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circMAN1A2 could serve as a novel serum biomarker for malignant tumors

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Abstract

Novel diagnostic and prognostic biomarkers of cancers are needed to improve precision medicine. Circular RNAs act as important regulators in cancers at the transcriptional and posttranscriptional levels. The circular RNA circMAN1A2 is highly expressed in nasopharyngeal carcinoma according to our previous RNA sequencing data; however, the expression and functions of circMAN1A2 in cancers are still obscure. Therefore, in this study, we evaluated the expression of circMAN1A2 in the sera of patients with nasopharyngeal carcinoma and other malignant tumors and analyzed its correlations with clinical features and diagnostic values. The expression levels of circMAN1A2 were detected by quantitative real-time PCR, and the correlations of clinical features with circMAN1A2 expression were analyzed by χ^2 tests. Receiver operating characteristic curves were used to evaluate the clinical applications of circMAN1A2. The results showed that circMAN1A2 was upregulated in nasopharyngeal carcinoma, oral cancer, thyroid cancer, ovarian cancer, and lung cancer, with areas under the curves of 0.911, 0.779, 0.734, 0.694, and 0.645, respectively, indicating the good diagnostic value of circMAN1A2. Overall, our findings suggested that circMAN1A2 could be a serum biomarker for malignant tumors, providing important insights into diagnostic approaches for malignant tumors. Further studies are needed to elucidate the mechanisms of circMAN1A2 in the pathogenesis of cancer.

KEYWORDS
circular RNA, diagnosis, malignant cancer, receiver operating characteristic, serum biomarker

1 | INTRODUCTION

Malignant tumors often have no obvious symptoms at early stages and can appear nonspecific, even if symptoms are observed. When patients show specific symptoms, tumors are usually already at a late stage. Nasopharyngeal carcinoma (NPC), oral cancer, thyroid cancer, ovarian cancer, and lung cancer are 5 common malignant tumors that show high rates of metastasis.[1-6] Insufficient early diagnosis and

poor prognosis, accompanied by high metastasis and recurrence, are the main causes of high mortality in malignant tumors.[7] Therefore, effective early diagnosis and new cancer treatment strategies play important roles in reducing the incidence of malignant cancers.

The early diagnosis of malignant cancers can be achieved using many methods, including blood detection, genetic testing, and cancer biomarker analysis.[8,9] In order to improve the early diagnosis of malignant tumors, many biomarkers have been identified. For example, plasma lipocalin-2 is upregulated in pediatric thyroid cancer and could have applications as a new biomarker.[10] Serum lactate dehydrogenase has also been shown to act as a significant marker of head and neck squamous cell carcinoma,[11] and Epstein-Barr virus (EBV) DNAs or miRNAs are significantly correlated with distant metastasis and death in patients with NPC.[12-18] Serum hsa-miR-1273 g-3p might also have potential applications as both a prognostic and diagnostic biomarker of recurrent epithelial ovarian cancer.[19] Squamous cell carcinoma antigen, a marker of squamous cell carcinoma, can be used to detect head and neck cancers.[20] However, there are clear limitations to the use of these biomarkers. Therefore, there is an urgent need to discover novel, effective diagnostic and prognostic biomarkers to treat malignant tumors.

Circular RNAs (circRNAs) are formed by linking a downstream 5′ splicing site to an upstream 3′ splicing site and are more stable than linear RNAs.[21,22] Circular RNAs were originally thought to be byproducts of splicing errors and were not widely studied. In recent years, many studies have found that a variety of circRNAs are frequently abnormally expressed in malignant cancers and might participate in the initiation and development of malignant cancers. For example, hsa_circ_0061140 regulates the miR-370/forkhead box M1 pathway to promote the epithelial-mesenchymal transition and thus enhances the growth and metastasis of ovarian cancer cells.[23] circFBLIM1 can act as a competing endogenous RNA to regulate the expression of filamin binding LIM protein 1 through sponging miR-346 in hepatocellular carcinoma.[24] Similarly, circGFRA1 regulates the expression of glial cell-derived neurotrophic factor family receptor-α1 through sponging miR-34a to exert regulatory functions in triple-negative breast cancer.[25] However, most circRNAs and their pathological functions in malignant tumors are still unknown. In particular, few studies have evaluated circRNAs as biomarkers in NPC, oral cancer, thyroid cancer, ovarian cancer, or lung cancer.

In this study, we carried out whole-transcriptome sequencing of 5-8F human NPC cells and identified 1 highly expressed circRNA, circMAN1A2, which had not been reported to date. Subsequently, we downloaded another NPC tissue RNA sequencing dataset from the GEO database and found that circMAN1A2 was also highly expressed in NPC tissues. Because the structure of circRNA is stable, we hypothesized that circMAN1A2 could have applications as a serum biomarker. Therefore, we focused on the circRNA circMAN1A2, which was first identified as an upregulated circRNA in the peripheral blood of patients with malignant tumors, and receiver operating characteristic (ROC) curves were used to evaluate the clinical applications of circMAN1A2. Our results provided novel insights into the potential applications of circMAN1A2

as an effective diagnostic biomarker and target for the treatment of malignant tumors.

2 | MATERIALS AND METHODS

2.1 | Serum collection

In total, 414 serum samples, including samples from 121 healthy controls, were collected in this study between March 2017 and January 2018 at Xiangya Hospital and the Affiliated Cancer Hospital of Central South University (Changsha, China). Participants in the healthy control group had no oncogenic diseases, infectious diseases, severe immune diseases, or other major diseases. Of the 293 patients with malignant tumors enrolled in this study, 100 had NPC, 55 had oral cancer, 57 had thyroid cancer, 36 had ovarian cancer, and 45 had lung cancer. No patients had received chemotherapy, radiotherapy, or surgery before treatment. Patient information, including patient's name, sex, age, hospitalization number, pathological type, pathological stage, identification number, and treatment status, was collected. We undertook statistical analyses of the expression levels of circMAN1A2 at various pathological stages, and the results showed that there were no significant differences (data not shown). Additionally, there were no significant differences in sex or age between the cancer group and control group (Table S1). This study was approved by the Ethical Committee of Central South University. Written informed consent was obtained from all patients and healthy controls.

2.2 | RNA extraction and quantitative RT-PCR

Total RNA was extracted from serum specimens using an miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Genrmany). Because there were no suitable internal housekeeping genes (internal control) to evaluate in serum, we used the pGL3 plasmid as a control to reduce error during RNA extraction. Other procedures were also used to improve the reliability of the study data. We added 1 ng (approximately 2×10^8 copies) of pGL3 to 200 μL serum samples, according to the manufacturer's protocol. Reverse transcription was carried out using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). Forward (F) and reverse (R) primers, synthesized by TSINGKE Biological Technology company (Changsha, Hunan, China), were as follows: circMAN1A2-F, 5′-AGATGGGCAAAGATGGATTGA-3′ and circMAN1A2-R, 5′-GCCTTCTCATGATCAGCTCG-3′; pGL3-F, 5′-TCCA TCTTGCTCCAACACCC-3′ and pGL3-R, 5′-TCGTCTTTCCGTGCTCC AAA-3′. The probe sequences were as follows: circMAN1A2-P, 5′-ROX-CAAAGATGGATTGAAGACAACCTTGATTTCAGTGTG-BHQ2-3′; and pGL3-P, 5′-HEX-ACGCAGGTGTCGCAGGTCTTCC-BHQ1-3′. Quantitative PCR (qPCR) using SYBR was carried out with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Quantitative PCR using TaqMan was carried out with iTaq universal probes Supermix (Bio-Rad). We used a Bio-Rad CFX96 Multicolor Real-time PCR Detection System (Bio-Rad). Fold changes in expression of circMAN1A2 were calculated using the comparative threshold cycle (Ct) method with the formula $2^{-(\Delta\Delta Ct)}$.

2.3 | Statistical analysis

Statistical analyses were undertaken using SPSS 13.0 and Graphpad Prism 5.0 software. Student's *t* tests were used to evaluate differences in the expression of *circMAN1A2* in serum from the patients and corresponding control groups. Results with *P* values of <0.05 were considered statistically significant. All statistical tests were repeated twice. The area under the ROC curve (AUC), sensitivity, and specificity for *circMAN1A2* were determined using ROC curve analysis.

3 | RESULTS

3.1 | *circMAN1A2* highly expressed in sera of patients with NPC

We used Quantitative PCR (qPCR) using SYBR to detect the expression of *circMAN1A2* in the sera of 71 patients with NPC and 51 healthy controls. The results showed that *circMAN1A2* expression levels were significantly higher in the sera of patients with NPC than in those of normal controls (Figure 1A, *P* < 0.001).

We then designed a TaqMan probe for *circMAN1A2* and further verified the expression of *circMAN1A2* using the more reliable Quantitative PCR using TaqMan experimental method. The results showed that the expression levels of *circMAN1A2* in the sera of patients with NPC were significantly higher than those in the normal control group (Figure 1B, *P* < 0.001). This confirmed our preliminary results.

Next, we undertook a correlation analysis using Quantitative PCR (qPCR) using SYBR and Quantitative PCR using TaqMan. The results showed that the experimental data for the 2 methods were well correlated, which verified the reliability of the experimental data (Figure 1C, *P* < 0.001).

Subsequently, we increased the number of samples in the control group to 121 cases and the number of serum specimens from patients with NPC to 100. The results showed that *circMAN1A2* expression levels were obviously higher in the sera of patients with NPC than in the normal control group (Figure 1D, *P* < 0.001). Taken together, these results indicated that *circMAN1A2* could be a serum biomarker for patients with NPC.

3.2 | *circMAN1A2* highly expressed in sera of patients with oral cancer, thyroid cancer, ovarian cancer, or lung cancer

According to the above results, *circMAN1A2* was significantly upregulated in the sera of patients with NPC. Furthermore, we collected additional serum specimens from patients with 4 other common types of cancer to verify the expression of *circMAN1A2* in these patients.

We used Quantitative PCR using TaqMan to detect the expression levels of *circMAN1A2* in the sera of patients with oral cancer, thyroid cancer, ovarian cancer, or lung cancer. We collected 55 cases of oral cancer and 121 healthy controls to verify the expression levels of
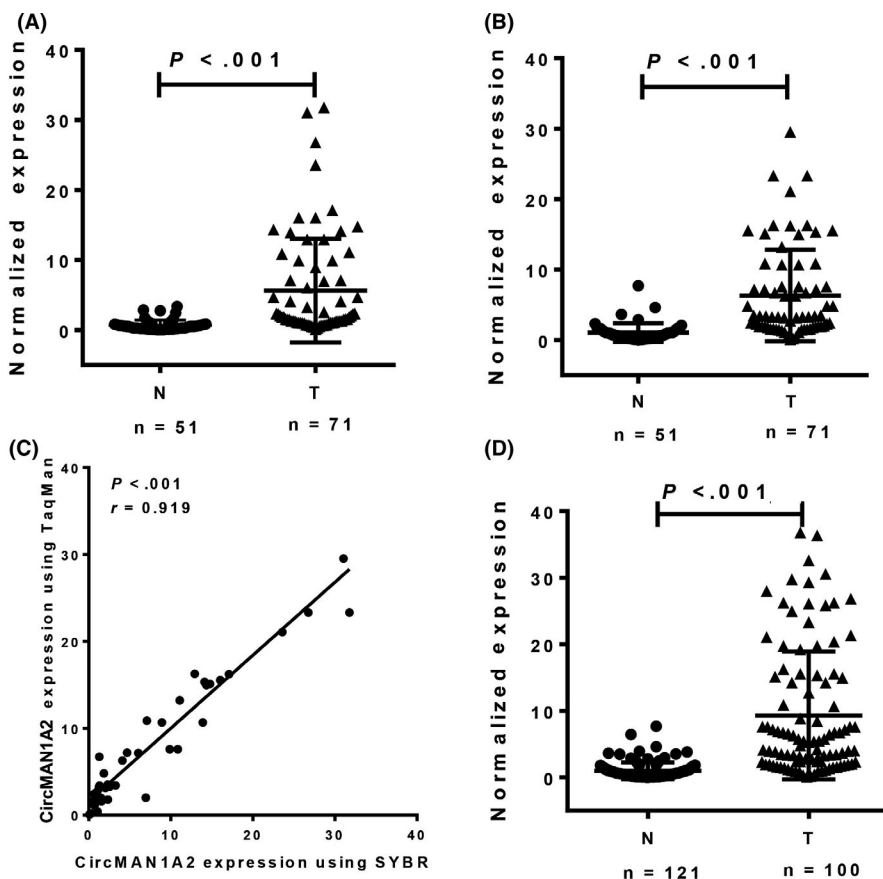
FIGURE 1 Expression of *circMAN1A2* in sera from patients with nasopharyngeal carcinoma (NPC) was detected by SYBR quantitative PCR (qPCR) and Quantitative PCR using TaqMan. A, Quantitative PCR (qPCR) using SYBR was used to detect the expression levels of *circMAN1A2* in the sera from patients with NPC (Tumor, T) compared with that in the normal control group (Normal, N). *P* < 0.001. B, Quantitative PCR using TaqMan was used to detect the expression levels of *circMAN1A2* in the sera of patients with NPC compared with that in normal controls. *P* < 0.001. C, Correlation analysis between Quantitative PCR (qPCR) using SYBR and Quantitative PCR using TaqMan. GraphPad Prism 5 software was used to analyze the correlations between the 2 experimental methods. *P* < 0.001, *r* = 0.919. D, Further analysis with larger sample sizes, *P* < 0.001

FIGURE 2 *circMAN1A2* was highly expressed in the sera of patients with oral cancer, thyroid cancer, ovarian cancer, or lung cancer. A-D, Relative expression of *circMAN1A2* in oral cancer (A), thyroid cancer (B), ovarian cancer (C), and lung cancer (D). $P < 0.01$
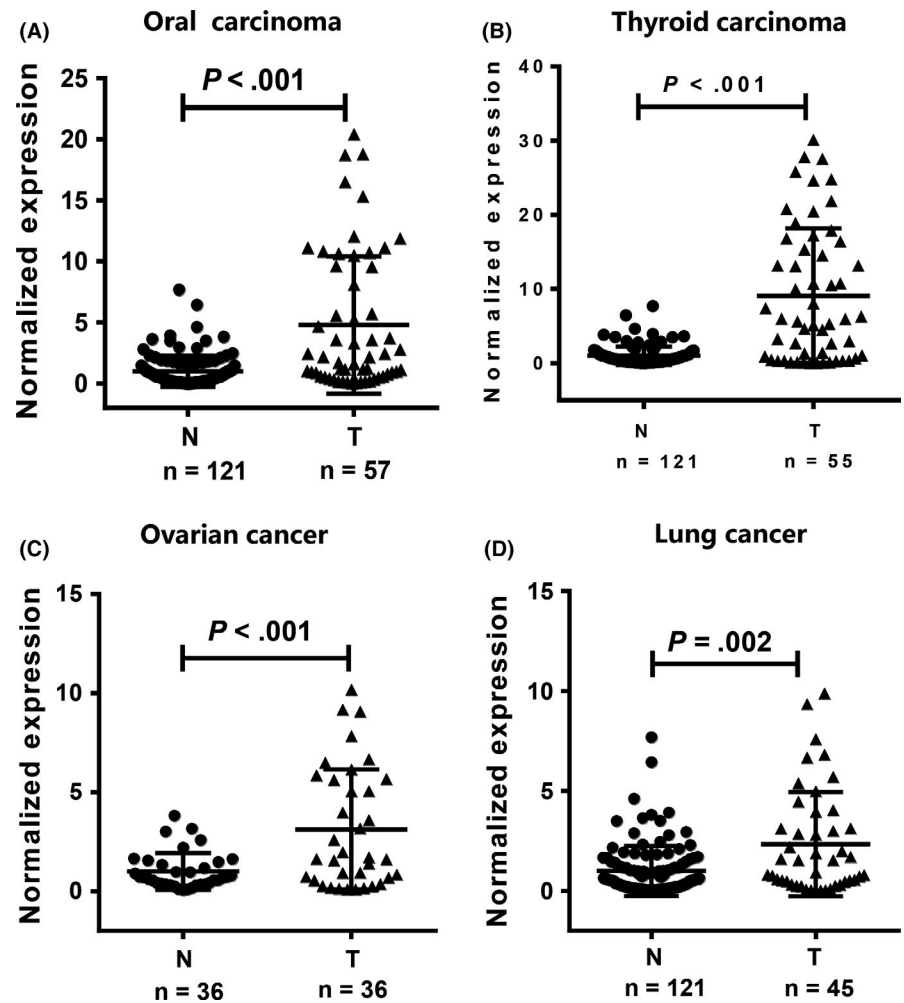
circMAN1A2. The results showed that *circMAN1A2* was significantly highly expressed in sera from patients with oral cancer compared with that in normal controls (Figure 2A, $P < 0.001$). Evaluation of *circMAN1A2* expression in 57 patients with thyroid carcinoma and 121 normal controls yielded similar results, indicating that *circMAN1A2* was highly expressed in thyroid carcinoma (Figure 2B, $P < 0.001$). Additionally, in samples from 36 patients with ovarian cancer and 36 normal controls, *circMAN1A2* was significantly highly expressed in the sera of patients with ovarian cancer (Figure 2C, $P < 0.001$). Finally, in 45 patients with lung cancer and 121 normal controls, patients with lung cancer showed significantly higher *circMAN1A2* levels (Figure 2D, $P = 0.002$).

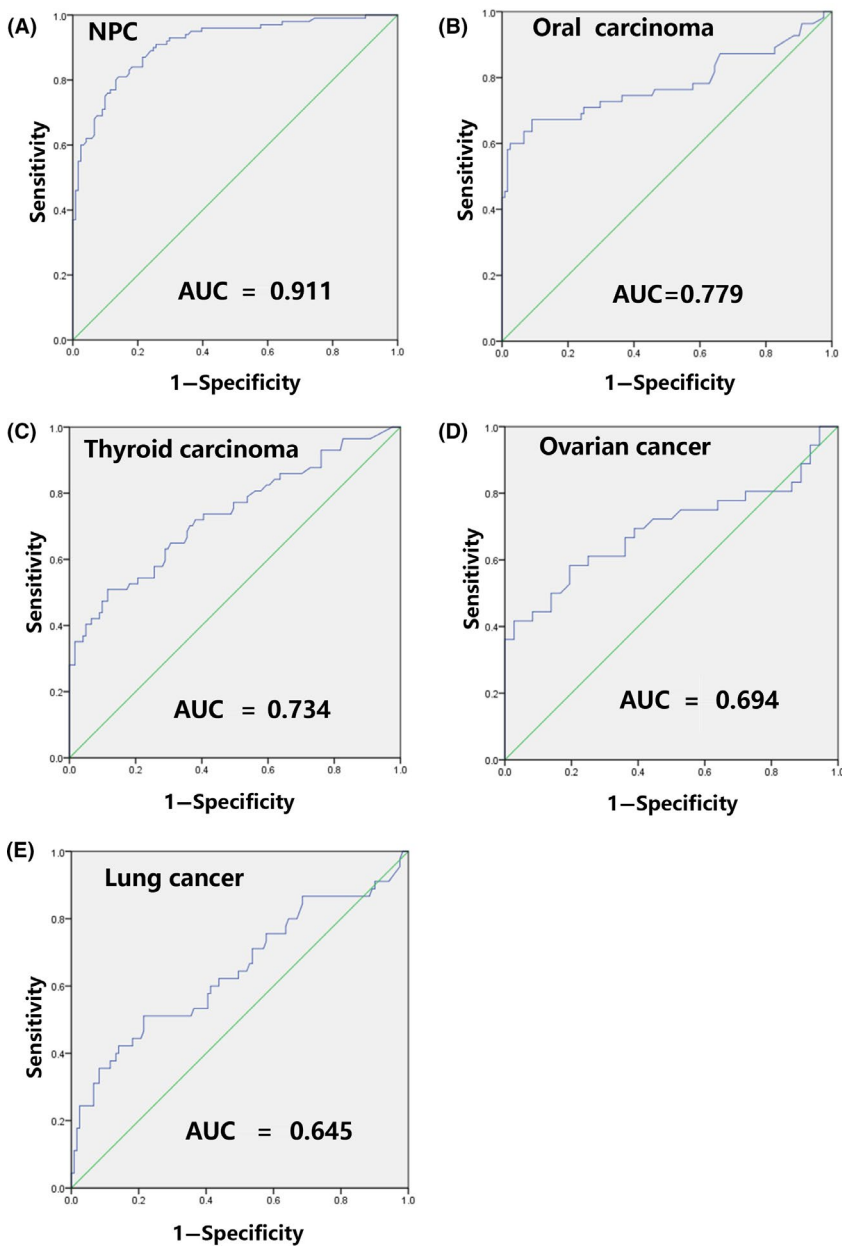## 3.3 | Receiver operating characteristic curve data for the clinical diagnostic value of *circMAN1A2*

Receiver operating characteristic curves can be used to evaluate the clinical value of novel biomarkers in the early diagnosis of cancer. The larger the area formed by the ROC curve and the oblique line, the more accurate the experimental data and the more valuable its clinical applications. In the ROC curve, specificity and sensitivity are combined to evaluate the clinical diagnostic value of biomarkers. Through ROC curve analysis, we can flexibly translate scientific research results into

practical clinical applications. When the AUC value is greater than 0.5, and as the AUC comes closer to 1, the specificity and sensitivity improve, and the clinical application value becomes greater. When the AUC value is between 0.5 and 0.7, the factor is assumed to have low clinical diagnostic value; in contrast, values between 0.7 and 0.9 and above 0.9 indicate moderate and high clinical diagnostic value, respectively.

Owing to the high expression of *circMAN1A2* in the sera of patients with NPC, oral cancer, thyroid cancer, ovarian cancer, or lung cancer, ROC curves were used to evaluate the clinical diagnostic value. The AUCs of *circMAN1A2* in patients with NPC (Figure 3A), oral cancer (Figure 3B), thyroid cancer (Figure 3C), ovarian cancer (Figure 3D), or lung cancer (Figure 3E) were 0.911, 0.779, 0.734, 0.694, and 0.645, respectively, indicating that *circMAN1A2* could serve as an effective diagnostic biomarker in these cancers (Table 1).

4 | DISCUSSION

Malignant tumors are a major threat to human health. Nasopharyngeal carcinoma, oral cancer, thyroid cancer, ovarian cancer, and lung cancer are common malignant tumors that show high rates of metastasis.[26-28] Moreover, the malignant progression and high mortality rates associated with these tumors are related

FIGURE 3 Receiver operating characteristic (ROC) curve data for the clinical diagnostic value of *circMAN1A2*. A-E, ROC curves of *circMAN1A2* in the serum of patients with nasopharyngeal carcinoma (NPC) (A), oral cancer (B), thyroid cancer (C), ovarian cancer (D), or lung cancer (E)

to their insidious onset, frequent metastasis and recurrence, and poor prognosis.[29-31] Treatment on the basis of early diagnosis is still the main approach for achieving long-term cancer-free survival.[32-36] Although many biomarkers of malignant tumors are currently used in the clinical setting, all of these biomarkers have limitations. Therefore, many studies have focused on identifying more effective biomarkers to improve the early diagnosis of malignant tumors.

Common serum markers include proteins, DNAs, and RNAs. Serum protein biomarkers include oncofetal proteins,[37-43] such as α-fetoprotein, carcinoembryonic antigen, tumor-associated antigens (eg CA19-9 and CA125), enzymes such as lactate dehydrogenase, and other special serum proteins (eg β2-macroglobulin). However, these biomarkers are mostly based on immune detection, which is not highly sensitive and is associated with a high false-positive rate.

Epstein-Barr virus DNA is closely correlated with distant metastasis in NPC. However, due to the latency of EBV infection, the virus might not be detected in some patients, and false positives are often observed. Some RNAs, including noncoding RNAs[44-51] and miR-NAs,[52-54] can also function as serum markers, but are unstable in serum. Because of the unique structure of circRNAs, they can be delivered by exosomes and are more stable in serum than circulating tumor DNAs or RNAs. Additionally, circRNAs are easier to extract and detect with higher specificity than proteins; accordingly, circRNAs are superior to current tumor biomarkers in many aspects and could be ideal serum biomarkers in cancers.

Circular RNAs were originally thought to be byproducts of splicing errors, but have since been shown to be widely expressed and exert regulatory functions in biological and pathological processes. In recent years, circRNAs have been reported to participate in the

TABLE 1 Receiver operating characteristic (ROC) curves analysis of *circMAN1A2* in malignant tumors

Cancer type	Area	SE[a]	Asymptotic significance.[b]	Asymptotic 95% confidence interval		Sensitivity	Specificity
				Lower bound	Upper bound		
Nasopharyngeal carcinoma	0.911	0.019	0.000	0.873	0.949	0.810	0.860
Oral cancer	0.779	0.046	0.000	0.690	0.869	0.673	0.909
Thyroid cancer	0.734	0.043	0.000	0.651	0.818	0.509	0.884
Ovarian cancer	0.694	0.065	0.005	0.567	0.821	0.583	0.806
Lung cancer	0.645	0.052	0.004	0.542	0.748	0.511	0.785

[a]Under the nonparametric assumption.
[b]Null hypothesis: true area = 0.5.

pathogenesis of multiple malignant tumors. Circular RNAs in serum or other bodily fluids have become promising biomarkers for clinical diagnostic and prognostic applications. These serum biomarkers are easy to detect, reproducible, noninvasive, and easily commercialized. For example, circulating *hsa_circ_0081001* can serve as a potential biomarker and therapeutic target for patients with osteosarcoma.[55] Moreover, *hsa_circ_0033155* could serve as a biomarker or therapeutic target in non-small-cell lung cancer.[56] *circPVT1* can serve as a new proliferative factor and prognostic marker in gastric cancer.[57] Additionally, *hsa_circ_0001874* and *hsa_circ_0001971* act as biomarkers for the diagnosis of oral squamous cell carcinoma,[58] and *circ-LDLRAD3* as a biomarker for the diagnosis of pancreatic cancer.[59] However, few studies have evaluated novel serum circRNAs in NPC, oral cancer, thyroid cancer, ovarian cancer, and lung cancer.

In this study, we found that the novel circRNA *circMAN1A2* was upregulated in NPC, oral cancer, thyroid cancer, ovarian cancer, and lung cancer, indicating that this circRNA might exert oncogenic effects in malignant tumors. Subsequently, we used 2 qPCR methods (Quantitative PCR (qPCR) using SYBR and Quantitative PCR using TaqMan) and compared their advantages and disadvantages. We then adopted the experimental method of Quantitative PCR using TaqMan, which was a more accurate and reliable method, for verification of gene expression. In addition, ROC curves were generated to evaluate the diagnostic value of *circMAN1A2* in malignant tumors. Analysis of the AUCs indicated that *circMAN1A2* could be a favorable diagnostic biomarker in malignant tumors.

Real-time fluorescence qPCR is a commonly used and reliable detection method for nucleic acids. There are 2 approaches that use qPCR: 1 based on fluorescent dyes and 1 that is targeted at specific fluorescent-labeled DNA sequences, called probes.[60-62] The invention of TaqMan probes has overcome the limitations of SYBR Green I, that is, its lack of specificity owing to its ability to bind to all DNA double-stranded structures.[63-66] During the extension stage of TaqMan-PCR, the DNA polymerase removes the TaqMan probe by hydrolysis, resulting in a fluorescent signal. Moreover, multiple fluorescence signals can be used to detect the expression of several genes simultaneously using multiple channels.[67] Quantitative PCR using TaqMan can be used to evaluate the normal control group and cancer groups in the same qPCR tube using the same cDNA

template.[68] Additionally, TaqMan probes can increase the specificity and sensitivity of qPCR and reduce system error in the experiment, providing a good basis for the evaluation of clinical application value.

In this study, we found that *circMAN1A2* was highly expressed in the peripheral blood of patients with cancer. Thus, we wondered whether the molecules were protected from RNA enzyme degradation by association with exosomes or extracellular vesicles. Importantly, many studies have shown that the communication between cancer cells and surrounding stromal cells can induce cancer metastasis.[69] Moreover, special messenger-exosomes in the cancer microenvironment play important roles in malignant cancer metastasis. Exosomes are comprised of a phospholipid bilayer, contain proteins, lipids, sugars, and nucleic acids,[70] and are mainly derived from polycystic vesicles. After the fusion of polycystic vesicles and serosa, the exosomes are released. Direct fusion, endocytosis, or a mechanism combining external secretion of surface markers can lead to regulation of receptor cells. Additionally, many recent studies have examined noncoding RNAs in exosomes, which can carry a variety of noncoding RNAs to receptor cells and facilitate communication between nonadjacent cells.[71-73] Exosomes can participate in the occurrence and development of cancer invasion, and the high expression of circRNAs observed in the peripheral blood of patients with cancer suggested that the target circRNA might be protected by exosomes or extracellular vesicles. Further studies are needed to assess these potential mechanisms.

Most circRNAs exert their functions through competitive endogenous RNAs.[74] Based on our preliminary analysis, we hypothesized that *circMAN1A2* might exert its role through this mode of regulation, that is, by binding to miRNAs and target genes. We predicted that there were 28 miRNAs bound to *circMAN1A2* (based on Miranda and regRNA2.0 databases), among which *hsa-miR-135a-3p* had the smallest minimum free energy (Table S2), suggesting that *circMAN1A2* was most likely combined with *hsa-miR-135a-3p*. miR-135a-3p is downregulated and serves as a tumor suppressor in ovarian cancer,[75] consistent with our hypothesis. We also predicted binding partners for *hsa-miR-135a-3p* using TargetScan and miRwalk; the top 6 scores were for *SLC4A8*, *IKZF4*, *SMAGP*, *SP1*, *ERBB3*, and *CBX5* (Table S3). These were all

oncogenes, with functions as a sodium-dependent bicarbonate transporter,[76] immune and inflammation regulator,[77-80] membrane transfer protein,[81] transcription factor,[82] tyrosine kinase receptor,[83] and heterochromatin protein,[84] respectively. These findings provided important clues for studying the functions and mechanisms of this novel circRNA.

In summary, in this study, we verified that *circMAN1A2* was significantly upregulated in the sera of patients with NPC, oral cancer, thyroid cancer, ovarian cancer, or lung cancer and had good clinical diagnostic value. We speculate that *circMAN1A2*[1] could be a serum biomarker for malignant cancers and could provide clues for the early diagnosis of malignant cancers. Further research on *circMAN1A2* will improve our understanding of malignant cancer progression. The functions and regulatory mechanisms of *circMAN1A2* in malignant cancer progression should be clarified in further studies.

DISCLOSURE

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.