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Long Noncoding RNA HOTAIR: A Prognostic Biomarker in HBV Egyptian Patients with Hepatocellular Carcinoma

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Abstract: Histologically, confirmed that hepatocellular carcinomas account for around 90% of all primary liver tumors. Hepatocellular carcinoma, which is caused by the hepatitis B virus (HBV), develops through both direct and indirect mechanisms. In the early phases of the disease, the patient's genome becomes unstable due to the integration of HBV DNA, and several genes that cause cancer undergo direct insertional mutagenesis. The expression patterns of long noncoding RNA (lncRNA) are useful for HCC prognosis since lncRNA expression changes significantly in many disorders, such as cancer and viral infections. This research aimed to examine the expression profile of HOTAIR, which stands for Hox transcript antisense intergenic RNA. Using quantitative real-time polymerase chain reaction (qRT-PCR), the expression pattern of HOTAIR was evaluated in serum samples from 50 patients with HBV infection and 50 patients with HCC on top of HBV infection. In comparison, 50 participants who appeared to be in good health and were age- and socioeconomically matched to the patient groups served as controls. To assess the investigated lncRNA's prognostic capacity, receiver operator curve (ROC) analysis was carried out.

Keywords: HBV, HCC, lncRNA, HOTAIR.

1. INTRODUCTION

N Hepatocellular carcinoma (HCC) is a global health concern that varies geographically, according to epidemiological statistics. Egypt is the fifteenth most populated nation in the world and the third most populous nation in Africa. According to Egypt's health authorities, HCC is the most difficult medical condition to treat. Over ten years, the number of HCC patients doubled[1].

Multiple stages of liver cancer move through the body, making it the most common cancer diagnosis and the third leading cause of cancer-related deaths worldwide. About 75–85% of primary liver malignancies are hepatocellular carcinomas (HCC), which usually begin as cirrhosis brought on by fibrotic chronic liver disorders (CLD).[2] There is a complex multistep process involved in the pathophysiology of HCC that leads to the genesis of hepatocyte malignant transformation, the earliest stage of hepatocyte malignant transformation.[3] Alpha-fetoprotein (AFP) levels in the serum can be utilized as a diagnostic indicator for HCC. Among those with chronic liver disease, those whose AFP serum levels remain consistently high are considered at high-risk for hepatocellular carcinoma (HCC). In patients with liver cirrhosis, changes in AFP levels may signal the onset of HCC, rapid viral hepatitis, or possible liver disease progression.[4] A new factor in cancer formation and a possible source of noninvasive cancer biomarkers, long non-coding RNA (lncRNA), has recently drawn notice.[5] Multiple lines of evidence indicate that cancer-associated lncRNA, such as HOX transcript antisense RNA (HOTAIR), HOXA distal transcript antisense RNA (HOTTIP), growth arrest-specific

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transcript 5 (GAS5), BRAF-Activated Non-Protein Coding RNA (BANCR), and Small Nucleolar RNA Host Gene 3 (SNHG3), are closely linked to the prognosis of HCC. Of these lncRNAs, HOTAIR has been the subject of the most research. The prognosis of several human malignancies may be negatively affected by HOTAIR.[6] This has led to HOTAIR's increased recognition as a novel tumor marker for HCC.[7] This study was set out to demonstrate that HOTAIR could be a biomarker for predicting HCC prognosis in Egyptian patients with HBV.

2. METHODS

N There were 150 participants in all for this prospective observational study. Of these, 50 individuals received an HBV diagnosis, and 50 more were driven to the hospital of Kasr El-Ainy, Cairo University in Egypt following an HBV with HCC diagnosis. Tests using quantitative real-time polymerase chain reaction (qRT-PCR) verified the presence of HBV. As a control group, the other fifty participants were healthy volunteers. The study excluded participants who had already experienced hepatitis C virus (HCV).

The study protocol was accepted by the Ethical Committee of Al-Azhar University in Cairo, Faculty of Pharmacy (Girls) (Approval No. 128). Written informed permission was given by each enrolled subject. Peripheral venous 5 mL blood sample was taken from each participant using the BD Vacutainer device. Serum separator tubes were utilized, and the blood was centrifuged for ten minutes at 4000 rpm after a fifteen-minute clotting time. The sera were then separated and kept for further examination at -80 °C.

2.1. Total RNA isolation:

Utilizing the QIAamp RNA Blood Mini Kit (Qiagen, Germany; Catalog No. 4374966), serum total RNA was extracted. This kit purifies total RNA using guanidine hydrochloride and a lysis reagent based on guanidine thiocyanate. The extraction process began with the addition of 100 µL of blood sample to 400 µL of buffer AVL-carrier RNA reagent at room temperature to ensure thorough mixing. This was accomplished by 15 seconds of pulse-vortexing. To ensure full lysis, the mixture was let to remain at room temperature for ten minutes. To encourage efficient binding, ethanol (96–100%) was injected, and then mix-vortexing was performed for 15 seconds. After passing the solution through the QIAamp mini-column, it was centrifuged at 6000 x g (8000 rpm) for one minute without stopping to moisten the rim. Add 500 µL of buffer AW1 (guanidine hydrochloride) and continue centrifugation for another minute. Centrifugation was then continued for three minutes after 500 µL of buffer AW2 was added to the mini-column. Next, 60 µL of buffer AVE was added to a clean 1.5 ml microcentrifuge tube containing the mini-column. The mixture was centrifuged for one minute after equilibrating to ambient temperature for one minute. Lastly, for later usage, the extracted RNA was stored at -80°C.

2.2 Determining the quantity of total RNA:

A spectrophotometer from NanoDrop Technologies, Inc., Wilmington, USA, called a NanoDrop® (ND)-1000, was used to measure the amount of RNA and determine its purity. 1½L of the extracted RNA was added to the NanoDrop-1000 to measure the samples. After that, readings were made and calculated according to Beer-Lambert's law. A260 = 1, which stands for absorbance at 260 nm, showed that the sample contained 44 µg/mL of nucleic acid.

2.3 Quantification of pure lncRNA via PCR:

In this stage of the study, the miScript SYBR® Green PCR kit and its corresponding protocol were utilized. Both the housekeeping control SNORD-68 and HOTAIR primers were used. In order to prepare, the samples were defrosted and subsequently mixed with 200 µL of water that did not contain RNase. The reaction mixture for the miScript Universal primer (10x), 12.5 µL of QuantiTect SYBR Green PCR Master Mix (2x), 5 µL of RNase-free water, and 2.5 µL of cDNA templates was used. The Rotor-Gene Q 72-well rotor (Qiagen, USA) was then used to quantify the targeted lncRNA, HOTAIR. Before initiating the 40 cycles of amplification, the mixture was heated to 95°C for 15 minutes to activate it. Denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds were the subsequent steps in the DNA synthesis. The primer sequences used were as follows: HOTAIR, 5′-GGAAAGATCCAAATGGGACCA-3′ (forward) & 5′-CTAGGAATCAGCACGAAGCAAA-3′ (reverse), and for SNORD-68, 5′-CTGACTTCAACAGCGACACC-3′ (forward) & 5′-TAGCCAAATTCGTTGTCATACC-3′ (reverse).

2.4 Calculation of Results:

Melting curves were examined in the qRT-PCR analysis after cycles were finished to verify and validate the targeted lncRNA's specific expression. Rotor-Gene Q software 2.1 (Qiagen) was used to automatically calculate the cycle threshold (Ct) values. Using the ΔCt method, SNORD-68's relative expression was assessed. This involved deducting the targeted lncRNA Ct values from the SNORD-68 Ct values and then computing the ΔΔCt values for the patient and control groups. The ΔCt values of the control group were subtracted from the ΔCt values of the patient group to finish this phase. Lastly, the fold changes (FC) show that the expression ratio (Rq) for the target lncRNA was calculated using the 2^-ΔΔCt technique.

2.5 The Statistical Analysis:

Mean ± standard deviation (SD) is the metric used to display the outcomes of the statistical analysis performed in this study using GraphPad Prism 8.02. To determine whether there was a relationship between the HOTAIR expression level and the biochemical markers studied in the HBV and HBV with HCC patient groups, Spearman's correlation test was employed. To find statistically significant differences, a significance level of $P<0.05$ was chosen.

3. RESULTS

3.1 The conventional results for biochemistry and demographics:

Participants in the current study included 50 HBV patients (aged 41.08±11.92) and 50 HBV with HCC patients (aged 44.30±11.74) with symptomatic indications and a verified diagnosis by clinical judgment and PCR results. The remaining 50 subjects were age-matched (46.42±12.08) healthy control participants. Table (1) enumerates their traits and typical biochemical results.

3.2 Evaluation of lncRNA-HOTAIR:

Patients with HBV and HCC on top of HBV had a statistically significant elevation in the strength of the HOTAIR expression, according to the clinicopathological findings (Table 2 and Figure 1). In addition, it revealed a noteworthy rise in HBV with HCC patients relative to HBV patients**.**

3.3 Correlation coefficient for the investigated lncRNA-HOTAIR:

Inc-RNA HOTAIR level and total and direct bilirubin showed a strong negative connection in the control group ($p<0.001$ & 0.001, respectively). Within the HBV group, AST, ALT, and α-fetoprotein ($p=0.001$, 0.035 & 0.003, respectively) demonstrated a robust positive correlation with Inc-RNA HOTAIR level, but alkaline phosphatase and Inc-RNA HOTAIR level showed a significant negative correlation ($p=0.020$) as displayed in Figure (2) and Table (3).

3.4 Validity of α-fetoprotein and lncRNA HOTAIR in the detection of HCC.

Our ability to depend on α-fetoprotein for HCC prediction is demonstrated by the ROC curve. At a threshold value of 7.8 ng/mL, α-fetoprotein distinguishes between the HBV with HCC group and the control group with 100% sensitivity and 100% specificity. At a threshold value of >112 ng/mL, it can also distinguish between the HBV and HBV with HCC groups with 100% specificity and 100% sensitivity, respectively, as seen in Figure (3) and Table (4). In addition, the Roc curve illustrates the reliability of lncRNA HOTAIR in predicting HCC. The HOTAIR assay successfully differentiates between the HBV with HCC and control groups down to a relative expression (Rq) of 1.8, with a sensitivity of 98% and a specificity of 100%. When the cutoff is greater than 2.9, HOTAIR can distinguish between the HBV with HCC and HBV groups with a specificity of 72% and a sensitivity of 84%, respectively, as demonstrated in Figure (4) and Table (5).

4. DISCUSSION

Currently, hepatocellular carcinoma ranking the fourth in Egypt and globally the sixth most common cause of cancer[8]. There is a strong correlation between HBV infection and HCC, and regions with an endemic HBV infection tend to have a greater prevalence of HCC.[9] HCC shows overexpression of HOTAIR, an oncogenic lncRNA. It comes from the Homeobox (HOX) gene cluster's source antisense strand and is crucial to the development of cancer. It has been noted that HOTAIR significantly affects HCC by controlling cell division, migration, invasion, and apoptosis. A worse prognosis, greater metastasis, and bigger tumor sizes are usually associated with elevated expression of HOTAIR[10].

Table 1. Description of the demographics and regular biochemistry results

	Control group	HBV group	HBV with HCC group
N	50	50	50
Age (range, year) Mean ± SD, years	21- 66 46.42± 12.08	21- 58 41.08±11.92	21 - 60 44.30 ± 11.74
Gender	Male =30 Female =20	Male = 34 Female = 16	Male = 35 Female = 15
AST (range, U/L) Mean ± SD, U/L	8 - 33 27.28±6.95	32-96 52.08±18.32[a]	20 – 260 61.28 ± 35.5[b,c]
ALT (range, U/L) Mean ± SD, U/L	7 – 55 25.76±8.08	40-72 52.64±9.09[a]	14 – 126 55.52 ± 27.22[b]
Total bilirubin (range,mg/dl) Mean ± SD, mg/dl	0.3 – 1 0.89±0.21	0.5-1.3 0.94 ± 0.56	0.2 – 4.6 2.01 ± 1.43[b,c]
Direct bilirubin (range, mg/dl) Mean ± SD, mg/dl	0.1-0.3 0.23±0.10	0.12-0.9 0.26±0.16	0 – 2.1 1.0 ± 0.74 [b,c]
Albumin (range,g/dl) Mean ± SD, g/dl	3.4-5.4 4.88 ± 0.16	3.2-4.9 4.04 ±0.54	2 – 4.7 3.38 ± 0.64 [b,c]
Alkaline phosphatase (range, U/L) Mean ± SD, U/L	44 – 147 82.68 ±28.3	39-114 74.68 ±25.98	32 – 127 94.58 ± 25.67 [c]
Serum Creatinine (range, mg/dl) Mean ± SD, mg/dl	0.86-1.21 0.96 ±0.26	0.5-1.5 0.97 ±0.31	0.4 – 2.5 0.97 ± 0.3
α - fetoprotein (range, ng/mL) Mean ± SD	0.82 – 7.80 2.64± 1.90	2.80- 119.0 31.29± 23.62[a]	287 - 698 504.44 ± 91.66 [b,c]
HCsAg and HCV-AB	Negative	Negative	Negative

a: statically significant between the control group and the HBV group, p<0.05
b: statically significant between the control group and the HBV with HCC group, p<0.05
c: statically significant between HBV group and HBV with HCC group, p<0.05

Table 2. Comparison between the studied groups regarding lncRNA-HOTAIR

LncRNA-HOTAIR	Control group	HBV group	HBV with HCC group
Range	0.86 – 1.80	1.7 – 4.5	1.4 – 6.6
Mean± SD	1.07± 0.17	2.79 ± 0.89[a]	4.28 ± 1.57[b,c]

a: statically significant between the control group and the HBV group, p<0.05
b: statically significant between the control group and the HBV with HCC group, p<0.05
c: statically significant between HBV group and HBV with HCC group, p<0.05

Currently ranking fourth in Egypt, HCC is the sixth most common cancer globally[8]. There is a strong correlation between HBV infection and HCC, and regions with an endemic HBV infection tend to have a greater prevalence of HCC.[9] HCC shows overexpression of HOTAIR, an oncogenic lncRNA. It comes from the Homeobox (HOX) gene cluster's source antisense strand and is crucial to the development of cancer.

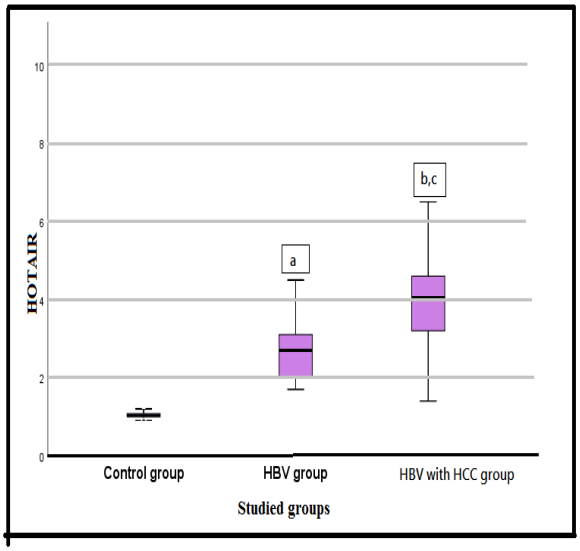

Figure 1. The expression fold of HOTAIR among the studied groups

The HBV group showed a 2.6 fold increase as compared to the control group while the HBV with HCC group showed a 4 fold increase as compared to the control group, 1.5 fold increase as compared to the HBV group.
a: statically significant between the control group and the HBV group, p<0.05
b: statically significant between the control group and the HBV with HCC group, p<0.05
c: statically significant between HBV group and HBV with HCC group, p<0.05.

It has been noted that HOTAIR significantly affects HCC by controlling cell division, migration, invasion, and apoptosis. A worse prognosis, greater metastasis, and bigger tumor sizes are usually associated with elevated expression of HOTAIR[10]. In the past, the focus of investigations into the molecular pathophysiology of HCC has been on the function of genes that code for proteins. There has been a recent uptick in interest in researching non-coding RNAs, which encompass both large and tiny non-coding RNAs. Despite the large amount of research that has been done on sncRNAs, especially microRNAs (miRNAs), there is still much to learn about the roles that lncRNAs play in HCC.

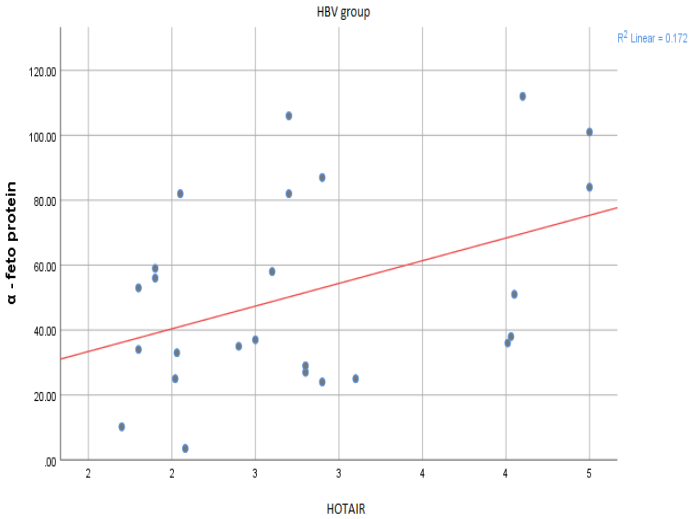

Figure 2. Correlation between α- fetoprotein and lncRNA HOTAIR in HBV group.

Nevertheless, several lncRNAs have been linked to the etiology of HCC, providing insight into its pathophysiology and establishing lncRNAs as promising markers for diagnosis, prognosis, and treatment in this kind of cancer[11].

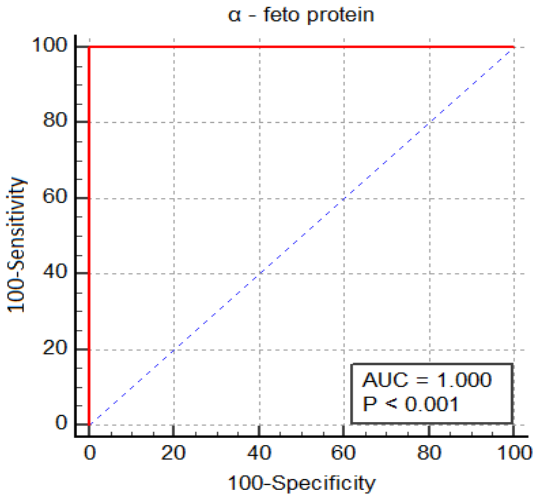

Figure 3. ROC curve of α-fetoprotein

Regarding demographic information, the average age in the HBV, HCC, and control groups was 45.90 ± 7.59, 40.60 ± 12.67, and 48.0 ± 12.85, respectively. Age was not a statistically significant factor that separated the groups under study. According to the gender distribution, there were 20 female patients and 30 male patients in the control group. Among the studied groups, there was no statistically significant difference in gender.

Table3. Correlation between lncRNA-HOTAIR levels and different parameters in the studied groups.

Parameters	INC-RNA HOTAIR					
	Control group (n=50)		HBV group (n=50)		HBV with HCC group (n=50)	
	r	P-value	R	P-value	r	P-value
AST (U/L)	-0.219-	0.127	0.454*	0.001	0.241	0.092
ALT (U/L)	-0.152-	0.293	0.300*	0.035	-0.006-	0.968
T.Bilirubin (mg/dl)	-0.480-*	0.000	0.003	0.984	-0.065-	0.652
D. Bilirubin (mg/dl)	-0.459-*	0.001	0.197	0.169	0.052	0.722
Albumin (g/dl)	0.178	0.216	0.241	0.092	0.184	0.202
Alkaline Phosphate (U/L)	0.265	0.063	-0.327-*	0.020	-0.102-	0.482
Creatinine (mg/dl)	0.049	0.738	0.187	0.193	0.098	0.500
α-feto protein (ng/ml)	0.038	0.793	0.415*	0.003	0.192	0.181

Statistically significant, r= Spearman's rho*

Table 4. Absolute sensitivity, specificity, and area under the curve of α-fetoprotein for diagnosis of HCC.

α-fetoprotein (ng/ml)	AUC	Cut off Point	Sensitivity	Specificity	PPV	NPV	p-value
HBV with HCC vs Control	1.00	>7.8	100%	100%	100%	100%	**<0.001**
HBV with HCC vs HBV	1.00	>112	100%	100%	100%	100%	**<0.001**

AUC: Area under Curve, PPV: Positive Predictive Value, NPV: Negative Predictive Value

Table 5. Absolute sensitivity, specificity, and area under the curve of lncRNA HOTAIR in the detection **of HCC.**

lncRNA HOTAIR (Rq)	AUC	Cut off Point	Sensitivity	Specificity	PPV	NPV	p-value
HBV with HCC vs Control	0.999	>1.8	98%	100%	100%	98.04%	<0.001
HBV with HCC vs HBV	0.819	>2.9	84%	72%	75%	81.8%	<0.001

AUC: Area under Curve, PPV: Positive Predictive Value, NPV: Negative Predictive Value

There was a majority of males in all of the groups examined, which is in line with our findings and those of another study that did not find any differences in the distribution of age or gender. Furthermore, the research revealed that there were no noteworthy disparities in the gender (p = 0.797) or age distributions between the control group and the HCC patients.[12] The present study found that AST levels in the control group dropped significantly more than in the HBV and HCC groups. Furthermore, there was a statistically significant difference in AST levels between the HCC and HBV groups (P = 0.001). The reduction in ALT levels was statistically significant when the control group was compared to the HBV and HCC groups.

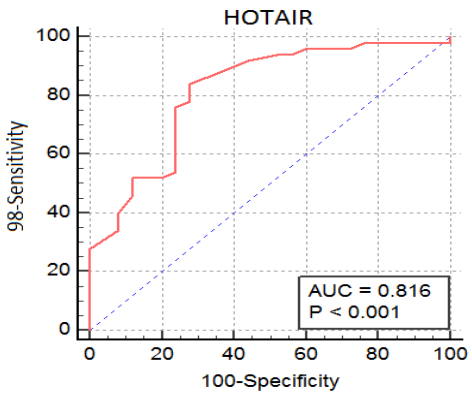

Figure 4. ROC curve of lncRNA HOTAIR

When compared to both the control group and the HBV group, the HCC group showed a significantly higher total bilirubin level. There was a statistically significant rise (P < 0.001) in direct bilirubin levels when comparing the HBV group to the control group and a similarly substantial increase (P < 0.001) when comparing the HCC group to the control group. The HCC group's albumin levels dropped significantly (P < 0.001) in comparison to the HBV group and the control group.

The HCC group had considerably greater levels of alkaline phosphatase than the HBV group (P < 0.001). Blood creatinine levels did not differ significantly (p > 0.05) across the study groups. The present results were in line with results from another study that revealed a statistically substantial variance in bilirubin, AST, and ALT levels between HCC patients and controls.[13] Furthermore, a separate investigation found that HCC patients had substantially higher ALT and AST values than cirrhosis patients.[14] The levels of AST, ALT, bilirubin, and albumin all differed significantly across the research groups.[15] In contrast to the control group, the HBV and HCC groups in the current study exhibited a noticeable rise in α-fetoprotein (P < 0.001). Not only that, but the HCC group showed a significantly higher increase compared to the HBV group (P < 0.001). Prior research has shown that α-fetoprotein levels were highly significant in patients with HCC when contrasted to those with HBV (p-value < 0.001), and our data support this.[16]

Our research indicates that the HCC and HBV groups had significantly higher levels of lncRNA-HOTAIR compared to the control group (P < 0.001). Furthermore, the HCC group showed a much higher increase (P < 0.001) when contrasted with the HBV group. The levels of lncRNA HOTAIR and alkaline phosphatase were shown to be significantly inversely related in the HBV group (p=0.020). The levels of lncRNA HOTAIR were strongly correlated with AST, ALT, and α-fetoprotein, respectively, with p-values of 0.001, 0.035, and 0.003, respectively.

The results of the present study demonstrate that α-fetoprotein can be trusted as a diagnostic tool for hepatocellular cancer. It can distinguish between the HCC group and the control group at a 7.8 ng/mL threshold with a sensitivity of 100% and a specificity of 100%. With a specificity of 100% for HCC and a sensitivity of 100% for HCV, α-fetoprotein can differentiate between the two groups when the threshold exceeds 119 ng/mL. When the threshold is above 112 ng/mL, α-fetoprotein can differentiate between the HCC and HBV groups with a specificity of 100% and a sensitivity of 100%, respectively. Another study indicated an AUC of 0.85 (95% CI: 0.80 - 0.90) for α-fetoprotein, and the ideal cutoff value for this was determined to be 20.85 ng/ml. Here are the results for patient diagnoses of HCC using this cutoff: 86.2% specificity, 89.9% positive predictive value, 64.7% negative predictive value, and 77.4% total accuracy.[17]

α-fetoprotein facilitates the efficient development, expansion, and dissemination of HCC tumors. Additionally, it prevents the immune system from attacking cancer cells by interfering with their processes.[18] Patients with HCC have a worse chance of surviving the disease-free and overall survival periods when their preoperative α-fetoprotein levels are high. Higher α-fetoprotein levels are associated with microvascular invasion, bigger tumor size, and poorly differentiated HCC.[19] However, several disadvantages were observed over the years, where it lacks accuracy as a diagnostic or screening marker, it shows variability in cutoff values. [18,19]

The study's findings demonstrated how useful the ROC curve is for determining how well lncRNA HOTAIR predicts HCC. Where lncRNA HOTAIR demonstrated great sensitivity (98%) and specificity (100%), successfully differentiating between HCC patients and the control group. lncRNA HOTAIR demonstrated 84% sensitivity and 72% specificity in differentiating HBV-infected individuals from HCC patients, which suggests that the higher expression of lncRNA HOTAIR is a significant predictor of HCC. Consistent with previous research, lncRNA markers were used to study the differentiation of cirrhosis patients from HCC. Results showed a statistically significant difference in HOTAIR between the HCC and cirrhotic groups These results suggest that HOTAIR shows promise as a diagnostic to differentiate between cirrhosis and HCC patients.[20]

Interestingly, when compared to normal controls, cells infected with HBV and tissues associated with HBV-related HCC exhibit a substantial increase in HOTAIR expression. Individuals with HBV-related HCC had considerably greater plasma levels of HOTAIR than those with chronic hepatitis B, whether or not they have cirrhosis. This suggests that HOTAIR may have the potential as a non-invasive diagnostic biomarker for the detection of HCC in individuals with HBV.[21] By increasing HBV promoter activity, HOTAIR facilitates HBV transcription and replication. It increases HBV replication by interacting with HBx and increasing HBx-mediated activation of the miR-539 promoter, which in turn decreases APOBEC3B expression.[22] The recurrence of tumors in HBV-related HCC patients following liver transplantation is predicted by HOTAIR overexpression. An unfavorable prognosis is associated with high HOTAIR levels. [23]

5. CONCLUSION

The level of HOTAIR substantially increases in HBV and HCC patients when compared to controls, thus it has the potential as a non-invasive diagnostic and prognostic marker making it a promising biomarker candidate for HBV-related HCC.

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Author contribution: Reham El Gabry: Conceptualization, Methodology, Investigation, Writing - Original draft. **Lalia A. Rashed**: Investigation, Formal Analysis. Mai Mehrez: Formal Analysis, Data curation. **Shereen Saeid Elshaer**: Conceptualization, Methodology, Writing - Review and editing.

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