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LRP4 promotes proliferation, migration, and invasion in papillary thyroid cancer

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ABSTRACT

Dysregulation of cell proliferation and death is considered the foundation of the malignant biological characteristics of cancer. In this study, we conducted a comprehensive analysis of a massively parallel whole transcriptome resequencing of paired papillary thyroid cancer and normal thyroid tissues from 19 patients. In addition, we found that *LRP4*, a member of the low-density lipoprotein receptor-related protein family, is significantly overexpressed in thyroid carcinoma. We demonstrated through quantitative real-time polymerase chain reaction (qRT-PCR) that *LRP4* is upregulated in papillary thyroid cancer (PTC) tissues. This observation was also consistent with data analyzed from The Cancer Genome Atlas (TCGA) cohort. Thus, the biological role of *LRP4* in the thyroid cancer in the present study was investigated using the PTC cell lines TPC1, BCPAP and KTC-1. In these cell lines, the mRNA level of *LRP4* was higher than normal thyroid cancer cell named HTORI3. In vitro experiments demonstrated that *LRP4* downregulation significantly inhibited the colony formation, proliferation, migration, and invasion of the three PTC cell lines. Knockdown of *LRP4* by small interfering RNA (siRNA) in those cell lines decreased the protein expression of N-cadherin, Enhancer of zeste homolog 2 (EZH2), and Zinc finger E-box-binding home-box 1 (ZEB1). Furthermore, *LRP4* knockdown significantly reduced the levels of phosphorylated PI3K in the PTC cell lines. In conclusion, the present study indicated that *LRP4* is a gene associated with PTC and might become a potential therapeutic target.

1. Introduction

Thyroid cancer is one of the most predominant endocrine malignancies with an increasing prevalence of morbidity. The U. S. National Cancer Institute estimated that the number of new thyroid cancers in 2017 was 56,870, with an estimated 2010 deaths [1]. When referring to the incidence of thyroid cancer in China, the annual estimated number is 90,000, while the number of estimated deaths is 6800 [2]. The majority of the thyroid cancers included papillary (PTC) and follicular thyroid carcinoma (FTC), with PTC comprising 85–95% of all thyroid cancers [3]. The prognosis of PTC is often favorable, and its 5-year survival are as high as 90% [4]. Nevertheless, approximately 20% of PTC patients had lymph node metastasis, and the number of those who underwent total thyroidectomy and experienced regional recurrence is approximately 10% [5]. There are certain clinicopathologic factors, such as age, extrathyroidal spread (ETS), lymph nodes metastasis and distant metastasis, that contribute to poor prognoses [6]. Therefore, early diagnosis and treatment are necessary to improve the patients' quality of life. Currently, fine-needle aspiration cytology with cytological evaluation remains the gold standard, but this procedure fails to discriminate benign and malignant cancer in up to one-third of cases. Additionally, molecular testing of thyroid nodules has evolved rapidly over the past decade and not only helps improve the accuracy of thyroid cytology for indeterminate cases but also potentially guides the extent of surgery as initial therapy for suspected thyroid malignancies [7,8]. Considerable studies have been made in understanding the molecular mechanisms of PTC over the past two decades. Among those studies, B-type Raf kinase (BRAF) V600E mutations could promote PTC tumorigenesis and progression by aberrantly activating the mitogen-activated pathway kinase(MAPK) pathway [9]. In addition, the BRAF V600E

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mutation in PTC has been widely used as a biological marker to diagnose and predict the prognosis of PTC due to its specificity. Furthermore, TERT, RAS and PI3K gene mutations also play a critical role in thyroid cancer progression [10,11]. Although some studies have made some progress regarding thyroid cancer diagnosis, the mechanism of thyroid cancer progression is complex and much less understood. *LRP4*, a member of the low-density lipoprotein receptor-related protein family, plays a significant role in the ischemic brain injury response, and its deficiency seems to be a protective factor in response to ischemic brain injury via the release of ATP and adenosine as well as $A_{2A}R$ signaling [12]. Remarkably, in the progression of neuromuscular junctions, *LRP4* can be a key factor in connection with neuromuscular disorders such as myasthenia gravis, Lambert-Eaton myasthenic syndrome, Isaacs' syndrome, congenital myasthenic syndromes, Fukuyama-type congenital muscular dystrophy, amyotrophic lateral sclerosis, and sarcopenia [13,14]. Furthermore, *LRP4* could also regulate synapse formation during CNS development [15]. Several human cancers are also associated with *LRP4* gene, for example, hepatocellular cancer (HCC) [16], neuroendocrine tumors [17]. In HCC, Agrin binds to *LRP4* to form a critical oncogenic axis [16]. Several findings in PTC have been revealed through sequencing technology and a common database. Hucz J et al. conducted microarray studies in differentiated papillary carcinoma and normal tissues and found that *LRP4* is significantly overexpressed in PTC tissue compared with normal thyroid tissues [18]. The same conclusion was confirmed in Jarzab B and Hucz J's work [18,19].

In our study, 19 paired PTC tissue samples and adjacent noncancerous samples were subjected to whole transcriptome sequencing and bioinformatics analysis to assess the mRNA expression profiles. We showed that *LRP4* is one of the most significantly upregulated genes, and 43 pairs of tumor samples with matched adjacent noncancerous tissues were subjected to qRT-PCR to validate the abnormal expression of *LRP4*. Cell culture experiments and western blot were performed to demonstrate the role of *LRP4* in PTC. Our study was designed to explore the role of *LRP4* in the proliferation and metastasis of thyroid carcinoma and to analyze the relationship of *LRP4* expression with the clinical and molecular features of PTC.

In a conclusion, we demonstrated that *LRP4* is a gene associated with PTC and might become a potential therapeutic target.

2. Materials and methods

2.1. Patients and samples

In all, 43 tissue samples, including primary PTC samples and paired parathyroid tissues, were obtained from patients who underwent surgical treatment at the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. In addition, none of the included patients underwent preoperative treatments, such as chemotherapy or radiotherapy. The samples were snap-frozen in liquid nitrogen immediately after resection and then stored at −80 °C before RNA extraction. The cases were retrospectively reviewed to confirm the histological diagnosis and to ensure abundant tumor samples for assessment by two senior pathologists. Informed consent for the scientific research of biological material was obtained from each patient. PTC mRNA expression data were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). All procedures performed in studies involving human participants adhered to the ethical standards of the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA isolated from patient tissue specimens and PTC cell lines was treated with a standard TRIzol protocol (Invitrogen). To assess RNA quality and quantity, the A260/A280 ratio and spectrophotometry values were used, respectively. Then, cDNA was synthesized with a ReverTra Ace qPCR RT Kit, and qPCR was carried out using SYBR Green Real-time PCR Master Mix (TOYOBO, QPK-201-201T) according to the User Guide's protocol. The primer sequences were used as followed; LRP4: forward primer 5′-GCCGCCAAGTCATTATCT-3′; reverse primer: 5′-TCAG-CACCTTCCTCTTACT-3′.GAPDH: forward primer 5′-GGTCGGAGT-CAACGGATTTG-3′; and reverse primer:5′-ATGAGCCCCAGCCTTCTCCAT-3′.

2.3. Cell culture

Two PTC cell lines (TPC1 and BCPAP) were provided by Professor Mingzhao Xing of the Johns Hopkins University School of Medicine, Baltimore, MA, USA. In addition, KTC-1 cells were purchased from Stem Cell Bank, Chinese Academy of Sciences. TPC1 and BCPAP cells were cultured in RPMI 1640 (Gibco C11875500BT) supplemented with 10% fetal bovine serum (FBS) (Gibco, 10099141), 1 × MEM nonessential amino acids (Gibco 11140-050) and 1 × sodium pyruvate (Gibco 11360-070). KTC-1 cells were cultured in the same medium described above except for the additional inclusion of Glutamax™ −I (Gibco, 35050-061). All cells were grown in the appropriate conditioned medium and maintained at 37 °C in an atmosphere of 5% CO_2/95% air. Cells were plated into six-well plates at 4×10^5 cells/well and incubated for 24 h in their respective growth medium.

2.4. Cell transfection

For cell interference, *LRP4* siRNA was synthesized at Shanghai GenePharma Company Limited. The sequences of the *LRP4* and negative control siRNA are as follows: *LRP4* (Sense: GCGAU-GAGGAUGGAUGUAUTT; Antisense: AUACAUCCAUCCUCAUCGCTT). Briefly, cells were seeded into 6-well plates. After 24 h, siRNA was transfected into cells using Lipo iMAX (Invitrogen, Grand Island, NY) according to the manufacturer's protocol, with a final siRNA concentration of 100 nM (TPC-1), 50 nM (BCPAP) or 75 nM (KTC-1). Cells were harvested 48 h after transfection for subsequent analysis of RNA expression. All knockdown experiments were performed in triplicate.

2.5. Cell proliferation assay

Cell proliferation was evaluated using the cell counting kit-8 (CCK-8, Beyotime, China) assay according to the manufacturer's protocol. Briefly, 1000 cells/well for TPC-1 and KTC-1 cells and 2000 cells/well for BCPAP cells were plated into a 96-well plate. Next, 10 μl CCK-8 solution was added to each well. After 4 h of incubation at 37 °C in the presence of 5% CO_2, the incubation was halted, after which the absorbance at 450 nm was measured using a spectrophotometer at 24 h, 48 h, 72 h, and 96 h after initial plating. For each group, data from five wells were pooled. All assays were performed in triplicate.

2.6. Colony formation assay

For the colony forming assay, the three si-*LRP4* treated cell lines or si-NC treated cell lines (2×10^3 cells for TPC1 and KTC-1, 4×10^3 cells for BCPAP) were seeded into 6-well plates, incubated for 8–14 days and then fixated with 4% PFA (paraformaldehyde;

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