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RESEARCH ARTICLE

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MicroRNA-203a-3p may prevent the development of thyroid papillary carcinoma via repressing MAP3K1 and activating autophagy

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Abstract

Background: Papillary thyroid carcinoma (PTC) grows slowly but has a great risk of metastasis. MicroRNAs are well known as vital tumor-related gene regulators. In PTC, the role of miR-203a-3p and the underlying mechanisms remain not completely understood.

Methods: We conducted CCK8 assay, wound healing assay, transwell experiment and flow cytometry analyses to investigate the function of miRNA-203a-3p. The interaction of miRNA-203a-3p with its gene MAP3K1 was characterized by quantitative real-time polymerase chain reaction, western blotting and luciferase assay.

Results: We found that the levels of miRNA-203a-3p were statistically decreased in PTC tissues. When mimics were delivered to TPC-1 and KTC-1 cells to upregulate miR-203a-3p, it was observed that cell proliferation, metastatic abilities and cell cycle process were prevented but cell apoptosis was enhanced. Furthermore, we proved the interaction between MAP3K1 and miR-203a-3p. Intriguingly, similar to miR-203a-3p mimics, siMAP3K1 showed a tumor-suppressive effect, and this effect could be reversed when miR-203a-3p was simultaneously inhibited. Finally, selected autophagy-linked proteins such as LC3 Beclin-1 were detected and found to be increased when miR-203a-3p was upregulated or MAP3K1 was inhibited.

Conclusion: Overall, miR-203a-3p inhibits the oncogenic characteristics of TPC-1 and KTC-1 cells via suppressing MAP3K1 and activating autophagy. Our findings might enrich the understanding and the therapeutic strategies of PTC.

KEYWORDS
autophagy, MAP3K1, microRNA-203a-3p, papillary thyroid carcinoma

1 | INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common (>90%) and least malignant thyroid cancer.[1,2] It can develop at any age, and frequently happens to children and young women (before 40 years old). Although PTC grows slowly and is usually confined to the thyroid gland for several years, the lesions can spread from the primary site to other parts of the gland and cervical lymph nodes through the

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lymphatic vessels, leading to metastasis and poor prognosis.[3] The incidence of PTC is increasing year by year.[4,5] Investigating the mechanisms of PTC occurrence and progression may facilitate the diagnosis and treatment of patients with PTC.

In recent years, microRNAs (miRNAs) have attracted the huge attention of scientists. MiRNAs are the most abundant non-coding RNAs. They only have ~20 nucleotides. It has been well known that miRNAs can post-transcriptionally and negatively regulate the expression of target mRNAs through base pairing to the 3′-untranslated regions (UTRs).[6,7] Tremendous studies have demonstrated that numerous miRNAs are involved in tumorigenesis and progression.[8–11] For example, the interest miRNA in the present study, miR-203a-3p showed abnormal expression in different cancer types, such as epithelial ovarian cancer, prostate cancer, gastric cancer and non-small cell lung cancer (NSCLC), and contribute to multiple steps of cancer development.[12–15] However, the function of miR-203a-3p in PTC and the underlying mechanisms remain not completely understood. The aim of our study is to prove the activity of miR-203a-3p in PTC and to uncover the possible molecular mechanisms. The mitogen-activated protein kinase (MAPK) pathway is frequently activated in cancerous cells. MAP3K1, a MAPK family member has been reported to have multiple functions in cell viability, cell death, epithelial–mesenchymal transition (EMT) and metastasis in tumors.

In the present study, following the investigation of the influence of miR-203a-3p on cell proliferation, metastatic ability, cell death and cell cycle of PTC cell lines, we further proved the direct interaction between MAP3K1 and miR-203a-3p, and the effect of MAP3K1 on cell death, viability, metastatic ability and cell cycle could be rescued by miR-203a-3p. Finally, it was demonstrated that the interest miR-203a-3p might exert its tumor-suppressive role through activating cell autophagy in PTC. Overall, our study uncovers a novel mechanism underlying the regulatory activities of miRNA-203a-3p.

2 | MATERIAL AND METHODS

2.1 | Tissue samples

From February 2017 to March 2018, we collected 54 PTC tissue pairs including the corresponding vicinal healthy tissues from Ningbo HwaMei Hospital. The detailed pathological parameters of patients with PTC were included in Table S1. The preparation and dealing with all the human specimens in the study were approved by the Ethics Review Board of the Ningbo HwaMei Hospital.

2.2 | Cell cultivation and transfection

Healthy thyroid cell line, Nthy-ori3-1, as well as five PTC cell lines: TPC-1, KTC-1, B-CPAP, 8505C and SW1736 were acquired from the Cell Bank/Stem Cell Bank (Shanghai, China) and cultured in DMEM medium containing 10% fetal bovine serum (FBS), penicillin and streptomycin in an incubator with 10% CO_2 at 37°C. MiR-203a-3p mimic/inhibitor, siMAP3K1, and the corresponding negative controls were purchased from Genepharma Company, and delivered to TPC-1 and KTC-1 cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific).

2.3 | cDNA synthesis and quantitative real-time polymerase chain reaction

RNAs were firstly isolated using the traditional TRIzol reagent, and quantified on a NanoDrop 2000 device (Thermo Fisher Scientific). Subsequently, for the quantification of mRNAs, cDNAs were synthesized with the M-MLV reverse transcriptase (Promega), while for the quantification of miRNAs, cDNAs were synthesized with TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). Regarding quantitative real-time polymerase chain reaction (qRT-PCR), the primers of miRNA-203a-3p and the endogenous control U6, MAP3K1 and the housekeeping gene GAPDH (shown in Table S2) were purchased from Beijing Genomics Institution. The qRT-PCR system was prepared and run on a Mastercycler Gradient as previously described.[16] The relative levels of miR-203a-3p and MAP3K1 were calculated by the $2^{-\Delta\Delta Cq}$ method.[17]

2.4 | Cell proliferation assay

In brief, TPC-1 or KTC-1 cells were transfected with miR-203a-3p mimic/inhibitor or siMAP3K1 or the corresponding negative controls in 96-well plates. Transfected cells were treated with CCK-8 solution at 4 points (24, 48, 72, and 96 h). The absorbance at 450 nm was measured with a microplate reader.

2.5 | Cell apoptosis assay

Cell apoptosis assay was conducted with the annexin V-FITC/PI apoptosis detection kit (Becton). Briefly, transfected TPC-1 or KTC-1 cells were washed with cold 1× PBS, and then resuspended with binding buffer, and finally stained with PI and Annexin V-FITC reagents in dark. The dead cells were analyzed by a flow cytometer (Beckman Coulter).

2.6 | Cell cycle evaluation

To check the influence of miR-203a-3p mimic/inhibitor and siMAP3K1 on the process of the cell cycle of TPC-1 and KTC-1 cells, cell cycle evaluation was carried out using the PI/RNase staining reagent (Becton), and were detected with a flow cytometer (Beckman Coulter) as previously described.[18]

2.7 | Migration and invasion assays

For migration assay, TPC-1 and KTC-1 cells were transfected with miR-203a-3p mimic/inhibitor or siMAP3K1, 24 h later, the wound was created by the yellow pipette tip on 6-well plates. After incubation in an FBS-free medium for 24h, the wound was photographed. For invasion assay, transfected TPC-1 and KTC-1 cells were loaded into the upper chamber of the Transwell inserts precoated with and without Matrigel (BD Biosciences). The upper chamber contained an FBS-free medium, while the lower chamber contained a medium with 20% FBS. After 48 h, the invasive cells were stained with 0.1% crystal violet and were further photographed by an optical microscope.

2.8 | Protein isolation and western blot

To quantify interested protein levels, cellular proteins were firstly lysed with the radioimmunoprecipitation lysis buffer. For the detection of protein expression by western blot, 20 μg of lysed cellular proteins, 10% of sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and polyvinylidene difluoride membrane, anti-rabbit secondary antibody (ab97051), anti-mouse secondary antibody (115–035-003E) and enhanced chemiluminescent substrate (Beyotime) were used. The specific procedure of western blot was as described previously.[19] The primary antibodies used for western blot include anti-MAP3K1 (ab224628), anti-GAPDH (ab9485), anti-Bcl-2 (ab182858), anti-Bax (ab32503), anti-CDK4 (ab108357), anti-cyclin D1 (ab16663), anti-Bcl-2 (ab32124), anti-MMP-2 (#40994), anti-MMP-9 (#13667), anti-vimentin (#46173), anti-LC3 (A7198), and anti-Beclin-1 (A17028). Finally, an infrared imaging system (Li-COR) was used to visualize the signal bands. The gray-scale values of all signal bands from the western blot were evaluated by Image J 1.8.

2.9 | Luciferase reporter test

To confirm the direct interaction between miR-203a-3p and MAP3K1, the 3′-UTRs of MAP3K1-wild-type or MAP3K1-mutant were firstly cloned into pmirGLO vectors (Promega). Subsequently, TPC-1 and KTC-1 cells were co-transfected with constructed pmirGLO vectors along with miR-203a-3p mimic or inhibitor. Twenty-four hours later, the Dual-Luciferase Reporter Assay kit (Promega) was used to detect the luciferase activity as well as the Renilla luciferase activity for normalization.

2.10 | Statistical analyses

The differences between subgroups were tested by Student's *t*-test or analysis of variance (ANOVA) with Tukey's honestly significant difference test. Two-way ANOVA with Post Hoc test was used for CCK-8 data analyses. Pearson's correlation analysis was used for association evaluation. $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered as statistically significant.

3 | RESULT

3.1 | MiR-203a-3p was downregulated in PTC

With the purpose of exploring the function of miR-203a-3p in PTC, qRT-PCR was firstly carried out to quantify the levels of miR-203a-3p in 54 PTC tissue samples and the corresponding vicinal healthy tissues. The relative expression levels of miR-203a-3p in PTC tissue samples were notably lower than those in the vicinal healthy tissue samples ($p < 0.01$; Figure 1A). Moreover, the relative expression levels of miR-203a-3p in Nthy-ori3-1, B-CPAP, TPC-1, 8505C, KTC-1 and SW1736 were measured. Unsurprisingly, we found that the relative expression levels of miR-203a-3p in the above five PTC cell lines were obviously downregulated compared to the healthy cell line Nthy-ori3-1, in particular in the cell lines of KTC-1 and TPC-1 (Figure 1B), therefore we chose these two PTC cell lines for further investigation. MiR-203a-3p mimic was synthesized to upregulate, whereas an inhibitor was used to downregulate the miR-203a-3p levels in the PTC cell lines. The bar charts in (Figure 1C,D) show the successful delivery of mimic and inhibitor into TPC-1 as well as KTC-1 cells.

3.2 | MiR-203a-3p inhibits the oncogenic characteristics of PTC

We carried out CCK8 assay, wound healing and transwell assays, as well as flow cytometry experiments to investigate the function of miR-203a-3p in the cell lines of KTC-1 and TPC-1. As shown in Figure 2A,B, we found that miR-203a-3p mimic suppressed cell viability, and in contrast, miR-203a-3p downregulation by inhibitor promoted cell viability. In addition, the upregulation of miR-203a-3p by mimic repressed the wound healing ability (Figure 2C) and invading ability (Figure 2D) of PTC cells, and the downregulation of miR-203a-3p by inhibitor exhibited an inverse effect (Figure 2C,D). Furthermore, we carried out the transwell assay without Matrigel, and consistent with the above results, we observed similar results in the two PTC cell lines (Figure S2A). For apoptosis assay with KTC-1 and TPC-1 cells, we found miR-203a-3p mimic increased the apoptotic rate, and inversely miR-203a-3p inhibition decreased the apoptotic rate (Figure 2E). For the cell cycle analysis (Figure 2F), miR-203a-3p mimic made more cells arrested at G2/M and S phases and fewer cells at G0/G1 phases, whereas miR-203a-3p inhibitor made increasing cells enter into G0/G1 phases and decreasing cells into G2/M and S phases. The above functional assays indicate that miR-203a-3p may behave as a tumor-suppressive regulator in PTC. Inspired by the above functional results, we decided to explore the underlying mechanisms of miR-203a-3p. Since cell apoptosis, EMT and cell cycle process are important events in tumor development, apoptosis-related proteins like Bcl-2 and Bax, EMT markers such as

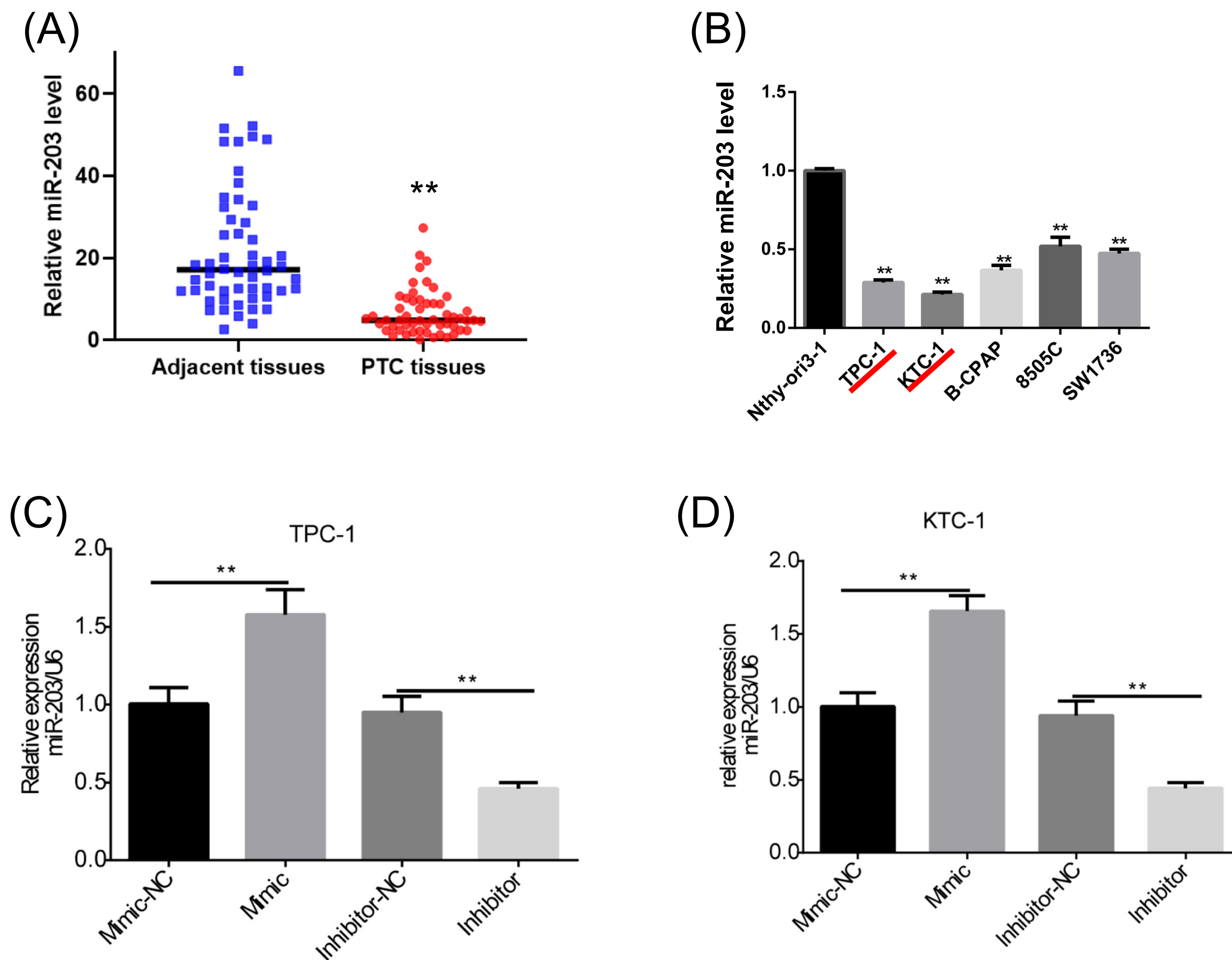

FIGURE 1 Expression levels of miR-203a-3p in PTC tissues and cell lines. The expression levels of miR-203a-3p in PTC tissues and the normal adjacent tissues (A). The expression levels of miR-203a-3p in healthy thyroid epithelial cell line Nthy-ori3-1, as well as five PTC cell lines, including TPC-1, KTC-1, B-CPAP, 8505C, and SW1736 (B). MiR-203a-3p mimic and inhibitor were used to upregulate and downregulate the miR-203a-3p levels, respectively, in TPC-1 (C) and KTC-1 (D) cells

MMP-2, Vimentin and MMP-9, and cell cycle-linked proteins like cyclin D1 and CDK4 were selected to be quantified by western blot. We found that in TPC-1 (Figure 2G), the levels of MMP-2, Vimentin and MMP-9, as well as CDK4 and cyclin D1, were decreased by miR-203a-3p overexpression and in contrast increased by miR-203a-3p downregulation. However, for Bcl-2 and Bax, miR-203a-3p mimic upregulated the levels of Bax, but downregulated the levels of Bcl-2. And miR-203a-3p inhibitor showed an inverse effect (Figure 2G). Consistently, almost the same phenomenon was found in KTC-1 (Figure 2H). These changes in protein levels well explain that miR-203a-3p influences PTC development via regulating related proteins.

3.3 | MiR-203a-3p directly binds to MAP3K1

It has been known that miRNAs post-transcriptionally regulate target mRNAs and inhibit protein translation. Following the investigation of the function of miR-203a-3p in PTC cells, TargetScan7.2 (Figure 3A) was used to predict the potential interacting mRNA MAP3K1 of miR-203a-3p. Subsequently, we found that different from the signature of miR-203a-3p in PTC tissues, the mRNA levels of MAP3K1 were remarkably elevated (Figure 3B). Intriguingly, the relative expression of miR-203a-3p and MAP3K1 was negatively correlated in PTC tissues (Figure 3C). Moreover, the mRNA (Figure 3D) and protein (Figure 3E) levels of MAP3K1 were much higher than those in the healthy cell line Nthy-ori3-1. The miR-203a-3p mimic could downregulate, and inversely the miR-203a-3p inhibitor upregulate the mRNA (Figure 3F,G) and protein (Figure 3H,I) levels of MAP3K1. Furthermore, it was found that the luciferase activity of MAP3K1-wt but not MAP3K1-mut could be remarkably inhibited with the co-transfection of miR-203a-3p mimic, and inversely elevated by a miR-203 inhibitor (Figure 3J,K). These results suggest that miR-203a-3p directly interacts with MAP3K1.

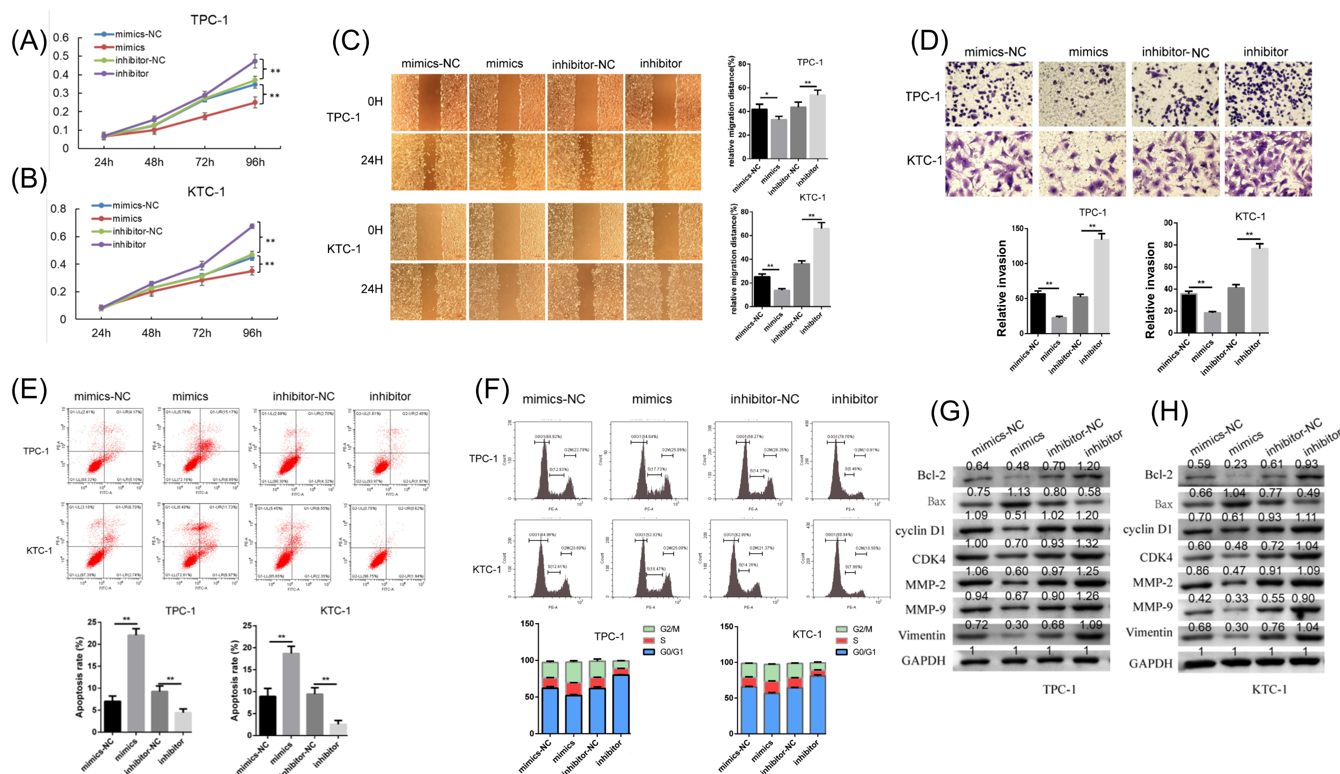

FIGURE 2 The function of the interest miR-203a-3p in PTC cells. The curves show the proliferation of transfected TPC-1 (A) and KTC-1 (B) cells. Migration abilities (C), invasive abilities (D), apoptosis (E) and cell cycle (F) were evaluated. The protein levels of MMP-2, Vimentin and MMP-9, and CDK4 and cyclin D1, as well as Bax and Bcl-2 were checked by western blot with two PTC cell lines of TPC-1 (G) and KTC-1 (H)

3.4 | The influence of MAP3K1 on PTC cells can be reversed by miR-203a-3p

Following the determination of the direct interaction of MAP3K1 and miR-203a-3p, we downregulated the MAP3K1 level by siMAP3K1, and investigated the effect of MAP3K1 on PTC development. We found that siMAP3K1 inhibited cell proliferation and at the same time the inhibition could be reversed by miR-203a-3p inhibitor in TPC-1 (Figure 4A) and KTC-1 (Figure 4B) cells. Furthermore, migration (Figure 4C) and invasion (Figure 4D) were also suppressed in the siMAP3K1 groups, and this suppression was obviously inversed by miR-203a-3p downregulation by inhibitor. Furthermore, the transwell assay without Matrigel showed similar results in TPC-1 and KTC-1 cells (Figure S2B). For apoptosis assay, siMAP3K1 enhanced cell apoptosis and the simultaneous downregulation of miR-203a-3p inhibited cell apoptosis (Figure 4E). Additionally, similar to the miR-203a-3p mimic, siMAP3K1 arrested more cells at G2/M and S phases, and miR-203a-3p downregulation by inhibitor recovered this arresting phenomenon (Figure 4F). Correspondingly, the EMT-related proteins (MMP-2, MMP-9 and Vimentin), the cell cycle-linked proteins like cyclin D1 and CDK4, as well as the apoptosis-related protein Bcl-2 were decreased by the knockdown of MAP3K1, and the decreasing influence was weakened by the introduction of miR-203a-3p inhibitor (Figure 4G,H). Different from Bcl-2, Bax was increased by siMAP3K1 and the increase was diminished by a miR-203a-3p inhibitor (Figure 4G,H). The above results indicate that the influence of MAP3K1 in PTC cells might be regulated by our interest in miR-203a-3p, which proves that MAP3K1 directly interacts with miR-203a-3p.

3.5 | MiR-203a-3p activates autophagy

Since it was reported that MAP3K1 was involved in cell autophagy, we further detected the levels of autophagy-related proteins, including LC3I/II and Beclin-1. It was found that in TPC-1 (Figure 5A) the upregulation of miR-203a-3p by mimic elevated, and in contrast, miR-203a-3p inhibitor decreased the amounts of LC3II/LC3I and Beclin-1. Similar to miR-203a-3p mimic, siMAP3K1 elevated the amounts of LC3II/LC3I and Beclin-1, and miR-203a-3p inhibitor weakened the increasing of LC3II/LC3I, as well as Beclin-1 (Figure 5A). And the results from TPC-1 (Figure 5A) and KTC-1 (Figure 5B) were observed alike, implying that miR-203a-3p represses PTC development by negatively regulating MAP3K1 and affecting autophagy.

4 | DISCUSSION

In PTC, numerous miRNAs have been reported to behave as tumor-suppressive miRNAs or onco-miRNAs.[20,21] MiR-203 was revealed to suppress PTC occurrence and progression in vitro and in vivo

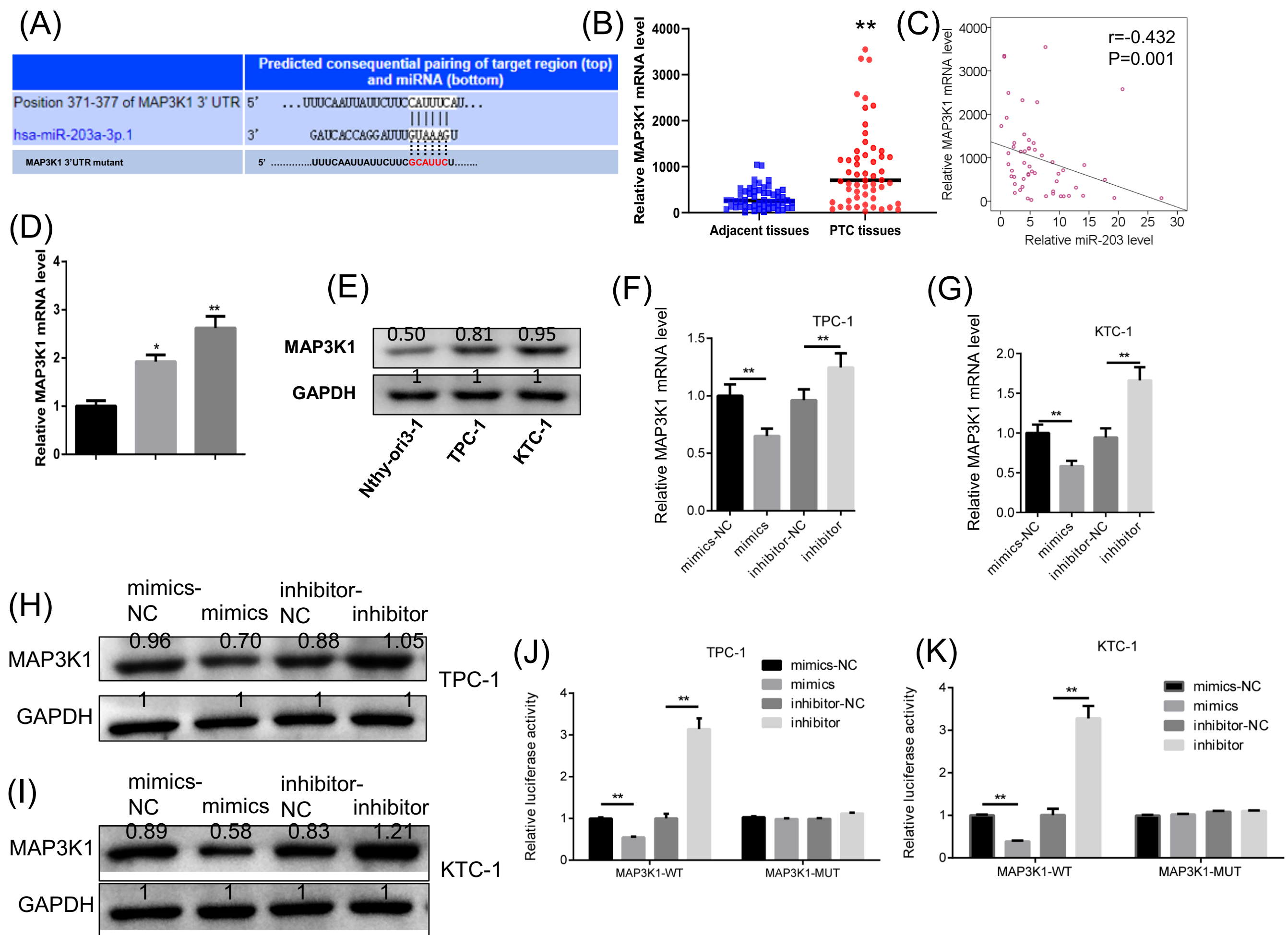

FIGURE 3 MAP3K1 directly interacts with miR-203a-3p. TargetScan7.2 screened a potential target MAP3K1 of miR-203a-3p (A). The chart shows the MAP3K1 mRNA levels in 54 PTC tissue samples and corresponding healthy specimens (B). The relative expression of MAP3K1 and miR-203a-3p was negatively associated in PTC tissue samples (C). The charts show the mRNA (D) along with protein (E) expression of MAP3K1. The charts show the mRNA as well as protein changes of MAP3K1 in two cell lines of TPC-1 (F, H) and KTC-1 (G, I). The change in luciferase activity was shown in the bar charts after TPC-1 (J) and KTC-1 (K) cells were co-transfected with MAP3K1-wt or MAP3K1-mut and miR-203a-3p mimic/inhibitor

by downregulating AKT3, and therefore regarded as a tumor-suppressive miRNA.[22,23] Also, miR-599 was reported to facilitate apoptosis and suppress cell proliferation by inhibiting the Hey2-dependent Notch signaling cascade in PTC.[24] Conversely, miRNA-146b-5p acts as an onco-miRNA to promote PTC development by targeting CCDC6 (coiled-coil domain containing 6).[25] From these investigations, we can get a clue that one miRNA is defined as a tumor suppressor or an onco-miRNA depending on the tumor-related mRNAs it binds to.

MiR-203 has been reported as a tumor suppressor or an onco-miRNA in PTC and also other cancer types. However, to the best of our knowledge, until now, the function of miR-203a-3p in PTC and the underlying mechanisms are not completely understood. In line with previous studies, the levels of miR-203a-3p were significantly decreased in our PTC tissue samples compared to the paired normal adjacent tissues, and this result was further confirmed with the healthy thyroid cell line, Nthy-ori3-1, as well as five PTC cell lines. Furthermore, we analyzed the data of miR-203a-3p in thyroid cancer derived from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO), and found the levels of miR-203a-3p in the tissues of thyroid cancer were significantly lower than those in normal tissues (Figure S1). Inspired by this phenomenon, we further investigated the influence of miR-203a-3p on cell viability, metastatic ability, cell cycle and cell death. We found that miR-203a-3p played a tumor repressive role in PTC via inhibiting oncogenic behavior. These findings were consistent with those in other cancer types, such as ovarian carcinoma,[26] hepatocellular carcinoma,[27] lung adenocarcinoma[28] and gastric cancer.[29]

Since miRNAs play their tumor-suppressive or tumor-promoting roles via regulating their target mRNAs in cancers, we further characterized one miR-203a-3p target MAP3K1. It has been well known that MAP3K1 exerts an oncogenic effect on different cancers through activating the downstream MAPK kinases.[30] Besides miR-203a-3p, several other miRNAs like miR-451 and miR-302 have been reported to target MAP3K1. For instance, miR-451 targeted and inhibited MAP3K1, leading to the repression of esophageal

FIGURE 4 The effect of MAP3K1 on PTC cells is reversed by miR-203a-3p. The curves show the proliferation of TPC-1 (A) and KTC-1 (B) cells. Migration abilities (C), invasive abilities (D), apoptosis (E) and cell cycle (F) were evaluated by siMAP3K1 and siMAP3K1 along with miR-203a-3p inhibitor. The protein levels of CDK4, cyclin D1, MMP-2, Vimentin and MMP-9, Bax and Bcl-2 were detected by western blot in different experimental subgroups of TPC-1 (G) and KTC-1 (H) cell lines

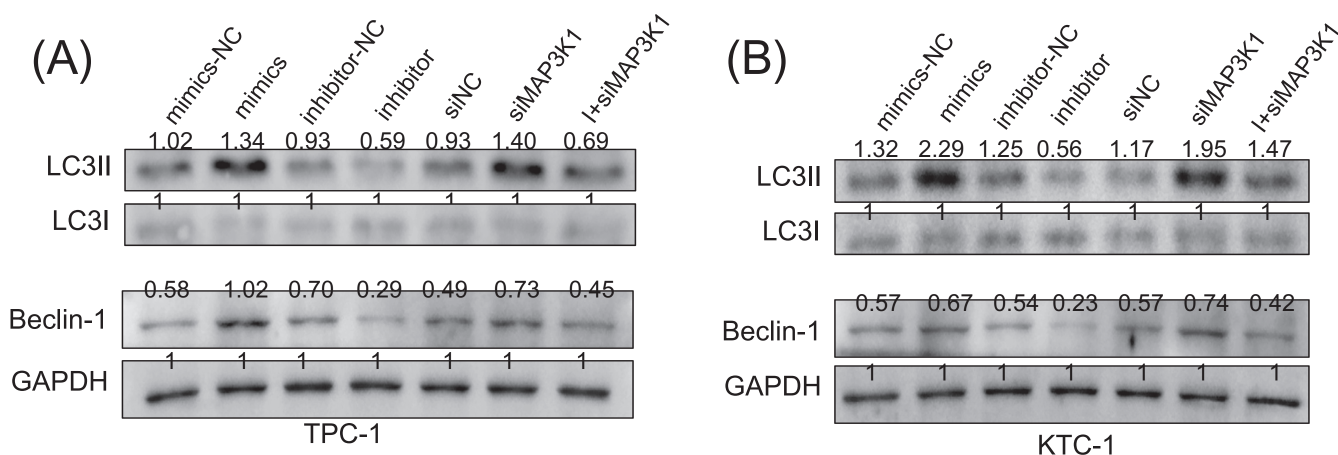

FIGURE 5 Cell autophagy is activated by a miR-203a-3p mimic or siMAP3K1. The levels of autophagy-associated proteins such as Beclin-1 and LC3II/I were tested by western blot. The gray values of western blot bands in different experimental subgroups of TPC-1 (A) and KTC-1 (B) cells were measured

carcinoma cell proliferation, and miR-302 could also interact with and suppressed MAP3K1, contributing to adriamycin therapy sensitization in breast cancer.[31,32] In line with the above investigations, we proved that miR-203a-3p could directly target MAP3K1, and further demonstrated that miR-203a-3p prevented the occurrence and development of PTC via negatively regulating its target MAP3K1. Most importantly, we firstly revealed that miR-203a-3p might prevent PTC via activating autophagy. Since one miRNA has several target mRNAs, and at the same time each mRNA has the potential to be targeted and regulated by multiple miRNAs, the miRNA-mRNA regulatory network is incredibly complicated. The present study just proved one possible target of miR-203a-3p, and unfolded one underlying mechanism about miR-203a-3p in PTC. Therefore, more studies should be performed to further investigate the mechanisms of miR-203a-3p in the occurrence and development of PTC and other cancer types.

In conclusion, miR-203a-3p may repress the occurrence as well as the progression of PTC by directly targeting MAP3K1 and activating autophagy.

AUTHOR CONTRIBUTIONS

Lei Dai conceived the study and prepared the data and the manuscript. Weidong Zhang and Xianjiang Wu conducted the experiments. Shuihong Zhou supervised the study.

CONFLICT OF INTEREST

No conflict of competing interest is declared.

DATA AVAILABILITY STATEMENT

The published article contains all data.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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