

Circular RNA circKIF4A facilitates the malignant progression and suppresses ferroptosis by sponging miR-1231 and upregulating GPX4 in papillary thyroid cancer

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ABSTRACT

Circular RNAs (circRNAs) are one type of non-coding RNA. They act as important role in regulating various biological processes in the malignant progression. But we don't clearly know the specific mechanism of the majority circRNAs in papillary thyroid tumor progression. In the current study, we explored circKIF4A and the result showed that it had high expression in papillary thyroid cancer. The functions of circKIF4A were explored by CCK-8, transwell, and mouse xenograft experiments. Knockdown of circKIF4A could suppress papillary thyroid cell growth and migration. In addition, RIP assays and dual luciferase vector reporter assays were further conducted. Our consequence showed circKIF4A facilitated the malignant progress of papillary thyroid tumor by sponging miR-1231 and upregulating GPX4 expression. In conclusion, our study proved that circKIF4A-miR-1231-GPX4 axis played a vital role in cancer proliferation and ferroptosis by competing endogenous RNAs. Therefore, targeting circKIF4A is very likely to be a potential method for treatment of papillary thyroid cancer in the future.

INTRODUCTION

Based on the cancer statistics in the world, thyroid cancer is the fifth most common malignancy. It was predicted that there would be 52,070 cases of thyroid cancer in 2020, with 37,810 cases in female and 14,260 cases in male [1]. There are various subtypes in thyroid cancer, and 85%-90% of them are papillary thyroid cancer [2]. Approximately 90% of patients can be cured with standard treatment [3]. However, locoregional recurrences or distant metastases occur in almost 10% of thyroid carcinoma cases, which is still a challenge in the treatment of thyroid cancer [4]. Once recurrence and metastasis occur, the prognosis and the life quality of these patients will become worse. Consequently, it is

extremely urgent to illustrate the specific mechanism of the carcinogenesis and invasion of papillary thyroid cancer.

Circular RNAs (circRNAs) are formed by covalently closed loops, which are regarded as novel endogenous noncoding RNAs [5]. They originate in the back-splice of pre-mRNAs (precursor mRNAs) without a head or a tail which are abundant in mammalian tissues [6]. Compared with linear counterparts, circRNAs show high stability *in vivo* because of their unique circular structure [7]. As important mediators of different biological process in the cell, circRNAs regulate the expression of vital genes via multiple comprehensive molecular mechanisms, including binding microRNAs (miRNAs), interacting

with proteins and encoding novel polypeptides [8]. Plenty of circRNAs has been discovered as regulators of a diversity of diseases, including Alzheimer's disease, heart failure, diabetes, and cancers [9–13]. Masses of circRNAs has been characterized and validated to be mediators of the malignant progress with the development made in high-throughput circRNA sequencing [14, 15]. For instance, the most thoroughly studied circRNA ciRS-7 promotes the growth, invasion, drug resistance, and immune escape of various tumors by sponging miR-7 [16–21]. CircFBXW7 is low-expressed in tumor tissues. By translating a 21kDa novel protein (FBXW7-185aa) and sponging miRNA, circFBXW7 can suppress cell multiplication and invasion in glioma and breast cancer [22, 23]. Additionally, circKIF4A and circRAD18 can compete endogenous RNAs to regulate triple-negative breast cancer [24, 25]. CircHIPK3 promotes the development of colorectal cancer via upregulating a series of robust oncogenes (FAK, IGF1R, EGFR, YY1) [26]. Nevertheless, we still wonder whether circRNAs are crucial in papillary thyroid cancer.

In the current study, we explored how circKIF4A affected papillary thyroid cancer. We found circKIF4A was high-expressed in papillary thyroid tumor, while suppression of circKIF4A led to the low growth and migration. Generally, we confirmed that the circKIF4A-miR-1231-GPX4 axis was associated with the malignant progress of papillary thyroid cancer.

RESULTS

circKIF4A is upregulated in papillary thyroid cancer with circular characteristics

First, we used RT-qPCR to analyze whether circKIF4A has different expression in 30 pairs of papillary thyroid cancer tissues and the nearby normal thyroid tissues. circKIF4A was found upregulated in papillary thyroid cancer (Figure 1A). Besides, compared to Nthy-ori3-1 (normal thyroid cell), especially in TPC-1 and KAT-5 cell lines, we found circKIF4A was upregulated (Figure 1B). Then, the circular structure and stability of circKIF4A was further examined by specific assays. In RNase R digestion experiment, circKIF4A was resisted to RNA exonuclease while linear KIF4A mRNA was digested after incubated with RNase R in TPC-1 cell (Figure 1C). In consistent, in actinomycin D assays, we found the circular form of circKIF4A had longer half-life span than the linear KIF4A mRNA (Figure 1D).

Knockout of circKIF4A attenuates the proliferation of papillary thyroid cancer cells

We used loss-of-function assays to figure out the function of circKIF4A. An siRNA was designed to silence

circKIF4A by targeting the back-splicing junction region of circRNA. Validating by RT-qPCR analysis, circKIF4A had decreased after siRNA transfection, which showed the efficacy of the knockdown assay (Figure 2A). We found downregulation of circKIF4A attenuated proliferation in CCK-8 assays (Figure 2B). Similarly, circKIF4A silencing suppressed cell colony forming ability, according to the colony formation assays (Figure 2C). Besides, we established mouse xenograft models to explore whether circKIF4A plays a role *in vivo*. We measured the tumor volumes at each time point, and the result showed that depletion of circKIF4A could inhibit tumor growth (Figure 2D). Reduction of the glutathione (GSH)/oxidized glutathione (GSSG) ratio was observed after knockdown of circKIF4A (Figure 2E).

Downregulation of circKIF4A inhibits the metastasis of papillary thyroid cancer cells

We next performed assays to investigate if the metastasis of papillary thyroid cancer is influenced by circKIF4A. The result showed that cells had low activity to migrate by silencing the expression of circKIF4A (Figure 3A). In consistent with this result, suppression of circKIF4A could also inhibit the metastasis in lung metastasis experiment *in vivo*, indicating that circKIF4A is vital in the metastasis of thyroid cancer (Figure 3B).

circKIF4A acts as a sponge of miR-1231 in papillary thyroid cancer

Next, we determined the subcellular location of circKIF4A by isolating of the cytoplasmic and nuclear portions of cellular RNA. The result showed that circKIF4A predominantly existed in the cytoplasm, which might indicate that it interacted with miRNA in the cytoplasm (Figure 4A). We furtherly used Circular RNA Interactome to predict whether there is a potential interaction between circRNA and miRNAs. Among the candidates, we found miR-1231 might have the ability to bind circKIF4A sequence (Figure 4B). By RT-qPCR analysis, we found there was a decreasing tendency of miR-1231 in papillary thyroid cancer cell lines (Figure 4C). The transfection of WT reporter and miR-1231 caused the deactivation of relative luciferase, according to dual luciferase reporter assays (Figure 4D). Then, we conducted RIP assays to prove the direct relationship between circKIF4A and miR-1231. We also noticed that miR-1231 was mainly enriched in the MS2bs-circKIF4A group (Figure 4E). Overexpression of miR-1231 could increase the GSH level which could be reversed by silencing circKIF4A in TPC-1 and KAT-5 papillary thyroid cancer cells (Figure 4F).

CircKIF4A promotes papillary thyroid cancer progression through circKIF4A-miR-1231-GPX4 axis

We used TargetScan to further find out the potential targets of miR-1231. We found GPX4 was a downstream target oncogene among the candidates (Figure 5A). GPX4 encodes a protein and protects cells against oxidative damage which plays an important role in multiple cancers, certainly including papillary thyroid

cancer [27–31]. By RT-qPCR analysis, we found GPX4 overexpressed in papillary thyroid cancer cell lines, detected (Figure 5B). The next step was to explore whether miR-1231 could bind the 3'-UTR of GPX4 mRNA directly. We found that the relative luciferase activity of cells was significantly decreased after transfecting miR-1231 and WT 3'-UTR-GPX4 vector. However, this phenomenon was not observed when mutant vector was transfected (Figure 5C).

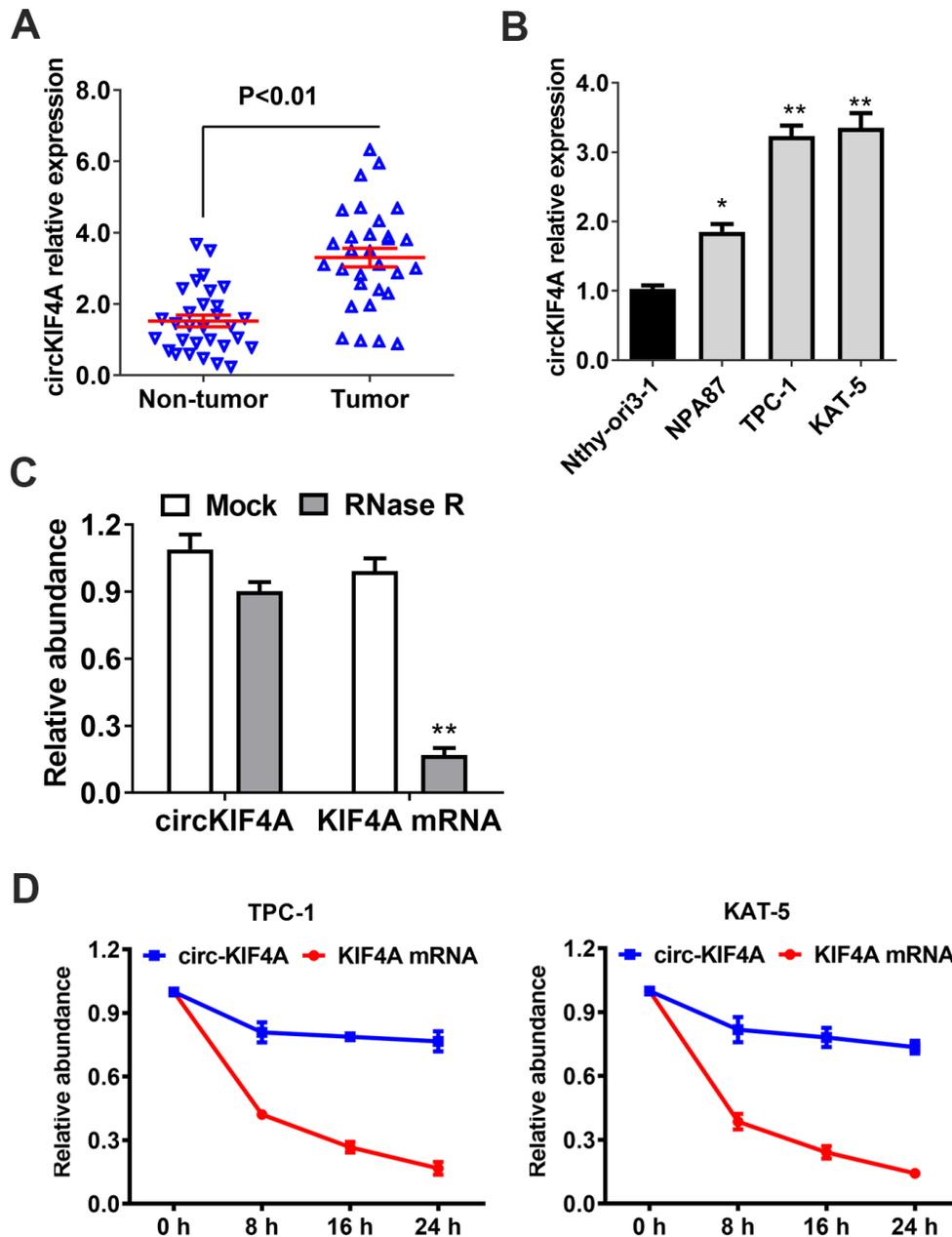


Figure 1. circKIF4A is upregulated in papillary thyroid cancer with circular characteristics. (A) circKIF4A expression in adjacent normal tissues and papillary thyroid cancer. (B) The relative expression of circKIF4A in cell lines. (C) RNase R assay examined the circular structure of circKIF4A in TPC-1 cell line. (D) Circular transcripts of KIF4A (circKIF4A) was more stable than its linear mRNA transcripts determined by actinomycin D treated assay.

Additionally, miR-1231 mimics significantly reduced the expression level of GPX4 mRNA, which indicates that GPX4 is downregulated by miR-1231 (Figure 5D). Moreover, AGO2 related RIP assays were carried out. We found that circKIF4A, GPX4 and miR-1231 gathered to AGO2 RNA binding protein (Figure 5E). Suppression of circKIF4A could significantly increase GPX4 enrichment to RNA

induced silencing complex (Figure 5E). Knockdown of circKIF4A could reduce the protein expression of GPX4, while inhibition of miR-1231 could lead to the reversion of the protein expression of GPX4, analyzed by western blot assay (Figure 5F). Knockdown of circKIF4A significantly decreased the expression level of GPX4 detected by western-blot analysis (Figure 5G).

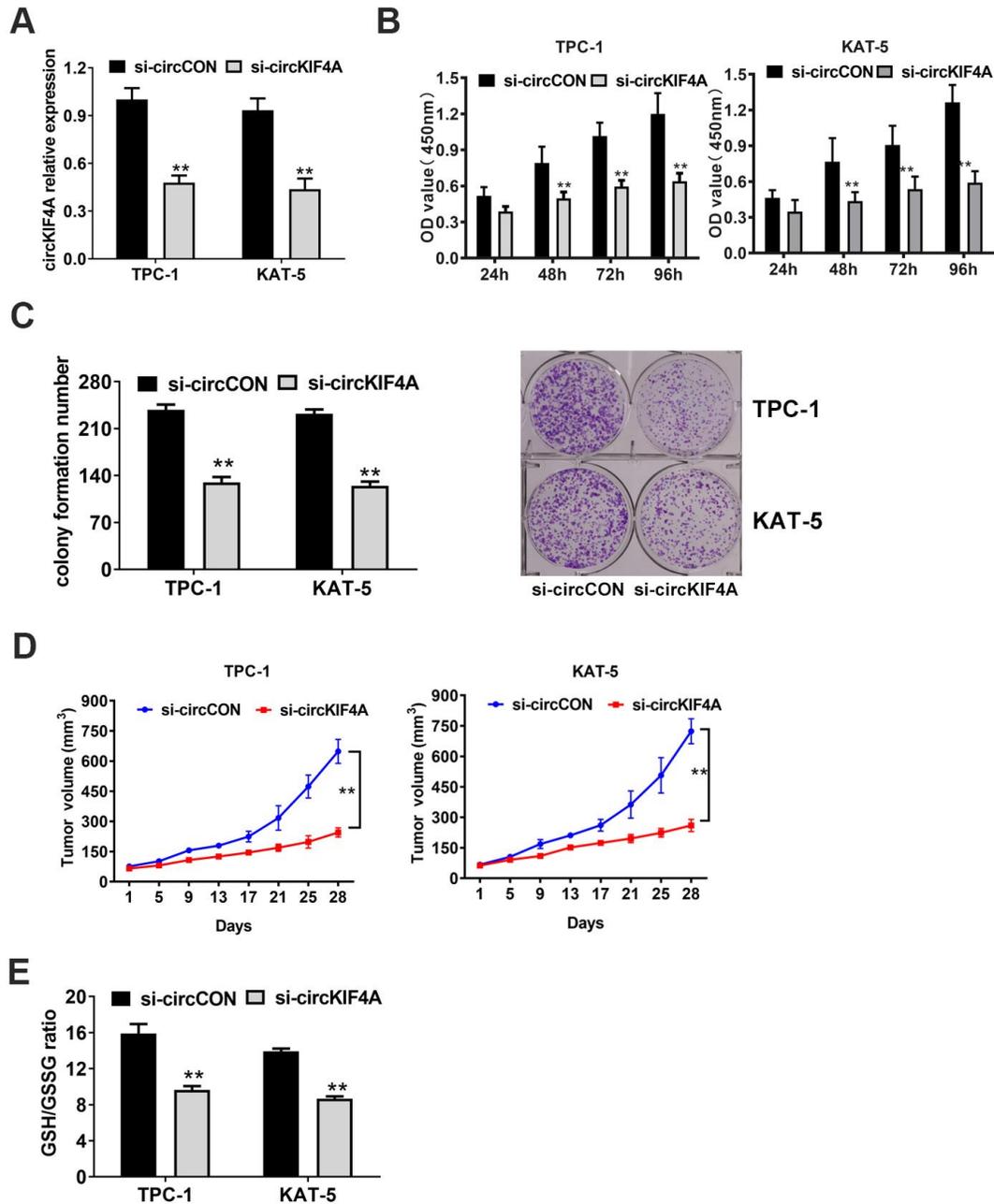


Figure 2. Knockdown of circKIF4A attenuates the proliferation of papillary thyroid cancer cells. (A) Knockdown effect of circKIF4A was assessed in TPC-1 and KAT-5 cell line. (B) CCK-8 assays evaluated cell proliferation after knockdown of circKIF4A. (C) Colony formation assays revealed that circKIF4A silencing suppressed cell colony formatting ability. (D) Mouse xenograft models were established. Tumor volume was estimated in every four days. (E) GSH/GSSG ratio was detected.

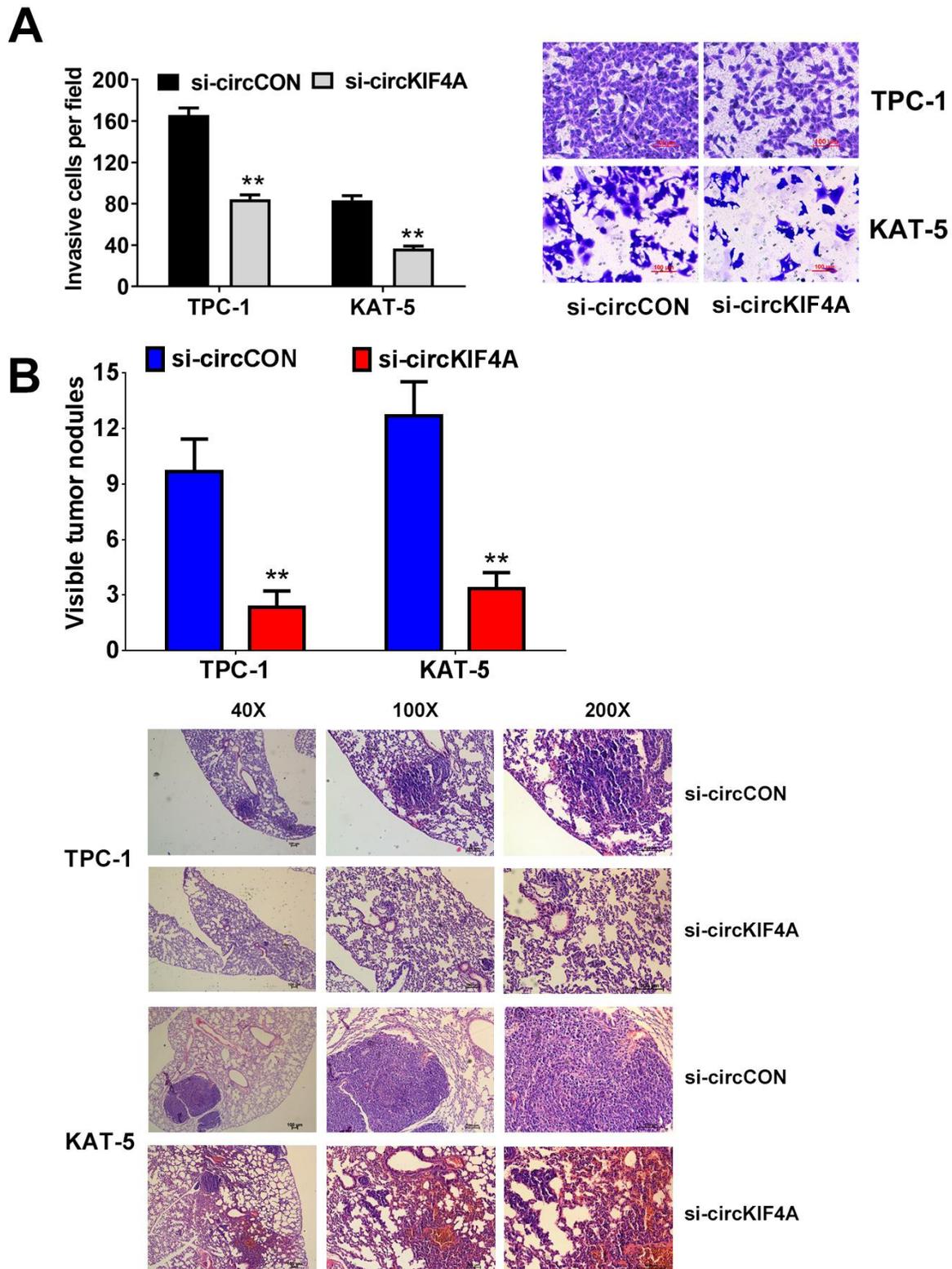


Figure 3. Downregulation of circKIF4A suppresses the metastasis of papillary thyroid cancer cells. (A) Transwell experiments were conducted in TPC-1 and KAT-5 cell line. (B) The number of lung metastases was counted and recorded. HE-stained sections of lung metastases were presented.

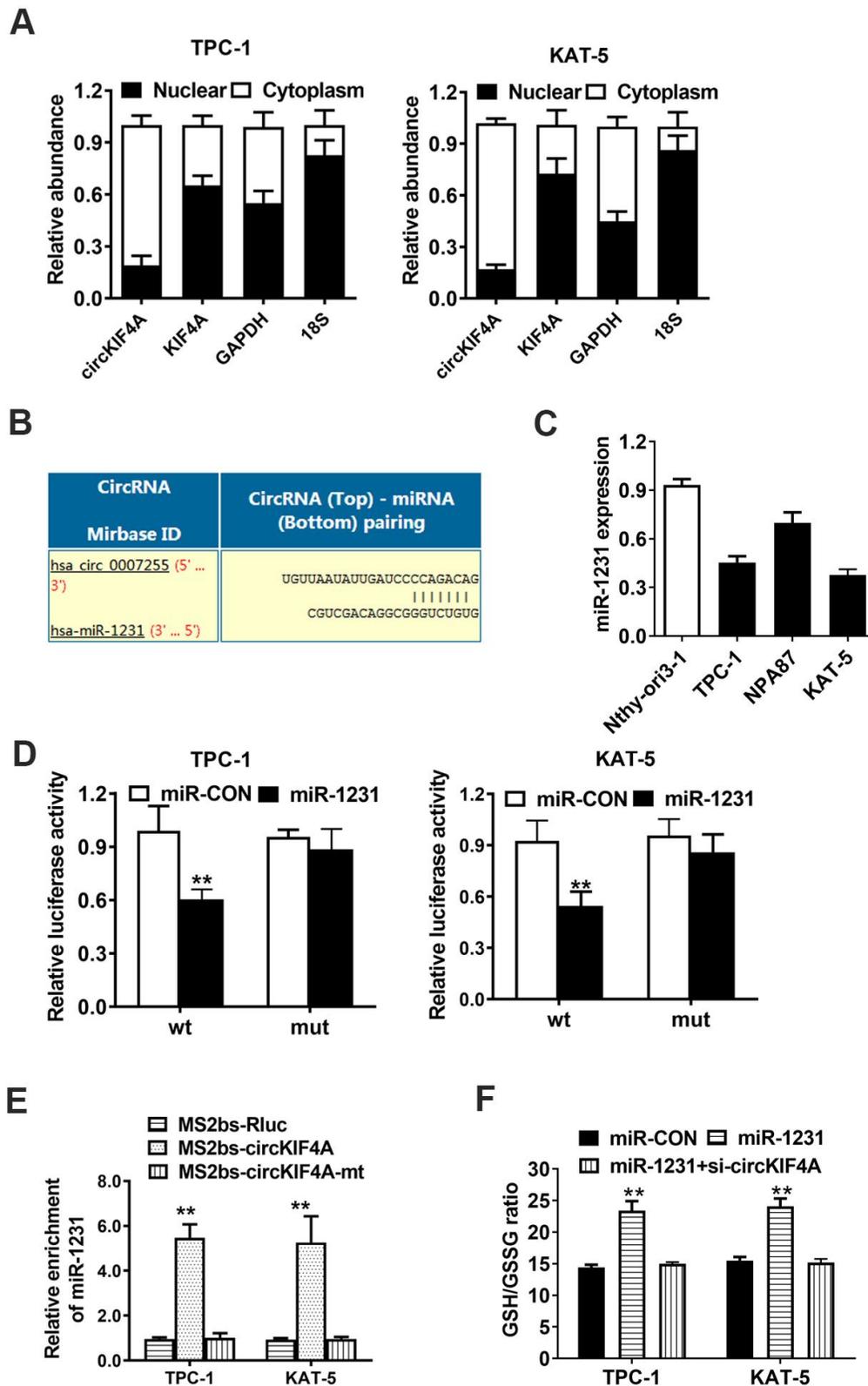


Figure 4. circKIF4A acts as a sponge of miR-1231 in papillary thyroid cancer. (A) 18S, GAPDH, circKIF4A and KIF4A in nuclear and cytoplasmic part analyzed by RT-qPCR. (B) Predicted interaction site of miR-1231 within the circKIF4A sequence. (C) miR-1231 expression in papillary thyroid cancer cell lines. (D) Luciferase reporter assay of TPC-1 and KAT-5 cells transfected with miR-1231 mimics and circKIF4A. (E) MS2-based RIP assay transfected with MS2bs-circKIF4A, MS2bs-circKIF4A-mt or Rluc control. (F) GSH/GSSG ratio was detected. GSH/GSSG ratio was increased after overexpression of miR-1231 which could be reversed by silencing circKIF4A.

DISCUSSION

CircRNAs have become popular topics in RNA biological field and have attracted the eyes of many scientists. The high-throughput sequencing technology and bioinformatics analysis enabled scientists to discover and investigate various kinds of circRNAs [14, 15].

Researchers have come to realized that these special non-coding RNAs with circular structures are integral parts in multiple biological processes rather than worthless product of pre-mRNA splicing [32]. circPRKCI is upregulated in lung adenocarcinoma which promotes tumorigenesis by sponging miR-589 and miR-545 [33]. By translating a novel 370-aa β -catenin

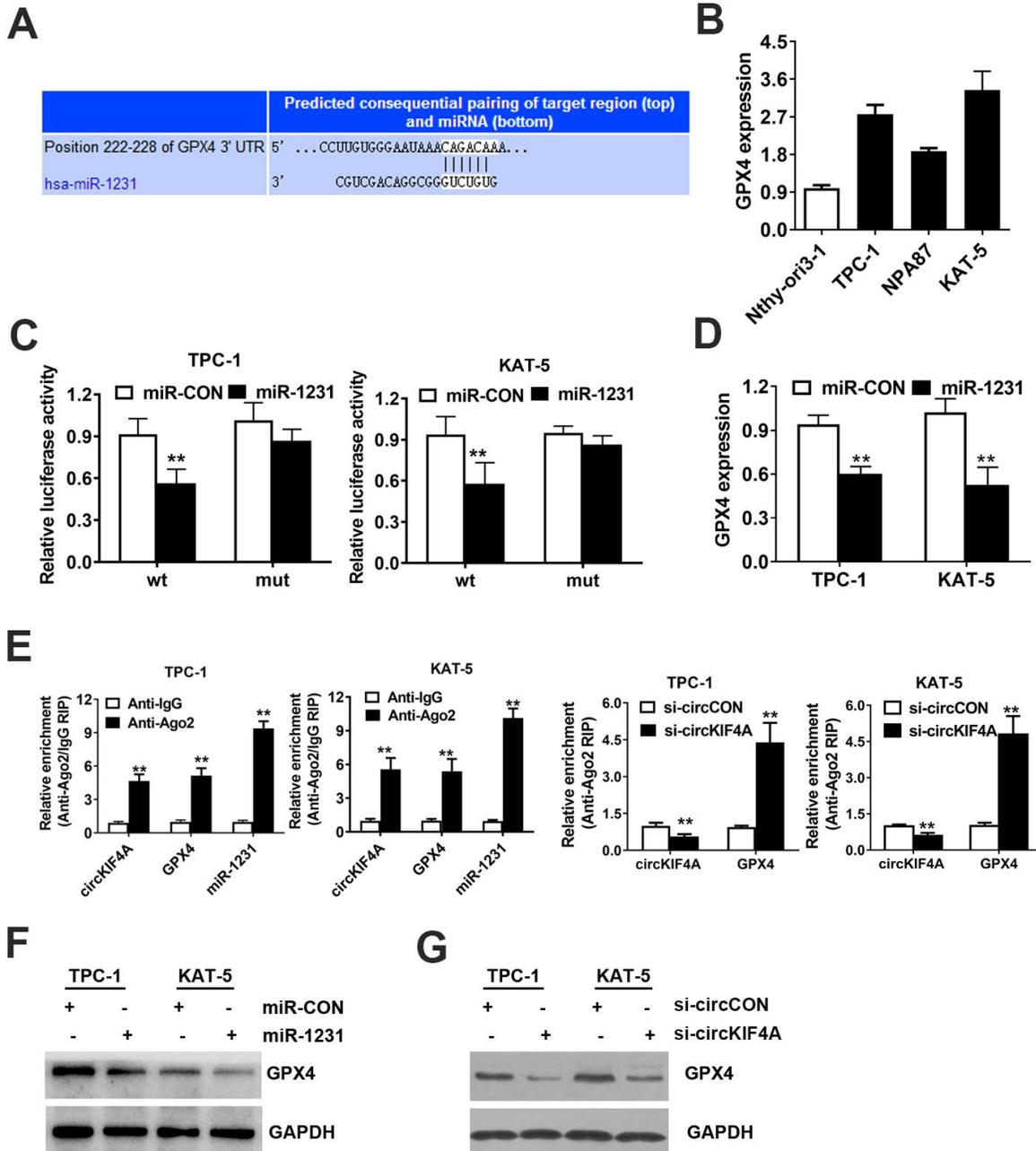


Figure 5. CircKIF4A promotes papillary thyroid cancer progression through circKIF4A-miR-1231-GPX4 axis. (A) Predicted interacting site of miR-1231 within the 3'-UTR of GPX4. **(B)** GPX4 expression in papillary thyroid cell lines. **(C)** Dual luciferase reporter assay of TPC-1 and KAT-5 cells. **(D)** Expression of GPX4 was reduced after overexpression of miR-1231. **(E)** Enrichment of circKIF4A, GPX4 and miR-1231 on AGO2 RNA binding protein assessed by RIP assay. **(F)** Overexpression of miR-1231 resulted in the reduction of GPX4 protein expression. **(G)** Knockdown of circKIF4A reduced the protein expression of GPX4.

isoform, a circRNA derived from CTNNB1 pre-mRNA promotes the proliferation hepatocellular carcinoma cell with the mechanism of activating the Wnt signaling pathway [34]. circPLK1 were identified as oncogenic drivers by reducing apoptosis in breast cancer [35]. However, there are only few studies investigating the roles and functions of circRNAs in papillary thyroid cancer. For example, circ_0006156 had higher expression in serum exosomes, and through the miR-1178/TLR4 axis, it could accelerate papillary thyroid cancer to bad prognosis [36]. Through the FGFR1 pathway, circRAPGEF5 modulates progression of thyroid cancer [37].

In this study, the result showed circKIF4A was a frequently upregulated novel circRNA. Besides, knockout of circKIF4A could significantly inhibit the development of cancer *in vitro* and *in vivo*. All the results showed that circKIF4A was able to directly sponge miR-1231 and promote papillary thyroid cancer progression by upregulating antioxidant protein GPX4 expression.

According to the reported studies, miR-1231 had suppressive effect in many cancers. For instance, miR-1231 is secreted by stem cells, it can prevent pancreatic cancer cells from growing and metastasizing [38]. By targeting EGFR expression, miR-1231 is downregulated in prostate cancer [39]. Additionally, miR-1231 can also lower the risk of papillary thyroid cancer cell [40]. As a target downstream of miR-1231, GPX4 protects cells against oxidative damage which is harmful to multiple cancers, certainly including papillary thyroid cancer. The GSH (glutathione)-GPX4 (glutathione peroxidase 4) system is considered to be a main cell protection system that suppresses ferroptosis [41, 42]. We confirmed GPX4 was the target of miR-1231 in papillary thyroid cancer. By competing endogenous RNA, circKIF4A could enhance the expression of GPX4.

In summary, we confirmed that the circKIF4A-miR-1231-GPX4 axis was associated with papillary thyroid cancer. It could compete endogenous RNA and suppress the progression and metastasis. Therefore, we might consider targeting circKIF4A as a novel method for treatment of papillary thyroid cancer.

MATERIALS AND METHODS

Clinical sample data and ethical standards

We collected fresh nearby normal thyroid tissues and primary papillary thyroid cancer samples from Sun Yat-Sen University Cancer Center. This study was approved by the Ethics Committee of the Sun Yat-Sen University Cancer Center. We collected all the written informed consent before doing this study.

Cell culture

The cell lines used in this study were all purchased from the ATCC. Cell lines were cultured according to the supplier's instructions. Cell authenticity was verified occasionally by the method of DNA fingerprinting.

CCK-8 assay

3000 si-circKIF4A and si-circCON cells were put into a 96-well plate. The cells were incubated for each time period in the incubator at 37° C. Afterwards, CCK-8 solution (10ul) was added and incubate for one hour before measurement. Basic information is listed in (Supplementary Table 1).

Western blot analysis

RIPA lysis and PMSF were used to isolate protein from cells. The protein was put into each well of the SDS-PAGE gel and separated. The protein was afterwards moved to the PVDF membranes for 2 hours at 300 mA. The membrane was treated with each antibody at 4° C overnight and then incubated with the specific secondary antibody at room temperature for 1 hour. Anti-GPX4 (1:1000, Abcam, USA) and anti-GAPDH antibody (1:1000, Affinity, USA) are used to detect certain protein.

RNase R digestion assay

After 3 ug extracted total RNA of TPC-1 papillary thyroid cancer cells were treated with RNase R (5 Uug) or control solution for 20 min at 37° C, the resulting RNA solution was purified and quantified by RT-qPCR analysis.

Actinomycin D assay

TPC-1 and KAT-5 cells were exposed with 2ug/ml actinomycin D to degrade the linear mRNA transcription for 0, 8, 16, and 24 hours. The TPC-1 and KAT-5 cells were harvested at certain time period and the linear KIF4A mRNA and circular circKIF4A were quantified by qPCR-analysis.

RT-qPCR analysis

Total RNA was extracted by TRIzol (Invitrogen). qRT-PCR assays were carried out using Takara SYBR PCR kit. Basic information is listed in (Supplementary Tables 2, 3).

Transwell assay

Totally, 5×10^4 cells were resuspended and added to the above chambers (serum-free medium) and medium

(medium with 20% FBS) was loaded to the lower chambers. Methanol was utilized to fix the left cells. The migrated cells were imaged and counted, after staining with crystal violet (1%).

Luciferase reporter assay

TPC-1 and KAT-5 cells were seeded 1×10^4 cells in each well in 96-well plates. circKIF4A and GPX4 3'-UTR was cloned into a CMV promoter-driven luciferase in a pCDNA3.0 vector after amplified from human genomic DNA. The putative miRNA binding site of circKIF4A and GPX4 3'-UTR was mutated in luciferase reporter assays. The constructed reporting vectors (circKIF4A-wt/mut or GPX4 3'-UTR-wt/mut) and miRNA inhibitors or mimics were both transfected into cells for 48 hours.

RNA immunoprecipitation (RIP)

Cells were transfected with MS2bs-circKIF4A, MS2bs-circKIF4A-mt and MS2bs-Rluc. After incubating for 48 hours, RIP was performed. The relative level of miR-1231 was determined after purification. The RIP assays for AGO2 were performed with an IP level anti-Ago2 antibody (Millipore). The abundance of circKIF4A, GPX4 mRNA and miR-1231 was tested after RNA purification.

Mouse xenograft model

KAT-5 and TPC-1 cells (2×10^7) were subcutaneously injected into nude mice (four mice/group, 4-week-old) and treated with intratumoral injection (50 μ L si-circCON, si-circKIF4A) every four days. The volume of tumors was estimated every four days according to the formula $0.5 \times \text{width}^2 \times \text{length}$. After 28 days, the tumors were weighed. For *in vivo* lung metastasis assay, cells (1×10^5) were injected through tail veins (four mice/group). The lungs were extracted after 8 weeks and the number of metastatic sites were quantified via microscopy of HE-stained sections.

Statistical analysis

All statistical analysis was performed with SPSS 22.0 software. All data are reported as the mean \pm standard deviation (SD). Groups were compared using Student's t test. Paired t test was used to compare the expression of circKIF4A in two groups. $P < 0.05$ was regarded as statistically significant.

Abbreviations

3'-UTR: 3'-untranslated regions; circRNA: circular RNA; GPX4: glutathione peroxidase 4; miRNAs:

microRNAs; SD: standard deviation; RIP: RNA immunoprecipitation.

AUTHOR CONTRIBUTIONS

Wenkuan Chen, Jianchang Fu designed the experiments. Quan Zhang interpreted the data and wrote the manuscript. Yingle Chen, Yudong Li carried out experiments. Li Ning, Dou Huang and Shumei Yan collected the human samples. All authors read and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPLEMENTARY MATERIALS

Supplementary Tables

Supplementary Table 1. The target sequences of siRNAs used in this study.

siRNA	Sequence (5' - 3')
si-circCON	UUCUCCGAACGUGUCACGUTT
si-circKIF4A	GAUCUAUAACGUAUUAUATT

Supplementary Table 2. Primer sequences for qRT-PCRs used in this study.

Target	Direction	Sequence (5' - 3')
18S	Forward	TTAATTCCGATAACGAACGAGA
	Reverse	CGCTGAGCCAGTCAGTGTAG
circKIF4A	Forward	GAGGTACCCTGCCTGGATCT
	Reverse	TGGAATCTCTGTAGGGCACA
GPX4	Forward	GAGGCAAGACCGAAGTAAACTAC
	Reverse	CCGAACTGGTTACACGGGAA
miR-1231	Forward	CCTCAACTGAATTGCCGACTC
	Reverse	CTCAACTGGTGTCTGGAGTC
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

Supplementary Table 3. The sequences of oligonucleotides used in this study.

Oligonucleotides	Sequence (5' - 3')
miR-1231 mimics	GUGUCUGGGCGGACAGCUGC
Control mimics	UCUACUCUUUCUAGGAGGUUGUGA