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Circulating lncRNAs HOTTIP and HOTAIR as Potential Biomarkers in Crigler-Najjar Syndrome: A Preliminary Report from Shiraz Liver Transplant Research Center

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Abstract

Crigler-Najjar syndrome (CNS) is a rare autosomal recessive disorder characterized by impaired bilirubin metabolism, leading to neurotoxic unconjugated bilirubin accumulation. Liver transplantation remains the only effective treatment, highlighting the need for novel diagnostic and therapeutic approaches. Long non-coding RNAs (lncRNAs) have emerged as potential biomarkers in various diseases, including cancer. This study aimed to evaluate the expression of lncRNAs HOTTIP and HOTAIR in CNS patients and healthy controls, exploring their potential as non-invasive diagnostic tools. Serum samples from CNS patients (n = 6) and healthy controls (n = 26) were analyzed using quantitative real-time polymerase chain reaction. While both lncRNAs showed decreased expression in CNS patients compared to controls, the differences were not statistically significant. However, receiver operating characteristic curve analysis revealed promising diagnostic performances for both lncRNAs. Correlations between lncRNA expression and clinical parameters were explored, revealing potential associations with disease progression. Overall, this study provides preliminary insights into the role of lncRNAs HOTTIP and HOTAIR in CNS and underscores the need for further research to validate their utility as biomarkers and therapeutic targets in this rare disorder.

Keywords

Crigler-Najjar syndrome; lncRNAs; HOTAIR; HOTTIP; liver

1. Introduction

Crigler-Najjar syndrome (CNS) is a rare autosomal recessive disorder of bilirubin metabolism with a prevalence of 1 in a million live births worldwide, affecting both genders equally [1]. Impaired function of uridine 5'-diphosphate glucuronyltransferase (UGT1A1), an intracellular enzyme primarily expressed in hepatocytes responsible for bilirubin glucuronidation activity, results in neurotoxic unconjugated bilirubin (UCB) accumulation [1, 2].

Crigler-Najjar syndrome has two distinct forms; type 1 is the most severe form due to the complete absence of UGT1A1 enzyme activity [1, 3]. Patients with Crigler-Najjar syndrome type 1 have total serum bilirubin levels ranging from 20 mg/dl up to 50 mg/dl and severe jaundice. If left untreated, it can lead to severe neurological damage, including kernicterus, and eventually death [3, 4]. Patients with Crigler-Najjar syndrome type 2 have residual UGT1A1 enzymatic activity, which is adequate for maintaining UCB concentration below the risk of developing severe neurologic impairment (these patients have total serum bilirubin levels of 3.5-20 mg/dl) [1, 4]. The diagnosis of Crigler-Najjar syndrome is based on thorough clinical evaluation, laboratory findings, and molecular genetic study [5]. Intensive phototherapy, plasmapheresis, and pharmacological treatment are among the therapeutic methods available for Crigler-Najjar syndrome patients; however, these treatment options have limited efficacy and significant impacts on patients' quality of life [4, 5]. Currently, liver transplantation is the only effective treatment for Crigler-Najjar syndrome patients that restores UGT1A1 activity, reduces serum bilirubin levels, and significantly increases the 5-year survival rate of Crigler-Najjar syndrome patients. Yet, it has severe

complications and shortcomings [4]. Therefore, understanding Crigler-Najjar syndrome molecular mechanisms is essential to identify new diagnostic and therapeutic targets.

Long non-coding RNAs (lncRNAs) are a subset of non-coding RNAs longer than 200 nucleotides [6]. They play significant roles in homeostatic and physiological functions such as cell cycle regulation, cell differentiation, and organogenesis by regulating transcription, translation, and chromatin modification (Figure 1) [7-9].

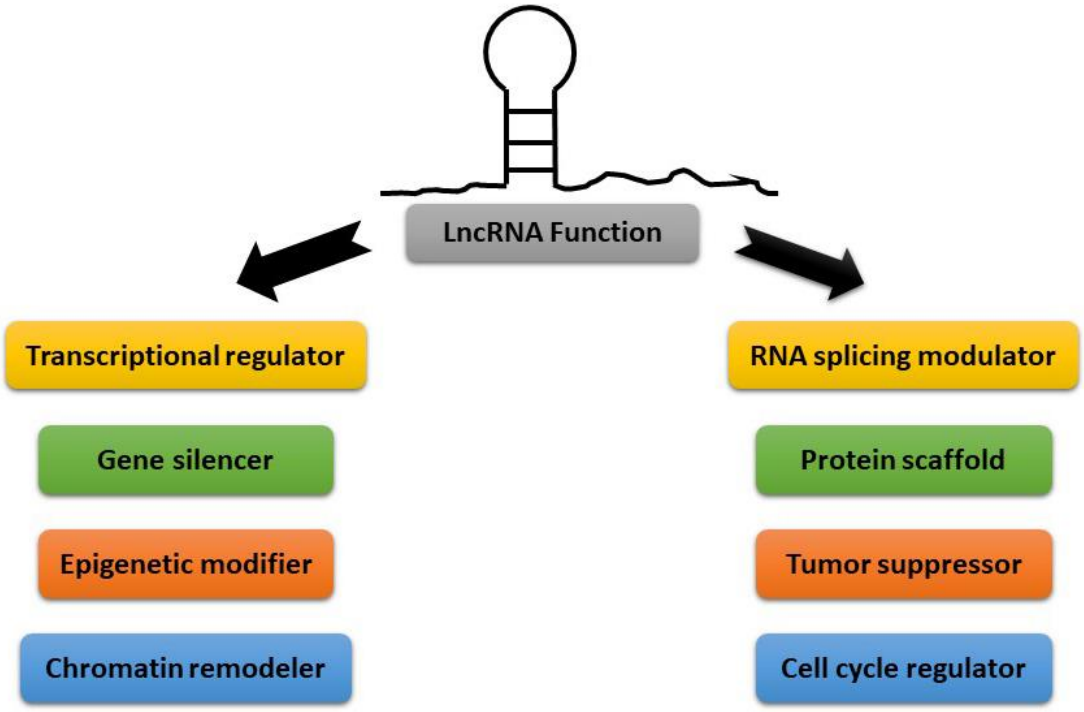


Figure 1 LncRNA function.

HOXA transcript at the distal tip (HOTTIP) is a lncRNA involved in the HOX gene network, leading to epigenetic changes and promoting tumorigenesis [9]. Additionally, HOTTIP is involved in developmental processes [10]. Several studies have identified HOTTIP as an oncogenic lncRNA and a non-invasive biomarker for various human malignancies, including colorectal, pancreatic, and gastric cancer, as well as renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC) [7, 10].

The lncRNA HOX transcript antisense RNA (HOTAIR) contributes to normal mammalian development, including skin, lumbosacral, urogenital system development, and osteogenesis [11]. HOTAIR plays a crucial role in initiating and progressing numerous human cancers by promoting cellular proliferation, migration, and invasion while inhibiting cancer cell apoptosis and regulating immune signaling and inflammation [11, 12]. Previous studies have explored the role of lncRNA HOTAIR in various cancers, such as breast, pancreatic, gastric, and non-small cell lung cancers, as well as cholangiocarcinoma and HCC, suggesting HOTAIR as a potential diagnostic and therapeutic target for these malignancies [12-14].

As lncRNAs can be detected in body fluids, recent studies have suggested that they could serve as a promising non-invasive biomarker for early diagnosing diseases like cancer [15].

The purpose of this study is to assess the expression of lncRNA HOTTIP and HOTAIR in the serum of Crigler-Najjar syndrome patients and compare the findings with those of healthy individuals to create a non-invasive diagnostic tool for this rare yet challenging disease.

2. Materials and Methods

2.1 Participants and Ethics Statement

This cross-sectional study involved 6 Crigler-Najjar syndrome patients and 26 healthy individuals. The patients were from the Shiraz Pediatric Liver Cirrhosis Cohort Study (SPLCCS) (IR.SUMS.REC.1398.142) [16], and their clinical and laboratory data were obtained from the pediatric liver cirrhosis registry (IR.SUMS.REC.1399.530). Blood samples were collected and stored at -80°C in the Shiraz Transplant Research Center biobank. The control group was comprised of patients admitted to surgery wards for minor procedures, such as tonsillectomy, with no history of liver disorders. Written informed consent was obtained from each individual's parents before they were enrolled in the study. The Ethics Committee of Shiraz University of Medical Sciences approved the study protocols (IR.SUMS.MED.REC.1401.457).

2.2 Circulating lncRNAs Extraction and Quantitative Real-Time Polymerase Chain Reaction

lncRNA was extracted from the serum samples of Crigler-Najjar syndrome patients and the control group using RNX-Plus (Cinnagen, Iran) following the manufacturer's instructions. Complementary DNA was synthesized by the RB-lncRNA Synthesis kit (RNA biotechnology company, Iran) with specific stem-loop primers using 300 ng total RNA in a 20-µL total reaction. The reaction included 100 pMol stem-loop primer for lncRNA, 10 mM dNTP, 100 pMol RT Primer, 5× Reverse Transcriptase Buffer, and M-MLV reverse transcriptase enzyme. It was conducted in a thermal cycler machine under the following conditions: 60°C for 10 minutes, 55°C for 45 minutes, 72°C for 20 minutes, and 12°C for 10 minutes. Real-time PCR was carried out using the Step-One ABI applied Biosystem (Life Technologies). U6 snRNA served as a control for the analysis of mi expression. Reactions were performed following these parameters: 95°C for 15 minutes, 95°C for 15 seconds, and 62°C for 60 seconds for 40 cycles. Each sample underwent three measurements for subsequent analysis, and the mean cyclic threshold (Ct) values were utilized. The Livak ($2-\Delta\Delta Ct$) method was used to determine the samples' lncRNA HOTAIR and HOTTIP expression.

2.3 Statistical Analysis

Data normality was assessed using the Shapiro-Wilk test, skewness, and kurtosis indices. Average data were expressed as mean ± standard deviation. Non-normal variables were presented as the median and interquartile range (IQR) (25 and 75 percentile) with numbers (%) for categorical variables. The Mann-Whitney and Chi-square tests were employed to compare the two groups. Furthermore, the relationship between lncRNAs' expression and laboratory and baseline data, as well as gender in the cases, was analyzed using Spearman's and Mann-Whitney tests. The receiver operating characteristic (ROC) curve for each lncRNA was plotted, and sensitivity, specificity, and area under the curve (AUC) were determined. The data was analyzed using SPSS software (Version 21, SPSS Inc., Chicago, United States). A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Clinical and Demographic Characteristics of the Participants

A total of 6 Crigler-Najjar syndrome patients and 26 healthy control subjects participated in this cross-sectional study. Their baseline demographic data are presented in Table 1. 50% of Crigler-Najjar syndrome patients were female with a median age of 2.5 years. In contrast, the median age of the control group was 6 years, with 38.5% being female. All Crigler-Najjar syndrome cases' parents were relatives, and 50% of cases had a family history of liver diseases. One out of six patients underwent liver transplantation, and no deaths were reported during the study. Crigler-Najjar syndrome patients exhibited significantly higher levels of total and direct bilirubin and liver enzymes (AST, ALT, ALKP) compared to the control group. The participants' laboratory findings are detailed in Table 2.

Table 1 Demographic features of case and control groups.

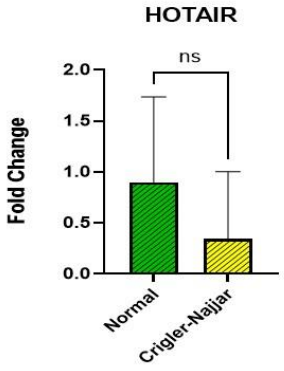
Variables	Case (n = 6)	Control (n = 26)
Age, y, med (Q1-Q3)	2.5 (0.5, 5.1)	6 (4.5, 7.25)
Gender, n (%)		
Male	3 (50.0)	16 (61.5)
Female	3 (50.0)	10 (38.5)

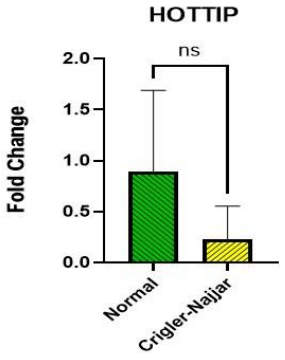
Table 2 Laboratory findings of case and control groups.

Variables	Case (n = 6)	Control (n = 26)	P value
AST (U/L)	41.5(32.5, 47.25)	22.5 (21.0, 28.52)	0.008
ALT (U/L)	19.5 (11.75, 30.0)	7.0 (6.0, 12.25)	0.001
ALKP (U/L)	812.21 (690.75, 892.5)	402 (353, 490)	<0.001
Albumin (g/dL)	4.7 (2.79, 5.10)	3.9 (3.67, 4.20)	0.381
Total Bill (mg/dL)	21.11 (4.58, 29.32)	0.4 (0.4, 0.5)	<0.001
Direct Bill (mg/dL)	0.44 (0.40, 0.65)	0.2 (0.1, 0.2)	<0.001
Hb (g/dL)	10.25 (9.06, 13.5)	12.3 (13.1, 13.9)	0.029
WBC per 1000	6.3 (5.09, 7.90)	6.95 (6.02, 8.12)	0.308
Platelet per 1000	361.2 (280.0, 455.5)	318.0 (280.2, 375.8)	0.408

3.2 Expression of HOTTIP and HOTAIR lncRNAs in CNS Patients

This research examined the serum levels of HOTTIP and HOTAIR lncRNAs in both the case and control groups. The findings indicated that lncRNA HOTTIP was reduced in Crigler-Najjar syndrome patients (mean fold change of 0.2334) compared to healthy individuals (mean fold change of 0.8895). However, the difference was not statistically significant ($p = 0.09$). Similarly, the expression of lncRNA HOTAIR was lower in Crigler-Najjar syndrome cases (mean fold change of 0.3492) than in healthy controls (mean fold change of 0.9016), but this disparity was not statistically significant ($p = 0.24$). These results are illustrated in Figure 2.





Unpaired t test HOTAIR	
P value	0.2494
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.231, df=9
Mean of column A	0.9016
Mean of column B	0.3492
Difference between means (B - A) ± SEM	-0.5524 ± 0.4486
95% confidence interval	-1.567 to 0.4624
R squared (eta squared)	0.1442

Unpaired t test HOTTIP	
P value	0.0965
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.856, df=9
Mean of column A	0.8895
Mean of column B	0.2334
Difference between means (B - A) ± SEM	-0.6561 ± 0.3535
95% confidence interval	-1.456 to 0.1437
R squared (eta squared)	0.2767

Figure 2 LncRNA HOTTIP and HOTAIR expression levels in CNS patients and healthy subjects.

3.3 ROC Curve

Receiver operating curves [17] were utilized to assess the diagnostic accuracy of HOTAIR and HOTTIP lncRNAs in Crigler-Najjar syndrome patients (Figure 3). For HOTAIR, to distinguish between Crigler-Najjar syndrome and the healthy group, an expression level of 0.266 was identified as the cut-off value, with a sensitivity of 83.3% and specificity of 100% (area under the curve (AUC) of 0.867 and p = 0.008). Regarding HOTTIP, to differentiate between Crigler-Najjar syndrome cases and the control group, a plasma level of 0.202 was determined as the cut-off value, with a sensitivity of 83.3% and specificity of 100% (AUC of 0.867 and p = 0.008).

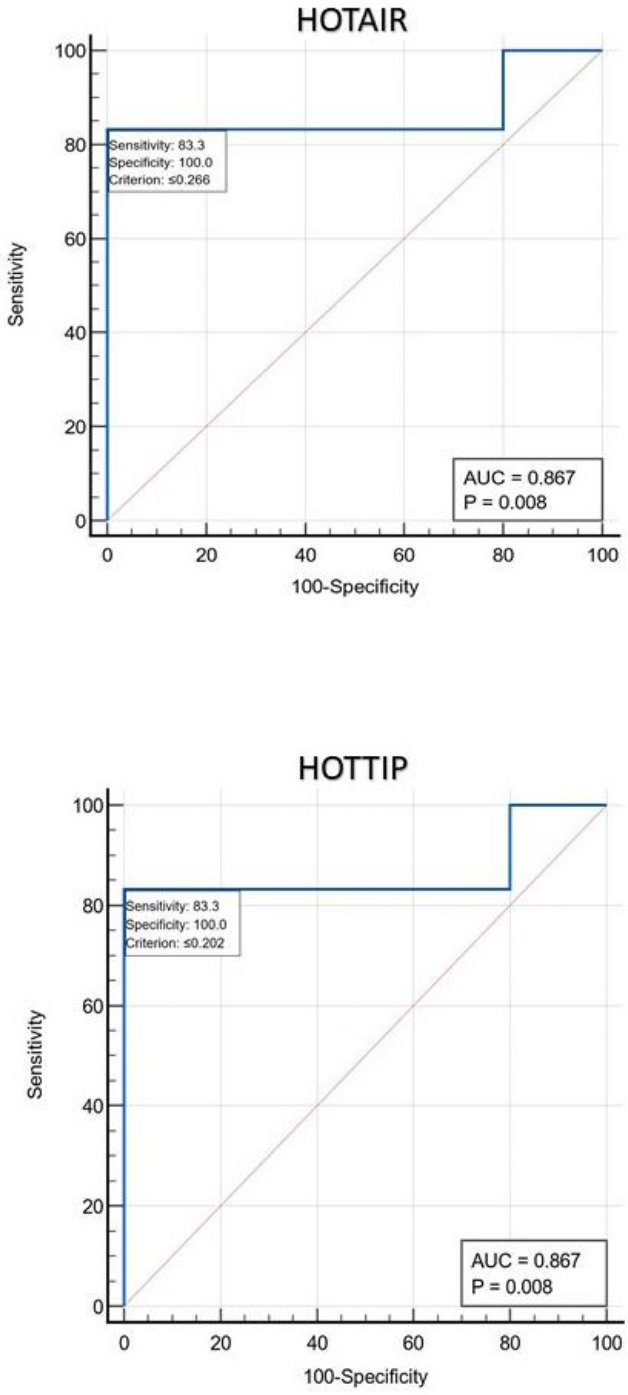


Figure 3 ROC curves for HOTAIR and HOTTIP lncRNAs.

3.4 Relationship between lncRNA HOTAIR and HOTTIP Expression and Baseline and Laboratory Data of CNS Patients

Furthermore, we examined the association between lncRNA HOTAIR and HOTTIP expression levels and the baseline and laboratory findings of Crigler-Najjar syndrome cases. Our findings indicated no link between HOTAIR and HOTTIP expression and patients' demographic data, such as gender, parental relationship, and history of liver transplantation. Additionally, our results showed

no correlation between serum levels of HOTAIR and HOTTIP and laboratory findings, including liver enzymes, total, and direct bilirubin among Crigler-Najjar syndrome cases. Nevertheless, we observed a significant inverse correlation between HOTAIR levels and serum albumin (r = -0.928, p = 0.008) and a direct correlation with platelet counts (r = 0.943, p = 0.005). The detailed data are presented in Table 3 and Table 4.

Table 3 Relationship between HOTAIR and HOTTIP expression and CNS patients' demographic data.

	HOTAIR		**HOTTIP**	
Gender	Mean ± SD	P. value	Mean ± SD	P. value
Male	0.13 ± 0.11	0.700	0.39 ± 0.41	0.200
Female	0.56 ± 0.95		0.07 ± 0.09	
Parents relevant				
***Cousin**	0.43 ± 0.82	0.667	0.27 ± 0.40	0.667
***Relative**	0.16 ± 0.13		0.15 ± 0.06	
Liver Disease				
Yes	0.56 ± 0.95	0.700	0.07 ± 0.09	0.200
No	0.13 ± 0.11		0.39 ± 0.41	
Liver transplantation				
Yes		0.667		0.667
NO				

*A cousin is not a direct ancestor or descendant, but a relative who shares a common ancestor. The term 'cousin' typically refers to a person's first cousin – the child of someone's aunt or uncle.

Table 4 Correlation between HOTAIR and HOTTIP levels and laboratory findings of Crigler-Najjar syndrome cases.

Variables	**HOTAIR**		**HOTTIP**	
	rho	P value	rho	P value
Age	-0.928	0.008	-0.754	0.084
AST	0.206	0.695	0.118	0.824
ALT	0.314	0.544	0.600	0.208
ALKP	0.429	0.397	0.600	0.208
Albumin	-0.928	0.008	-0.638	0.713
Total protein	-0.600	0.208	0.143	0.787
Total Bill	-0.600	0.208	0.143	0.787
Direct Bill	0.486	0.329	0.543	0.266
INR	-0.500	0.667	-0.500	0.667
PT	-0.500	0.667	-0.500	0.667
Hb	-0.754	0.084	-0.464	0.354
WBC	-0.371	0.468	0.429	0.397
Platelet	0.943	0.005	0.657	0.156

4. Discussion

Crigler-Najjar syndrome (CNS) is an infrequent condition caused by a complete or partial loss of UGT1A1 activity leading to unconjugated hyperbilirubinemia [18]. Despite current standard treatments like phototherapy and liver transplantation, Crigler-Najjar syndrome poses a significant burden and dramatically impacts patients' quality of life. Therefore, innovative diagnostic and therapeutic approaches are essential [2, 18]. Long non-coding RNAs (lncRNAs) are increasingly recognized for their regulatory roles in various biological processes and diseases such as cancer [11, 19]. Hence, we conducted this research to assess the levels of lncRNAs HOTAIR and HOTTIP in individuals with Crigler-Najjar syndrome compared to healthy controls. Our findings revealed a decrease in the expression of lncRNAs HOTAIR and HOTTIP in the plasma of Crigler-Najjar syndrome patients compared to the control group. However, this decrease did not reach statistical significance. Previous studies have indicated that HOTTIP is one of the most significantly upregulated lncRNAs in hepatocellular carcinoma (HCC) and is associated with poor overall survival [7, 17]. Suppression of HOTTIP has been linked to the inhibition of HCC cell proliferation, tumor formation, reduced cell migration, and decreased pulmonary metastasis [7]. Kim et al. have suggested that HOTTIP, as a serum small extracellular vesicle-derived lncRNA, shows promising potential as a biomarker for early HCC diagnosis [20]. Additionally, individuals with higher levels of HOTTIP expression in tumors face an increased risk of post-transplantation recurrence [9].

LncRNA HOTAIR was significantly overexpressed in tumor tissue and peripheral blood of HCC patients, and patients with lower levels of lncRNA HOTAIR had more prolonged overall survival and progression-free survival [14]. HOTAIR enhances human liver cancer stem cell growth and knockdown of lncRNA HOTAIR induces apoptosis and prevents HCC cell proliferation, epithelial-mesenchymal transition, and migration [21, 22]. Additionally, overexpression of HOTAIR was significantly associated with tumor size, TNM stage, and postoperative recurrence in patients with cholangiocarcinoma (CCA), and its knockdown led to decreased invasion and increased apoptosis of CCA cells [13]. Tang and colleagues demonstrated that HOTAIR modulates autophagy through the miR-20b-5p/ATG7 axis in hepatic ischemia-reperfusion injury (IRI) [23].

A study conducted by Roshdy et al. on patients with chronic liver disease induced by hepatitis C virus (HCV) with or without cirrhosis and HCC revealed that lncRNA HOTAIR and HOTTIP were significantly upregulated in diseased groups and were able to distinguish between cirrhotic and HCC cases compared to healthy controls. Furthermore, they reported a significant inverse correlation of HOTAIR and HOTTIP with albumin ($r = -0.304$, $p = 0.007$ and $r = -0.392$, $p = 0.001$ respectively) [15]. Our results also demonstrated that HOTAIR had an inverse correlation with patients' age but no association with patients' gender. A recent meta-analysis indicated that HOTAIR expression had no association with the age and gender of patients with HCC but showed a significant relation with tumor stage and overall survival [24]. These discrepancies in results might be due to the limited sample size and different liver pathologies evaluated in these experiments. Additionally, several studies have revealed the contribution of HOTAIR and HOTTIP to liver fibrosis [19, 25]. HOTAIR might enhance liver fibrosis by regulating DNMT1, MEG3, and the p53 pathway in hepatic stellate cells (HSC) and induce the expression of fibrosis-related genes such as matrix metalloproteinase 2 (MMP2) and MMP9 [19, 26]. Previous investigations have shown that HOTTIP promoted mouse hepatic fibrosis by activating HSC as a competing endogenous RNA (ceRNA) for miR-148a and miR-150 [27-29].

The limitation of this study is the small sample size of patients, although this limitation is inherent when studying such a rare disease. This study is preliminary. Further studies in larger populations are needed to confirm or rule out the significant role of HOTTIP and HOTAIR as potential biomarkers in Crigler-Najjar Syndrome.

5. Conclusion

In conclusion, the results of this study showed a decrease in lncRNAs HOTAIR and HOTTIP in the serum of patients with Crigler-Najjar syndrome. However, it did not reach statistical significance. The absence of an increase in the level of ncRNA in patients with CNS may confirm that no oncogenic process is expected during the evolution of the CNS. This is the first study to investigate the expression of lncRNAs HOTAIR and HOTTIP in Crigler-Najjar syndrome patients compared to healthy controls. As mentioned earlier, HOTAIR and HOTTIP are implicated in various liver diseases, such as HCC, hepatic ischemia-reperfusion injury (IRI), and liver fibrosis. They have been proposed as potential diagnostic and therapeutic targets. Therefore, further research with a larger population is necessary to elucidate their precise roles in the pathology of Crigler-Najjar syndrome and assess the potential of these two lncRNAs as non-invasive biomarkers for diagnosing and treating Crigler-Najjar syndrome.

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Author Contributions

All of the authors contributed substantially to the concept and design of the study. Material preparation, data collection, and analysis were performed by N.M., M.M., N.A., K.F., M. Mo, S.P., M.A., E.S., M.H., S.A.M., T.K., and M.D. The primary draft of the manuscript was written by N.M. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no conflict of interest.

Data Availability Statement

All data are available in manuscript.

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