lncHOTAIR and SNHG-7 expression in correlation to miR-34a expression in gastric cancer pathogenesis

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Abstract

Background: gastric cancer (GC) is one of the common cancers with a high mortality rate and complex pathogenesis. In recent years many studies focused on the roles of non-coding RNA in GC biology. Long noncoding RNAs (lnc-RNAs) like HOTAIR and SNHG-7 are a class of non-coding RNA that can affect the cell's biology by sponging of micro-RNAs. Recent studies reveal the relation between these lnc-RNAs and miR-34a expression which can affect various molecular pathways in malignant cells.

Methods: GeneAll Trizol RNA extraction kit (Korea) was used to extract total RNA according to the supplied procedures and gene expression was evaluated. The qRT-PCR assay was performed to evaluate the expression levels of miR-34a, HOTAIR, and SNHG-7 in GC and paired marginal tissue specimens. The AUC of the ROC curve was estimated based on their expression in GC and gastric normal tissues to evaluate their diagnostic accuracy.

Results: Expression levels of miR-34a were higher in adjacent marginal samples compared to GC tissue samples. We noted significantly higher levels of HOTAIR expression in GC samples compared to non-tumor adjacent tissue samples and the expression of HOTAIR in GC tissues was negatively correlated with mir-34a. Similarly, we noted GC tissue samples showed higher levels of SNHG-7 expression and that SNHG-7 expression was negatively correlated with mir-34a. Regarding the clinicopathological factors, miR-34a expression was lower in patients with advanced GC, while enhanced expression of HOTAIR and SNHG-7 was noted in these patients.

Conclusion: Our findings suggested that miR-34a, HOTAIR, and SNHG-7 expression levels have high potential as diagnostic markers for discriminating GC patients from normal cases. In Addition, there is a negative correlation between miR-34a with two other genes which suggests the regulatory effects of HOTAIR and SNHG-7 on miR-34a.

Introduction

The incidence and mortality rate of gastric cancer (GC) varies among countries and populations, the lower socioeconomic groups have the higher rates among the others. Even though the mortality associated with GC has decreased in industrialized nations, it remains a major cancer-related public health burden [1]. Despite the advancements in diagnosis and treatment of GC, the prognosis is dismal due to late diagnosis, as the early stages of GC are clinically silent. Consequently, understanding the genetics and molecular pathogenesis of GC is a matter of importance for effective therapy and reduction of mortality rate.

Various studies have shown that Long noncoding RNAs (lncRNAs) are involved in the pathogenesis and progression of different types of cancer, including GC [2, 3]. lncRNAs are non-protein-coding RNAs with more than 200 nucleotides long. Previously, lncRNAs were considered transcriptional noise but recent studies have proven their participation in carcinogenesis. Accumulating studies have revealed their importance in many cellular processes such as cell cycle and cell differentiation regulation, epigenetic

regulation, gene imprinting, organogenesis, and stem cell pluripotency [4–6]. Deregulated lncRNA expression has an association with promoting or suppressing the development of GC [7, 8].

Hox transcript antisense intergenic RNA (HOTAIR) is a long lncRNA with six exons and 2.2 kb nucleotides which is transcribed from the HOXC locus. The overexpression of HOTAIR is proven to be associated with promoted metastasis and invasiveness and consequently poor prognosis of different types of cancers such as colon, breast, hepatocellular, lung, and pancreatic cancer [9–13]. Recent studies have confirmed the oncogenic role of HOTAIR upregulation in GC progression [14, 15].

Small nucleolar RNA host gene 7 (SNHG7) is another recognized oncogenic lncRNA that has been proven to have a positive correlation with metastasis and poor prognosis of different types of cancers including gastric, bladder, breast, prostate, and hepatocellular cancer [16–19]. According to literature, SNHG7 and HOTAIR act through distinct signaling pathways, one of these pathways, which exacerbates cell invasion and migration in GC, is the regulation of microRNAs (miRNAs) [20, 21].

MiRNAs are small non-protein-coding RNAs with 21–23 nucleotides long. MiRNAs regulate gene expression post-transcriptionally. Studies have proven that the altered expression of certain miRNAs causes the development and progression of cancers [22]. MiR-34a is a subtype of the miR-34 family, which is a class of miRNAs. Previous studies have shown that miR-34a expression is altered in different kinds of cancer including breast, lung, prostate, and head and neck squamous cell carcinomas [23–26].

Previous studies have suggested that miR-34a has a notable role in inhibiting GC oncogenesis through different pathways [27, 28]. Furthermore, in a recent study done by Zhang et al, results have proven that downregulation of miR-34a in GC is associated with poor prognosis and a high recurrence rate [20].

According to the previous studies, HOTAIR directly bonds to miR-34a and regulates certain pathways in different types of cancers. Knockdown of the HOTAIR upregulates miR-34a which affects the resistance of GC cells [34, 35]. Similar to HOTAIR, SNHG-7 acts as an oncogenic by competing with miR-34 to regulate certain signaling pathways during cancers. SNHG-7 was identified as a direct target of miR-34a [36]. In this research, we aim to determine the HOTAIR and SNHG-7 lncRNAs expression in correlation to miR-34a expression in gastric cancer pathogenesis.

Methods

Tissue samples

From April 2020 to April 2021, 40 pairs of fresh-frozen gastric cancer and marginal non-tumor tissue specimens were obtained from patients with primary GC, who underwent surgery at the Imam Riza Hospital. No local or systemic chemotherapy or radiation was received by the patients before surgery. This study was approved by the Ethics Committee of Tabriz University of Medical Sciences. Written informed consent was obtained from all of the patients.

Samples were assessed by a pathologist, and GC diagnosis was confirmed in all of the patients. GC staging was done according to the tumor node metastasis (TNM) system of the Union for International Cancer Control/American Joint Committee on Cancer [29]. Until used, specimens were kept frozen within liquid nitrogen.

Inclusion criteria included patients with a diagnosis of GC who underwent surgery, and exclusion criteria were previous targeted therapy such as chemotherapy, radiation, and intervention therapy for GC. Data regarding age, sex, tumor size, lymph node metastasis, distant metastasis, and TNM stage, were documented.

RNA extraction

GeneAll Trizol RNA extraction kit (Korea) was used to extract total RNA according to the supplied procedures and gene expression was evaluated. The purity and concentration of the extracted RNA were measured at the wavelengths of A260 and A280 using the Thermo Fisher NanoDrop spectrophotometer (USA), considering the absorbance at this range. 1% agarose gel was used for electrophoresis of the extracted RNA and the integrity was evaluated.

qRT-PCR

The qRT-PCR assay was performed to evaluate the expression levels of miR-34a, HOTAIR, and SNHG-7 in GC and paired marginal tissue specimens. According to the supplied protocols, 1000 ng of extracted RNA was converted to complementary DNA (cDNA), using the BioFACT cDNA synthesis kit (Korea). Afterward, their expression levels, in the StepOnePlus Real-Time PCR System (Applied Biosystems, USA), were quantified using BioFACT™ 2× Real-Time PCR Master Mix (Korea). GAPDH was used as an internal control. Table 1 shows Primer sequences. Amplification was achieved through the following steps: 15 minutes of initial denaturation at 95 °C, 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. A melting curve analysis was performed in the end. The specificity of the amplicons was confirmed by gel electrophoresis. Each reaction was performed triplicated with a total volume of 20 µl. Data analysis was performed using the Livak method ($2^{-\Delta\Delta CT}$).

ROC Curve Analysis

To investigate miR-34a, HOTAIR, and SNHG-7 expression as a potential detection biomarker and evaluate its diagnostic accuracy, using GraphPad 6 Prism (GraphPad Software, CA, USA), the area under the curve (AUC) of the receiver operating characteristic (ROC) curve was estimated based on their expression in GC and gastric normal tissues.

Statistical Analysis

Statistical analysis was done using GraphPad 6 Prism (GraphPad Software, CA, USA). All data are reported as the mean ± standard deviation. To evaluate the differences in miR-34a, HOTAIR, and SNHG-7

expression status between GC tumors and paired marginal tissues, and Unpaired t-test was employed. A *p*-value <0.05 is considered statistically significant.

Results

The clinical and histopathological characteristics of the patients are shown in table 2.

miR-34a Expression in GC Tissues

Expression levels of miR-34a in GC and paired adjacent non-tumor internal specimens of 40 patients were assessed using qRT-PCR. Results are shown in figure 1-a. We found significantly higher levels of miR-34a expression in adjacent marginal samples compared to GC tissue samples with a *p*-value equal to 0.001. These results indicated that decreased miR-34a expression may be related to the oncogenesis of GC. Furthermore, our findings showed a correlation between miR-34a expression and lymph node (LN) metastasis, distant metastasis, and TNM stage (*p*-value: 0.015, 0.007, and 0.032, respectively). (table-3)

HOTAIR function in GC Tissues

Expression levels of HOTAIR were assessed as described above, using qRT-PCR. Results are shown in figure 1-b. We noted significantly higher levels of HOTAIR expression in GC samples compared to non-tumor adjacent tissue samples with a *p*-value <0.001. These results suggested that HOTAIR played an important role in the pathogenesis of GC. In addition, as shown in table 3, there was a statistically significant relationship between HOTAIR expression levels and LN metastasis, distant metastasis, and TNM stage (p-value: 0.005, 0.004, and 0.041, respectively), showing higher levels of HOTAIR expression in patients with advanced GC. However, no significant correlation was found between HOTAIR expression and other clinicopathological factors (table 3).

SNHG-7 function in GC Tissues

Expression levels of SNHG-7 were assessed as described above, using qRT-PCR. Results are shown in figure 1-c. Similarly, we noted a statistically significant difference in SNHG-7 expression between the two groups, and GC tissue samples showed higher levels of SNHG-7 expression with a *p*-value <0.001. These results suggested that SNHG-7 played an important role in the pathogenesis of GC, as well. Plus to significant up-regulation there was a significant relationship between SNHG-7 expression levels and tumor size, LN metastasis, distant metastasis, and TNM stage (p-value: 0.034, 0.021, 0.037, 0.032, respectively), showing higher levels of SNHG-7 expression in patients with advanced GC. There was no significant correlation between SNHG-7 expression and other clinicopathological factors (table 3).

Furthermore, we investigated the correlation between SNHG-7 and miR-34a in regulating the progression of GC. Results are shown in figure 2-b. We noted that the expression of SNHG-7 in GC tissues was negatively correlated with mir-34a (*p*-value = 0.01).

Correlation between SNHG-7 expression and miR-34a

we investigated the correlation between HOTAIR, SNHG-7, and miR-34a expression levels due to our results there is a negative correlation between miR-34a and two other genes expression which proposes the regulatory effects of HOTAIR (p-value < 0.0001) and SNHG-7(p-value = 0.01) on miR-34a expression in tissues with gastric cancer. Results are shown in Figures 2-a and 2-b.

High potential of miR-34a, HOTAIR, and SNHG-7 as diagnostic biomarkers for GC

ROC curve analysis was performed to evaluate the diagnostic potential of miR-34a, HOTAIR, and SNHG-7 for discrimination of GC patients from normal cases. The area under curve (AUC) value of ROC curve analysis was estimated 0.770 (p-value = 0.0001), 0.770 (p-value = 0.0001), and 0.910 (p-value <0.0001), respectively. These results suggested the high potential of miR-34a, HOTAIR, and SNHG-7 expressions as diagnostic biomarkers for GC (figure 3). However, our results should be confirmed in a larger size of samples.

Discussion

Despite the advancements and aggressive therapy, the survival rate of patients diagnosed with GC remains unsatisfactory due to late diagnosis. Consequently, understanding the genetics and molecular pathogenesis of GC is a matter of importance for effective therapy and reduction of mortality rate. Recently, lncRNAs and miRNAs have become an emerging hotspot in the research fields of GC. A better understanding of the molecular pathogenesis of GC helps early detection and more effective targeted therapies, which consequently results in the reduction of incidence and mortality rate of GC.

This research examined the HOTAIR, SNHG-7, and miR-34a expression levels in GC and investigated their correlation. Furthermore, we examined their correlation with clinicopathological factors of patients, including age, sex, tumor size, LN metastasis, distant metastasis, and TNM stage of GC.

Our results revealed decreased levels of miR-34a expression in GC tissue samples and that downregulated expression of this molecule was associated with LN metastasis, distant metastasis, and high TNM stage. Resulting from that, lower levels of miR-34a correlate with the advancement and oncogenesis of GC. These findings are in accordance with previous studies. Zhang et. al. and Cao et. al. proved that the expression of miR-34a was downregulated in GC cell lines and this downregulation was significantly correlated with metastasis, tumor size, disease stage, and prognosis [27, 30]. Sun et. al. and Peng et. al. showed that promoted miR-34a expression suppresses the proliferation of GC cells [28, 31].

Regarding the HOTAIR molecule function, we noted significantly higher levels of HOTAIR expression in GC tissue samples. Upregulated levels of HOTAIR were associated with LN metastasis, distant metastasis, and high TNM stage. These results are consistent with previous studies showing that enhanced expression of HOTAIR correlates with advancement and poor prognosis of GC [14, 32]. Elsayed et. al. proved that HOTAIR is a potential biomarker for GC [15]. Chao et. al. showed that inhibition of HOTAIR suppresses GC cell activities [33]. Additionally, previous studies have demonstrated that HOTAIR interactions with miRNAs interfere with cellular activities during cancer development. According to the

literature, HOTAIR causes oncogenesis by reducing the expression levels of miRNAs [22]. In this research, we further investigated the HOTAIR expression in correlation to mir-34a. MiR-34a is a subtype of the miR-34 family, which is a class of miRNAs. Our results were in accordance with previous studies, indicating a negative correlation between HOTAIR and mir-34a expression levels in GC [21].

Furthermore, our results showed higher levels of SNHG-7 expression in GC tissue cells and a significant relationship between SNHG-7 expression levels and tumor size, LN metastasis, distant metastasis, and high TNM stage. Suggesting that SNHG-7 plays an important role in the pathogenesis of GC, as well. Additionally, an investigation of the correlation between SNHG-7 and miR-34a in regulating the progression of GC showed a negative correlation between the two. In accordance with the results of our study, Zhang et. al. proved that enhanced expression of SNHG-7 is associated with the development and poor prognosis of GC. Their results also showed that SNHG-7 increases GC cell invasion and migration by suppressing the miR-34a-Snail-EMT axis [20].

Our results proved that both HOTAIR and SNHG-7 target the miR-34a. knockdown of the HOTAIR and SNHG-7 upregulates miR-34a. These results were the same s results reported in the previous studies [34–36]. As miR-34a is downregulated in GC cell lines, promoted expression of miR-34a by these lnc-RNAs results in the inhibition of resistance of GC cells. miR-34a is an important factor in the pathogenesis of GC, indicating that both HOTAIR and SNHG-7 play an important role as well. In conclusion, our findings suggested that miR-34a, HOTAIR, and SNHG-7 expression levels have high potential as diagnostic markers for discriminating GC patients from normal cases.

Declarations

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Author's contribution

P.V and M.A conceived and designed the research. M.D conducted experiments. V.Z and A.C contributed new reagents or analytical tools. D.S analyzed the data. A.F and H.Z wrote the manuscript. All authors read and approved the manuscript and all data were generated in-house and no paper mill was used.

Conflict of Interest

The authors declare no conflict of interest.

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Tables

Table 1

primer list

Target	Sequences
miR-34a target sequences	**5'- UGGCAGUGUCUUAGCUGGUUGU -3'**
HOTAIR	**F** **-3'**GGTAGAAAAAGCAACCACGAAGC**5'-**
	R **-3'**ACATAAACCTCTGTCTGTGAGTGCC**5'-**
SNHG-7	**F** **-3'** GTGACTTCGCCTGTGATGGA**5'-**
	R **-3'** GGCCTCTATCTGTACCTTTATTC**5'-**
GAPDH	**F** **5'-AAGGTGAAGGTCGGAGTCAAC-3'**
	R **5'-GGGGTCATTGATGGCAACAA-3'**

Table 2

Clinical and pathological characteristics of tumor samples

Properties		Number of cases (%)
Age	>55	27 (67.5%)
	<55	13 (32.5%)
Sex	female	15 (37.5%)
	male	25 (62.5%)
Tumor size	<5cm	19 (47.5%)
	>5cm	21 (52.5%)
Lymph node metastasis	Positive	29 (72.5%)
	Negative	11 (17.5%)
Distant metastasis	Positive	22 (55.0%)
	Negative	18 (45.0%)
TNM stage	II	14 (35.0%)
	III & IV	26 (65.0%)

Table 3

correlation between miR-34a, HOTAIR, and SNHG-7 expression levels and clinicopathological factors

Clinical Feature	miR-34a Expression Level	HOTAIR Expression Level	SNHG-7 Expression Level
Age	Pvalue:0.780	Pvalue:0.720	Pvalue:0.480
Sex	Pvalue:0.610	Pvalue:0.420	Pvalue:0.350
Tumor size	Pvalue:0.054	Pvalue:0.072	Pvalue:0.034
LN metastasis	Pvalue:0.015	Pvalue:0.005	Pvalue:0.021
Distant metastasis	Pvalue:0.007	Pvalue:0.004	Pvalue:0.037
TNM stage	Pvalue:0.032	Pvalue:0.041	Pvalue:0.032

Figures

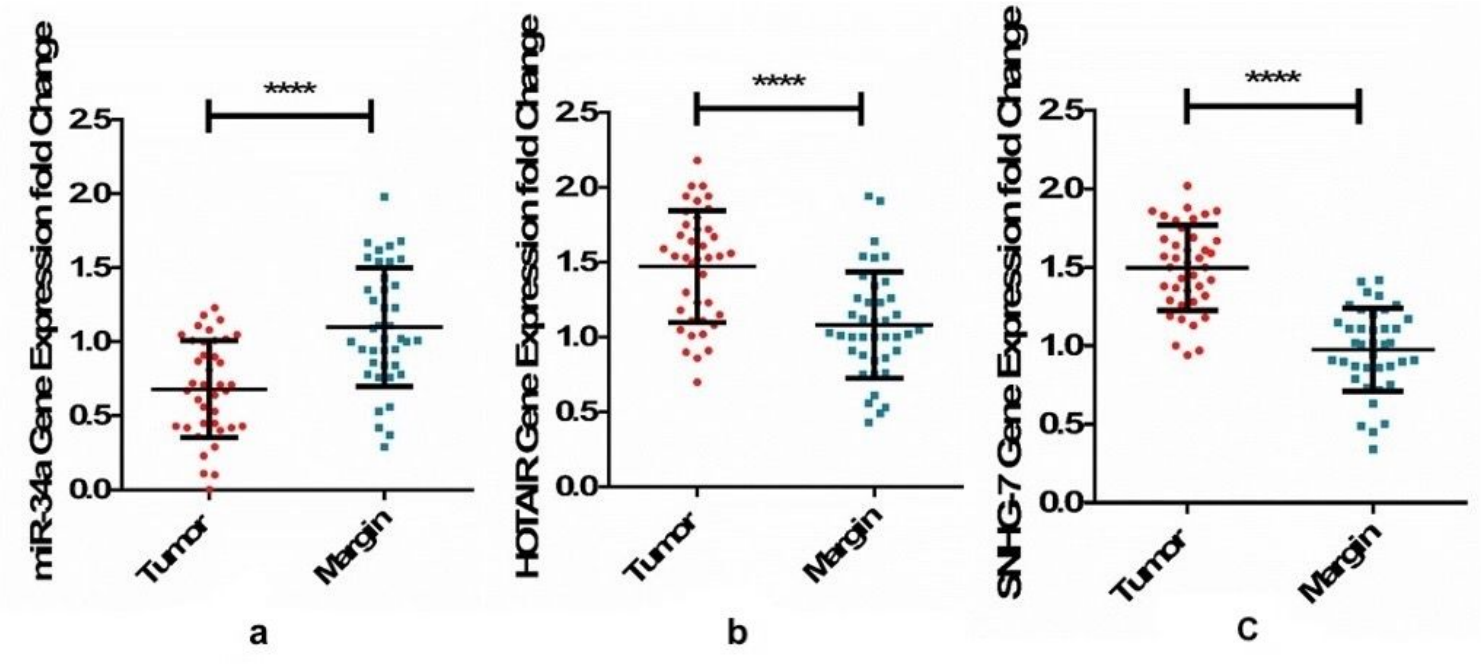

Figure 1

a. Expression levels of miR-34a in GC and paired margin samples b. Expression levels of HOTAIR in GC and paired margin samples c. Expression levels of SNHG-7 in GC and paired margin samples

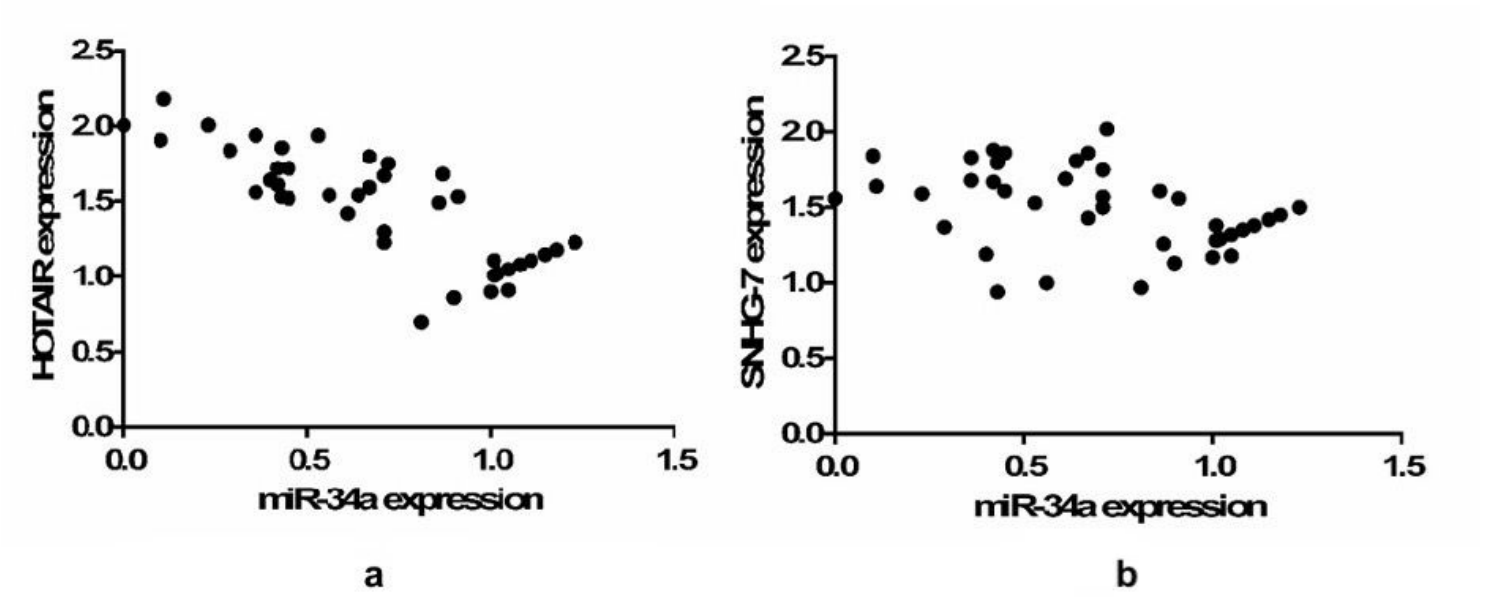

Figure 2

a. The correlation between HOTAIR and mir-34a expression in GC tissues (*p*-value <0.0001) **b**. The correlation between SNHG-7 and mir-34a expression in GC tissues (*p*-value= 0.01)

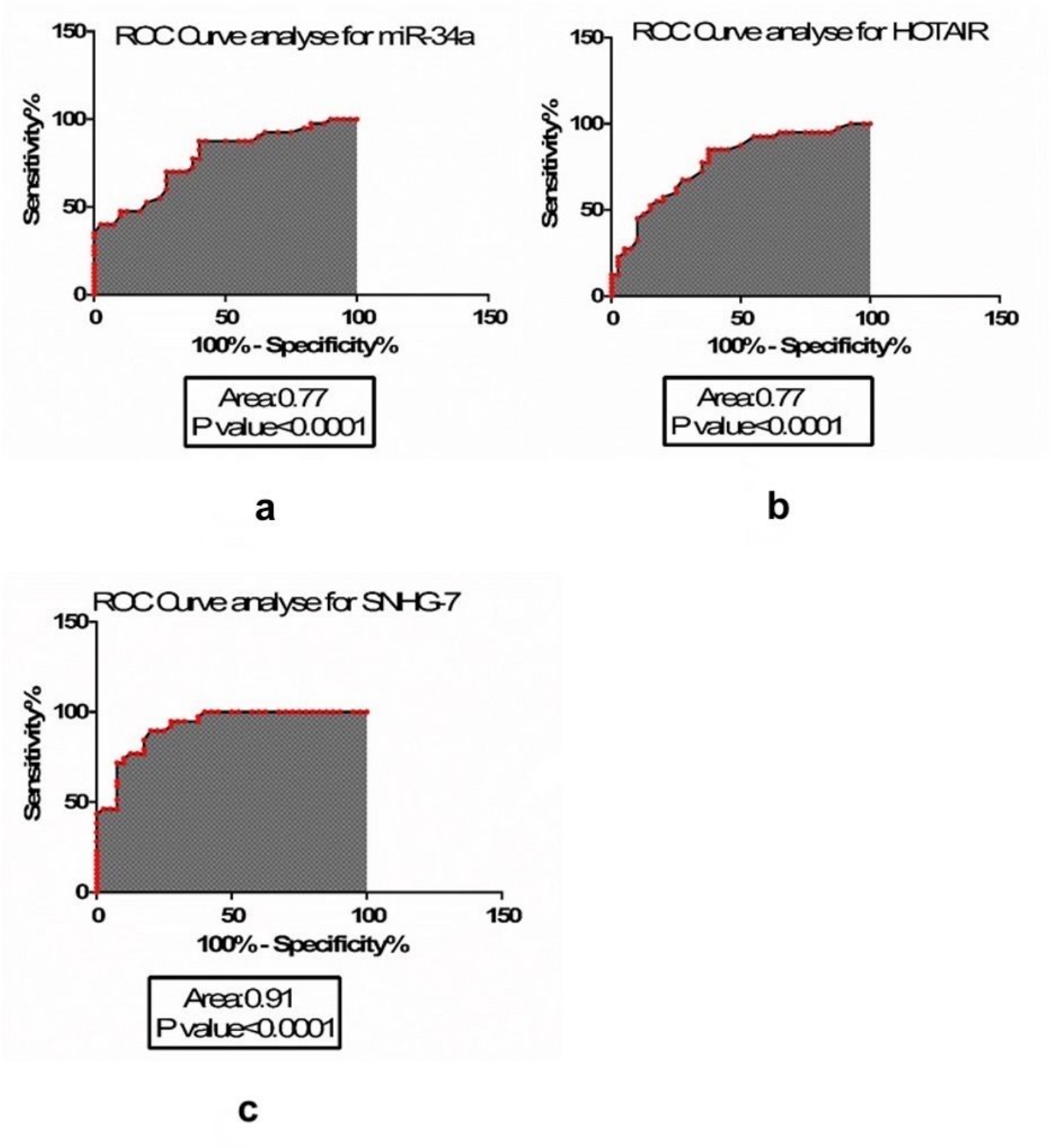

Figure 3

a. ROC curve analysis to evaluate the diagnostic potential of miR-34a **b**. ROC curve analysis to evaluate the diagnostic potential of HOTAIR **c.** ROC curve analysis to evaluate the diagnostic potential of SNHG-7