Inhibition of breast cancer cell proliferation and tumorigenesis by long non-coding RNA RPPH1 down-regulation of miR-122 expression

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Abstract
Background: Recent studies showed that long non-coding RNA (lncRNA) plays an important role in many life activities. RPPH1 is one of the lncRNA genes that are expressed differently between breast cancer and normal tissues by the lncRNA gene chip. Our study was conducted to examine the regulation of lncRNA RPPH1 in breast cancer.

Methods: Two cell lines, MCF-7 and MDA-MB-231, were selected to be the research objects in this study; RPPH1 overexpression and knockdown models were established by transforming vectors. Real-time polymerase chain reaction, MTT assay, clone formation and cell flow cytometer assay were used to test the function of RPPH1. Dual-luciferase assay was used to detect a target relationship between RPPH1 and miR-122.

Results: RPPH1 overexpression promoted cell cycle and proliferation and increased colony formation. In the RPPH1 overexpression model, there was a target relationship between RPPH1 and miR-122, and some of the downstream genes of miR-122, including ADAM10, PKM2, NOD2 and IGF1R, were increased. Moreover, we found that lentivirus-mediated interference of lncRNA RPPH1 inhibited tumour growth in nude mice.

Conclusion: Breast cancer progression can be promoted by directly targeting miR-122 through lncRNA RPPH1. This study provided evidence that can serve as the molecular basis for improving treatment options for patients.

Keywords: lncRNA RPPH1, miR-122, Breast cancer, Targeted regulation, Cell proliferation

Background
Breast cancer occurs in mammary epithelial tissues of malignant tumours and is one of the world’s three most commonly diagnosed cancers [1]. According to the World Cancer Research Fund International 2012 report, the United States, China and India share almost one-third of the burden of disease, accounting for approximately 25% of all new cancer cases diagnosed. By 2021, the incidence rate of breast cancer in women aged 55–69 years in China is estimated to increase from less than 6/10,000 to 1/1000, reaching a total of 250,000 cases [2]; in such situation, breast cancer can become the most common threat to women’s physical and mental health. Therefore, studies on the prevention and cure of breast cancer are of great significance in our country.

Long non-coding RNA (lncRNA), which measures more than 200 bp functional non-encoding RNAs in length [3], was once considered in the evolutionary process to be non-functional genome accumulation of ‘junk sequences’. However, in-depth research in recent years found that lncRNA plays an important role in dosage compensation, epigenetic regulation [4] and regulation of cell cycle and differentiation [5]. At present, some lncRNA genes, such as LOC554202 [6], BCAR4 [7], MALAT1 [8] and GASS [9] have been reported to play an important role in the occurrence and development of breast cancer.
Ribonuclease P RNA component H1 (RPPH1) is the RNA component of the RNase P ribonucleoprotein, an endoribonuclease that cleaves tRNA precursor molecules to form the mature 3′ termini of the tRNA sequences [10]. RPPH1 has also been used as an internal control for RNA quantification [11–13]. Recent deep sequencing studies showed that RPPH1 was upregulated in human gastric cancer tissues [14] and in the neocortex of patients with seizures [15]. A biochemical study has shown that RNase P takes part in the maturation IncRNA MALAT1 [16]. Although these data suggested that RPPH1 may be involved in disease progression in animals and humans, the regulatory mechanisms of RPPH1 expression remain largely unknown. In this study, we intended to explore the interactions of RPPH1 in breast cancer.

Methods

Cell lines, cell culture and tissue collection

The breast cancer cell lines MDA-MB-231, HCC-1937, MDA-MB-453 and MCF-7 were purchased from the Shanghai Cell Bank of the Chinese Academy of Science. The cells were cultured in RPMI 1640 medium (HyClone, Hudson, NH, USA) supplemented with 10% fetal bovine serum at 37 °C and with 5% CO2. Twenty paired breast tissues from patients were collected by surgical resection at Xiangya Hospital between May 2010 and November 2014; the diagnosis of breast cancer was confirmed by histopathologic evaluation. The study was approved by the ethics committee of the Xiangya Hospital of Central South University.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from the cell lines and tissues using Trizol reagent (Dongshe biotech, Guangzhou, China). Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, Waltham, MA, USA) was utilised for reverse transcription. Real-time PCR was performed using an ABI PRISM7300 Sequence Detection System (Applied Biosystems) with SYBR Green PCR mixture (Dongshe biotech). The sets of primers are shown in Table 1. The relative expression level was determined using the 2−ΔΔCt analysis method, where β-actin and U6 were used as the internal standard. All reactions were run in triplicate and all experiments were carried out in three independent times.

In-situ hybridisation assay (ISH)

The breast tissue chip containing breast carcinoma tissues and normal tissues was purchased from Auragen (Changsha, China). The sequence of the IncRNA RPPH1 probe was purchased from BG1 Tech company (Shenzhen, China). A hybridisation probe mixture (1:500) was added, and the operation followed the instructions of the in-situ Hybridisation Kit (Auragen) manufacturer. Finally, the stained chip was observed under optical microscope (Optec, ChongQin, China).

Vector construction and transfection

To construct the pRNAT-U6.1/Neo-shRPPH1, which served as the RPPH1 knockdown vector, an RPPH1 shRNA fragment that contained the target gene, a loop ring and the BamHI and HindIII sites was inserted into the pRNAT-U6.1/Neo vector. The target sequence of the RPPH1 shRNA was 5′-AAGTGAGTTCAATGGCTGAGG-3′. To construct the pcDNA3.1-oe RPPH1 vector, which was the RPPH1 overexpression vector, the target gene fragment was cloned into the site between the HindIII/Xhol of the pcDNA3.1 vector. Construction of both vectors with the target gene and correct insertion without nucleotide mutation or non-specific bands were confirmed.

For transfection of the RPPH1 knockdown and overexpression vectors, MCF-7 and MDA-MB-231 cells were seeded into a 60-mm dish at 37 °C and with 5% CO2 until the confluence of the cells was about 50–60%. The 2.5 μg negative control vector, RPPH1 overexpression vector, random sequence interference plasmid and RPPH1 knockdown vector were transfected using the lipo6000 reagent (Beyotime Biotechnology, China).
for 4 h in the culture without fetal bovine serum (FBS), before transferring into the complete medium for 48 h.

**MTT assay**

The cells were collected and adjusted to a concentration of $5 \times 10^4$ cells/well before they were cultured in a 35-mm dish at 37 °C with 5% CO$_2$ for 24, 48 and 72 h. Thereafter, 10 μL of MTT was added to the cells; the solution was maintained at 37 °C with 5% CO$_2$ for another 4 h before MTT was removed. The value of optical density was measured at 570 nm while the cells were suspended in 150 μL of dimethyl sulfoxide.

**Colony formation assay**

The clonogenicity of a single cell was detected by colony assay. Cells were collected by adding 0.25% trypsin and were adjusted to a concentration of 300 cells/petri dish, which was then loaded with 2 mL of pre-heated culture media before being cultured at 37 °C with 5% CO$_2$ for 2–3 weeks. Colony formation was terminated until the colony was visible to the naked eye. Thereafter, the number of clones was counted and the rate of colony formation was calculated as follows: Colony formation rate = (number of colonies/inoculation cell number) × 100.

**Cell cycle analysis**

The cell cycle was measured by flow cytometer. The cells were collected by trypsin (Auragene) and were washed twice with PBS before fixation in 500 μL of 75% precooled ethanol at 4 °C overnight. Thereafter, the cells were washed by PBS twice, bathed in water with 100 μL of RNase A (Auragene) at 37 °C for 30 min then incubated in the dark with 500 μL of PI (Solarbio, China) at 4 °C for 30 min. Thereafter, the stained cells were analysed in a FACScanto II (BD Biosciences, USA) and the results were recorded at an excitation wave length of 488 nm.

**Luciferase reporter assay**

The possibility of miR-122 and RPPH1 target binding was predicted using an online software miRcode (http://www.mircode.org/). The predicted RPPH1 mRNA binding site region was cloned into a psi-CHECK2 vector. MCF-7 and MDA-MB-231 cells were co-transfected with the vectors and the miR-122 mimics or scramble control. The supernatants were collected 48 h later; luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Fitchburg, WI, USA).

**Tumour formation in nude mice**

To assess the tumour forming potential of the parental and transfected cell lines, the in vitro cells in the log growth phase were trypsinised, washed in a serum-free medium and quantified by coulter counter before being injected into nude mice at a concentration of $2 \times 10^6$ cells. Each mouse was monitored once a week for tumour development. Sites were scored as positive for tumour growth when they reached 5 mm in diameter. Tumour volume was calculated using the formula: $V = 0.2618 \times a \times b \times (a + b)$, where $a$ represented the maximum longitudinal diameter and $b$ represented the maximum transverse diameter. Four weeks later, the mice were sacrificed. The entire animal study was approved by the ethics committee of the Xiangya Hospital of Central South University.

**Statistical analysis**

Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) or SPSS 18.0 software (SPSS, Chicago, IL, USA). Data were shown as mean ± standard deviation and were analysed using unpaired two-tailed Student’s t test or one-way analysis of variance with Bonferroni t post-test, depending on the conditions and group number. A $P$ value of less than 0.05 was considered to indicate statistical significance.

**Results**

**The expression of IncRNA RPPH1 was higher in breast cancer**

To explore the potential role of IncRNA RPPH1 in breast cancer, 20 pairs of cancer-site tissues with adjacent normal tissues were collected from clinical operations. The expression of the IncRNA RPPH1 gene was significantly increased in cancer sites when compared with adjacent sites, as demonstrated by qPCR (Fig. 1a), and in the breast tissue chip, as demonstrated by ISH (Fig. 1b). Moreover, the expression of IncRNA RPPH1 in the cytoplasm and nucleus of cancer tissue cells was higher than that in normal tissues (Fig. 1c). Among the cell lines of MDA-MB-231, HCC-1937, MDA-MB-453 and MCF-7, IncRNA RPPH1 expression was the highest in MCF-7 and the lowest in MDA-MB-231 (Fig. 1d). Therefore, MCF-7 and MDA-MB-231 were chosen for further study.

**LncRNA RPPH1 increased cell proliferation and promoted cell cycle in breast cancer cells**

Cell models of IncRNA RPPH1 overexpression and knockdown, which were named RPPH1 and shRPPH1, respectively, were established by transfecting...
pcDNA3.1-oeRPPH1 and pRNAT-U6.1/Neo-shRPPH1 vectors, respectively. The following results were similar between the cell lines MDA-MB-231 and MCF-7. First, qPCR detected a dramatically higher gene expression of lncRNA RPPH1 in the RPPH1 group compared with the shRPPH1 group (Fig. 2a), indicating successful construction of the lncRNA RPPH1 overexpression and knockdown models. Further evaluation by the MTT assay of lncRNA RPPH1 in the breast cancer cell lines showed that cell proliferation was increased in the overexpressed vector, but suppressed in the knockdown model (Fig. 2b). In both cell lines, the rate of clone formation in RPPH1 was increased when compared with the control group and almost doubled when compared with the shRPPH1 group (Fig. 2c). Lastly, flow cytometer assay showed that the S+G2 phase of the cell cycle decreased in the RPPH1 group, but increased in the shRPPH1 group (Fig. 2d); indicating that RPPH1 regulates the cell cycle mainly in the G1 phase.

RPPH1 inhibited tumour formation
Further evaluation of the effect of RPPH1 in solid tumour formation in nude mice showed that lentivirus-mediated interference of shRPPH1 lncRNA resulted to obvious changes in tumour size in vivo (Fig. 3a). Statistical evaluation of tumour volume showed that the interactions of RPPH1 can decrease the size of breast cancer in nude mice (Fig. 3b).

miR-122 is a target gene of lncRNA RPPH1
Prediction of the possible target binding gene site of lncRNA RPPH1 using the online software microRNA.org showed that RPPH1 had a target binding capacity with miR-122, which has complementary pairing of eight bases (Fig. 4c). As shown in Fig. 4a, qPCR to detect the expression level of target microRNA in the 20 cases of breast cancer and the corresponding adjacent tissues showed that the expression of miR-122 was higher in cancer tissues than in the adjacent tissues. Correlation analysis revealed a high value of $R^2 = 0.8431$, indicating that miR-122 may possibly be the target binding gene of RPPH1 in breast cancer. The level of miR-122 expression had an almost opposite trend with the level of RPPH1 expression in the four kinds of breast cancer cell lines, as well as in the RPPH1 overexpression and knockdown models (Figs. 1a, 4b). Then, we used dual-luciferase
experiment to verify the targeting effect relationship. The luciferase activity significantly decreased in the co-transfected RPPH1-psi-CHECK2 plasmid and in the miR-122 mimics in MDA-MB-231 and MCF-7 cell samples, compared with the other groups. These results indicated that lncRNA RPPH1 and miR-122 in MDA-MB-231 and MCF-7 cells had a targeting effect relationship (Fig. 4c).

**RPPH1 regulated breast cancer via miR-122**

Because the target gene of RPPH1 was discovered to be miR-122, we added miR-122 mimics into the RPPH1 overexpressed model to detect the biological functions of MCF-7 and MDA-MB-231 cells. MTT assay showed that the proliferation of both cells was suppressed in the presence of the miR-122 mimics (Fig. 5a). In addition, the
miR-122 mimics reversed the cell cycle promoting ability of the RPPH1 plasmid (Fig. 5b). Clone formation assay showed that the miR-122 mimics inhibited the capacity for clone formation in the RPPH1 cell group (Fig. 5c).

MiR-122 regulated several genes in breast cancer cells
MiR-122, which has been reported to have a role in cancers, has diverse target genes; we chose seven of these to explore the downstream genes of miR-122 when RPPH1 was overexpressed in breast cancer cells. As illustrated in Fig. 6a, the miR-122 target genes ADAM10, PKM2, NOD2 and IGF1R were highly expressed in the cells of the RPPH1 overexpression model, whereas the expression of Bcl-w, VEGFC and NDRG3 had no significant changes. Testing of the RNA expression levels of the four upregulated genes in the tumour xenografts showed that decreased expression of the ADAM10 and IGF1R genes in the RPPH1 knockdown group (Fig. 6b).

Discussion
In this study, we first found that lncRNA RPPH1 was significantly upregulated in breast cancer tissues compared with adjacent normal tissues; these findings corresponded with the result of microarray ISH. In addition, qPCR detected high expression of RPPH1 in four kinds of breast cancer cell lines. Based on these results, we assumed that RPPH1 plays an important role in the development of breast cancer and its high expression may help promote tumour deterioration; therefore, RPPH1 may be a promising prognostic biomarker for breast cancer.

To further explore the functions of RPPH1 in breast cancer, we speculated that RPPH1 played a significant role in tumour biology. First, RPPH1 expression in chosen representative breast cancer cell lines was investigated and compared with that in non-tumour breast cell lines. In agreement with our findings in breast cancer tissues, we found that the MCF-7 and MDA-MB-231 cell lines exhibited high RPPH1 expression, compared with the HBL-100 cell line. We then determined whether RPPH1 expression influenced the proliferation of breast cancer cells. Indeed, overexpression of RPPH1 enhanced cell proliferation and increased clone formation, whereas the knockdown of RPPH1 significantly inhibited cell proliferation in both MCF-7 and MDA-MB-231 cell lines. Moreover, we demonstrated that the mechanism may be associated with arrest of the G1 phase of the cell cycle, which indicated that RPPH1 has an ability to regulate the cell cycle. These results revealed that RPPH1 may affect breast cancer progression by affecting cell proliferation and the cell cycle.

RPPH1 is a well-known RNA subunit of RNase P, which is responsible for tRNA maturation in all three domains of life [17]. A previous study demonstrated that one of our tested ceRNA pathways, RPPH1/miR-330-5p/CDC42, may be involved in the compensatory behaviour of brain neurons to combat synaptic loss during AD pathogenesis [18]. However, the downstream pathway in breast cancer is currently not known. To explore the molecular mechanism through which RPPH1 contributes to cell proliferation and causes apoptosis in breast cancer cells, we investigated the potential target genes involved in cell proliferation and the cell cycle through microarray analysis. qPCR analysis demonstrated that the miR-122 mRNA levels were decreased in breast cancer cell lines and in cancer-site tissues, compared with the adjacent tissues. Moreover, the expression of miR-122 increased after RPPH1 knockdown and decreased after RPPH1 overexpression. Indeed, the dual-luciferase assay demonstrated that RPPH1 and miR-122 had a relationship of targeted binding. Therefore, we identified miR-122 as a target gene of RPPH1.

Further study by addition of miR-122 mimics into the RPPH1 overexpression model showed that the biological function of RPPH1 in breast cancer was related to the
regulation of miR-122 and that the cell biological functions, including proliferation, cell cycle and clone formation were changed to some extent. In addition, we confirmed that RPPH1 and miR-122 interacted with each other.

MiR-122, which plays a role in tumour suppression in various cancers, has been investigated extensively. In this study, we focused on the potential effectiveness of miR-122 in breast cancer. Ergun et al. [19] suggested that miR-122-5p was a potential regulator of ADAM10 and trastuzumab resistance. Sercan Biyun Wang et al. [20] found that miR-122 took the crucial role of a tumour suppressor by targeting IGF1R and regulating the PI3K/Akt/mTOR/p70S6K pathway in breast cancer. Another study demonstrated that miR-122 was strongly correlated with the clinical outcomes of breast cancer, including response to neoadjuvant chemotherapy and relapse with metastatic disease. In particular, higher circulating miR-122 levels estimated the occurrence of metastasis in stage II and III breast cancer cases [21]. Based on the results of previous studies, we chose the miR-122 target genes ADAM10 [19], Bcl-w [22], VEGFC [23], PKM2 [24], NOD2 [25], IGF1R [20] and NDRG3 [26] to explore the downstream genes in breast cancer. Based on the qPCR results in the RPPH1 function cell models, we may infer that RPPH1 promoted breast cancer cell proliferation and cell cycle progression through down-regulation of microRNA 122 and influence the expression of ADAM10, PKM2, NOD2 and IGF1R genes. The next step should be selecting one of the significant genes to clarify the specific pathway that interacts with RPPH1. Finally, investigation of tumour formation in nude mice in this study showed that lentivirus-mediated RPPH1 IncRNA interference can reduce the size of solid tumours.

**Fig. 4** miR-122 is a target gene of lncRNA RPPH1. a In the 20 paired clinical samples, miR-122 expression in cancer tissues is lower than that in adjacent tissues and the relativity expression rate between miR-122 and RPPH1. b In the breast cancer cell lines and models of RPPH1 overexpression and knockdown, the expression of miR-122 is opposite to that of RPPH1. c The binding area and results of expression in the dual-luciferase assay, n = 3, **P < 0.01 vs. control.
Conclusion

Overall, our results demonstrated that \textit{RPPH1} functions as a tumour promoter and plays an important role in advancing tumorigenesis by targeting miR-122 and may serve as a novel and potential therapeutic, diagnostic or prognostic target in breast cancer.

Abbreviations

- lncRNA: long non-coding RNA
- RPPH1: ribonuclease P RNA component H1
- qPCR: RNA extraction and quantitative real-time polymerase chain reaction
- ISH: in-situ hybridisation assay
- PBS: phosphate-buffered saline
- NCT: neoadjuvant chemotherapy

Authors' contributions

Both authors participated in the research design, data analysis and manuscript drafting and revision. Both authors read and approved the final manuscript.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.
Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Consent for publication
Not applicable.

Ethics committee approval and consent to participate
This study was approved by the ethics committee of Xiangya Hospital of Central South University.

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