Upregulation of long non-coding RNA LOC284454 may serve as a new serum biomarker for head and neck cancers

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Abstract

Purpose: Identification of effective diagnostic and prognostic biomarkers of cancer is necessary for improving precision medicine. Long non-coding RNAs(lncRNAs) play an important regulatory role in tumorinitiation and progression. The lncRNA LOC284454 is distinctly expressed in various head and neck cancers(HNCs), as demonstrated by our previous bioinformatics analysis. However, the expression levels and functions of LOC284454 in cancer are still unclear.

Methods: We investigated the dysregulation of lncRNAs in HNCs using the GEO database and found that LOC284454 was highly expressed in HNCs. Serum samples from 212 patients with HNCs and 121 normal controls were included in this biomarker study. We measured the expression of LOC284454 in the sera of HNC patients and normal controls using RT-qPCR. Receiver operating characteristics (ROC) analysis is an important statistical method that is widely used in clinical diagnosis and population screening. ROC was used to analyze the clinical value of LOC284454 in the early diagnosis of HNCs.

Results: LOC284454 was significantly upregulated in the sera of patients with nasopharyngeal carcinoma, oral cancer, and thyroid cancer. LOC284454 upregulation had good clinical diagnostic value in these cancers, as evaluated by area under the ROC curve values of 0.931, 0.698, and 0.834,respectively.

Conclusions: LOC284454 may be a valuable serum biomarker forHNCsfacilitating the early diagnosis of malignant cancers.Further studies are needed to elucidate the mechanisms underlying the involvement of LOC284454 in HNCs.This study provides the first evidence that LOC284454 may be a serum biomarker for HNCs.

Highlights

1. This is the first study examining *LOC284454* expression in HNCs.
2. This study provides the first evidence of *LOC284454* as a serum biomarker for HNCs.

Background

Head and neck cancers(HNCs), including cancers of the oral cavity, tongue, hypopharynx, nasopharynx, larynx, and thyroid, are the sixth most common cancers worldwide, with an estimated incidence of more than 500,000 new cases each year[1–3]. Most patients are in an advanced stage of HNCs at the time of diagnosis, with cervical lymph node involvement and/or distant metastasis. In these patients, the risk of metastasis and recurrence is significantly increased, and the mortality rate rises sharply.

Effective biomarkers for early diagnosis and prognosis are important for reducing the mortality of HNCs. Liquid biopsy is currently an effective and non-invasive method. Some serum markers, such as Epstein Barr virus DNA and microRNA (miRNA), lactate dehydrogenase, and antigens have been recognized fortheir clinical value[4–11]. However, they also have some limitations. Identifying serum biomarkers with high sensitivity and specificity is an urgent goal.

Long non-coding RNAs(lncRNAs) are transcripts longer than 200 nucleotides that do not encode proteins[12–18]. In recent years, many studies have shown that a variety of lncRNAs are frequently expressed in malignant cancers and may participate in the initiation and development of malignant cancers[19–26]. For example, the AFAP1-AS1 lncRNA promotes the proliferation, migration, and invasion of cervical cancer, colon cancer and nasopharyngeal carcinoma (NPC) through different mechanisms[27–29]. Additionally, PVT1 lncRNA induces radioresistance by regulating DNA repair and cell apoptosis, while promoting the proliferation of thyroid cancer through polycomb enhancer of zeste homolog 2/thyroid-stimulating hormone receptor[30–32]. However, the functional importance of most lncRNAs has not yet been elucidated, including their rolesin human tumors. Only a few lncRNAs have been reported to have clinical implications for early screening and prognosis.

Presently, we examined the expression level of LOC284454 in patients with HNCs and evaluated its clinical significance as a serum biomarker for early diagnosis.

Methods

Serum collection

In total, 333 serum samples were collected from Affiliated Cancer Hospital of Central South University between January 2018 andMarch 2019. The samples were collected from 121 normal donors and 212 HNC patients. Of the 212 HNC serum, 100 were NPC, 55 were oral cancer, and 57 were thyroid cancer serum samples. The patients hadnot received any radio-chemotherapy or surgery before diagnosis.Detailed clinical data were available for all patients. We analyzed the *LOC284454* expression level as well as the clinical characteristics of the patients and found that *LOC284454* had no significant correlation with pathological stages(data not shown), gender, and age distribution(Supplemental Table1). This study was approved by the Ethical Committee of Central South University. Written informed consent was obtained from all patients and healthy donors.

RNA extraction and RT-qPCR

Serum RNA was extracted using miRNeasy Serum/Plasma Kit(Qiagen,Germany). Since there is no suitable internal housekeeping gene for RT-qPCR, we added an external parameter, pGL3, to the RNA extraction process for removing systematic errors. The pGL3 (1 ng, approximately 2×10^8 copies) was added to serum samples according to the manufacturer's protocol using an miRNeasy Serum/Plasma Kit(Qiagen, Germany). The extracted serum RNA was reverse transcribed using a Revert Aid First Strand cDNA Synthesis Kit(Thermo Fisher Scientific, USA). Forward(F) and reverse(R) primers were synthesized by TSINGKE Biological TechnologyCompany(China), as follows: *LOC284454*-F, 5'-ATTACAGGTGGCTCAGGTGT-3', *LOC284454*-R, 5'-CTTCAGTGTGCCTCCTCAGT-3'; and pGL3-F, 5'-TCCATCTTGCTCCAACACCC-3', pGL3-R, 5'-TCGTCTTTCCGTGCTCCAAA-3'. The probe sequences were as follows:*LOC284454*-P,5'-FAM-CGTGCCTGGCTTTTCTCCACTATCTTG-BHQ1-3' and pGL3-P, 5'-HEX-ACGCAGGTGTCGCAGGTCTTCC-BHQ1-3'. Conventional SYBR-qPCR was performed using iTaq universal

SYBR Green Supermix(Bio-Rad, USA). TaqMan-qPCR was performed using iTaq Universal Probes Supermix(Bio-Rad,USA). All RT-qPCR procedures were performed using a Bio-Rad CFX96 Multicolor Real-time PCR Detection System. TaqMan-qPCR allowed the simultaneous detection of two probes in the same tube(Bio-Rad,USA).

Statistical analysis

Data were analyzed using SPSS 13.0 (SPSS Inc., USA) and GraphPad 5.0 (GraphPad, USA). Student's t-tests were used to evaluate differences between two groups of samples. P-values< 0.05 were considered statistically significant. All the test results obtained were from three independent replicates.The area under the curve (AUC), sensitivity, and specificity were obtained by receiver operating characteristic (ROC) curve analysis.

Results

*LOC284454*is upregulated in NPC, oral cancer, and thyroid cancer

We explored the dysregulation of lncRNAs in HNCs using the GEO database. *LOC284454* was significantly upregulated in several cancers, including NPC, oral cancer, and thyroid cancer. In NPC, we integrated three sets of gene expression profiles, including GSE53819, GSE68799, and one set of data from our research group(GSE61218). GSE30784 and GSE33630 were used to analyze oral cancer and thyroid cancer, respectively. The expression levels of *LOC284454* were significantly higher in NPC(Figure 1a, P<0.001), oral cancer(Figure 1b, P<0.001), and thyroid cancer(Figure1c, P<0.001), compared tonon-tumor tissues.

*LOC284454*expression is significantly increased in serum of patients with NPC

SYBR-quantitative polymerase chain reaction(qPCR) was used to detect the expression of *LOC284454* in the sera of 76 NPC patients and 51 healthy donors. *LOC284454* expression level was significantly higher in the serum of patients with NPC(Figure2a, P<0.001).

Next, to eliminate systematic errors and make the results are more reliable, we designed a TaqMan probe for *LOC284454* and tested the same serum samples using TaqMan-qPCR. This examination also revealed significantly higher expression of *LOC284454*in the serum of the NPC patients (Figure 2b, P<0.001). This was consistent with previous conventional RT-qPCR results. Subsequent correlation analysis of the results obtained by SYBR-qPCR and TaqMan-qPCR demonstrated a good positive correlation between the two methods, which verified the reliability of this data(Figure 2c, P<0.001).

We also verified the expression of *LOC284454* in a larger cohort of 121 normal controls and 100 NPC patients. The expression of *LOC284454* in the serum of NPC patients was significantly higher than that of the normal control group(Figure 2d, P<0.001). Taken together, these results suggested that *LOC284454* may be a potential serum marker for NPC.

LOC284454 is highly expressed in sera of patients with oral cancer and thyroid cancer

The results of the microarray analysis suggested that *LOC284454* may be dysregulated in oral cancer and thyroid cancer. Therefore, we next detected the expression of *LOC284454* in the serum of patients with these cancers using TaqMan-qPCR. *LOC284454* was significantly upregulated in the sera of patients with oral cancer and thyroid cancercompared with those of the normal controls(Figure 3a& 3b, $P<0.001$). Notably, a significant difference was evident in the proportion of men and women with thyroid cancer(43 females and 14 males). To exclude gender effects, we analyzed the expression of *LOC284454* in 43 female thyroid cancer patients and 36 normal women. The expression of *LOC284454* was higherin the tumor sera than inthe normal group(Figure 3c, $P=0.024$). The collective results demonstrated that the expression level of *LOC284454* in the sera of patients with oral and thyroid cancers was significantly higher than in normal controls.

Diagnostic value of serum *LOC284454* for HNC patients

ROC is commonly used to assess the diagnostic value of biomarkers. AUC refers to the area enclosed by the curve and the 45-degree diagonal line, which is used to quantify the diagnostic value. An AUC value <0.5 indicates almost no diagnostic value. AUCs of 0.5~0.7, 0.7~0.9, and >0.9 indicate low, moderate, and high diagnostic value, respectively. Values exceeding 0.9 also indicatehigh specificity and sensitivity. When the sensitivity-(1-specificity) value is the largest, its corresponding row is the best cut-off point.

The AUC values of *LOC284454* in NPC(Figure 4a), oral cancer(Figure 4b), and thyroid cancer(Figure 4c) were 0.931, 0.698, and 0.834, respectively, indicating that *LOC284454*might be an appropriate diagnostic biomarkers for these cancers(Table 1).

Discussion

HNCs rank as the sixth most common type of cancers worldwide. The cancers are often at an advanced stage at the time of diagnosisand display frequent recurrence and metastasis. Thus, prognosis and patient survival are poor. Radiotherapy and chemotherapy have largely improved the treatment of HNCs in recent decades[33–38]. However, the 5-year survival rate is still very low. Improving the accuracy of early diagnosis could significantly improve the disease-free survival rate of patients.

Compared with other detection methods, liquid biopsy has become the preferredchoice for disease screening because of its non-invasiveness, low cost, easeof use, and high stability. Some biomarkers for HNCs, including proteins, miRNAs, and EBV DNA, have been identified using liquid biopsies[39, 40]. However, each of these markers has its own disadvantages, including low positive rates, high false positive rate, need for experienced operators, and instrumental limitations. Therefore, finding effective early diagnostic markers in serum is critical for the treatment of HNCs.

LncRNAs have been reported to participate in the pathogenesis of HNCs. LncRNAs circulating in the serum or other bodily fluids present promising biomarkers for clinical diagnostic and prognostic

applications. For example, serum MALAT1, AFAP1-AS1, and AL359062 can function as diagnostic and prognostic biomarkers for NPC [41]. Notably, the upregulation of the ATB lncRNA can accurately predict papillary thyroid carcinoma and its prognosis[42]. However, few studies have examined novel serum lncRNAs in HNCs.

The LOC284454 lncRNA is located on 19p13.12 and the miR-23-a ~ 27a ~ 24 − 2 cluster is present upstream of the same transcript.LOC284454 is a nuclear localized and chromatin associated lncRNA.LOC284454 RNA is found only in primates and is highlyconserved.In our previous study, we demonstrated that LOC284454 promotes migration and invasion of NPC cells invitro and in vivo, and is associated with skeletal remodeling and adhesion signal pathways[43]. In this study, based on the feasibility of SYBR-qPCR and TaqMan-qPCR tests of serum LOC284454, we found that compared with healthy controls the expression of LOC284454 was higher in NPC, oral cancer, and thyroid cancer, indicating that LOC284454 might be very important for the diagnosis of HNCs. To confirm this, we used ROC curve analysis to evaluate the diagnostic value of LOC284454.The AUC values of LOC284454 in NPC, oral cancer, and thyroid cancer were 0.931, 0.698, and 0.834, respectively, indicating that LOC284454 might be an appropriate diagnostic biomarker for these cancers. Even though we found that LOC284454 is highly expressed in NPC, oral cancer, and thyroid cancer, there is no evidence that this can be generalizedto all cancers. The description that LOC284454 is significantly reduced in prostate, uterus, breast, and kidney cancer[44] suggests instead that LOC284454 is relatively specific and is highly expressed in HNC.

Real-time PCR can sensitively detect small changes in nucleic acids based on fluorescent dyes and fluorescentlylabeled probes. In TaqMan-PCR, a fluorescent reporter group and a fluorescence quenching group are labeled on both ends of the probe[45–48]. When amplified, the 5'-3' exonuclease activity of the Taq enzyme degrades the probe. The fluorescent reporter group and the fluorescence quenching group are separated, so that the fluorescence monitoring system can receive the fluorescent signal, and the accumulation of fluorescent signal is completely synchronized with the formation of the PCR product [49–51]. Since the qPCR instrument has a multicolor fluorescent channel, the experimental group and the control group are allowed to react in the same tube with the same cDNA template, which can reduce systematic errors and improve the specificity and sensitivity of the experiment[52]. This is also one of the highlights of this study and might be very useful for future detection of biomarkers.

We found that LOC284454 is highly expressed in the peripheral blood of HNCs. Why it remains stable in the peripheral blood is unclear. We suspect that this may be related to exosomes or vesicles. Exosomes can encapsulate proteins, lipids, and nucleic acids,remain stable in the tumor microenvironment, and are important in tumor metastasis[53]. Recent studies have shown that non-coding RNAs exist in exosomes. Exosomes can carry non-coding RNAs to non-adjacent cells for information communication and can participate in tumor development[54–57]. More research is needed to elucidate these mechanisms.

In summary, our results verified that LOC284454is significantly upregulated in the sera of patients with NPC, oral cancer, and thyroid cancer based on SYBR-qPCR and TaqMan-qPCR. Moreover, ROC curve data

indicate that LOC284454 could be used as a novel diagnostic biomarker for HNCs. Further research should focus on follow-up processes to study the prognostic value of LOC284454. It is hoped that the development of new technologies, such as digital PCR, will make it easier to detect phenotypic specific molecular changes, and will increase the sensitivity and specificity of biomarkers.

Conlusions

In this study, we investigated the dysregulation of lncRNAs in HNCs using the GEO database and found that LOC284454 was highly expressed in HNCs(nasopharyngeal carcinoma, oral cancer, and thyroid cancer). We measured the expression of LOC284454 in the sera of HNC patients via Taqman RT-qPCR. We then used ROC curve to analyze the clinical value of LOC284454 in the early diagnosis of HNCs. LOC284454 upregulation had good clinical diagnostic value in nasopharyngeal carcinoma, oral cancer, and thyroid cancer, as evaluated by area under the ROC curve values of 0.931, 0.698, and 0.834, respectively. LOC284454 may be a valuable serum biomarker for HNCs facilitating the early diagnosis of malignant cancers. Further studies are needed to elucidate the mechanisms underlying the involvement of LOC284454 in HNCs. This study provides the first evidence that LOC284454 may be a serum biomarker for HNCs.

Abbreviations

LncRNAs: Long non-coding RNAs; HNCs: Head and neck cancers; ROC: Receiver operating characteristics; miRNA: microRNA; NPC: Nasopharyngeal carcinoma; AUC: The area under the curve

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Central South University and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Consent to publish

Each author of this article has read the manuscript and agreed to publish.

Availability of data and material

The authors declared that all the data were available on line or from the corresponding author on reasonable request.

GEO databases: https://www.ncbi.nlm.nih.gov/gds/?term=

Competing interests

There are no potential conflicts of interest.

Acknowledgment

Not applicable.

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Authors' contributions

WX and ZYZ designed and revised the manuscript. CMF, JPW and YYT wrote the manuscript and drew figures. SZ, FX, CG, YHZ, ZL, XLL, YL and GYL participated in the design of the manuscript. All the authors read and approved the final version of the manuscript.

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Tables

Table 1. ROC curves analysis of LOC284454 in nasopharyngeal carcinoma, oral cancer and thyroid cancer

cancer types	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		Sensitivity	Specificity
				Lower Bound	Upper Bound		
nasopharyngeal carcinoma	0.931	0.017	0.000	0.899	0.964	0.770	0.959
oral cancer	0.698	0.047	0.000	0.606	0.791	0.582	0.810
thyroid cancer	0.834	0.032	0.000	0.771	0.898	0.877	0.620

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Figures

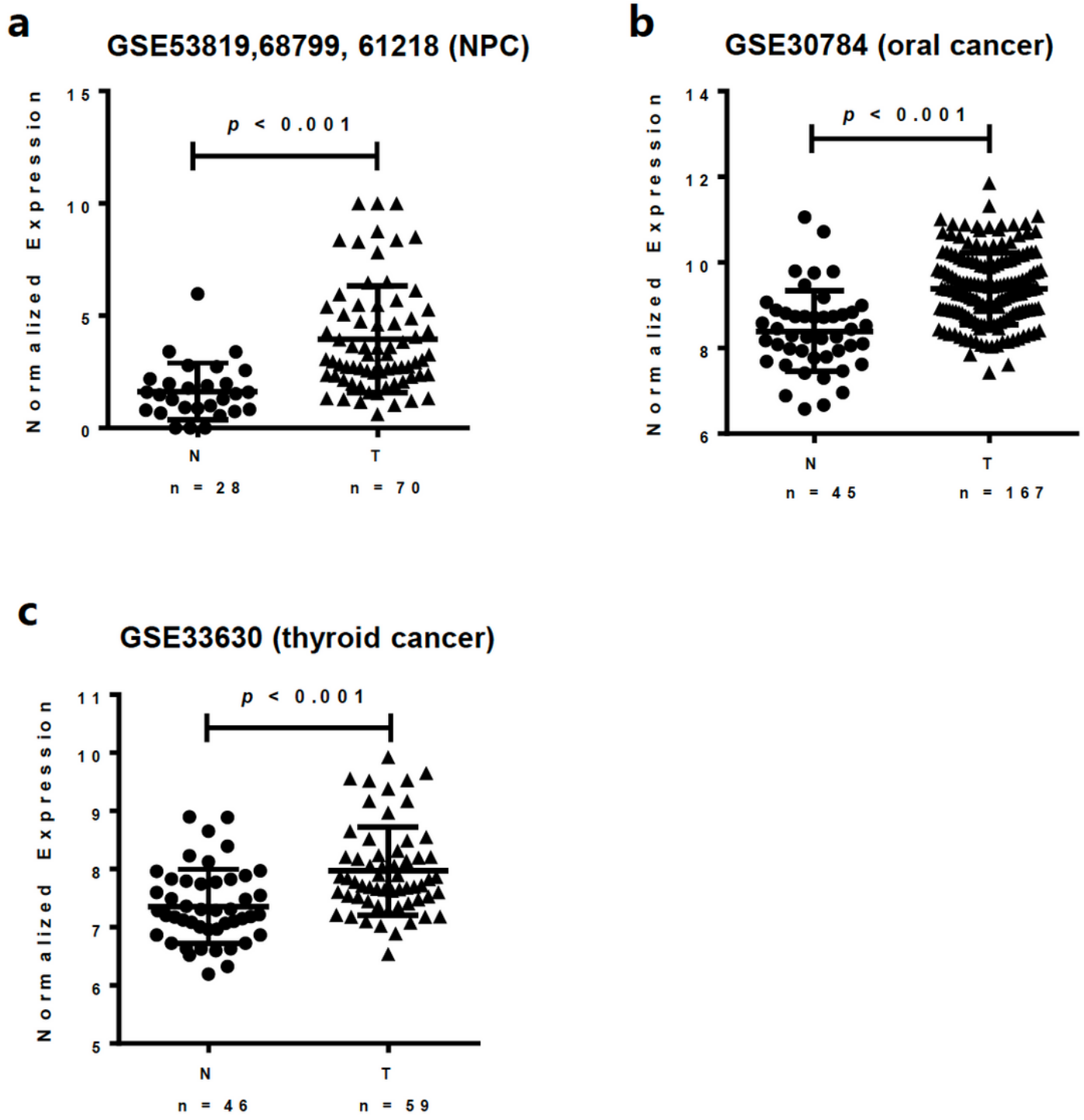

Figure 1

Upregulation of LOC284454 in head and neck cancers in the GEO database. LOC284454 was significantly upregulated in several head and neck cancers, including nasopharyngeal carcinoma(a), oral cancer(b), and thyroid cancer(c).

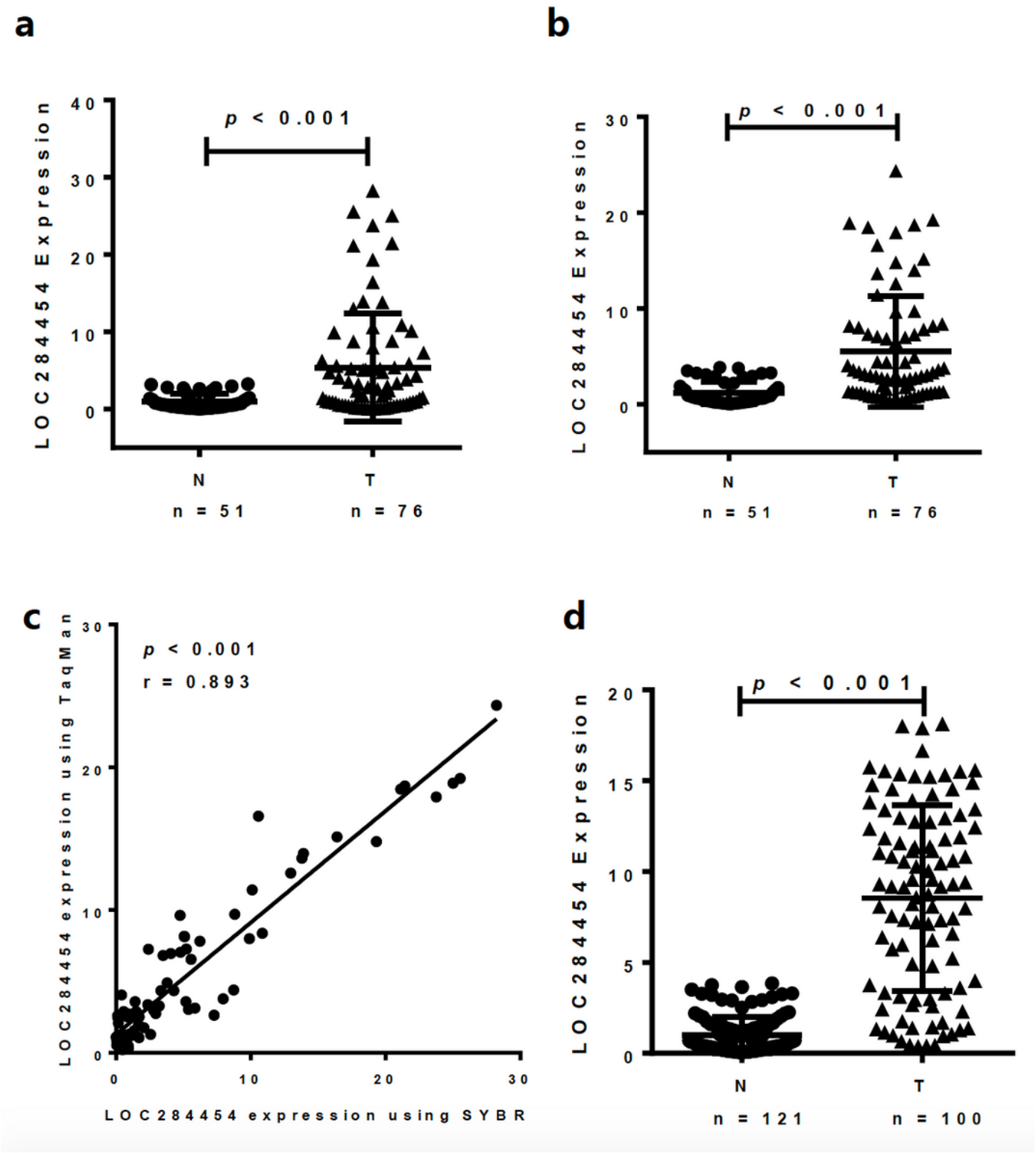

Figure 2

LOC284454 expression is significantly higherin sera of patients with NPC. (a). SYBRGreen quantitative polymerase chain reaction(qPCR) assay was used to detect the expression of LOC284454 in the sera of 76 NPC patients and 51 healthy donors. (b). A TaqMan probe for LOC284454 was used to detect the expression of LOC284454 in the same samples. (c). Correlation analysis of the results obtained by SYBR-

qPCR and TaqMan-qPCR. (d). Verification of the expression of LOC284454 in 100 NPC and 121 normal control samples.

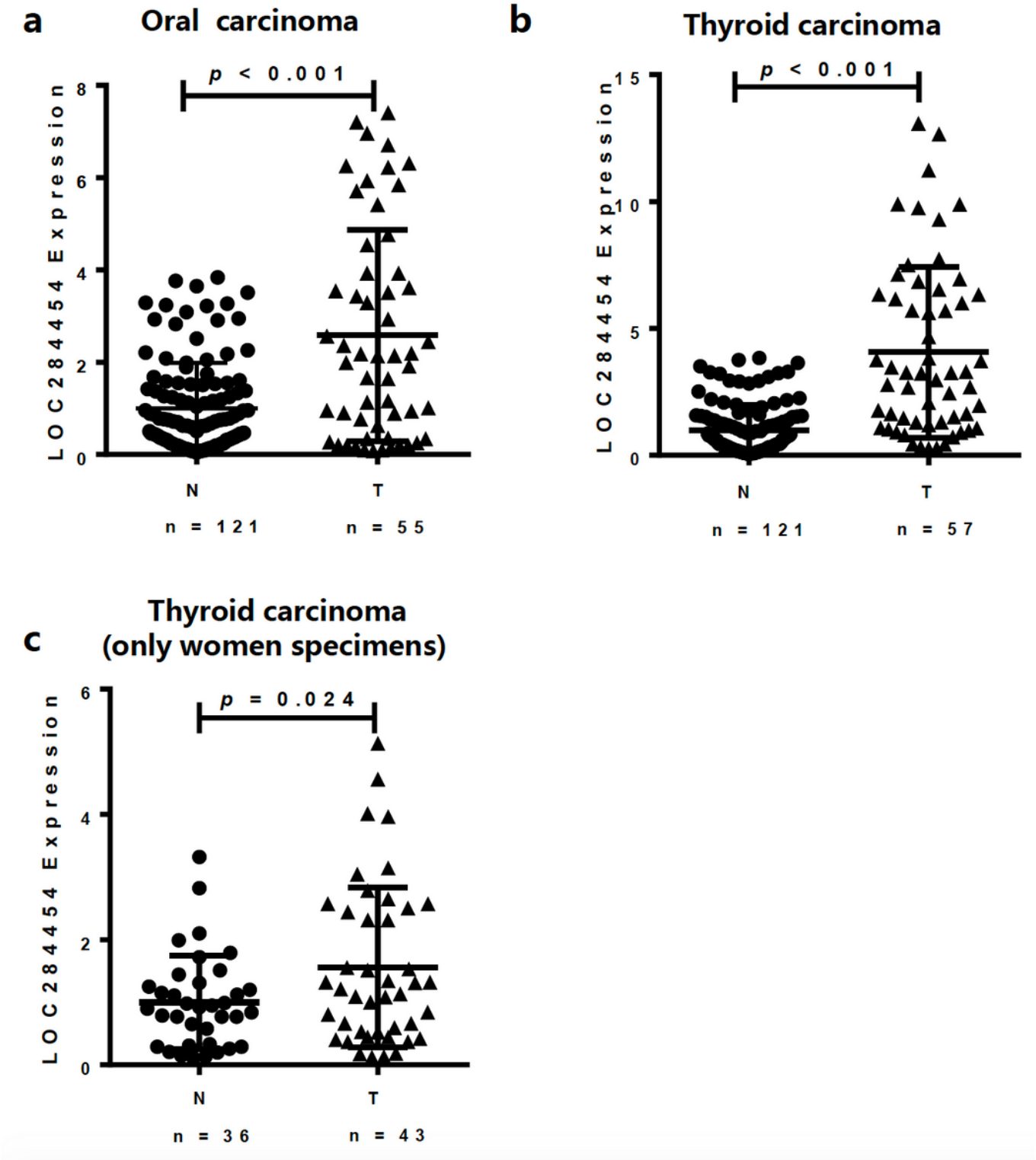

Figure 3

LOC284454 is highly expressed in sera of patients with oral cancer and thyroid cancer. TaqMan-qPCR to detect the expression of LOC284454 in oral cancer (a) and thyroid cancer (b). (c). Expression of LOC284454 in 43 female thyroid cancer patients and 36 normal women.

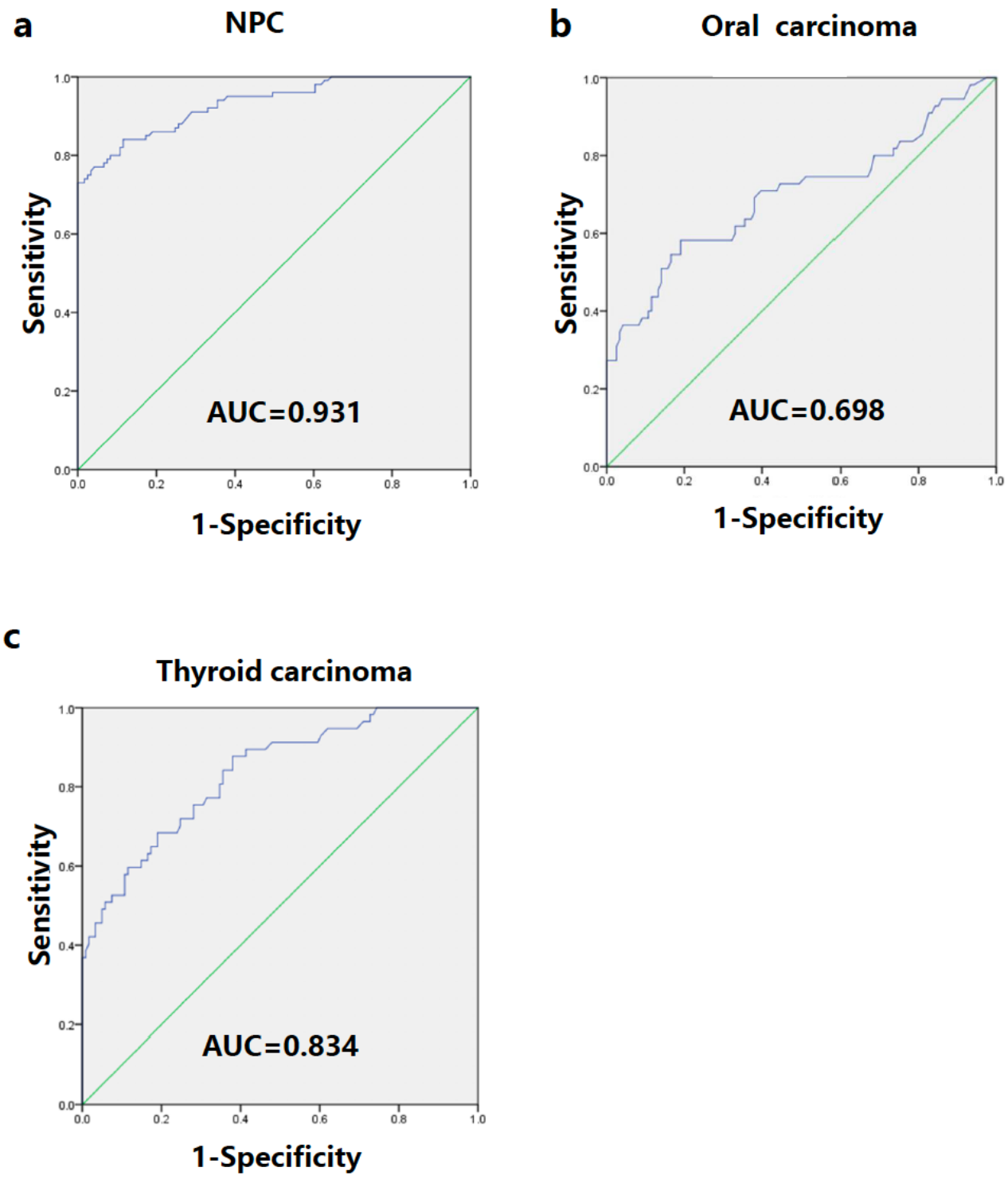

Figure 4

Diagnostic value of serum LOC284454 for HNC patients ROC analysis was performed to evaluate the diagnostic value of LOC284454. The AUC values of LOC284454 in NPC(a), oral cancer(b), and thyroid cancer(c) were 0.931, 0.698, and 0.834, respectively.