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ARHGAP36 regulates proliferation and migration in papillary thyroid carcinoma cells

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Abstract

The diagnosis and treatment of recurrence and metastasis in papillary thyroid carcinoma (PTC) are still clinical challenges. One of the key factors is the lack of specific diagnostic markers and therapeutic targets for recurrence and metastasis. Single-cell RNA sequencing (scRNA-seq) has emerged as a powerful approach to find specific biomarkers by dissecting expression profiling in human cancers at the resolution of individual cells. Here, we investigated cell profiles of the primary tumor and lymph node metastasis and paracancerous normal tissues in one PTC patient using scRNA-seq, and compared individual cell gene expression differences. The transcriptomes of 11,805 single cells were profiled, and malignant cells exhibited a profound transcriptional overlap between primary and metastatic lesions, but there were differences in the composition and quantity of non-malignant cells. *ARHGAP36* was one of the genes that were highly expressed in almost all of the primary and metastatic malignant cells without non-malignant or normal follicular cells and was then confirmed by immunostaining in a sample cohort. Compared with the paracancerous normal tissue, the expression of *ARHGAP36* in primary and metastatic carcinoma tissues was significantly higher as assayed by qRT-PCR. *ARHGAP36* knockdown significantly inhibited the proliferation and migration of PTC cells *in vitro* and involved several proliferation and migration-associated signaling pathways by RNA seq. Our study demonstrated that *ARHGAP36* is exclusively expressed in the malignant cells of primary PTC, as well as metastatic lesions, and regulates their proliferation and migration, meaning it can be used as a potential diagnostic marker and therapeutic target molecule.

Key Words

- ▶ *ARHGAP36*
- ▶ papillary thyroid carcinoma
- ▶ migration
- ▶ proliferation
- ▶ single-cell RNA sequencing

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Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy, accounting for 85% of all thyroid cancers, with a growing incidence rate every year (Siegel *et al.* 2020). The diagnosis of PTC has benefited from the widely used practice of fine-needle aspiration cytology (FNAC) in clinical practices (Haugen *et al.* 2016).

However, 15–30% of thyroid FNACs are classified as a group of indeterminate lesions, which offers a challenge in terms of interpretation and clinical management (Cibas & Ali 2017). Although most patients with PTC have good overall survival after treatment using established methods, the prevalence of PTC still raises concern because of the

presence of a nearly 30% recurrence rate and 8.6% cause-specific mortality for a three-decade period (Dong *et al.* 2019). Therefore, it is an urgent and necessary task to find diagnostic markers and new therapeutic targets of recurrence and metastasis in PTC.

It has been established that pathogenesis and progression of PTC were associated with the genetic and epigenetic alterations, the former includes the driver point mutations of BRAF, RAS and so on, as well as diverse gene fusion (RET/PTC, ETV6-NTRK3, etc.), and the latter includes MiR146b, MiR21, and Linc00941, etc. (Cancer Genome Atlas Research Network 2014, Yakushina *et al.* 2018, Gugnoni *et al.* 2020). Most of them perform biological functions through MAPK and PI3K/AKT signaling pathways in PTC cells (Xing 2013). Some of these markers have been used as diagnostic markers (BRAF V600E mutation) and therapeutic targets of recurrence and metastasis (BRAF and MAPK kinase inhibitor) in clinical practice, but there are still obvious limitations in the diagnostic efficiency as well as the therapeutic side effects (Cabanillas *et al.* 2019).

Human cancers are a heterogeneous disease and are intricate ecosystems composed of diverse cells, whose precise characterization is masked by bulk genomic methods (Suva & Tirosh 2019). Single-cell RNA sequencing (scRNA-seq) techniques have emerged as powerful tools for dissecting expression profiling in human cancers at the resolution of individual cells (Stark *et al.* 2019). They have been used in head and neck cancers (Puram *et al.* 2017), breast cancers (Azizi *et al.* 2018), pancreatic cancers (Peng *et al.* 2019), renal cancers (Young *et al.* 2018), and other malignant tumors, and have resolved the precise expression profiles of their respective cell subsets. Based on this, scRNA-seq can help identify specific molecular markers and therapeutic targets of PTC.

ARHGAP36, a RhoGTPase activating protein, has been identified from a genome-scale cDNA overexpression screen (Rack *et al.* 2014). Bulk normal tissue samples were analyzed by RNA-seq from 95 human individuals representing 27 different tissues, and the results showed that *ARHGAP36* was restricted in expression toward adrenal tissue, but not toward any other tissues, such as the thyroid gland (Fagerberg *et al.* 2014). It has previously been shown that *ARHGAP36* is upregulated in a subset of medulloblastoma and is responsible for tumorigenesis via the Hedgehog (Hh) signaling pathway (Eccles *et al.* 2016, Beckmann *et al.* 2019). *ARHGAP36* expression is also increased in pheochromocytomas (Croise *et al.* 2016). However, the meticulous expression and function of *ARHGAP36* in various subpopulations of PTC micro-

ecosystems have not, to date, been revealed in the literature.

In this study, we investigated the landscape of cells and transcriptional profiles of malignant cells in primary and metastatic PTCs by scRNA-seq, and found that *ARHGAP36* was one of the genes that were restricted in expression to primary and metastatic malignant cells. There was almost no expression in non-malignant cells and normal follicular epithelial cells. Then, the expression characteristics of *ARHGAP36* were validated with immunohistochemistry and qRT-PCR in a PTC tissue cohort, and gene knockdown experiments showed that *ARHGAP36* promoted the proliferation and migration of PTC cells through related signaling pathways. These results suggest that *ARHGAP36* can be used as a potential diagnostic marker and therapeutic target molecule for recurrence and metastasis of PTC.

Materials and methods

Patients and tissue collection

Specimens for scRNA-seq were collected from a 45-year-old female PTC patient, whose primary PTC (Ca), lymph node metastasis (LN), and matched paracancerous normal tissue (PCa) samples were harvested at the time of initial surgery. The samples were completely immersed in MACS tissue preservation solution (Miltenyi Biotec, #130-100-008) and delivered to the laboratory within 2 h at 4°C. In addition, the primary carcinoma, metastatic carcinoma, and adjacent tissues of 20 patients with PTC were collected and cut into two parts. One was fixed in 10% neutral formalin, embedded in paraffin, and cut into 4 µm slices for immunohistochemical staining, while the other section was immediately snap-frozen in liquid nitrogen and stored in liquid nitrogen until RNA extraction. None of the patients received any preoperative treatments. All of the samples were confirmed by two pathologists independently via pathological diagnosis. The use of human PTC tissue specimens in this study was approved by the Ethics Committee of Shanghai Jiaotong University Affiliated Sixth People's Hospital (IRB: SH6THHOSP-YS-2019-037), and written informed consent was obtained from all of the patients or their guardians before sample collection.

Tissue dissociation

Tissues were washed twice with PBS and cut into 1 mm³ pieces using sterile scalpel blades. Sample dissociation

was performed according to the instructions of a human tumor dissociation kit (Miltenyi Biotec, #130-095-929) using a gentle MACS Octo automatic tissue processor (Miltenyi Biotec, #130-096-427). After standing for 2–3 min, the supernatant was decanted and the large lumps were removed with a filter membrane. Following cell centrifugation, the supernatant portion was poured out and discarded. The cells were resuspended with erythrocyte lysis buffer, cultured at room temperature for 2–3 min, and then centrifuged at 120 *g* for 3 min at 4°C. Samples were finally resuspended in PBS. Cell viability was evaluated using trypan blue (Invitrogen). The cell suspension used for single-cell sequencing was diluted with PBS plus 0.04% BSA (Sigma) to achieve a concentration of around 10⁶ cells per microliter.

Single-cell RNA-seq

Cells suspensions (300–600 living cells per microliter determined by Count Star) were washed in PBS with 0.04% BSA and resuspended before loading cells onto the Chromium single cell controller (10× Genomics) to capture single cells in droplets according to the manufacturer's protocol. Libraries were prepared using Single Cell 3' Library Gel Bead Kit V2 (10× Genomics). Finally, sequencing was performed on the Illumina Novaseq 6000 sequencer with a sequencing depth of at least 132,920 reads per cell and 150 bp (PE150) paired-end reads (performed by CapitalBio, Beijing).

Single-cell RNA-Seq data processing

Cell Ranger (10× Genomics) software was utilized to analyze the raw sequencing data (version 1.3.1). Transcript counts in each cell were quantified using barcoded UMIs and 10× cell barcode sequences. The gene expression matrices were loaded to the R package Seurat version 3.0 for quality control and downstream analyses. Based on the number of genes detected, nUMIs, and the mitochondrial gene content, poor-quality cells were further filtered out. Single cell gene-expressions were visualized as t-distributed stochastic neighbor embedding-based (tSNE) overlays and marker gene heatmaps. The normalized expression matrix, tSNE coordinates, and clustering identified via the FindClusters in Seurat were used to analyze for the transcription data.

Immunohistochemistry staining

Before staining, the paraffin sections with a thickness of 4 μm were baked for 2 h at 60°C. The paraffin sections

were deparaffinized and rehydrated, then treated with 3% H₂O₂ in methanol, and the antigen was recovered in sodium citrate buffer at 750 W in a microwave oven for 10 min. The sections were blocked with 10% BSA in PBS for 60 min and incubated with the primary antibody anti-ARHGAP36 (Invitrogen) at 4°C overnight. The sections were then stained with the appropriate HRP-labeled polymer-conjugated secondary antibody for 60 min. The immune complexes were visualized by exposure to the DAB substrate for 3–5 min. The nuclei were counterstained with hematoxylin. Three random immunostaining images from each specimen were captured.

The staining score of ARHGAP36 protein was the sum of the staining intensity and the percentage of positive cells (Ding *et al.* 2019). The staining intensity of ARHGAP36 was scored as 0 (negative), 1 (weak, light yellow), 2 (moderate, yellow-brown), or 3 (strong, brown). The percentage of positively stained cells was scored by two observers blinded to clinical data in contrast to the total section area (TSA=100%) and defined as 0 (no), 1 (< 10%), 2 (10–50%), or 3 (>50%). The final scores were calculated and divided into negative (0), weak (1–2), moderate (3), and strong (4–6) staining groups. Moderate and strong groups were considered a positive expression.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA from tissues and cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocols, and reverse transcribed to cDNA using the MLV reserve transcriptase from Promega. The qRT-PCR analysis was performed in triplicate on the Mx3005P qRT-PCR system (Stratagene) using the SYBER Green master mix (BIORAD). The housekeeping gene GAPDH was used as an internal control. Data were obtained as Ct values which were used to calculate ΔCt values. Relative gene expressions were calculated by the 2^{−ΔΔCt} method. The primer sequences used were as follows: the endogenous control *GAPDH*: Forward, 5'-TGACTTCAACAGCGACACCCA-3'; Reverse, 5'-CACCTGTTGCTGTAGCCAAA-3'; *ARHGAP36*: Forward, 5'-CCCTGCCACAGTGATACCC-3'; Reverse, 5'-ACGGACCACATTGACAGAAGC-3'.

Cell culture and transfection

The human PTC cell lines K1 and TPC-1 were provided by Dr Zhongling Qiu (Department of Nuclear Medicine, Shanghai Jiao Tong University Affiliated Sixth People's Hospital), and authenticated for correct origin by short

tandem repeat (STR) analysis (Fu *et al.* 2019). TPC-1 cell line harbored *RET/PTC1* gene rearrangement, while K1 cell line harbored *BRAF* and *PI3KA* mutation, and no mutations in the remaining genes (Meireles *et al.* 2007). Cell lines were maintained in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 µg/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO₂ at 37°C.

TPC-1 and K1 cells were assigned into the control (CON) group (cells without any treatment), the si-negative control (NC) group (cells transfected with si-negative control), and the si-*ARHGAP36* group (cells transfected with si-*ARHGAP36*). Si-*ARHGAP36* and si-negative control were obtained from GeneChem (Shanghai, PR China). TPC-1 and K1 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer's instructions when the cells had achieved 80% confluence. siRNA sequences are listed as follows: si-*ARHGAP36*, 5'-GGGCTGCTGCTCCTTGCTCAAA-3'; si-negative control, 5'-TTCTCCGAACGTGTCACGT-3'.

MTT assay

Cells were seeded into 96-well plates (Corning Costar Corp) in a density of 2×10^3 cells/well and incubated at 37°C with 5% CO₂ for 24 h. At 48 h after si-*ARHGAP36* transfection, 10 µL of MTT solution (Sigma-Aldrich) was added to the plated cells, and incubation was continued for additional 4 h. After dissolving intracellular formazan crystals in DMSO (150 µL/well; Sigma-Aldrich) for 10 min, the absorbance at 490 nm was measured using an E_{max} precision microplate reader (Molecular Devices, Sunnyvale, CA, USA). The experiments were repeated at least three times.

Cell migration assay

Transwell assay was carried out using transwell chambers (Corning Costar Corp). Briefly, 5×10^4 cells were seeded into the upper chamber of plates in basal medium without FBS, while the lower chamber was filled with DMEM containing 10% FBS. After 24 h, the migratory cells on the bottom of the membranes were fixed and stained. Randomly chosen fields were photographed and the number of migrated cells per field was counted. Experiments were performed in triplicate.

RNA-sequencing analysis (RNA-Seq)

A stable negative control (NC) and *ARHGAP36* knockdown (KD) TPC-1 cell line were used for RNA-seq analysis by

GeneChem (Shanghai, PR China). The NanoDrop 2000 Nucleic Acid and Protein Analyzer (Thermo Scientific) were used to check the total RNA concentration and purity. Sequencing experiments were performed in triplicate for each of the two biological samples. Quality control of raw RNA-Seq data was performed using FastQC software; low-quality sequences were discarded. Normalization and differential expression were conducted by DESeq2 and 997 genes were selected for the over ± 2 fold differential expression with the thresholds of adjusted *P*-value < 0.05 and $|\log_2 \text{FoldChange}| > 1$. Genes significantly differentially expressed underwent enrichment analysis, performed on GO biological processes and KEGG pathways through clusterProfiler package in R/Bioconductor.

Statistical analysis

All of the experiments were performed at least three times. Statistical data were processed using GraphPad Prism 5.0 software as the mean $\pm$ s.d. of results from at least three independent experiments. Statistical analyses were performed using the Student's *t*-test and one-way ANOVA. *P* < 0.05 was considered to be statistically significant.

Results

Landscape of cells and transcriptional profiles of malignant cells in primary and metastatic PTC

We profiled the transcriptomes of 11,805 single cells from the matched Ca, LN, PCa samples and clustered the cells with consistent transcription, and then annotated the identity of cell clusters using the established marker genes (Fig. 1). Among them, the normal follicular epithelial cells from PCa were identified by TG, EpCAM marker genes, the malignant follicular epithelial cells from Ca and LN by TG, EpCAM, and KRT19 marker genes, and interstitial cell subsets from the three samples by their respective marker genes, such as CD4⁺ T cells (*CD4*, *MS4A6A*, *MSR1*), CD8⁺ T cells (*CD2*, *CD3D*, *CD8*), B/plasma cells (*CD19*, *CD22*, *CD79B*), vascular endothelial cells (*VWF*, *CD34*), fibroblasts (*ACTA2*, *FN1*), and CAFs (*FAP*, *POSTN*, *CXCL12*). It revealed that the majority of PCa were normal follicular epithelial cells (455/626), while in Ca and LN, malignant follicular epithelial cells (229/6923, 361/4256), that is the real cancer cell population in cancer tissue, only accounted for a small number (Fig. 1A, B and C). The aggregate analysis of all cells from the matched samples was further used to ascertain the relationship between these cell populations and the dynamics of their phenotypic changes during

Figure 1

Profiling of the cellular composition of PTC tumors using scRNA-seq. (Ca, primary PTC samples; LN, lymph node metastasis samples; PCa, paracancerous normal tissue samples). (A) The t-SNE plot of primary PTC tissues comprised of seven distinct cell populations. (B) The t-SNE plot of metastatic PTC tissues comprised of six distinct cell populations. (C) The t-SNE plot of paracancerous normal thyroid tissues comprised of six distinct cell populations. (D) The aggregate analysis of three samples. 2D t-SNE plots depicting 11,805 single cells derived from primary tumors, lymph node metastases, and paracancerous normal tissue in one PTC patient. Single cells are colored by cell types. (E and F) Heatmap of genes significantly expressed in a malignant epithelial cell (including primary and metastatic malignant cells) compared with normal follicular epithelial cells (adjusted *P*-value < 0.05, log₂ fold-change > 1). NFE, Normal Follicular Epithelial cell; ME, Malignant Epithelial cell; VE, Vascular Endothelium cell; MF, Myofibroblast cell; CAF, Cancer-associated fibroblast. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0230>.

PTC progression (Fig. 1D). It showed that the normal follicular epithelial cells in PCa formed a separate cluster, while malignant follicular epithelial cells in Ca and LN exhibited a profound transcriptional overlap. There were significant differences in gene expression profiles between malignant and normal follicular epithelial cells (Fig. 1E and F). The interstitial cells among the matched samples have an overall concordance in their identity and representation, albeit with some distinctions in terms of the cell percentage per subset (Fig. 1D). For example, B cell subsets were only found in Ca, while fibroblasts and CAFs were significantly enriched in LN. Besides, there are few interstitial cells in PCa.

***ARHGAP36* expression in individual cells and its validation in a PTC tissue cohort**

We found that *ARHGAP36* was exclusively expressed in the malignant cells of primary and metastatic cancers, but not in the non-malignant and normal follicular epithelial cells in adjacent normal samples (Fig. 2A, B and C). In a PTC sample cohort, immunohistochemical staining of the primary tumor and lymph node

metastasis showed that the expression of *ARHGAP36* was localized to malignant follicular epithelial cells (Fig. 2D). Compared with adjacent tissues, the expression of *ARHGAP36* in primary and metastatic tumors was significantly higher as shown by qRT-PCR ($P < 0.001$; Fig. 2E).

Biological function of *ARHGAP36* expression in PTC cells

We generated two *ARHGAP36* knockdown PTC cell lines using stable transfection of siRNAs, including in TPC-1 and K1 cells, and *ARHGAP36* expression was significantly decreased in infected cells, as shown by qRT-PCR (Fig. 3A). Cell proliferation by MTT and migration by Transwell assays were significantly suppressed in si-*ARHGAP36*-transfected TPC-1 /K1 cells compared with the negative control group (Fig. 3B and C).

Involvement of signaling pathways in *ARHGAP36* expression in PTC cells

We next performed RNA-seq-based gene expression profiling of TPC-1 cells treated with siRNAs targeting

Figure 2

ARHGAP36 is highly expressed in both primary and metastatic malignant epithelial cell populations. (A) *ARHGAP36* specifically expressed in the malignant epithelial cell subpopulation on the kmeans-7-clusters t-SNE projection of primary PTC tissues. (B) *ARHGAP36* specifically expressed in the malignant epithelial cell subpopulation on the kmeans-6-clusters t-SNE projection of lymph node metastasis samples. (C) *ARHGAP36* hardly expressed in the normal follicular epithelial cell subpopulation on the kmeans-6-clusters t-SNE projection of paracancerous normal thyroid tissues. (D) Representative immunohistochemical staining with anti-*ARHGAP36* in tissue specimens from PTC and normal tissues (original magnification 200 $\times$); statistical results are shown on the right. (E) RT-qPCR analysis of *ARHGAP36* mRNA levels in PTC tissues ($n = 20$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0230>.

Figure 3

Knockdown of *ARHGAP36* inhibits proliferation and migration of PTC, K1 and TPC-1 cells. (A) RT-qPCR analysis of *ARHGAP36* knockdown efficiency in K1 and TPC-1 cells. (B) MTT assays of viability in transfected cells. (C) Comparison of staining and numbers of migratory cells in Transwell migration assays. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0230>.

ARHGAP36 (si*ARHGAP36*), compared with those treated with a non-targeting siRNA. Principal component analysis (PCA) using the first and second principal components is shown in Fig. 4A. There was a clear difference between *ARHGAP36* knockdown cells and control samples. We found a significantly altered expression for 997 genes (645 upregulated and 352 downregulated), and a volcano plot shows that *ARHGAP36* knockdown leads to a massive transcriptional response (Fig. 4B). Furthermore, Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathways of the set of differentially expressed genes showed that *ARHGAP36* depletion was related to pathways involved in proliferation and migration of cancer cells, including cytokine – cytokine receptor interaction, transcriptional misregulation, TNF signaling, NOD-like receptor signaling, IL-17 signaling, cell adhesion molecules (CAMs) and neuroactive ligand-receptor interaction pathways (Fig. 4C).

Figure 4

Overview of RNA profiles (KD, knockdown; NC, negative control). (A) Results of principal component analysis (PCA). Data show that there was a significant difference between the sample groups and between the sample repeats. (B) Volcano map of differentially expressed RNAs. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for differentially expressed RNA genes. The top 20 significantly enriched pathways and their scores ($P < 0.05$) are listed as the y-axis and the x-axis, respectively. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0230>.

Discussion

Like other malignancies, intratumoral heterogeneity in the primary and metastatic PTCs is a major challenge in clinical management. This heterogeneity is not only limited to histopathological diversity but also is manifested as variations in several genetic and/or epigenetic alterations, as well as the numbers of interactions between a tumor and its surrounding microenvironment (Chmielik *et al.* 2018). Among the new technologies currently emerging for studying cancer microniches, scRNA-seq is often used to cluster cells and identify their heterogeneity based on the expression profiles of single cells in a tumor (Puram *et al.* 2017, Azizi *et al.* 2018, Young *et al.* 2018, Peng *et al.* 2019, Stark *et al.* 2019, Suva & Tirosh 2019). Here, we applied this approach to explore the cell landscapes of primary and matched metastatic PTC and their differences in gene expression between malignant cells and normal follicular cells. Our analyses highlight a multicellular ecosystem of PTC, and show transcriptional overlap between malignant cells in primary and matched metastatic lesions, while non-malignant cells had differences in cell composition and quantity. This difference supports the notion that a different microenvironment occurs between metastasis and primary carcinoma. Indeed, a previous study of head and neck squamous cell carcinomas also demonstrated analogous cell profiles in primary and metastatic lesions (Puram *et al.* 2017). Compared with previous studies (Chmielik *et al.* 2018), our work first dissected the landscape of PTC cells using ScRNA-seq and represented an important step toward understanding the intrinsic characteristics of primary and metastatic PTCs as a different entity.

Among our key findings was the identification of the *ARHGAP36* gene, which was restricted to malignant cells, regardless of whether they were from primary or metastatic lesions. To date, this gene has been found only in the brain and adrenal tumors (Rack *et al.* 2014, Eccles *et al.* 2016, Beckmann *et al.* 2019). It is particularly important that in normal tissues, the gene expression is almost exclusively restricted to the adrenal gland, followed by the brain (Fagerberg *et al.* 2014). Based on this fact, as a putative proto-oncogene, our study revealed, for the first time, that *ARHGAP36* is specifically expressed in malignant cells of PTC, and suggests that it may be a potential diagnostic marker both primary and metastatic cancers.

Due to the strong expression of *ARHGAP36* in both primary and metastatic PTCs, we were driven to investigate its pro-proliferation and pro-migration function. As anticipated, *in vitro* results show that *ARHGAP36* could promote the proliferation and migration of PTC cells. This suggested that, as in medulloblastoma (Rack *et al.* 2014, Beckmann *et al.* 2019), *ARHGAP36* plays a role as a proto-oncogene in PTC. In this case, *ARHGAP36* may be a potential therapeutic target for PTC recurrence and metastasis. However, further *in vivo* studies are still needed.

ARHGAP36 is the activator of *Gli*, which is a key member of the Hh pathway (Rack *et al.* 2014). The Hh pathway is essential for embryonic development and tissue homeostasis, and its misactivation can cause developmental abnormalities and cancers (Raleigh & Reiter 2019). Thus far, the meticulous regulation of the Hh pathway by *ARHGAP36* has been studied (Nam *et al.* 2019, Zhang *et al.* 2019). Numerous studies suggest that the Hh pathway is involved in the growth and invasion of thyroid carcinomas (Xu *et al.* 2017), and the impression in the literature is that Hh is more common in poorly differentiated and anaplastic thyroid carcinomas. In the present study, ScRNA-seq showed that Hh pathway members were not expressed in PTC malignant cells, but rather, in some non-malignant cells. By knocking down *ARHGAP36* in PTC cells and then performing RNA sequencing, we found that cell proliferation and migration-related pathways were significantly affected, including cytokine – cytokine receptor interaction, transcriptional misregulation in cancer, CAMs, neuroactive ligand-receptor interaction pathways and so on. Member genes of the Hh pathway, however, were not involved. In combination with previous studies suggesting that there is a non-Hh pathway through which *ARHGAP36* functions (Rack *et al.* 2014, Beckmann *et al.* 2019), We believe that *ARHGAP36* relies on cytokines, CAMs, transcriptional misregulation and other non-Hh signaling pathways to perform its functions in PTC cells. In addition, our data show that cancer associated fibroblasts (CAFs) and fibroblasts in metastatic PTC have clear expression of *Gli* isoforms. Whether *ARHGAP36-Gli* is involved in the dialogue between cancer cells and CAFs remains to be studied.

In summary, our work provides important insights into PTC biology and an atlas of micro-ecosystems involved in primary and metastatic PTCs. By means of sc-RNA seq, our resolution of the expression and function of *ARHGAP36* in PTC opens a new avenue

for the identification of specific genes in individual cells of other human tumors. Although further studies are needed, *ARHGAP36* may be a potential diagnostic marker and therapeutic target of recurrence and metastasis in PTC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Author contribution statement

Z Y initiated and organized the study. T Y, W Q, and J S performed experiments and bioinformatics analyses, T Y, W Q, and Y F statistical analyses. T Y and Z Y wrote the manuscript. All authors read and approved the final manuscript.

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