

Clinical Potential of HOTAIR, MALAT1, and UCA1 lncRNAs As a Biomarker to Achieve More Accurate Prognostic Predictions for Liver Cancer

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Research Article

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Abstract

Background: Hepatocellular carcinoma (HCC) is a major worldwide health concern and is the third leading cause of cancer death. Recent studies have focused on the association between long non-coding RNAs (lncRNAs) and cancer, lncRNAs showed to have an important role in the prognosis, diagnostic, and investigation of liver cancer. Therefore, this study focuses on the expression profiles of *HOTAIR*, *MALAT1*, and *UCA1* lncRNAs considering the clinicopathological characteristics of patients with liver tumors.

Methods and Results: The expression profiles of *HOTAIR*, *MALAT1*, and *UCA1* lncRNAs were evaluated using qRT-PCR in the paired liver tumor and the adjacent non-tumor samples. After RNA extraction from tissue samples, cDNA synthesis and the RT-qPCR method were performed. Livak method ($2^{-\Delta\Delta Ct}$) was used for calculating the expression level of lncRNAs. Principal-component analyses followed by receiver operating characteristic (ROC) curve analyses were performed to evaluate the diagnostic potential of the selected lncRNAs. Our results showed that *HOTAIR*, *MALAT1*, and *UCA1* were overexpressed significantly in patients with liver cancer compared to the healthy groups ($P < 0.001$). Moreover, the expression of *HOTAIR* was enhanced significantly compared to the expression of *MALAT1* and *UCA1* in patients with liver cancer ($P < 0.001$). This study showed that there were no significant associations between lncRNAs expression and the clinical characteristics ($P > 0.05$). Significantly elevated circulating lncRNAs were found to be liver cancer-specific and showed differentiation of liver cancer samples from the controls. Kaplan-Meier analysis revealed no significant correlations between the lncRNAs expression and overall survival.

Conclusion: Based on our findings, the studied lncRNAs were not correlated with clinicopathological characteristics of the liver cancer patients although the overexpression of these lncRNAs might provide novel molecular biomarkers in HCC cases.

Background

One of the most common types of cancer is liver cancer. The incidence of liver cancer differs vastly over the world and is more prevalent in sub-Saharan Africa and eastern Asia [1]. Most of the primary liver cancers (PLC) are derived from the intrahepatic bile ducts epithelial lining [2]. HCC encompasses nearly 90 percent of all cases of primary liver malignancy and is the biggest cause of cancer-related death [3, 4]. Despite the advancement of various therapy strategies, such as surgical resection, radiation therapy, and chemotherapy, the outcomes are unsatisfactory as HCC molecular mechanism is still unknown [5]. Therefore, various novel and reliable biomarkers are required to identify, predict and treat liver cancer. As a result, many types of research on unraveling the molecular mechanisms of liver cancer have been conducted, and various mechanisms have been recognized [1].

Recent advances in cancer transcriptome profiling and the documents supporting long non-coding RNAs (lncRNAs) function, several differentially expressed lncRNAs are correlated with various types of cancer including breast, lung, colorectal, prostate, and liver cancer [6]. Accumulating studies of cancer-

associated lncRNAs have been reported on the critical role of lncRNAs in tumorigenesis and the development of various tumors, several cancer types, and metastasis [6–8]. Furthermore, numerous researches have shown the associations between some lncRNAs, such as prostate cancer-associated non-coding RNA 1 (*PRNCR1*), the metastasis-associated HOX antisense intergenic RNA (*HOTAIR*), metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), urothelial cancer-associated 1 (*UCA1*), colon cancer-associated transcript 2 (*CCAT2*) and different cancers, such as prostate cancer [9], liver cancer [10], breast cancer [11], lung cancer [12], gastric cancer [13] and esophageal squamous cell carcinoma (ESCC) [14]. According to the literature, various cancer types have been affected by tumorous clinical-pathological features, such as age, gender, lymph node metastasis, clinical stage, and tumor size [15]. Recently, many researchers are searching for novel biomarkers that could help the diagnosis or prognosis of cancers. One of the main advantages of lncRNAs is their high stability as cancer diagnostic and prognostic biomarkers. Different studies have reported significant correlations between lncRNAs expression and clinical characteristics [13, 16–18].

In the present study, we propose that the lncRNAs expression profile may be used as a clinical marker for the diagnosis or prognosis of liver cancer therefore in this study we evaluated the potential usefulness of three lncRNAs (*HOTAIR*, *MALAT1*, and *UCA1*) in liver cancer.

Methods

Subjects

We studied a total of 15 patients. Fifteen liver tumor and non-tumor (healthy) tissue samples from patients without preoperative chemotherapy or radiotherapy were obtained from Isfahan General Hospital (Isfahan, Iran) and were histologically evaluated based on the type and the grade of cancer.

Rna Extraction And Cdna Synthesis

Samples were transferred to RNA later immediately after resection and stored at -20°C until used for RNA extraction. The RNXTM-Plus solution was used to extract total RNA (SinaClon, IRAN) except for an extended 1h treatment with *DNase*I. Two methods were used to assess RNA purity, concentration, and integrity, Thermo Scientific NanoDrop™ 1000 Spectrophotometer and electrophoresed on 2% agarose gel. For reverse transcribed (1 mcg of RNA for complementary DNA), the random hexamer priming and PrimeScript™-RT reagent kit (TaKaRa, Japan) was used according to the manufacturer's protocol. This cDNA was quantified using spectrophotometry.

Quantitative Real-time Pcr

All samples were analyzed using a rotor gene 6000 Corbett detection system and the qPCR was quantified using SYBR®Premix Ex Taq™ II kit (TaKaRa, Japan) according to the manufacturer's instructions. Thermal cycling conditions were applied for 5 min at 95°C followed by 40 cycles at 95°C for 15s and 60°C for 1 min. No template control (NTC) containing H₂O was included in each run. To verify the

specificity of PCR products, a melting curve analysis was performed. Besides, PCR products were analyzed in terms of size and specificity using agarose gel electrophoresis. For qPCR analysis, all samples were normalized to *GAPDH*. Forward and reverse primer sequences are indicated in Table 1. The qPCR tests were run in triplicate, and the results were provided as the mean \pm the standard error of the mean (SEM). The relative lncRNA concentration was calculated using the mean value in each triple ($\Delta\text{Ct} = \text{Ct mean lncRNA} - \text{Ct mean GAPDH}$). To calculate expression fold changes, $2^{-\Delta\Delta\text{Ct}}$ methods were used [19, 20].

Table 1
Sequences of primers used in this study

| Primers | Primer Sequences (5' to 3') | Ta (°C) | Product Size (bp) |
|---------------|--|---------|-------------------|
| <i>HOTAIR</i> | GGTAGAAAAAGCAACCACGAAGC ACATAAACCTCTGTCTGTGAGTGCC | 58 | 170 |
| <i>MALAT1</i> | GAAGGAAGGAGCGCTAACGA TACCAACCACTCGCTTTCCC | 62 | 197 |
| <i>UCA1</i> | ACGCTAAC TGGCACCTTGTT TGGGGATTACTGGGGTAGGG | 53 | 187 |
| <i>GAPDH</i> | GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTC | 60 | 226 |

Statistical analysis

The Student's t-test was applied to compare lncRNAs expression in clinical samples. The receiver operating characteristic (ROC) curves were used to assess the diagnostic value of the identified lncRNAs on liver cancer by calculation sensitivity and specificity for each possible cutoff point of the individual lncRNAs. This was performed univariately for each individual lncRNA. The patients were divided based on the median value of the lncRNAs expression into two groups; cases with high lncRNAs and cases with low lncRNAs expression. To examine correlations between lncRNA expression and clinical features, the Chi-square test and Fisher's exact test were utilized. The time between the date of surgery and date of death or last follow-up was taken as overall survival (OS). We investigated the association between the expression levels of lncRNAs with survival through Kaplan-Meier analysis to assess the prognostic value of lncRNAs as biomarkers for liver cancer. The log-rank test was used to determine the significance between gene expression levels and the patient outcome in liver cancer patients. The Cox proportional hazards regression model was also utilized to assess the predictive value of lncRNA levels in patients with liver cancer. To perform all statistical tests, REST 2009 and Graph Pad Prism statistical software, version 7.00 (Graph Pad, San Diego, CA, USA) were utilized. A P-value of <0.05 was considered statistically significant for all tests [19, 20].

Results

Expression of HOTAIR, MALAT1, and UCA1 in liver cancer

The expression levels of three lncRNAs, (*HOTAIR*, *MALAT1*, and *UCA1*), in 15 pairs of liver cancer and matched adjacent non-cancerous tissues were measured by real-time PCR. The expression levels of *HOTAIR*, *MALAT1*, and *UCA1* were upregulated compared to their average expression in healthy tissues ($P < 0.0001$) (Fig. 1). Fig. 1 also indicates that the expression level of *HOTAIR* was increased significantly compared to the expression of *MALAT1* and *UCA1* in patients with liver cancer ($P < 0.001$).

Correlations Between Lncrnas Expressions

We used the Chi-square test and Fisher's exact test to evaluate correlations between the lncRNAs expressions. The patients were divided based on the median value of the lncRNAs expression into two groups of low and high lncRNAs expression cases. Table 2 shows the correlations between lncRNAs expressions for these two groups. The Chi-square test and Fisher's exact test indicated that there were no significant associations between lncRNAs expressions ($P > 0.05$).

Table 2 Correlation between the lncRNAs expressions

| Gene | <i>HOTAIR</i> | | <i>MALAT1</i> | | <i>UCA1</i> | |
|---------------|---------------|-------|---------------|-------|-------------|------|
| | Low | High | Low | High | Low | High |
| <i>HOTAIR</i> | Low | | 26.7 | 26.7 | 20 | 33.3 |
| | High | | 26.7 | 20 | 33.3 | 13.3 |
| P value | | | 0.782 | | 0.189 | |
| <i>MALAT1</i> | Low | 26.7 | 26.7 | | 26.7 | 26.7 |
| | High | 26.7 | 20 | | 26.7 | 20 |
| P value | | 0.782 | | | 0.782 | |
| <i>UCA1</i> | Low | 20 | 33.3 | 26.7 | 26.7 | |
| | High | 33.3 | 13.3 | 26.7 | 20 | |
| P value | | 0.189 | | 0.782 | | |

Correlations Between Lncrnas Expressions And Clinical Characteristics

Tables 3, 4 and, 5 show the correlations between *HOTAIR*, *MALAT1*, and *UCA1* expression and clinical characteristics, respectively. We found that there were no significant correlations between lncRNAs expressions and the clinical characteristics ($P > 0.05$).

Table 3
Correlation between *HOTAIR* expression and the clinical characteristics

| Variables | Cases (%) | <i>HOTAIR</i> IncRNA | | P value |
|--------------------------------|-----------|----------------------|------|---------|
| | | Low | High | |
| Age 55±8.54 (47–74) | | | | 0.833 |
| ≤55 | 60 | 33.3 | 26.7 | |
| >55 | 40 | 20 | 20 | |
| Sex | | | | 0.185 |
| Male | 73.3 | 46.7 | 26.7 | |
| Female | 26.7 | 6.7 | 20 | |
| Tumor size (cm) | | | | 0.782 |
| <3 | 46.7 | 26.7 | 20 | |
| ≥3 | 53.3 | 26.7 | 26.7 | |
| Angioinvasion | | | | 0.714 |
| Yes | 33.3 | 20 | 13.3 | |
| No | 66.7 | 33.3 | 33.3 | |
| Differentiation | | | | 0.535 |
| Poor differentiated | 33.3 | 20 | 13.3 | |
| Moderate differentiated | 60 | 26.7 | 33.3 | |
| Well differentiated | 6.7 | 6.7 | 0 | |

Table 4

Correlation between *MALAT1* expression and the clinical characteristics

| Variables | Cases (%) | <i>MALAT1</i> IncRNA | | P value |
|-------------------------|-----------|----------------------|------|---------|
| | | Low | High | |
| Age 55±8.54 (47–74) | | | | 0.833 |
| ≤55 | 60 | 33.3 | 26.7 | |
| >55 | 40 | 20 | 20 | |
| Sex | | | | 0.310 |
| Male | 73.3 | 33.3 | 40 | |
| Female | 26.7 | 20 | 6.7 | |
| Tumor size (cm) | | | | 0.782 |
| <3 | 46.7 | 26.7 | 20 | |
| ≥3 | 53.3 | 26.7 | 26.7 | |
| Angioinvasion | | | | 0.143 |
| Yes | 33.3 | 26.7 | 6.7 | |
| No | 66.7 | 26.7 | 40 | |
| Differentiation | | | | 0.535 |
| Poor differentiated | 33.3 | 20 | 13.3 | |
| Moderate differentiated | 60 | 26.7 | 33.3 | |
| Well differentiated | 6.7 | 6.7 | 0 | |

Table 5
Correlation between *UCA1* expression and the clinical characteristics

| Variables | Cases (%) | <i>UCA1</i> IncRNA | | P value |
|-------------------------|-----------|--------------------|------|---------|
| | | Low | High | |
| Age 55±8.54 (47–74) | | | | 0.833 |
| ≤55 | 60 | 33.3 | 26.7 | |
| >55 | 40 | 20 | 20 | |
| Sex | | | | 0.876 |
| Male | 73.3 | 40 | 33.3 | |
| Female | 26.7 | 13.3 | 13.3 | |
| Tumor size (cm) | | | | 0.782 |
| <3 | 46.7 | 26.7 | 20 | |
| ≥3 | 53.3 | 26.7 | 26.7 | |
| Angioinvasion | | | | 0.464 |
| Yes | 33.3 | 13.3 | 20 | |
| No | 66.7 | 40 | 26.7 | |
| Differentiation | | | | 0.535 |
| Poor differentiated | 33.3 | 13.3 | 20 | |
| Moderate differentiated | 60 | 33.3 | 26.7 | |
| Well differentiated | 6.7 | 6.7 | 0 | |

Liver Cancer-specific Tumor Marker

ROC analysis was performed to distinguish the optimal cutoff value for lncRNAs to differentiate liver cancer cases from controls from which the sensitivities of circulating *HOTAIR*, *MALAT1*, and *UCA1* were defined to be 100%, 100%, and 100%, at the specificities of 100%, 93.33%, and 100% with an area under the ROC curve of 1.000, 0.998 and 1.000, respectively (Fig. 2).

Correlation Between The Lncmas Expression And Patient Survival

The log-rank test in liver cancer patients was applied. To evaluate the predictive value of the lncRNAs levels in liver cancer patients the Cox proportional hazards regression model was also used. Clinicopathological factors and OS were then analyzed in the high and the low lncRNAs expression groups, but no significant differences were found between groups ($P > 0.05$, Table 6 and Fig. 3).

Table 6
Log-rank test for all patients undergoing liver Cancer

| Variables | Overall Survival | | |
|--|------------------|-----------------|-------|
| | HR | 95% CI | P |
| HOTAIR (Low vs High) | 0.495 | 0.09698 – 2.002 | 0.318 |
| MALAT1 (Low vs High) | 0.895 | 0.1944 – 3.993 | 0.877 |
| UCA1 (Low vs High) | 1.454 | 0.3533 – 6.858 | 0.592 |
| Age (≥ 55 vs ≥ 55) | 0.321 | 0.05506 – 1.105 | 0.106 |
| Sex (Male vs Female) | 0.366 | 0.04357 – 1.604 | 0.159 |
| Tumor size (<3 cm vs ≥ 3 cm) | 0.370 | 0.08635 – 2.124 | 0.319 |
| Angioinvasion (Yes vs No) | 1.003 | 0.1945 – 5.180 | 0.997 |
| Differentiation (Poor vs Moderate and Well) | 0.505 | 0.1005 – 1.985 | 0.347 |

Discussion

LncRNAs are transcribed RNA molecules over 200 bases in length that lack significant protein-coding potential. There are correlations between lncRNAs and DNA-binding proteins, like chromatin-modifying complexes [15, 18]. These transcripts play critical roles in physiological processes [15]. LncRNAs were initially considered to be spurious transcriptional noise. However, in the past few years, thousands of lncRNAs were recognized, and the functional roles of them in epigenetics have been argued [13, 16]. LncRNAs, functioning as regulatory agents, have been defined for various complex cellular processes, including differentiation, cellular signaling, genomic imprinting, alternative splicing, angiogenesis, epigenetic regulation, cell death, cell proliferation, and growth [7, 8]. Increasing evidence has indicated that dysregulation in expression levels of many lncRNAs are associated with developmental processes and disease states most notably in cancer [7, 17].

One of the first reported and characterized lncRNAs involved in cancer progression and associated with metastasis was *HOTAIR* [6, 8, 21–23]. *HOTAIR* was originally discovered by Rinn *et al.* through a custom tiling array of the HOXC locus (12q13.13) [18, 24, 25]. Several studies assessed the relative expression of *HOTAIR* in different cancers, such as breast cancer [7, 11, 16, 17, 23], liver cancer [10], non-small-cell lung cancer (NSCLC) [7, 26], colorectal cancer [17, 27], gastrointestinal stromal tumor [7, 17, 28] and pancreatic cancer [7, 17, 29]. Moreover, *HOTAIR* is proposed to increase tumor invasiveness and metastasis [7, 17, 23]. The relative *HOTAIR* overexpression was reported and was shown to affect the tumor behavior in these cancers [7, 10, 11, 16, 17, 23, 26–29]. Furthermore, Gupta RA *et al.* showed high upregulation of *HOTAIR* in both primary and metastatic breast tumors [6]. Correlations between overexpression of *HOTAIR* in HCC and different clinicopathological factors including larger tumor size, lymph node

metastasis, tumor recurrence after liver transplantation (LT), and shorter disease-free survival after surgical resection (DFS) or LT has been reported [10, 18, 30, 31]. HOTAIR lncRNA can increase the carcinogenic activity of HCC cells by interacting with tumor suppressor microRNAs and suppressing the RNA binding motif protein, hence accelerating the epithelial-mesenchymal transition [25].

One of the long intergenic non-coding RNA (lincRNA) is *MALAT1* with >8,000 nts, located on chromosome 11q13 [32]. *MALAT1* is correlated with high metastatic potential and poor patient prognosis in a variety of cancers, such as lung cancer, hepatocellular carcinoma, breast, prostate, uterus, and esophageal squamous [4, 6, 12, 33]. This lncRNA is associated with cancer in terms of proliferation, metastasis, invasion, and apoptosis [32]. Association between high expression of *MALAT1* and melanoma metastasis has been reported by Tian *et al* [34]. Moreover, Dong *et al.* revealed that *MALAT1* can play an important role in tumor proliferation and metastasis via the phosphoinositide 3-kinase (PI3K)/Akt pathway [35]. It has been shown that overexpression of *MALAT1* in tumor tissues or sera may cause advanced tumor stages and reduced overall survival of HCC patients and can signify a higher risk of tumor recurrence following liver transplantation [3, 36]. In addition, the correlation between overexpression of *MALAT1* in HCC and chemoresistance to multiple agents including 5-fluorouracil, mitomycin C, and Adriamycin via a hypoxia-inducible factor (HIF)-1 α -MALAT1-microRNA(mir)-216b pathway has been reported [37]. Also, the key role of *MALAT1* in HCC progression by inducing serine/arginine-rich splicing factor 1 (SRSF1) upregulation and mammalian target of rapamycin (mTOR) activation was demonstrated [38].

UCA1 gene is placed in chromosome 19p13.12, having three exons and encoding two transcripts. In several studies upregulation of *UCA1* in some cancers including bladder cancer, breast cancer, colorectal cancer, prostate cancer, and osteosarcoma has been shown [15, 39–43]. Alireza Fotouhi Ghiam *et al.* reported the important role of *UCA1* as one of significant mediators of radiation response in prostate cancer [41]. Moreover, according to Wen *et al.* *UCA1* can perform as a potential biomarker for diagnosis and prognosis of osteosarcoma [40]. Ultimately, Wang *et al.* revealed the significance of *UCA1* in predicting tumor lymph node metastasis [44]. According to Feng Wang *et al.* *UCA1* overexpression in HCC tissues has been linked to a variety of factors, including TNM stage, metastasis, and postoperative survival. *UCA1* has the ability to reverse the inhibitory effect of miR-216b on HCC cell growth and metastasis, which could be related to the derepression of fibroblast growth factor receptor 1 (FGFR1) expression acting as a miR-216b target gene and the activation of the ERK signaling pathway [15].

In this study, we first assessed the expression of *HOTAIR*, *MALAT1*, and *UCA1* in liver cancer tissues. From the results obtained in our study, *HOTAIR*, *MALAT1*, and *UCA1* lncRNA in liver cancer tissues showed to have increased expression levels compared to the healthy tissues. Our findings are in agreement with previous study studies [4, 8, 15, 18]. In various reports, significant correlations between *HOTAIR*, *MALAT1*, and *UCA1* expression levels and clinical-pathological characteristics, such as lymph node metastasis, clinical stage, and tumor size, in some cancers including gastric cancer [13], HCC [18], ESCC [33], and osteosarcoma [40] were found. However, our results did not show significant correlations between *HOTAIR*, *MALAT1*, and *UCA1* expression levels and the clinical-pathological characteristics, our findings

also indicated *HOTAIR*, *MALAT1*, and *UCA1* can serve as a biomarker for the diagnosis of liver cancer. According to various studies *HOTAIR*, *MALAT1*, and *UCA1* may be as a biomarker in gastric cancer, papillary thyroid cancer, and osteosarcoma, respectively [13, 40, 45]. Our findings are in line with these studies. Some studies have reported significant relationships between OS and the relative expression of these lncRNAs (*HOTAIR*, *MALAT1*, and *UCA1*) [4, 12, 15, 18, 33], but the OS of patients with high expression of these lncRNAs in liver cancer tissues was not significantly lower in our investigation.

Abbreviations

PLC: Primary liver cancers; HCC: Hepatocellular carcinoma; ESCC: Esophageal squamous cell carcinoma; lncRNAs: Long non-coding RNAs; PRNCR1: Prostate Cancer Associated Non-Coding RNA 1; HOTAIR: HOX Antisense Intergenic RNA; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; UCA1: Urothelial cancer associated 1; CCAT2: Colon cancer-associated transcript 2; OS: Overall survival; NTC: No template control; SEM: Standard error of the mean; ROC: Receiver operating characteristic; NSCLC: Non-small-cell lung cancer; lincRNA: Long intergenic non-coding RNA

Declarations

Ethics approval and consent to participate

All applicable international, national, and institutional guidelines for the care of human were followed. All patients and legally authorized representative of patients who were dead during this study signed the informed consent. The study protocol was approved by the ethics committee of the Cellular and Molecular Research Center of Shahrekord University of Medical Sciences.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EM, AA, and FR, designed the study and writing-original draft. ME, FACH, EHL, HK, HJ, FSSH, EM, AA, and FR performed the experiments and analyzed the data. All authors read, critically revised and approved the manuscript.

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Figures

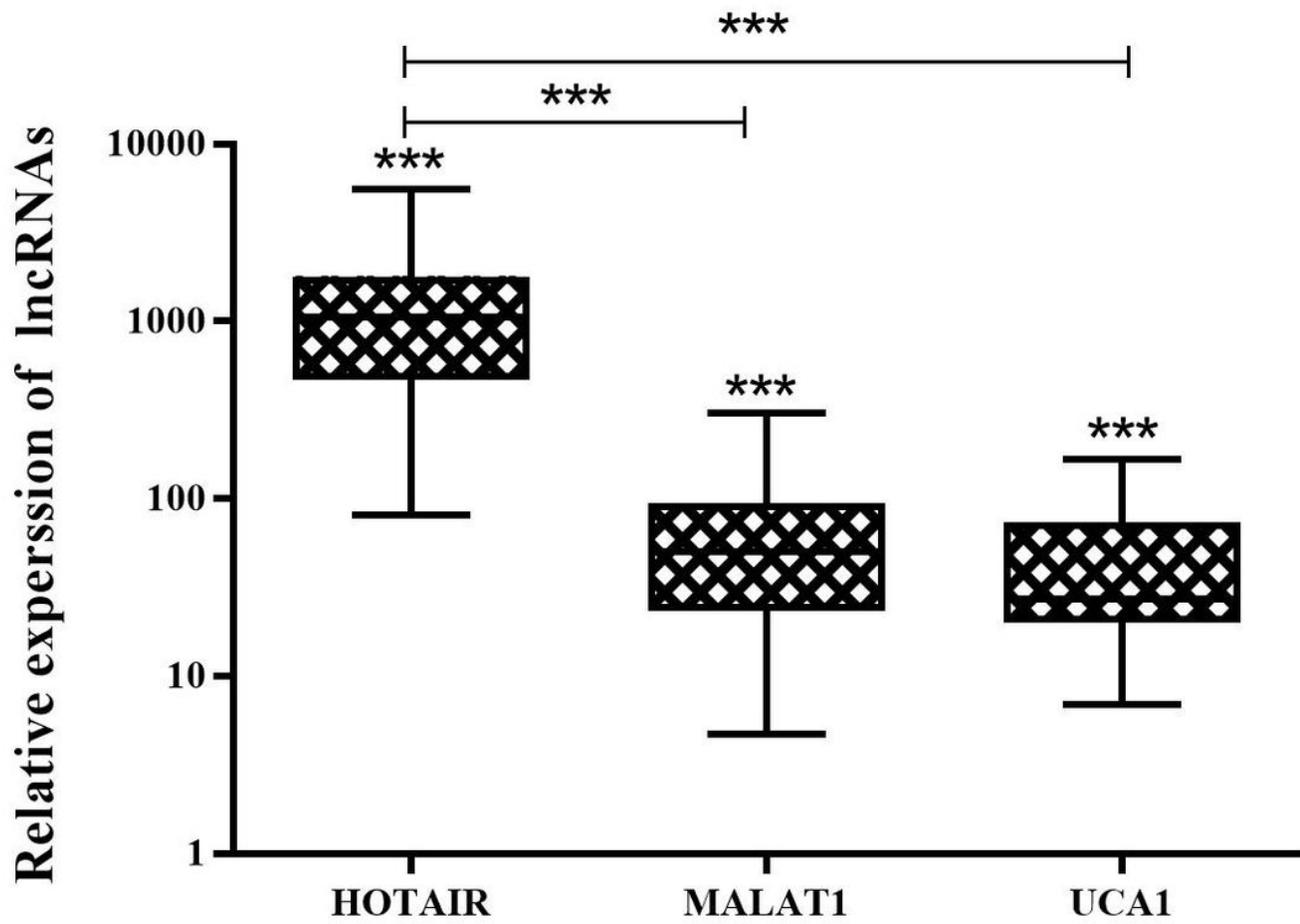


Figure 1

Relative expression of HOTAIR, MALAT1, and UCA1 lncRNAs and Comparison of the three lncRNAs in liver cancer cases. ***significant at the 0.001 level. Error bars show the minimum and maximum variables

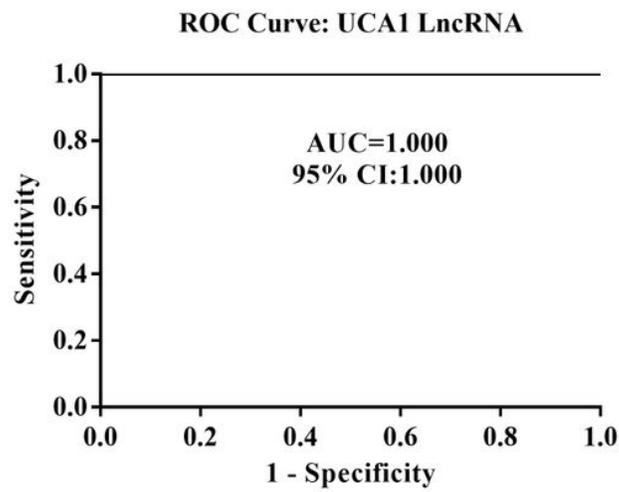
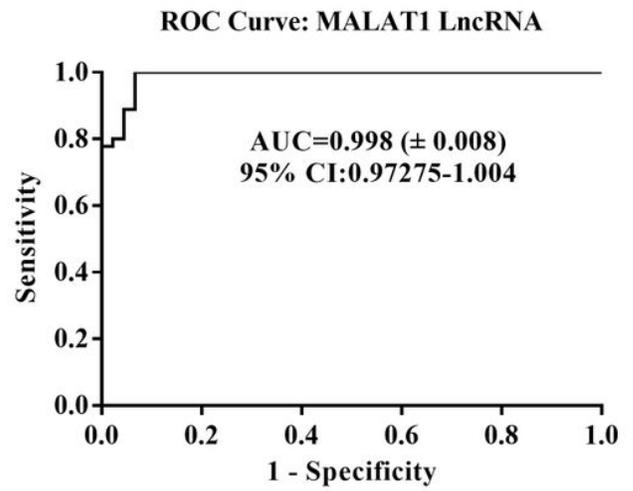
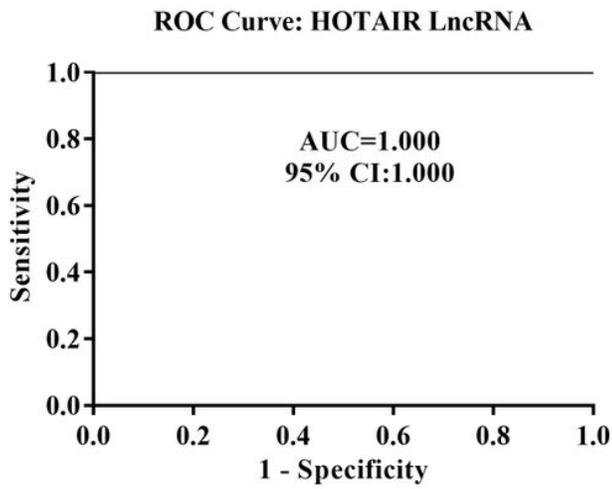


Figure 2

Receiver-operating characteristic (ROC) curve analyses of three-lncRNAs signature to discriminate liver cancer patients from healthy controls

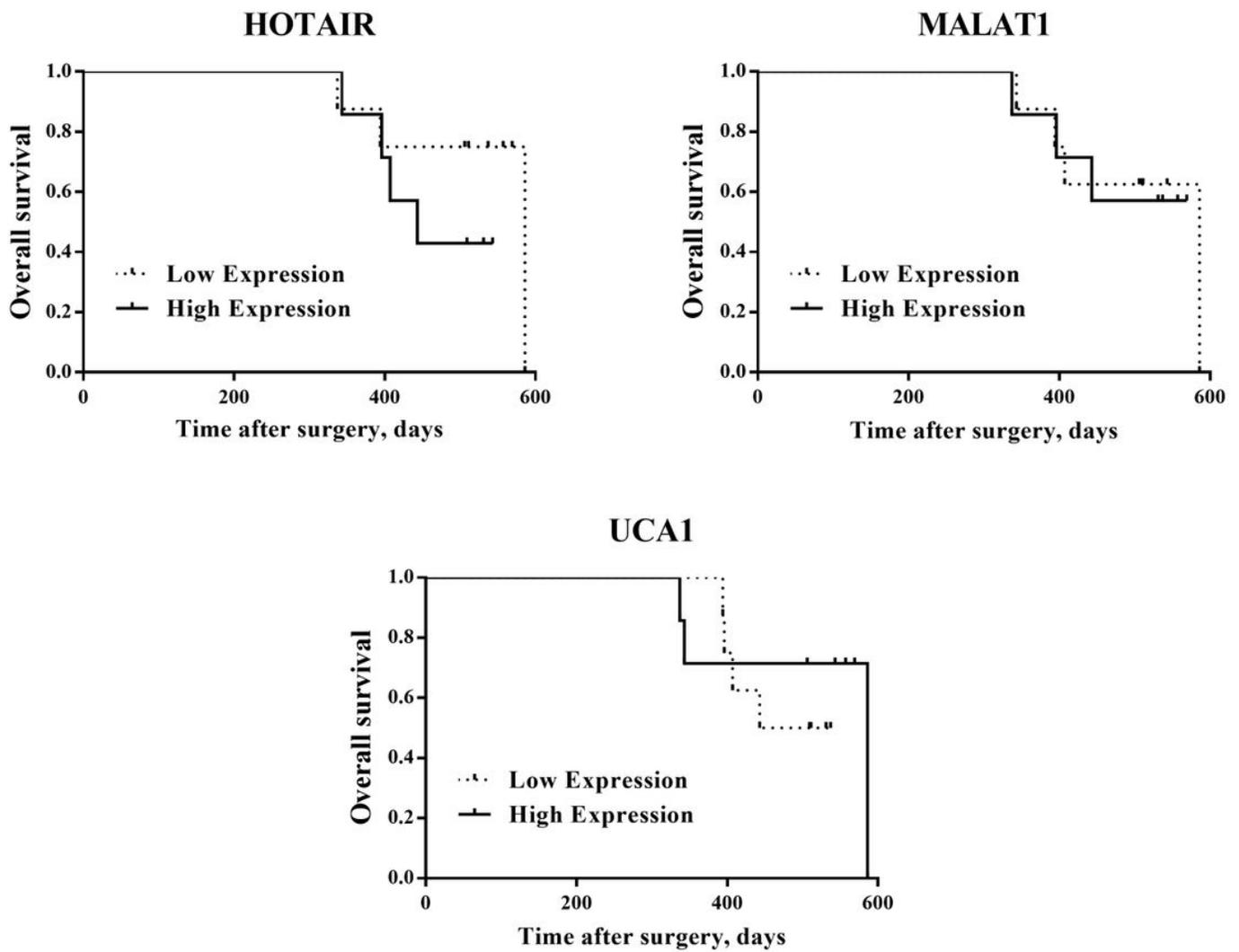


Figure 3

Kaplan–Meier survival curves for associations of lncRNAs with survival. The lncRNAs expression and overall survival (OS) in all patients: the lncRNAs low versus high, $P > 0.05$ (log-rank test)