosome 12q13.13 between the HoxC11 and HoxC12 genes [20] and one of the most significant administrative RNAs in human cells. It was first described by Rinn et al. [21] as a spliced and polyadenylated RNA with six exons and 2.2 kb nucleotides. HOTTIP gene is located at the homeobox A (HOXA) locus (chromosomal locus 7p15.2) which encodes the 3764 bp transcript. Therefore, the lncRNA is termed 'HOXA transcript at the distal tip' (HOTTIP) [22, 23].

HOTAIR promotes the carcinogenic activity of HCC cells via multiple mechanisms, such as the suppression of RNA-binding motif protein, triggering of epithelial-mesenchymal transition, and interaction with miRNAs [24]. The expression of HOTAIR was found to be stimulated by c-Myc in gallbladder cancer cells [25]. c-Myc also may have a pivotal role in hepatocarcinogenesis [25, 26]. HOTAIR was also noticed to be regulated by IκB kinase [27]. Suppression of HOTAIR was noticed to promote the inhibition of cell proliferation and invasion [28]. HOTTIP is overexpressed in numerous cancers [23, 29, 30] and in HCC [31]. The knockdown of HOTTIP in pancreatic cancer was found to lead to cell proliferation arrest and impaired cell invasion via inhibiting epithelial-mesenchymal transition [32].

Previous research has shown that circulating non-coding RNAs (nRNA) could be used as a non-invasive biomarker for the early detection of cancers [33–35], such as lncRNAs, can be detected in body fluids and may have diagnostic and prognostic roles in cancer. Recent studies have investigated the clinical implication of cell-free lncRNAs in cancer patients [36, 37].

Aim of the work

The aim of the study is to develop a non-invasive diagnostic tool based on measuring the plasma levels of different lncRNAs markers namely HOTAIR and HOTTIP in order to detect HCV genotype 4-induced HCC at the early stages of the disease.

Methods

Study design

We are planning a study of subjects in which we will regress their values of the patient's against control. Prior data indicate that the standard deviation of control is 0.6 and the standard deviation of the regression errors will be 1.9. If the true slope of the line obtained by regressing patients against control is 1.7, we will need to study 25 subjects for each group to be able to reject the null hypothesis that this slope equals zero with probability (power) 90%. The Type I error probability associated with this test of this null hypothesis is 0.05.

Patient's criteria

Patients enrolled in this study were admitted to the Gastroenterology and Hepatology Department in affiliated hospital of our institution from November 2016 to August 2018. Diagnosis of patients was based on full medical history, thorough clinical examination, abdominal ultrasonography and laboratory assessment including CBC, and liver function tests, serological and HCV genotyping by HybProbe probes with the light cycler carousel-based system.

This study was approved by our institution Ethics Review Board and informed consent was obtained from all the subjects included in this study according to the roles

of the Declaration of Helsinki 1975. Approval of local ethical committee (REC number 01/19).

Inclusion criteria

All included patients were suffering from chronic hepatitis C genotype (4), persisting more than 6 months (HCV RNA positive). They did not receive any specific treatment for HCV during the last 6 months.

All chronic liver disease (CLD) who developed malignancy on top of previous HCV infection. Diagnosis of cirrhosis was depending upon ultrasonographic criteria (surface irregularity, coarse echo pattern, portal vein diameter, splenic size, presence or absence of ascites), laboratory findings of hypoalbuminemia and hypoprothrombinemia, in addition to APRI score (APRI score calculated regarding AST to Platelet Ratio Index (APRI) = [AST Level (IU/L)/AST (upper limit of normal) (IU/L)]/platelet count (109/L)×100, (normal < 0.05, CLD without cirrhosis 0.5–1.5 and cirrhosis ≤ 1.5) [38] and esophageal or gastric varices diagnosed by endoscopy as a sign of portal hypertension in indicated patients. Diagnosis of HCC was depending upon the presence of focal hepatic lesions diagnosed by abdominal ultrasound and confirmed by triphasic spiral computed tomography (CT) and/or magnetic resonance imaging according to the American Association for the Study of Liver Diseases (AASLD) 2011 guidelines [39].

Exclusion criteria

The exclusion criteria included any concomitant cause CLD such as patients with history of schistosomiasis, chronic viral diseases other than HCV, dual HBV and HCV infection, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis, biliary disorders, malignancies other than HCC, regular intake of hepatotoxic drugs, alcohol abuse, diabetes, and HCV-infected patients receiving direct-acting antiviral or immunomodulatory interferon-α therapy.

Based on the inclusion and exclusion criteria, 100 patients were included in this study. Seventy-five patients with chronic hepatitis C were classified into two major groups: group I, CLD without HCC (n = 50), and group II, CLD with HCC (n = 25). Group I was further subdivided into two subgroups: (Ia) (CLD without cirrhosis) (n = 25) and (Ib) (CLD with cirrhosis) (n = 25). In addition, twenty-five age- and sex-matched healthy adults served as a control group.

Specimen collection and handling

About 7 ml peripheral venous blood were collected under strict aseptic conditions by clean venipuncture using vacuum blood collection tubes and distributed as 2.5 ml in EDTA tubes for complete blood picture, 2.5 ml in another sterile EDTA tube (stored at −80°C) for viral RNA extraction for HCV genotyping, miRNA, and mRNA extraction. In addition, 2 ml in a plane tube were allowed to clot at 37 °C, and then centrifuged at 3000 rpm for 10 min and the collected serum was stored at −80°C to be used for performing liver and kidney functions, and other specific serological tests.

Laboratory investigations

All individuals were subjected to general investigations including; haemogram, using an automated cell counter (Celltac 5, Nihon Kohden, Tokyo, Japan). A battery of liver function tests was performed using standard methods. Alpha-fetoprotein (AFP) was measured using an autoanalyzer (Hitachi 736, Hitachi Japan). Coagulation tests were measured using Stago Compact Max, USA. Serological diagnosis of HCV infection was done using Murex anti-HCV, version IV, Murex Diagnostics limited, Dartford, England. HCV-RNA by PCR. Hepatitis B surface antigen and HBV core antibody were examined by enzyme-linked immunosorbent assay (ELISA) system assay. Autoimmune hepatitis was investigated by detecting anti-nuclear antibodies (ANA) using the immunospec ANA screen ELISA test system. A serological examination of schistosomiasis (*Schistosoma mansoni* Ab) was done using antibody detection, an in-house ELISA.

HCV genotyping: Viral RNA Extraction was done using a high pure viral RNA kit (version 18, 2011), cat. no: (11858882001) "(https://www.roche.com)". cDNA synthesis (transcriptor first strand) was done according to the cDNA synthesis kit (transcriptor first strand) (version 6.0, 2010), cat. no: (04379012001) "(https://www.roche.com)". HCV genotyping detection was done using hot start reaction mix detection for PCR using HybProbe probes with the light cycler carousel-based system (version 15, 2011), cat. no: (03003248001) "(https://www.roche.com)".

Target gene expression: Total RNA extraction was done according to a high pure RNA isolation kit (version 12, 2011), cat. no: (11828665001) "(https://www.roche.com)". Gene expression detections were performed using light cycler EvoScript RNA SYBR green I master (version 2, 2017), easy to use reaction mix for one-step RT-qPCR cat. no: (07800134001) "(https://www.roche.com)". The primer sequences are illustrated in Table 1.

Analysis of results depending on the SYBR green I filter combination (465–510) on light cycler EvoScript RNA SYBR green I master and comparative CT methods were applied to analyze data. Housekeeping gene B-actin was used as an endogenous control to normalize the amount of total mRNA in each sample of HOTAIR and HOTTIP between different samples. Genes expression was calculated relative to the control samples (used as the calibrator sample) using the formula 2_ΔΔCT and were expressed as fold change "(https://bitesizebio.com)".

Statistical analysis

The data were analyzed using Microsoft Excel 2010 and statistical package for social science (SPSS version 24.0)

Table 1 Primers of genes included in the study

Gene	Sequence	Tm	Reference
HOTAIR			
Forward	5'-GCA GTA GAA AAA TAG ACA TAG GAGA-3'	58oc	"(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4774541/)"
Reverse	5′-AAT GAT AGG GAC ACA TCG GGG AAC T-3′	58oc	
HOTTIP			
Forward	5′-GTG GGG CCC AGA CCC GC-3′	58oc	"(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4637691/)"
Reverse	5′-AAT GAT AGG GAC ACA TCG GGG AAC T-3′	58oc	
B-actin (used as an endogenous control to normalize the amount of total mRNA in each sample)			
Forward	5-GCACCACACCTTCTACAATG-3	58oc	"(http://hgsv.washington.edu)"
Reverse	5-TGCTTGCTGATCCACATCTG-3	58oc	

for Windows (SPSS IBM., Chicago, IL). Continuous normally distributed variables were represented as mean± SD with a 95% confidence interval, while non-normal variables were summarized as median with 25 and 75 percentile, and using the frequencies and percentage for categorical variables; a p value < 0.05 was considered statistically significant. To compare the means of normally distributed variables between groups, the Student's t test was performed, and the Mann-Whitney test was used in non-normal variables. $\chi 2$ test or Fisher's exact test was used to determine the distribution of categorical variables between groups. The diagnostic performance of HOTAIR and HOTTIP was assessed by ROC curves. The area under the ROC (AUROC) was used as an index to compare the accuracy of tests. The cut-off for the diagnosis of the group of the study was taken from the point of maximum combined sensitivity and specificity. The sensitivity and specificity for relevant cut-offs were also displayed. Spearman's rank correlation coefficient (r) was done to show the correlation between non-parametric parameters, while Pearson's correlation for parametric parameters. Effect modification was evaluated by stratification, statistical interaction and was assessed by including main effect variables and their product terms in the logistic regression model.

Results

Individual demographic and routine laboratory characteristics of the studied groups are shown in Table 2.

Depending on the fold change low, the fold change results showed that the gene expressions of HOTAIR and HOTTIP constantly upregulated in the studied groups (Table 3).

Gene expression of HOTAIR in all studied diseased groups was highly significantly (p < 0.001) upregulated compared with the control group. There was no significant upregulation in subgroup Ia when compared with subgroup Ib, while group II showed a significant upregulation when compared with subgroup Ia and subgroup Ib (p < 0.001 and p < 0.01) respectively (Fig. 1) (Table 4).

In contrast, HOTTIP expression, a highly significantly (p value < 0.001) upregulated in all diseased groups when compared with the control group. But there was no significant difference in subgroup Ia when compared with subgroup Ib. While it was obvious, the expression of HOTTIP in group II was significantly (p value < 0.01) upregulated compared with the subgroup Ia, subgroup Ib, and the group I (Fig. 2) (Table 4).

The correlation study revealed a significant direct moderate correlation of HOTAIR with HOTTIP (r = 0.652 and p value = 0.001 (Fig. 3) and fair correlation between HOTAIR with AFP (r = 0.481 and p value = 0.001) (Fig. 4). While there were inverse fair correlations with albumin (r = −0.304 and p value = 0.007) (Table 5).

HOTTIP expression showed a significant direct fair correlation with AFP (r = 0.400 and p value = 0.001) (Fig. 5), while there were inverse fair correlations with albumin (r = −0.392 and p value = 0.001) (Table 5).

Receiver operating curves (ROC) were established to show the diagnostic performances of the HOTAIR and HOTTIP genes in the studied groups (Table 6).

ROC curve

In HOTAIR gene, for discrimination of cirrhotic group from without cirrhosis grou, it was found that plasma HOTAIR at the cut-off value of 4.7, with sensitivity of 48.0% and specificity of 72.0% with areas under curve (AUC) of 52.0 (p value = 0.8, 95% confidence interval C.I 35.6%–68.4%) and accuracy of 60.0% (Fig. 6a), for discrimination of HCC group from cirrhotic group, it was found that plasma HOTAIR at the cut-off value of > 7.0, with sensitivity of 64.0% and specificity of 76.0% with areas under curve (AUC) of 69.8 (p value = 0.007, 95% confidence interval C.I 55.5%–84.1%) and accuracy of 60.0% (Fig. 6c), and for discrimination of HCC group from CLD patients, it was found plasma HOTAIR at the cut-off value of > 9.2, with sensitivity of 64.0% and specificity of 86.0% with areas under curve (AUC) of 74.2 (p value = 0.001, 95% confidence interval C.I 61.9%–86.5%) and accuracy of 60.0% (Fig. 6d) (Table 6).

Table 2 Socio-demographic characteristics, laboratory investigations, and ultrasound finding among patients of the studied groups

		Control *n* = 25	Group I (CLD without HCC) *N* = 50		Group II (CLD with HCC) *n* = 25
			Subgroup Ia (CLD without cirrhosis) *n* = 25	Subgroup Ib (CLD with cirrhosis) *n* = 25	
Age		47.1 ± 8.5	47.7 ± 9.9	58.9 ± 9.3aa, bb	56.3 ± 7.8aa, bb
Sex Female/male		2(08.0%)/23(92.0%)	15(60.0%)/10(40.0%)aa	12(48.0%)/13(52.0%)aa	6(24.0%)/19(76%)bb, cc
US finding	Cirrhosis	0(0.0%)	0(0.0%)	25(100.0%)	7(28.0%)cc
	Splenomegaly	0(0.0%)	3(12.0%)	19(76.0%)bb	22(88.0%)bb,c
	Ascites	0(0.0%)	0(0.0%)	21(84.0%)	25(100.0%)c
ALT (umol/dl)		27.5 (15.5–31.8)	40.0 (17.0–51.0)a	46.5 (24.3–84.8)a	55.5 (34.5–82.5)aa, bb
AST (umol/dl)		32.0 (24.3–38.0)	38.0 (28.0–46.0)	44.5 (32.0–69.5)aa	80.0 (37.3–111.0)aa, bb, c
AFP (ng/ml)		2.3 (1.5–3.1)	2.2 (1.4–4.5)	8.8 (6.5–16.8)aa, bb	224.0 (44.3–597.5)aa, bb, cc
Albumin (umol/dl)		4.1 ± 0.5	4.1 ± 0.5	3.1 ± 1.1aa, bb	2.6 ± 0.7aa, bb, c
Total bilirubin (umol/dl)		0.9 (0.8–1.1)	0.6 (0.4–0.9)aa	1.4 (0.7–3.6)a, bb	1.9 (1.3–5.0)aa, bb, c
Direct bilirubin (umol/dl)		0.3 (0.2–0.4)	0.2 (0.1–0.3)a	0.6 (0.2–1.9)a, bb	1.1 (0.4–2.5)aa, bb
ALP (IU/L)		74.3 ± 19.0	84.9 ± 30.2	103.2 ± 35.6aa, b	202.4 ± 88.0aa, bb, cc
PT (s)		12.4 (11.4–12.8)	15.4 (13.2–18.8)aa	17.8 (15.0–20.4)aa	16.3 (14.5–19.0)aa
PC (%)		89.6 (78.2–100.0)	64.0 (55.0–80.0)aa	52.5 (44.0–73.8)aa	71.5 (55.0–75.3)aa
INR (s)		1.1 (1.0–1.1)	1.5 (1.0–1.6)aa	1.5 (1.2–1.8)aa	1.4 (1.2–1.5)aa
HB (g/dl)		12.6 ± 1.4	12.4 ± 1.6	10.4 ± 2.2aa, bb	11.2 ± 2.4a, b
WBCs (/mm3)		6.2 ± 2.3	7.0 ± 2.8	7.3 ± 2.6	7.8 ± 3.1
Platelets (/mm3)		241.5 ± 56.5	228.2 ± 67.3	112.9 ± 52.9aa, bb	136.1 ± 82.2aa, bb
APRI score		0.34 ± 0.13	0.61 ± 0.17	1.69 ± 0.31	2.1 ± 1.3

Age, albumin, alkaline phosphates (ALP), hemoglobin (HB), white blood cells (WBCs) and platelets are represented as mean ± SD; the data were analyzed by *t* test. But sex and ultrasound (U/S) findings are represented as frequency and percent; the data were analyzed by X^2 test. While alanine aminotransferase (ALT), aspartate aminotransferase (AST), alpha-fetoprotein (AFP), total bilirubin, direct bilirubin, prothrombin time (PT), prothrombin concentration (PC) and international normalized ratio (INR) are represented as median and interquartile range (25%–75%); the data were analyzed by Mann-Whitney *U* test
p value bearing (b) initial is significantly different comparing with HCV group
p value bearing (c) initial is significantly different comparing with cirrhotic group
p value bearing (#) initial is significantly different comparing with CLD group
1 initial *p* value <0.05 is significant, 2 initial *p* value <0.01 is highly significant

In HOTTIP gene, for discrimination of cirrhotic group from without cirrhosis group, it was found plasma HOTTIP at the cut-off value of 13.4, with sensitivity of 32.0% and specificity of 100.0% with areas under curve (AUC) of 53.9 (*p* value = 0.640, 95% confidence interval C.I 37.1%–70.8%) and accuracy of 66.0% (Fig. 7a), for discrimination of HCC group from cirrhotic group, it was found plasma HOTTIP at the cut-off value of > 34.8, with sensitivity of 36.0% and specificity of 92.0% with areas under curve (AUC) of 65.8 (*p* value = 0.033, 95% confidence interval C.I 51.3%–80.4%) and accuracy of 64.0% (Fig. 7c), and for discrimination of HCC group

Table 3 Biomarkers fold change in the studied groups

Biomarkers	Control *n* = 25	Group I (CLD without HCC) *n* = 50				Group II CLD with HCC *n* = 25	
		Subgroup Ia (CLD without cirrhosis) *n* = 25		Subgroup Ib (CLD with cirrhosis) *n* = 25			
		Fold-change	Type of Regulation	Fold-change	Type of Regulation	Fold-change	Type of Regulation
HOTAIR	1	4.1536	Up	10.724	Up	20.508	Up
HOTTIP	1	3.9372	Up	11.46	Up	22.788	Up

The fold change results depend on the fold change low: fold-change (2^(−Delta Delta Ct)) is the normalized gene expression (2^(−Delta Ct)) in the test sample divided the normalized gene expression (2^(−Delta Ct)) in the control sample. (Fold-change values less than 1 indicate a negative or downregulation https://www.biostars.org/))

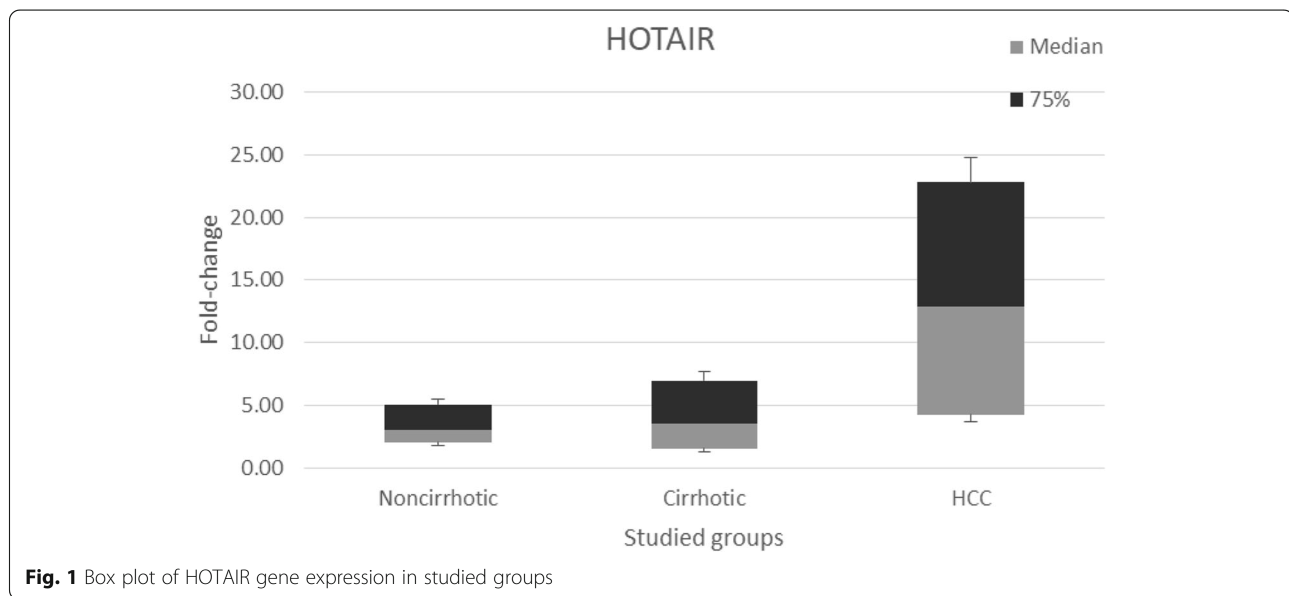

Fig. 1 Box plot of HOTAIR gene expression in studied groups

from CLD patients, it was found plasma HOTTIP at the cut-off value of > 16.6, with sensitivity of 48.0% and specificity of 88.0% with areas under curve (AUC) of 68.3 (*p* value = 0.007, 95% confidence interval C.I 55.0%–81.7%) and accuracy of 74.7% (Fig. 7d) (Table 6).

Univariate logistic regression analysis performed to characterize the two markers (HOTAIR and HOTTIP) as a predictor and/or prognostic parameter are shown in Table 7.

– Cirrhosis risk according to gene expression

Regarding univariate logistic regression analysis, the HOTAIR and HOTTIP were considered predictor and/or prognostic parameters for cirrhotic progression. An increase in 1 ° of HOTAIR increased the odds of being cirrhosis by a factor of 1.111 with *p* value = 0.05, but an increase in 1 ° of HOTTIP increased the odds of being cirrhosis by a factor of 1.07 with *p* value = 0.05.

– HCC risk according to gene expression

The HOTTIP was considered a predictor and/or prognostic parameter for HCC progression. An increase in 1 ° of its expression level increased the odds of being HCC by a factor of 1.029 with *p* value = 0.05. But there is no significance for HOTAIR in the characterization of HCC progression (OR = 1.027, *p* value = 0.1).

Concerning CLD as a general group (subgroups Ia and Ib) had an increase in 1 ° of HOTAIR which increased the odds of being HCC by a factor of 1.047 with *p* value = 0.01. For HOTTIP, an increase in 1 ° of HOTTIP which increased the odds of being HCC by a factor of 1.05 with *p* value = 0.003.

Discussion

LncRNAs play a central role in the regulation of differentiation, cell development, and proliferation [40]. Moreover, specific expression of lncRNA in the

Table 4 Biomarkers gene expression in the studied groups

Biomarkers	Control $n = 25$	Group I CLD without HCC		Group II CLD with HCC $n = 25$
		Subgroup Ia (CLD without Cirrhosis) $n = 25$	Subgroup Ib (CLD with Cirrhosis) $n = 25$	
HOTAIR	0	3.1(2–5.45)^{aa}	3.6(1.45–9.4) ^{aa}	12.9(3.95–23.35) $^{aa, bb, c, \#\#}$
HOTTIP	0	2.4(1.15–5.8)^{aa}	4.6(0.2–19.95) ^{aa}	14.8(1.55–42.05) $^{aa, b, c, \#}$

All parameters are represented as median with interquartile range (25%–75%) of the fold change of the studied groups, the data were analyzed by Mann-Whitney *U* test
p value bearing (a) initial is significantly different comparing with control group
p value bearing (b) initial is significantly different comparing with HCV group
p value bearing (c) initial is significantly different comparing with cirrhotic group
p value bearing (#) initial is significantly different comparing with CLD group
$^{1\ initial}$*p* value < 0.05 is significant, $^{2\ initial}$ *p* value < 0.01 is highly significant

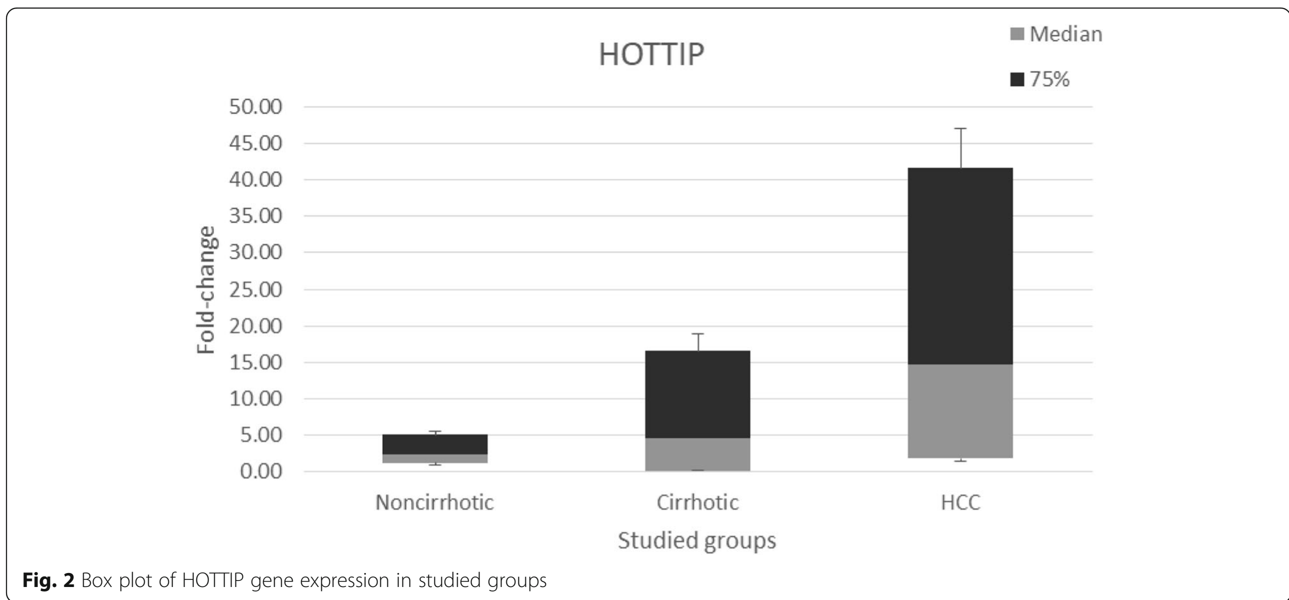

Fig. 2 Box plot of HOTTIP gene expression in studied groups

development of tumors has been revealed and can be used as a promising biomarker to diagnose and monitor tumors, and lncRNAs also could be collected easily from body fluids and tumor tissues [41]. The aim of our study is to develop a non-invasive diagnostic tool based on measuring the plasma levels of different long non-coding RNAs in order to detect HCV-induced HCC at the early stages of the disease.

Our results revealed a significant upregulation of HOTAIR gene in cirrhotic and HCC patients when compared with controls ($p < 0.001$). Similarly, a significant increase was noticed on comparing HCC with chronic HCV without and with cirrhosis patients ($p < 0.001$, $p < 0.01$) respectively. No significant difference was found between chronic HCV with cirrhosis and chronic HCV without cirrhosis. Our results are in agreement with Eman et al. [42] and Nande et al. [43] who found a similar increase in HOTAIR expression in patients with gastric cancer and non-small cell cancer patients. Gupta et al. reported that HOTAIR was overexpressed in breast cancer and its high expression in primary breast tumors was a significant predictor of subsequent metastasis and mortality [44]. HOTAIR expression was found to be higher in HCC tissues than in adjacent non-cancerous tissues [13, 45–47].

Regarding HOTTIP gene, a significant upregulation was revealed in cirrhotic and HCC patients when compared with controls ($p < 0.001$). In addition, there is a

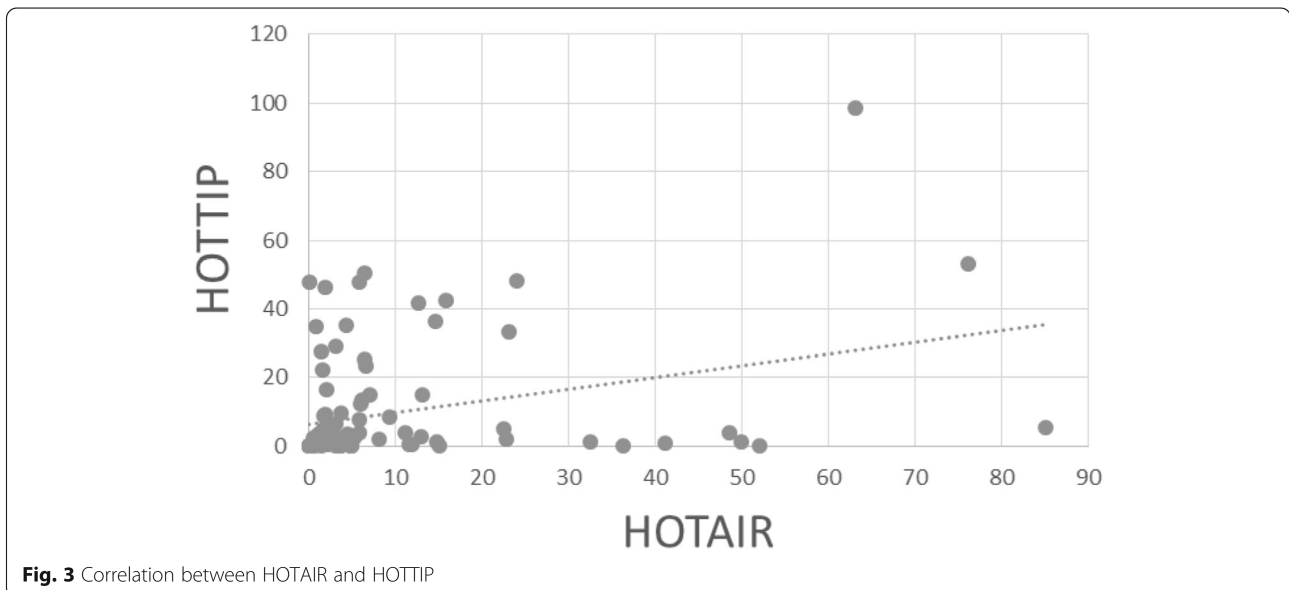

Fig. 3 Correlation between HOTAIR and HOTTIP

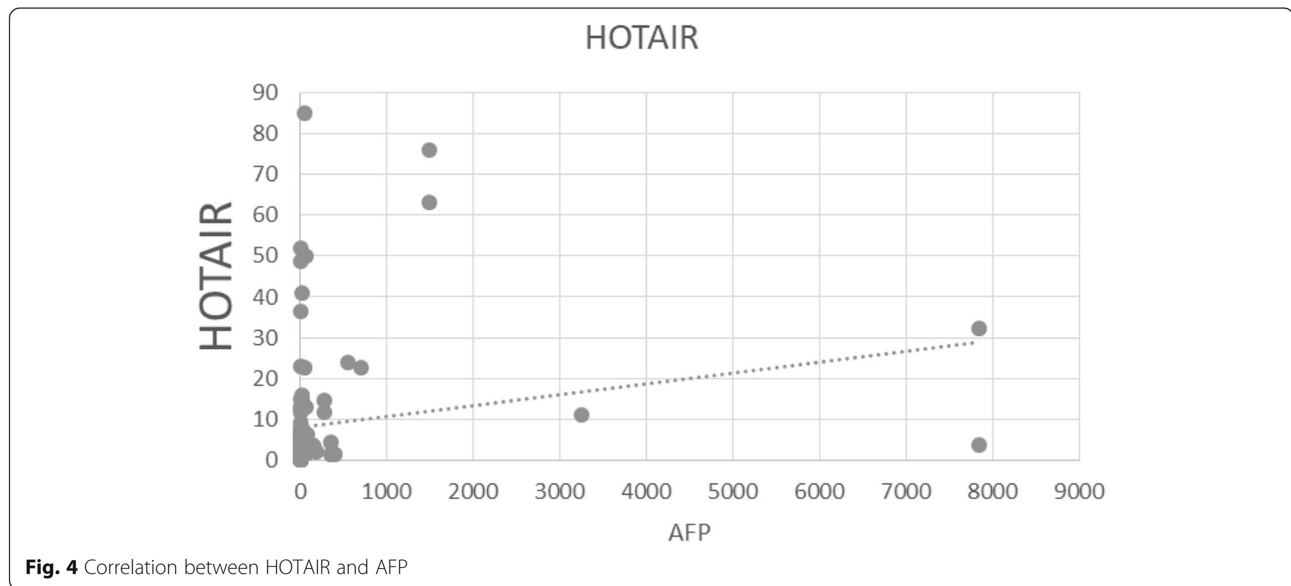

Fig. 4 Correlation between HOTAIR and AFP

Table 5 Correlation study of the studied genes

	HOTAIR		HOTTIP	
	Correlation coefficient	Sig. (2-tailed)	Correlation coefficient	Sig. (2-tailed)
HOTAIR			0.652**‡	0.001
HOTTIP	0.652**‡	0.001		
Age	0.231*‡	0.044	0.202	0.078
Sex	−0.211	0.065	−0.221	0.054
AFP	0.481**‡	0.001	0.400**‡	0.001
Albumin	−0.304**‡	0.007	−0.392**‡	0.001
T. bil	0.116	0.316	0.133	0.248
D. bil	0.164	0.154	0.123	0.286
ALT	0.394**‡	0.001	0.228*‡	0.046
AST	0.200	0.081	0.188	0.102
ALP	0.402**‡	0.001	0.452**‡	0.001
UREA	0.227*‡	0.047	0.307**‡	0.007
CREAT	0.187	0.103	0.222	0.052
PT	0.483**‡	0.001	0.458**‡	0.001
PC	−0.411**‡	0.001	−0.306**‡	0.007
INR	0.379**‡	0.001	0.340**‡	0.002
HB	−0.172	0.135	−0.200	0.08
WBCs	0.104	0.369	0.175	0.128
Platelets	−0.418**‡	0.001	−0.330**‡	0.003

All parametric parameters were analyzed by Pearson's correlation test, while all nonparametric parameters were analyzed by Spearman rho (rank correlation)
‡(*r*) Initial is calculated regarding Pearson's correlation test
Results of correlation studies depending on the correlation coefficient values (*r*) as follows: weak < 0.24; fair 0.25–0.49; moderate 0.5–0.74; strong 0.75–1
Alkaline phosphates (ALP), hemoglobin (HB), white blood cells (WBCs), ultrasound (U/S), alanine aminotrasferase (ALT), aspartate aminotransferase (AST), alpha-fetoprotein (AFP), total bilirubin (T.Bil), direct bilirubin (D.Bil), prothrombin time (PT), prothrombin concentration (PC) and international normalized ratio (INR)
*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

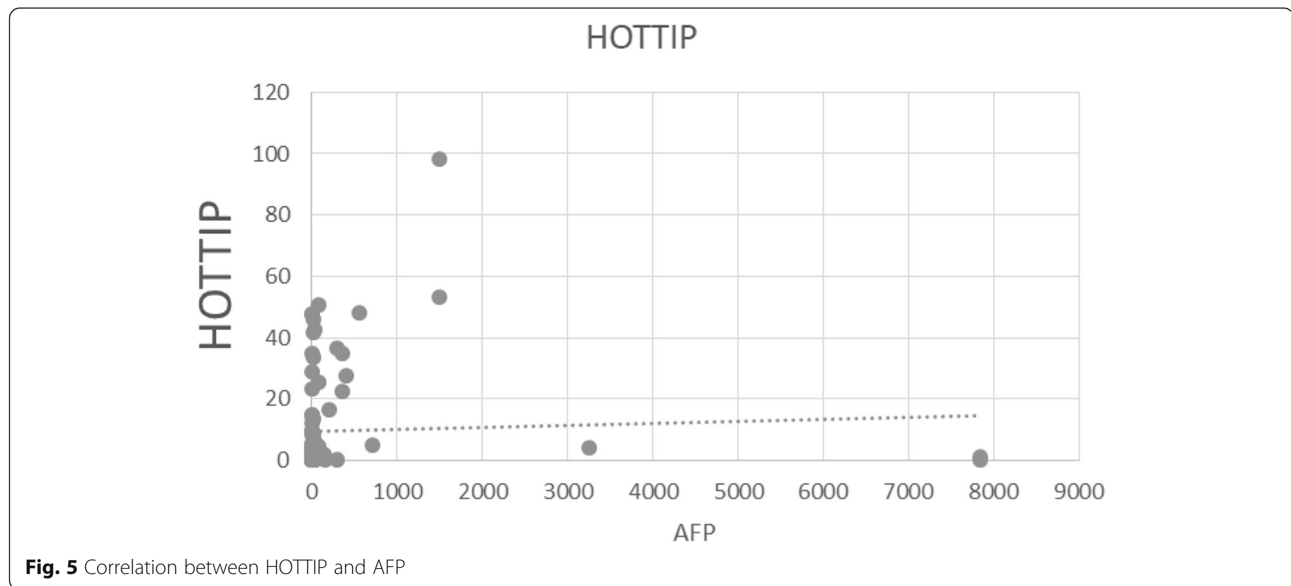

Fig. 5 Correlation between HOTTIP and AFP

significant increase in HCC patients when compared with chronic HCV without and with cirrhosis patients ($p < 0.01$). The contribution of HOTTIP as having oncogenic roles has been revealed in tumorigenesis; recent advances have confirmed that HOTTIP is frequently overexpressed in many tumors to play oncogenic roles in cancer development and progression, including HCC [31]. HOTTIP expression was noticed to increase the HCC cell proliferation [48] and was associated with HCC progression and poor clinical outcome [31, 49].

The diagnostic performance of HOTAIR and HOTTIP gene expressions as markers in cirrhotic patients at different cut-off points using ROC curve showed that no significant difference between cirrhotic and non-cirrhotic patients regarding HOTAIR at the cut-off value of 4.7, with sensitivity of 48.0% and specificity of 72.0% with areas under curve (AUC) of 52.0 (p value = 0.8, 95% confidence interval C.I 35.6%–68.4%) and accuracy of 60.0%, also no significant difference between cirrhotic and non-cirrhotic patients regarding HOTTIP at the cut-off value of 13.4, with sensitivity of 32.0% and specificity of 100.0% with areas under curve (AUC) of 53.9 (p value = 0.640, 95% confidence interval C.I 37.1%–70.8%) and accuracy of 66.0%. The results indicated that HOTAIR and HOTTIP could not be used for discrimination between cirrhotic and non-cirrhotic patients.

In case of HCC, for discrimination of HCC group from cirrhotic group, it was found that a highly significant difference between HCC and cirrhotic patients regarding HOTAIR at the cut-off value of > 7.0, with sensitivity of 64.0% and specificity of 76.0% with areas under curve (AUC) of 69.8 (p value = 0.007, 95% confidence interval C.I 55.5%–84.1%) and accuracy of 60.0%, also a significant difference between HCC and cirrhotic patients regarding HOTTIP at the cut-off value of > 34.8, with sensitivity of 36.0% and specificity of 92.0% with areas under curve (AUC) of 65.8 (p value = 0.033, 95% confidence interval C.I 51.3%–80.4%) and accuracy of 64.0%. The results indicated that HOTAIR and HOTTIP can be used to discriminate between HCC patients than cirrhotic patients.

Table 6 Diagnostic performances of HOTAIR and HOTTIP to discriminate (cirrhotic and HCC) patients from CLD patients

Biomarkers in the studied groups		Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	95% CI	*p* value
HOTAIR	Cirrhotic vs non-cirrhotic	4.7	48.0	72.0	63.2	58.1	60.0	52.0	35.6–68.4	0.8
	HCC vs non-cirrhotic	> 9.2	64.0	96.0	94.1	72.7	80.0	78.6	65.6–91.7	< 0.0001**
	HCC vs cirrhotic	> 7.0	64.0	76.0	72.7	67.9	70.0	69.8	55.5–84.1	0.007**
	HCC vs CLD without HCC	> 9.2	64.0	86.0	69.6	82.7	78.7	74.2	61.9–86.5	0.001**
HOTTIP	Cirrhotic vs non-cirrhotic	13.4	32.0	100.0	100.0	59.5	66.0	53.9	37.1–70.8	0.64
	HCC vs non-cirrhotic	> 13.4	52.0	100.0	100.0	67.6	76.0	70.8	56.2–85.4	0.005**
	HCC vs cirrhotic	> 34.8	36.0	92.0	81.8	59.0	64.0	65.8	51.3–80.4	0.03*
	HCC vs CLD without HCC	> 16.6	48.0	88.0	66.7	77.2	74.7	68.3	55.0–81.7	0.007**

PPV Positive predictive value, *NPV* Negative predictive value and *AUC* Area under curve, **p value* <0.05 is significant, ***p value* <0.01 is highly significant

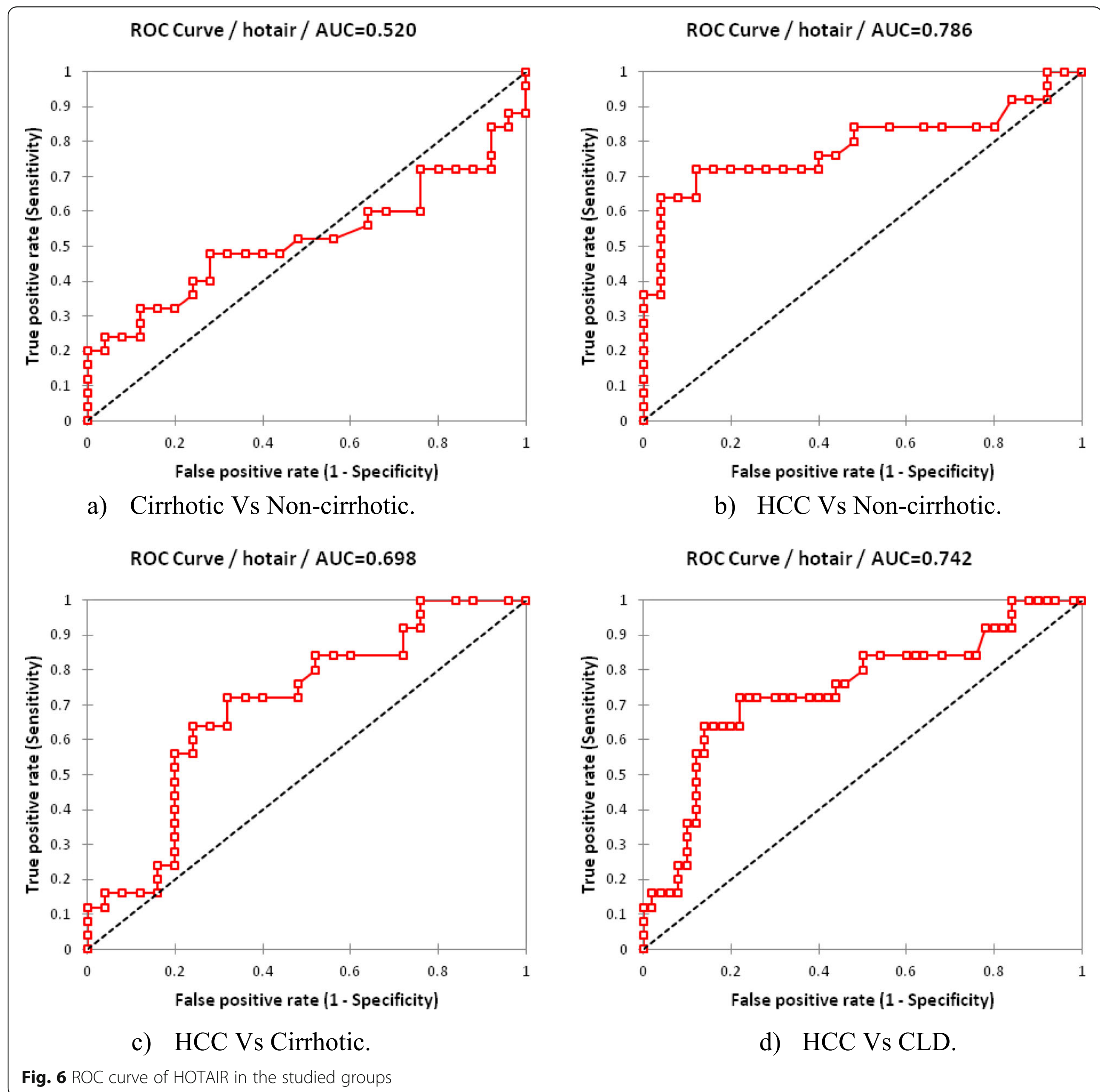

Fig. 6 ROC curve of HOTAIR in the studied groups

Concerning for discrimination of HCC patients from CLD (cirrhotic and non-cirrhotic) patients, it was found that a highly significant difference between HCC and CLD patients regarding HOTAIR at the cut-off value of > 9.2, with sensitivity of 64.0% and specificity of 86.0% with areas under curve (AUC) of 74.2 (*p* value = 0.001, 95% confidence interval C.I 61.9%–86.5%) and accuracy of 60.0%, also a highly significant difference between HCC and CLD patients regarding HOTTIP at the cut-off value of > 16.6, with sensitivity of 48.0% and specificity of 88.0% with areas under curve (AUC) of 68.3 (*p* value = 0.007, 95% confidence interval C.I 55.0%–81.7%) and accuracy of 74.7%. These results indicated that HOTAIR and HOTTIP can be used for discrimination between HCC and CLD without HCC.

Interestingly, according to regression analysis, the expression levels of HOTAIR and HOTTIP were considered as significant predictors associated with the changes of the cirrhotic progression and cirrhosis risk assessment (*p* value = 0.05 and *p* value = 0.05) respectively. Ge et al. suggested that the miR-192/-204-

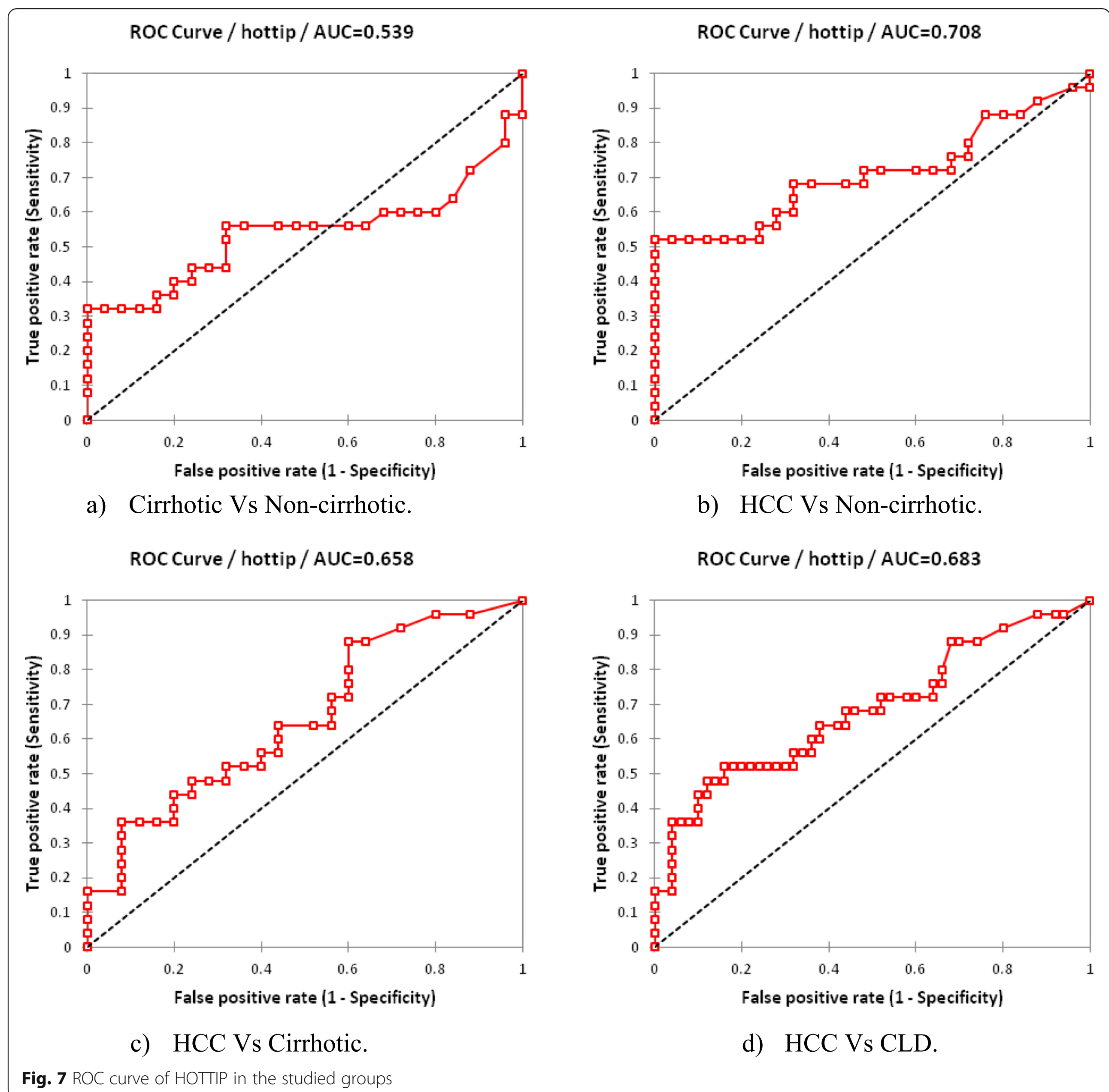

Fig. 7 ROC curve of HOTTIP in the studied groups

HOTTIP axis was a significant molecular pathway during tumorigenesis of HCC and demonstrated the prognostic and potential therapeutic roles of HOTTIP [50], in contrast, Quagliata et al. reported that the patients with higher lncRNAs HOTTIP/HOXA13 expression had poorer prognosis in liver cancer [32]. Also, overexpression of HOTAIR was found to be strongly associated with unfavorable prognosis for patients with HCC [51]. Evidently, the expression levels of HOTTIP increased the odds of being cirrhosis when selected as significant predictors associated with the chances of diagnosis HCC versus cirrhosis patients (p value = 0.05) and concerning CLD as a general group (non-cirrhotic and cirrhotic) had the expression levels of HOTAIR and HOTTIP were considered as predictor parameters associated with the chances of diagnosis for HCC progression and HCC risk assessment (p value = 0.01and p value = 0.003) respectively.

Correlation analysis revealed a significant correlation between HOTAIR and HOTTIP (r = 0.652 and p = 0.001). A significant direct correlation of HOTAIR and HOTTIP with AFP (r = 0.481 and p value = 0.001 and r = 0.400 and p value = 0.001) respectively. A significant inverse correlation of HOTAIR and HOTTIP with

Table 7 Univariate analysis showing the predictive power of different biomarkers for cirrhosis and HCC diagnosis

	Biomarker	OR	95% C.I	*p* value
Cirrhotic vs non-cirrhotic	HOTAIR	1.111	0.998–1.236	0.05*
	HOTTIP	1.07	0.999–1.147	0.05*
HCC vs non-cirrhotic	HOTAIR	1.141	1.026–1.270	0.01*
	HOTTIP	1.103	1.030–1.182	0.005**
HCC vs cirrhotic	HOTAIR	1.027	0.994–1.062	0.1
	HOTTIP	1.029	0.998–1.062	0.05*
HCC vs CLD without HCC	HOTAIR	1.047	1.011–1.084	0.01*
	HOTTIP	1.05	1.017–1.084	0.003**

OR Odd ratio, *C.I* Confidence interval
p value calculated depend on logistic regression analysis, **p value* <0.05 is significant, ***p value* <0.01 is highly significant

albumin ($r = -0.304$ and p value = 0.001and $r = -0.392$ and p value = 0.001) respectively.

Conclusions

Our results suggested that the gradual overexpression of HOTAIR and HOTTIP gene expressions with the progression of disease prompts us to use it as a non-invasive diagnostic and prognostic biomarkers for HCV-induced HCC in HCV genotype 4 patients.

Abbreviations

AASLD: American Association for the Study of Liver Diseases; AFP: Alpha fetoprotein; ALP: Alkaline phosphatase; ALT: Alanine aminotrasferase; ANA: Anti-nuclear antibodies; APRI: AST, platelet ratio index; AST: Aspartate aminotransferase; AUC: Area under the ROC; CLD: Chronic liver disease; CT: Computed tomography; CT: Threshold; ELISA: Enzyme-linked immunosorbent assay; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HOTAIR: Hox transcript antisense intergenic RNA; HOTTIP: HOXA transcript at the distal tip; HOXC: Homeobox gene C cluster; INR: International normalized ratio; LNCRNAs: Long non-coding RNAs; PC: Prothrombine concentration; PT: Prothrombine time; US: Ultrasound

Acknowledgements

We wish to thank Dr. Rady E. El-Araby (Central Lab, Theodor Bilharz Research Institute (TBRI), Ministry of Scientific Research, Egypt) for statistical analysis.

Authors' contributions

FRF made the work design, performance of lab tests, finalized the manuscript and submission. MSF participated in the molecular genetic testing. EGE consultant and drafted the manuscript. OMM patients selection and categorization, integrated the clinical and laboratory data. AAM revised the manuscript, collection of references and consultant. MDE participated in the study design, followed the patients clinically, and supplied the clinical data. FME participated in the study design, supervised the clinical part and finalized the clinical data. All authors approved the manuscript.

Funding

This work was funded by Theodor Bilharz Research Institute (Internal project NO: 23 K) to submit chemicals but no external fund was received.

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by Theodor Bilharz Research Institute Ethics Review Board and an informed consent was obtained written from all the subjects included in this study according to the roles of the Declaration of Helsinki 1975. Approval of local ethical committee (TBRI-REC number 01/19). Patients enrolled in this study were admitted to Gastroenterology and Hepatology Department, Theodor Bilharz Research Institute, Giza, Egypt from November 2016 to August 2018.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Received: 14 October 2019 Accepted: 22 January 2020
Published online: 20 February 2020

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