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Overexpression of PURPL and downregulation of NONHSAT062994 as potential biomarkers in gastric cancer

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ABSTRACT

Aims: Long non-coding RNAs (LncRNAs) play central roles in the formation and development of gastric cancer (GC). The aim of this study was to evaluate the expression of PURPL and NONHSAT062994 and the relationship between their expressions with clinical characteristics in GC.

Main methods: PURPL and NONHSAT062994 LncRNAs and p53 gene expression levels were analyzed both in 50 pairs of cancerous and adjacent noncancerous tissue samples in GC patients using qRT-PCR and in four sets of data obtained from Gene Expression Omnibus (GEO) database. Chi-square ($\chi 2$) test was used to determine the relationship between PURPL, NONHSAT062994 RNA levels and the clinicopathological characteristics of GC. Receiver operating characteristic (ROC) curves were drawn to represent sensitivity and specificity of PURPL and NONHSAT062994 expression as markers of GC.

Key findings: Expression of PURPL was significantly upregulated in 50 GC samples as well as in GC tissues from GSE13911 and GSE27342 datasets. Our results demonstrated that PURPL RNA level in GC was significantly related to tumor size and histopathological grade. p53 expression at both protein and mRNA levels were significantly decreased in GC tissues compared to adjacent control samples.

NONHSAT062994 expression was downregulated in 50-pair GC and GC tissues from GSE13915 dataset. However, NONHSAT062994 showed no consistently differential expression in GSE2637dataset. NONHSAT062994 was significantly associated with histological grade and tumor size.

Significance: Overall, these results suggest that PURPL and NONHSAT062994 may play critical roles in the progression of GC and therefore might be considered as candidate tumor markers for therapeutic goals.

1. Introduction

Cancer is the main cause of death in industrialized countries and the second leading cause of death in developing countries [1]. In 2008, about 12.7 million cases of cancer and 7.6 million deaths were reported worldwide [2]. Gastric cancer (GC) is the fifth most common cancer and is the third most lethal cancer [3]. The prevalence of GC in Iran is high, approximately 26.1 per 100,000, while the prevalence of this cancer has declined dramatically throughout the world [2]. Diagnosis in advanced stages of this cancer has made it the fatal cancer in Iran [4]. Therefore, the discovery of non-invasive biomarkers is essential for the early diagnosis and treatment of GC. Transcriptome studies have shown that transcription of more than 80% of human genome causes production of non-coding RNAs [5]. Long non-coding RNAs (lncRNAs) are non-coding RNAs with a 5' terminal methylguanosine cap and with a long more than 200 nucleotides [6]. So far, about 6000 lncRNAs with no specific role have been identified in the human genome [7].

LncRNAs are classified into different groups based on their chromosomal location and effect on gene expression. For example, some lncRNAs are intergenic, while others are intronic. Some of lncRNAs regulate the gene expression at the transcriptional level, while others function is in the post-transcriptional level (8). Long length of lncRNAs allows them to bind with other biological macromolecules such as mRNAs, mirRNAs, DNA, and proteins [8]. Interestingly, lncRNAs are able to be transmitted to distant cells by exosomes [9]. This cell-to-cell transmission impacts on important processes such as genetic and epigenetic regulation, angiogenesis, tumorigenesis, and drug resistance, therefore exosomal lncRNAs have been considered as therapeutic goals and tumor markers in some studies [10].

LncRNAs, with impacts on chromatin regeneration [11],

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transcription regulation [12,13], and RNA editing [14] have important cellular functions such as differentiation, growth, proliferation, immune escape, autophagy, and apoptosis [15–19]. Similarly, critical roles of LncRNAs in regulation of signaling pathways, survival, genomic imprinting, proliferation, migration and metastasis by regulating epithelial-to-mesenchymal transition (EMT) in GC have been reported [22,23]. In addition, many lncRNAs act as tumor suppressors and oncogenes, so aberrant lncRNA expression may participate in carcinogenesis [21]. Thus, it is possible to draw the conclusion that LncRNAs have a central role in the onset and progression of GC [20].

Over the past decades, accumulating evidence has reported that many lncRNAs such as MALAT1, HOTAIR, UCA1, ZFAS1, ATB, FENDRR, BANCR, SPRY4-IT1, and LOC100130476 play important roles in GC occurrence and development [22]. Each of these lncRNAs is involved in various pathological processes in GC, for example, MALAT1 impacts on EMT and angiogenesis, and its expression level is correlated with lymphatic metastasis, distant metastasis and the Tumor-Node-Metastasis (TNM) stage of tumor [24,25]. SPRY4-IT1 is involved in EMT, epigenetic regulation and its RNA level is correlated with lymphatic metastasis, distant metastasis, and TNM stage [26,27].

LncRNA NONHSAT062994 is a newly discovered lncRNA located on human chromosome 19 with a length of 585 nucleotides. Little research has been conducted on the role of NONHSAT062994 but it has recently been reported that anomalous expression of this lncRNA is involved in colorectal cancer and the formation of Human Earlobe Keloids [28,29]. On the other hand, RP11-46C20.1, LOC643401/LINC01021 or PURPL are unstable nuclears: lncRNAs that are upregulated in cell in response to DNA damage, while PURPL and p53 directly affect the expression of each other by auto-regulatory feedback loop [30–34].

Considering the aberrant expression of these lncRNAs in cancer formation, in the present study we determined NONHSAT062994 and PURPL expression at the RNA level in GC tissues and adjacent noncancerous tissues and investigated the correlation of these lncRNAs expressions with clinicopathological parameters.

2. Materials and methods

2.1. Expression analysis in GEO database

Microarray data from online Gene Expression Omnibus (GEO) datasets (http://www.ncbi.nlm.nih.gov/geo/) were checked for GC and four datasets including GSE13911 [35] (38 tumor biopsy and 31 matched normal tissues), GSE27342 [36] (80 tumor biopsy and 80 matched normal tissues), GSE2637 [37] (11 tumor biopsy and 3 unmatched normal tissues), and GSE13195 [38] (25 tumor biopsy and 25 matched normal tissues) were found involving the expression data for PURPL and NONHSAT062994. Then GEO2R, an online tool, was applied to analyze the expression data of PURPL and NONHSAT062994 and the raw data were downloaded in Series Matrix Files for analyzing the clinicopathologic relationship. Inclusive information of GSE series are tabulated in Table 1.

2.2. Patients and tissue specimens

The biological samples were obtained from the Iran National Tumor Bank, Cancer Institute (Tehran University of Medical Sciences, Tehran-Iran). From GC patients, 50 fresh-frozen GC samples and corresponding noncancerous matched tissue samples were collected immidiately after resection and stored in liquid nitrogen until further gene expression analysis. The histopathological characteristics of all subjects who underwent surgical operation, including clinical age, gender, tumor size (cm), tumor/node/metastasis (TNM) staging, histological grade, lymph node metastasis, vascular metastasis, and necrosis were recorded. Other malignancies or inflammatory diseases have been considered as exclusion criteria in the present study.

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated from biological samples using RNX-Plus (Sinaclon, Tehran, Iran), according to the manufacturer's instructions. The concentrations of total RNAs were evaluated and subsequently cDNAs were synthesized using RevertAid™ fist strand cDNA synthesis kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol.

For evaluation of relative gene expression, the cDNA products were analyzed by the Roche Light Cycler 96 System (Roche Life Science Deutschland GmbH, Germany) using the RealQ Plus 2 × Master Mix Green (Ampliqon, Odense, Denmark). Primer sequences were synthesized by Takapou Zist Company (Takapou Zist, Tehran, Iran). The primer sequences were as follows: PURPL (forward: 5′-CGTGTGAAAA GAACCCAGGTA-3’; reverse: 5′-CGCCTGGTAAAACAACCAGT-3′), NONHSAT062994 (forward: 5′-TCTGGTCCCGTGGATTTCTG-3’; reverse: 5′-ATCGCCATCACTGTCCTTCTG-3′), p53 (forward: 5′- TAACAG TTCCTGCATGGGCGGC-3’; reverse: 5′- AGGACAGGCACAAACACGC ACC-3′) and β-actin as a housekeeping gene (forward: 5′-ACAGAGCC TCGCCTTTGC-3’; reverse: 5′-ATCACGCCCTGGTGCCT-3′). Relative RNA levels of target genes were normalized versus Ct values obtained for the internal control β-actin. The $2^{-\Delta\Delta CT}$ formula was used for calculation of lncRNAs fold change expression [39].

2.4. Western blot analysis

Western blotting technique was used to evaluate p53 expression at the protein level. Twenty mg of tissue was powdered with liquid nitrogen, then added to 600 µl of radioimmunoprecipitation assay (RIPA) buffer. RIPA buffer was then supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). The tissues lysate agitated for 2 h at 4 °C. After centrifugation, their supernatants were separated. The total protein content of the supernatants was measured by bicinchoninic acid (BCA) method. In the next step, 40 µg of the protein extracts was separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes by electrical current. Then blocking was performed twice for 40 min at room temperature with 5% nonfat skimmed milk powder (Santa Cruz Biotechnology, Inc, Texas, USA) dissolved in tris buffered saline with tween 20 (TBST). The membrane was incubated with primary antibodies: p53 (STJ96288, St. John's Laboratory, London, UK) and β-Actin (ab119716, Abcam, Cambridge, UK). Secondary horseradish peroxidase-conjugated antibody (ab6721; Abcam, Cambridge, UK) was used for chemiluminescent detection (Bio-Rad, Feldkirchen, Germany). To normalaize the expression of protein in this study, β-Actin was used as internal control. Densitometry analysis of the bands was performed

Table 1
Inclusive information of the GEO datasets.

Series	Accession	Type platform
GSE13911	Homo sapiens	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array
GSE27342	Homo sapiens	GPL5175 [HuEx-1_0-st] Affymetrix Human Exon 1.0 ST Array [transcript (gene) version]
GSE2637	Homo sapiens	GPL2009 18k cDNA chip NCCS
GSE13195	Homo sapiens	GPL5188 [HuEx-1_0-st] Affymetrix Human Exon 1.0 ST Array [probe set (exon) version]

with the Image J software (https://imagej.nih.gov/ij/).

2.5. Statistical analysis

Statistical analysis and graphing were performed using both the Statistical Package for Social Sciences version 13 (SPSS; Chicago, IL, USA) and Prism5.00 (GraphPad Software, LaJolla, CA). Based on the results of the Kolmogorov-Smirnov test, the expression difference between two groups was analyzed by Mann–Whitney U or independent T-test. All data were expressed as mean ± standard deviation (SD) and p-values less than 0.05 were considered statistically significant. The chi - square test was performed to assess the relationship between expression levels of PURPL and NONHSAT062994RNA along with the clinical characteristics of GC. In order to perform this test, we need to make data in a qualitative form. Therefore, the median of relative expression data was calculated ($2^{-\Delta\Delta CT}$) in the GC tissues and based on data divided into two groups: high expression and low expression. Receiver operating characteristic (ROC) curves were drawn for the evaluation of the possibility of lncRNAs for GC detection.

3. Results

3.1. Expression of PURPL and NONHSAT062994 in GEO datasets

GSE13911, GSE27342, GSE2637, and GSE13195 datasets which were covering gene expression data from tumor biopsies and matched adjacent noncancerous tissues were analyzed to evaluate the relative expression of PURPL and NONHSAT062994 in GC. As shown in Fig. 1a and Fig. 1b, PURPL were found overexpressed in GSE13911 and GSE27342 GC tissues compared with adjacent noncancerous tissues ($p = 0.001$, $p < 0.002$, respectively). In addition, as shown in Fig. 1c and d, downregulation of NONHSAT062994 RNA in GSE13195 and GSE2637 datasets was observed in GC tissues compared with adjacent normal tissues ($p = 0.0001$, $p = 0.61$, respectively).

3.2. NONHSAT062994, PURPL, and p53 expression in GC tissues

The PURPL RNA levels increased significantly in GC tissues compared with adjacent control samples, with an average increase of 14.91-fold ($p < 0.001$; Fig. 2a). Conversely, the RNA level of NONHSAT062994 was found higher in the matched noncancerous samples than in GC tissues (Fig. 2b). p53 expression at both protein and mRNA levels decreased significantly in GC tissues compared to adjacent control samples (Fig. 2 c, d, and e).

3.3. Association of NONHSAT062994 and PURPL expression and clinicopathological feature in GC

The association of NONHSAT062994 and PURPL expression with clinicopathological characteristics were investigated. Age ($p = 0.586$), gender ($p = 0.902$), TNM stage ($p = 0.366$), lymph node metastasis ($p = 0.229$), vascular metastasis ($p = 0.902$), and necrosis ($p = 0.329$) showed no significant correlation with NONHSAT062994 RNA level, but the relative RNA level of NONHSAT062994 was significantly correlated with the tumor size ($p = 0.004$), and tumor histological grade ($p = 0.004$) as shown in Table 2. NONHSAT062994 expression level in GSE13195 had no significant association with age ($p = 0.848$), gender ($p = 0.513$), TNM stage ($p = 0.098$), metastasis ($p = 0.548$), and lymph node metastasis ($p = 0.548$).

PURPL expression was significantly associated with the tumor size ($p = 0.005$) and histological grade ($p = 0.006$). However, as shown in Table 3, the expression of this lncRNA had no significant association

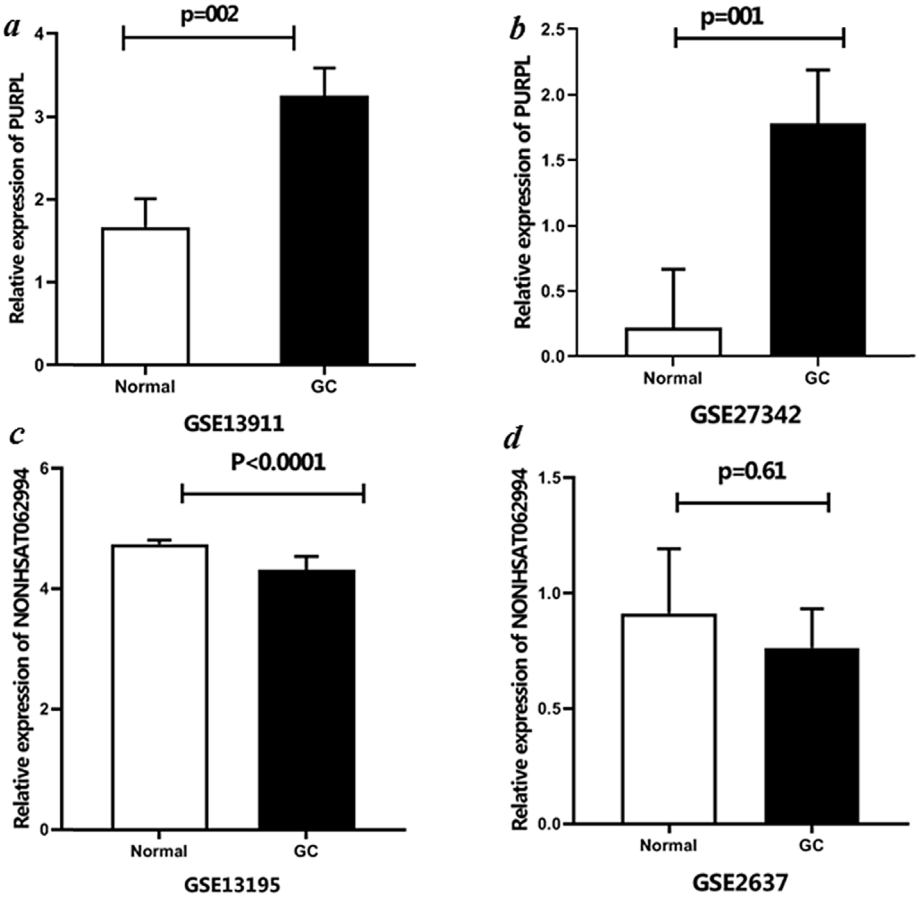

Fig. 1. PURPL and NONHSAT062994 RNA level in GEO dataset. (a) GSE27342, (b) GSE13911, (c) GSE13195, (d) GSE2637. Data were analyzed by Student's *t*-test.

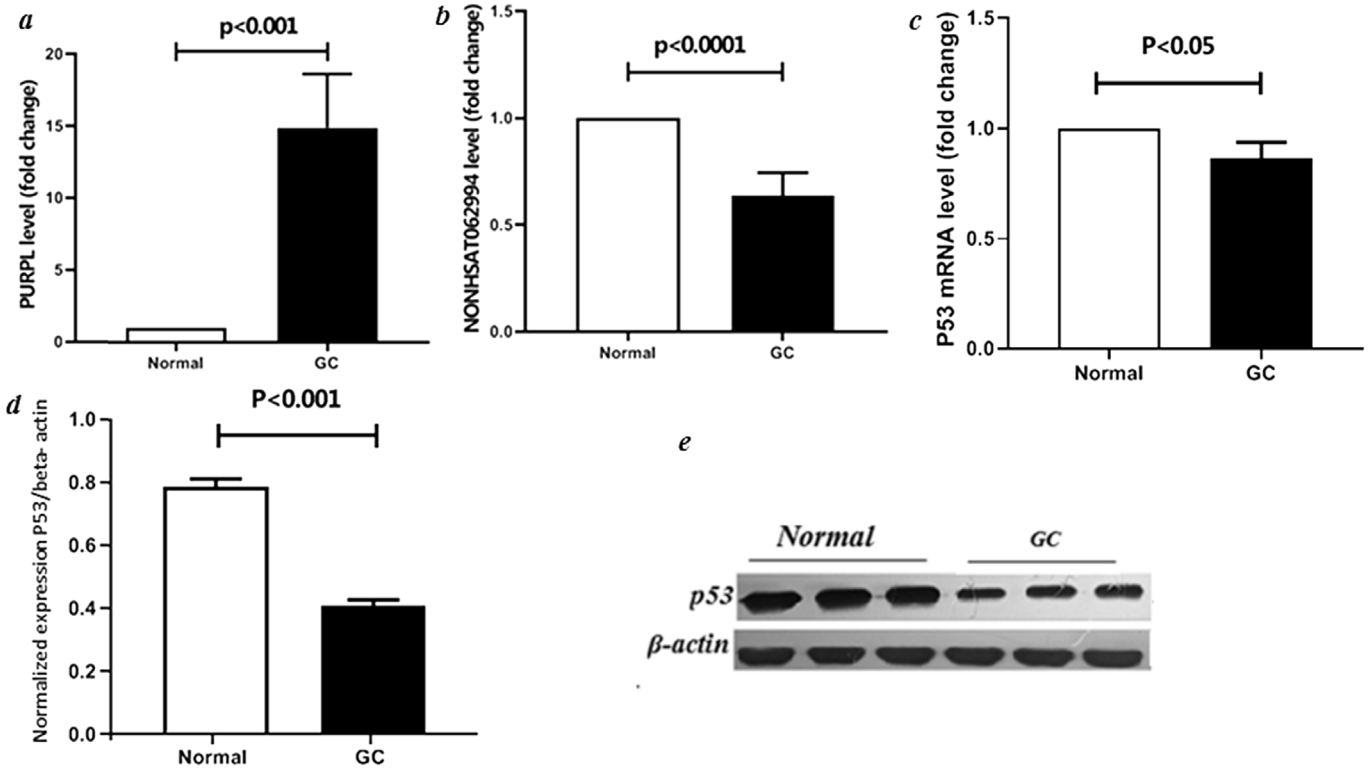

Fig. 2. Expression of NONHSAT062994, PURPL and p53 in GC tissues and naocancerous gastric tissues detected by qRT-PCR (a, b and c). Western blot analysis and quantitative densitometry measurement of the p53 protein expression (d and e). Data were analyzed by Student's *t*-test.

Table 2
The assosiation between NONHSAT062994 expression and clinicalpathological characters in GC pationts.

Characteristics	Number	Expression of lncRNA NONHSAT062994		X^2	p
		Low expression (%)	High expression (%)		
Age(years)					
< 63	23	12 (52.2)	11(47.8)	0.279	0.586
≥ 63	27	12 (44.4)	15 (55.4)		
Gender					
Female	15	7 (46.7)	8 (53.3)	0.015	0.902
Male	35	17 (48.6)	18 (51.4)		
Tumor size(cm)					
< 6	25	17 (68.2)	8 (32.0)	8.432	**0.004***
≥ 6	23	17 (73.9)	6 (26.1)		
Histological grade					
1&2	39	22 (56.4)	17 (43.6)	5.024	**0.004***
3&4	11	2 (18.2)	9 (81.8)		
TNM stage					
Low (I, II)	8	5 (62.5)	3 (37.5)	0.818	0.366
High	40	18 (45.0)	22(55.0)		
Lymph node metastasis					
Yes	39	2 (51.3.5)	19 (48.7)	1.447	0.229
No	10	3 (30.0)	7 (70.0)		
Vascular metastasis					
Yes	42	20 (47.6)	22 (52.4)	0.015	0.902
No	8	4 (50.0)	4 (50.0)		
Necrosis					
Yes	6	4 (66.7)	2 (33.3)	0.952	0.329
No	44	20 (45.5)	24 (54.5)		

All data were analyzed by χ2 test. The median of relative expression data was calculated ($2^{-\Delta\Delta CT}$) in the GC tissues and based on data divided into two groups: high expression and low expression.

Table 3
The association between PURPL expression level and clinicalpathological feature in GC pationts.

Characteristics	Number	Expression of lncRNA PURPL		X^2	P
		Low expression (%)	High expression (%)		
Age (years)					
< 63	23	14 (60.9)	9 (39.1)	0.410	0.552
≥ 63	27	14 (51.9)	13 (48.1)		
Gender					
Female	15	10 (66.7)	5 (33.3)	0.989	0.320
Male	35	18 (51.4)	17 (48.6)		
Tumor size (cm)					
< 6	25	19 (76.0)	6 (24.0)	8.270	**0.005***
≥ 6	23	8 (34.8)	15 (65.2)		
Histological grade					
1&2	39	22 (56.4)	17 (18.2)	5.024	**0.027***
3&4	11	2 (81.8.0)	9 (18.2)		
TNM stage					
Low (I, II)	8	6 (75.5)	2 (25.0)	1.417	0.242
High	40	21 (50.0)	19 (50.0)		
Lymph node metastasis					
Yes	39	21(53.8)	18(46.2)	0.122	0.727
No	10	6 (60.0)	4(40.0)		
Vascular metastasis					
Yes	42	24 (57.1)	18 (42.9)	0.139	0.709
No	8	4 (50.0)	4 (50.0)		
Necrosis					
Yes	6	4 (66.7)	2 (33.3)	0.315	0.575
No	44	24 (54.5)	20 (45.5)		

All data were analyzed by χ2 test. The median of relative expression data was calculated ($2^{-\Delta\Delta CT}$) in the GC tissues and based on data divided into two groups: high expression and low expression.

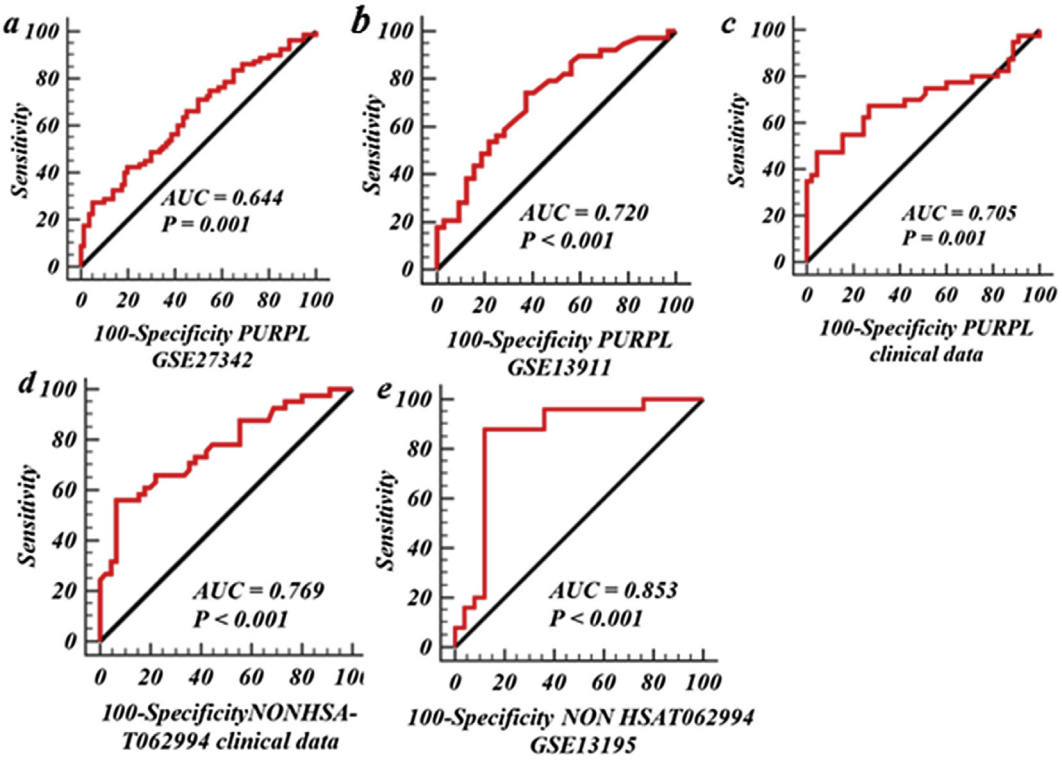

Fig. 3. ROC of PURPL and NONHSAT062994 RNA levels for GC detection in different GEO data and in the clinical data.

with age (p = 0.522), gender (p = 0.320), TNM stage (p = 0.424), lymph node metastasis (p = 0.727), vascular metastasis (p = 0.709), and necrosis (p = 0.575). In GSE27342 dataset also, no significant association was observed between age (p = 0.115), gender (p = 0.879), and TNM stage (p = 0.433) with PURPL expression.

3.4. ROC curve analysis

ROC curve analysis was performed to find out whether the expression levels of PURPL and NONHSAT062994 might be considered as potential tumor markers for GC. The area under curve (AUC) of ROC analysis for PURPL as plotted for GC tissues vs. control tissue was obtained as [0.644 (95% CI, 0.564–0.718)] in GSE27342 dataset, [0.720 (95% CI, 0.601–0.820)] in GSE13911 dataset, and [0.705 (95% CI, 0.596–0.799)] in 50 pairs of GC patient samples as shown in Fig. 3a–c. Similarly, the AUC of NONHSAT062994 for GC tissues compared with control tissues in GSE13195 and 50-pair GC samples was measured [0.853 (95% CI, 0.724–0.937)] and [0.769 (95% CI, 0.655–0.853)] respectively as shown in Fig. 3d and e.

4. Discussion

A search for lncRNA in PubMed delivers an outstanding number of studies. In 2005, only 94 articles had been published in PubMed, while in 2018 the number of publications has skyrocketed to 4038, increasing approximately by 43 folds. LncRNAs seem to attract remarkable attention in the near future as vital RNAs in cancer occurrence and development. Among thousands of lncRNAs, the biological functions of only a few lncRNAs have been identified to the present day [40–42].

High expression of PURPL reduces instability and protein levels of p53, so this lncRNA acts as a pro-survival gene [30]. MYBBP1A is a binding protein for p53, which by forming the MYBBP1A-p53 complex increases protein level of p53 whereas PURPL prevents the formation of this complex and decreases protein level of p53 [30]. Recently, experiments have revealed that the upregulation of PURPL in cancer cell lines decreases the expression of p53, an important tumor suppressor gene, and finally leads to tumorigenicity [30].

For the first time, the current study showed that the PURPL expression level was upregulated in GC tissues compared with normal matched gastric tissues. The analysis of GEE13911 and GEE27342 datasets showed that the PURPL expression level in GC tissues was significantly higher than that of matched normal tissues.

In line with previous studies, our findings showed that the expression of PURPL increased significantly in the tumor tissues compared to normal samples [30,34]. Fu et al. have reported significant upregulation of PURPL both in liver cancer tissues (compared to noncancerous tissues) and various liver cancer cell lines [43]. In the present study, it is shown that p53 of both protein and mRNA levels was downregulated in GC tissues compared with normal matched gastric tissues. The essential role of p53 as a transcription factor which arrest cell cycle, when DNA is damaged, has been identified for decades. LncRNAs are able to regulate p53 and its subset targets through multiple mechanisms such as interacting with chromatin, RNA, and protein [43]. Although the results of our study showed an increase expression of PURPL and a decrease expression of p53 in cancer tissues simultaneously, further studies are required to understand the exact mechanism of interaction between these two genes in gastric cancer.

We found that the PURPL level in GC was significantly related to histological grade and tumor size. It is also interesting to note that in the liver cancer, the expression of this lncRNA is related to tumor size and tumor differentiation [44]. Suppressing the PURPL expression in liver cancer cell lines induced apoptosis, inhibited cell proliferation and arrested cell cycle progression [44]. Together, these results allow us to draw this conclusion that PURPL plays an important role in prognosis and treatment of GC although further studies are required to reveal the exact mechanism of how PURPL functions.

The ROC curve results showed a relatively good specificity and sensitivity for PURPL RNA level in discriminating between gastric cancers and non cancer tissues, indicating that the PURPL expression level may be used for diagnosis of gastric cancer.

In vitro and in vivo studies have shown that NONHSAT062994 alone is not able to prevent cancer formation, rather, this is carried out

by the effects on downstream pathways. Downregulation of NONHSAT062994 expression in the cell increases the expression of Akt, and then c-Myc and Cycline D1, and ultimately leads to tumorigenicity [21].

In the current work, for the first time, we showed that the NONHSAT062994 expression was downregulated in GC tissue. It has been reported that NONHSAT062994 has significantly downregulated in cell lines and human colorectal cancer (CRC) tissues [28]. In consistence with our results, GSE13915 dataset analysis showed that NONHSAT062994 expression in GC tissues was significantly lower than normal matched gastric tissues. Furthermore, GEE2637 analysis showed that expression levels of NONHSAT062994 in GC tissues were lower than normal matched adjacent tissues, but the difference did not reach statistical significance. The small sample size in GSE2637 is probably the reason for this difference with our results.

An important observation in the current study is that the expression level of NONHSAT062994 was significantly associated with tumor size and histological grade. In colorectal cancer, researchers demonstrated that NONHSAT062994 expression is associated with the tumor size [28]. Tumor size is strongly related to long-term survival, prognosis and malignancy potential [45–47]. However, the expression of genes was very low, but their undesirable expression has an effect on the cell cycle [48]. For example, some tumor suppressive LncRNAs by impact on important factors such as p53, p21 and matrix metalloproteinases regulate apoptosis, cell proliferation, and metastasis [49].

Our ROC curve analysis showed that the AUC value of NONHSAT062994 in GSE13195 50-pair GC patients was 0.769 (95% CI 0.655–0.853). The overexpression of NONHSAT062994 diminished CRC cell proliferation and significantly inhibited tumor growth. It has been reported that lower NONHSAT062994 levels were closely related to the overall survival of colorectal cancer patients [28].

Overally, these results propose that PURPL and NONHSAT062994 may play critical roles in GC formation and progression and, thus, can be used as tumor markers for therapeutic goals in GC.

5. Conclusion

In summary, our study for the first time revealed the expression profile of PURPL and NONHSAT062994 in GC. Our findings demonstrated that PURPL and NONHSAT062994 expression were significantly aberrant in GC tissue and their expression was correlated with sex, tumor size, and histological grade. Therefore, it can be suggested that PURPL and NONHSAT062994 might be used as potential tumor markers and therapeutic targets in GC.

Declaration of competing interest

The Authors declare that they have no conflict of interest.

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