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Concurrent *PIK3CA* and *TERT* Mutation Promote the Proliferation and Invasion of Thyroid Carcinoma Cells and may be Caused by Up-regulating the Expression of GABPA/GABPB1

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Abstract

Our previous research demonstrated that *TERT* and concurrent *PIK3CA* mutations predict worse overall survival in patients with poorly differentiated thyroid carcinoma and anaplastic thyroid carcinoma. However, the molecular mechanism underlying the synergistic oncogenic operations of the two oncogenes is unclear. This study aimed to explore further the effect of *TERT* and *PIK3CA* co-mutation on the malignant biological phenotype of thyroid carcinoma and its possible mechanism. *PIK3CA* E545K mutation plasmid was transfected into thyroid anaplastic cancer cell line (C643) with *TERT* promoter mutation, then CCK-8 and transwell invasion assays were used to investigate the ability of cell proliferation and invasion, respectively. RT-qPCR and western blot were performed to detect the expression of PIK3CA, TERT, GABPA and GABPB1. GABPA/GABPB1 siRNA plasmid was transfected with C643 cells, then the ability of cell proliferation and invasion were identified. We also detected the expression of PIK3CA and TERT. C643 cells carry *TERT* promoter mutation C228T. Concurrent *PIK3CA* E545K and *TERT* mutation markedly enhanced the proliferation and invasion of C643 cell *in vitro*, with significantly increased mRNA/protein expression of PIK3CA, TERT, GABPA and GABPB1. Knocking down GABPA markedly inhibited cell proliferation. Knocking down of GABPB1 significantly decreased the proliferation and invasion of C643 cells, with much lower expression of PIK3CA and TERT. *TERT* and *PIK3CA* co-mutations promote the proliferation and invasion of thyroid anaplastic carcinoma cells and may be caused by up-regulating the expression of GABPA and GABPB1.

Keywords: Thyroid carcinoma, TERT, PIK3CA, Proliferation, Invasion, GABPA, GABPB1

Introduction

Most thyroid carcinomas are differentiated thyroid cancer (DTC) and have an excellent prognosis. Poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) are rare but more aggressive and have a worse prognosis, with a mean survival time of 3.2 years and 6 months, respectively. Our previous study demonstrated that the genetic alterations detected in PDTC and ATC were mainly in *BRAF, RAS*, *TP53*, *TERT*, and *PIK3CA*. Mutation in *BRAF*, *TP53*, *TERT* or *PIK3CA* were predictors of poor overall survival (OS). Moreover, *TERT* and concurrent *PIK3CA* mutations predict poor overall survival in PDTC/ATC patients [1]. However, the molecular mechanism underlying the synergistic oncogenic operations of the two oncogenes is unclear.

PIK3CA mutation promotes tumor progression by regulating cell growth, proliferation, and dedifferentiation through the PI3K/AKT/mTOR signaling pathway. *PIK3CA* mutations are significantly associated with low OS in ATC [1-3]. *TERT* promoter mutation C228T or C250T produces new ETS binding sites, promoting TERT transcription. *TERT* promoter mutation in thyroid carcinoma is an independent adverse prognostic factor, significantly correlated with tumor recurrence, metastasis, and iodine refractory [1,4].

As a member of the ETS family, GA binding protein (GABP) transcription factors are composed of the DNA-binding

GABPA subunit and a transactivating GABPB subunit. GABPB1 and GABPB2 are paralogues encoding different β subunits of GABPB [5]. GABPB1L is the long protein isoform of GABPB1 [6]. High expression of GABPB1L promotes the formation of the GABPA-GABPB1L complex, which specifically binds and activates the mutant *TERT* promoter [7,8]. GABPB1L plays a role as an oncogene in malignant tumors, including thyroid cancer [7,9].

As for the molecular mechanism underpinning the synergistic oncogenic operations of *PIK3CA* and *TERT* oncogenes. Experiments were conducted using an anaplastic thyroid carcinoma C643 cell line, which carries the *TERT* promoter mutation [10]. We hypothesize that concurrent *PIK3CA* and *TERT* mutation may boost the proliferation and invasive ability of anaplastic thyroid carcinoma by up-regulating the expression of GABPA and GABPB1.

Materials and Methods

Sanger sequencing

The mutation hotspot regions within the *TERT* promoter in the C643 cell line were analyzed using direct Sanger sequencing. Isolation of DNA from cultured cells was performed with the Genomic DNA Mini Preparation Kit (D0063, Beyotime Biotechnology, Shanghai, China). qPCR was performed under the following conditions: 93°C for 5 min, (93°C 40s, 58°C 30s and 72°C 60s) ×40 cycles, and finally 72°C for 7 min. DNA sequences with a size of 2031bp were generated. Sequence analysis was performed and analyzed with the SeqStudio Gene Analyzer (Applied Biosystems/Thermo Fisher, Shanghai, China). The primer sequences used were available in **Supplementary File 1**.

Cell culture and cell transfection

The human ATC cell line C643 was purchased from Procell Life Science & Technology Co., Ltd (Shanghai, China). C643 cells were cultured in RPMI-1640 medium (E600028, Sangon Biotech, Shanghai, China) containing 10% fetal bovine serum (Gibco, New York, USA) and 1% penicillin-streptomycin (15140-122, Gibco, New York, USA), and maintained at 37°C with 5% CO_2.

The siRNAs targeting *PIK3CA*, *GABPA* and *GABPB1* were designed and synthesized by Beyotime Biotechnology (Shanghai, China). siRNA sequences are listed in **Supplementary File 1.** A plasmid containing the variant cDNA sequence (*PIK3CA* E545K (glutamate to lysine), which is one of the three common hotspot mutations of *PIK3CA* gene) was constructed. C643 cells were cultured to reach 90% confluence (**Blank group**). siRNA mediated targeting of PIK3CA **(si-PIK3CA group)** and its negative control **(si-NC group)** were transfected into C643 with TERT C228T mutation. Then plasmid with *PIK3CA* E545K mutation and negative control were respectively transfected into the above cells (PIK3CA E545K group and PIK3CA E545K-NC group). siRNA transfections were conducted using RNA TransMate reagent (E607402, Sangon Biotech, Shanghai, China). Plasmid DNA transfection was performed with Lipofectamine™ 3000 Transfection Kit (L3000015, Thermo Fisher, Shanghai, China) according to the manufacturer's instructions.

CCK-8 assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8) (E606335, BBI, Xianggang, China). Cells (Blank, si-NC, si-PIK3CA, PIK3CA E545K-NC and PIK3CA E545K) were respectively seeded in 24-well culture plates (3×10^4/well) with 3 repeats for each condition and placed at 37°C, incubated overnight in a 5% CO_2 incubator. After 24 hours, the cells were treated with 10 ul CCK-8 solution for another. 10 ul CCK-8 was added into each plate well at 24, 48, and 72 hours, and incubated at 37°C for an hour. A microplate reader (Infinite 200, TECAN, Switzerland) was used to measure the OD value of each well at 450 nm.

Transwell invasive assay

The concentration of the cell was 5×10^5/ml. Inoculate the constructed group cells into the wells of the Matrigel plate. After incubation at 37°C for 24 and 48 h, take out of the transwell chamber and discard the culture medium in the hole. Stain with 0.1% crystal violet for 20 minutes, gently remove cells that have not migrated through the well with a cotton swab, and wash with PBS 3 times. The cells were observed in 200× visual fields and counted. The cell number/200× field in different groups were compared using the statistical T test.

Western blot analysis

Cells were lysed with RIPA buffer with PMSF (R00105, Solarbio, Beijing, China). The proteins were quantitated by BCA Protein Assay Kit (C503021, Sangon Biotech, Shanghai, China). Twenty microliter protein lysates were electrophoresed in 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane (IPVH00010, Millipore, Massachusetts USA) using Mini-PROTEAN® Tetra Cell system (1658001, Bio-Rad) and PowerPac™ Universal power supply (164-5070, Bio-Rad). The proteins were blocked in 5% non-fat dried milk at room temperature for 2h, followed by incubation with the indicated dilutions of the primary antibodies overnight at 4°C. After washing three times (10 min for each) with TBST, the secondary anti-bodies (anti-rabbit or anti-mouse IgG-HRP) were added and incubated for a further 2 h at room temperature. The immunoreactive signals were visualized by ECL (Sangon Biotech, Shanghai, China) under Gel Document Tianneng (Zhejiang, China). The protein expression levels were quantified on the blots using Gel-Pro-Analyzer software. GAPDH was used as the loading control protein to verify the amount of total loading protein.

Primary antibodies against PIK3CA (1:1,000 dilution, rabbit anti-human PIK3CA antibody, 4249T, Cell Signaling Technology, Danvers, USA), TERT (1:500 dilution, mouse anti-human TERT antibody, NB100-37, Novusbio, Beijing, China), GABPA (1:500 dilution, mouse anti-human GABPA antibody, 5B6, Novusbio, Beijing, China), GABPB1 (1:1,000 dilution, rabbit anti-human GABPB1 antibody, GTX103464, GeneTex, Texas, USA) were used.

RT-qPCR assay

Total RNA was extracted from C643 cells employing the total RNA isolation kit (SuperfecTRI, Pufei Biotech Co., Ltd, Shanghai, China). cDNA was synthesized using an M-MLV kit (Promega, Ruibo Biotechnology Co., Ltd, Guangzhou, China) following the protocol provided by the manufacturer. qPCR was performed with the following profile: 95°C for 5 min and a total number of 40 cycles with a denaturation temperature of 95°C for 12s and an annealing temperature of 60°C for the 40s. A melting curve was obtained after amplification to provide evidence for a single reaction product. Human ACTB/GAPDH was employed as a housekeeping gene. The samples were run in triplicate and the mean value was calculated for each case. CT value of target genes minus CT value of ACTB/GAPDH gene is Δ Ct.

Statistical analyses

Statistical analyses were conducted with SPSS version 23.0. Comparisons between groups were performed via independent *t-test*. Statistical significance was defined as two-sided values of $P<0.05$. All experiments were repeated at least three times or two times. Graphical representations were performed on GraphPad Prism 7.00.

Results

Thyroid anaplastic carcinoma C643 cells and cell models construction

TERT C228T promoter mutation was detected in the thyroid anaplastic carcinoma C643 cell line by Sanger sequence (**Figure1A**), and a higher PIK3CA mRNA expression level was observed in C643 cells (**Figure 1B**). To reduce the background expression of PIK3CA, C643 cells were transfected with PIK3CA siRNA-2 (**si-PIK3CA group**) and its negative control (**si-NC group**). The mRNA and protein of PIK3CA of the si-PIK3CA group was significantly reduced than that of the control group (**Figure 1C and Figure 3E**). At the same time, the ability of cell proliferation and invasion is weakened (**Figure 2**). To explore whether cells with concurrent *PIK3CA* and *TERT* mutation are more aggressive, PIK3CA E545K plasmid were transfected into C643 siPIK3CA group (**PIK3CA E545K group**, corresponding to the concurrent mutation of *PIK3CA* and *TERT*) and its negative control (**PIK3CA E545K-NC group**, corresponding to the *TERT* mutation). The mRNA and protein of PIK3CA of the PIK3CA E545K group was significantly increased than that of the control group (**Figure 1D and Figure 3E)**.

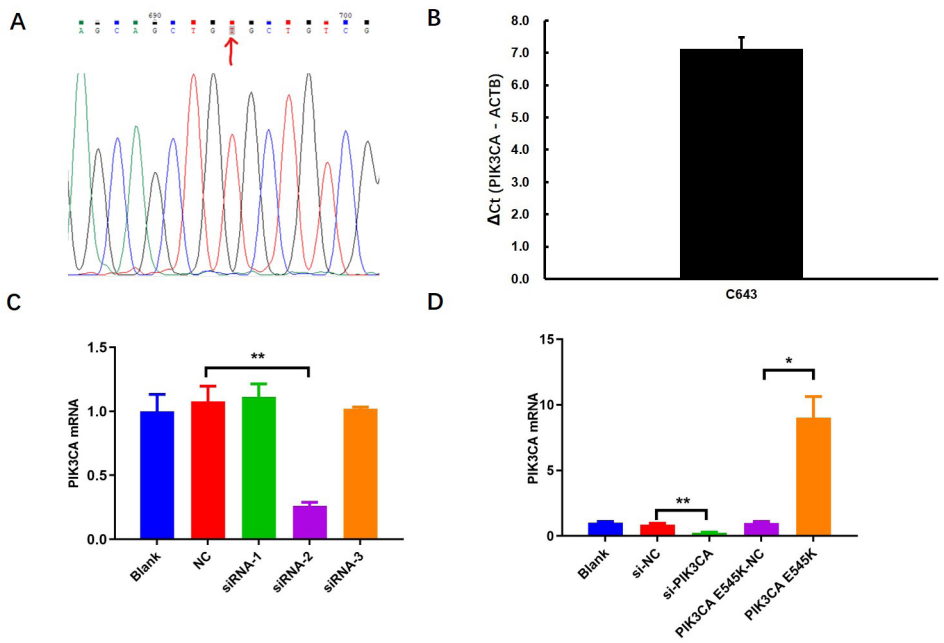

Figure 1. Thyroid anaplastic carcinoma C643 cells and transfection efficiency assays. A. *TERT* promoter mutation C228T was detected in the C643 cell line by the Sanger sequence. **B**. PIK3CA mRNA expression level in C643 cells. The value of ΔCT(CTPIK3CA-CTACTB) was less than 12, which indicated the expression of PIK3CA gene was high in C653 cells. **C**. PIK3CA siRNA-2 showed the highest transfection efficiency and was used in further experiments. **D**. The transfection efficiency of PIK3CA E545K expression was detected.

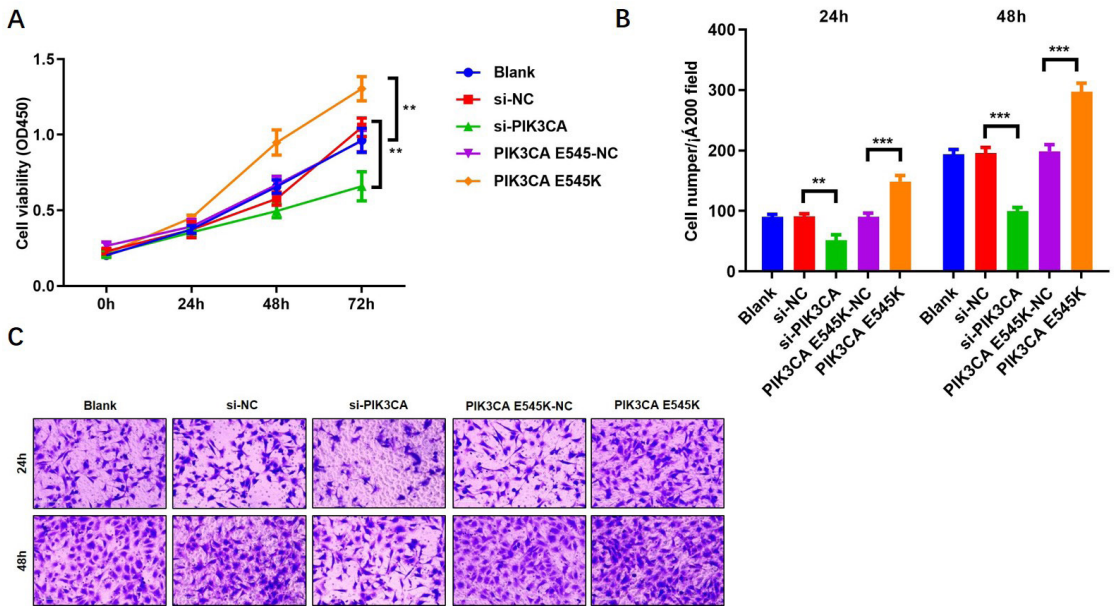

Figure 2. Concurrent *PIK3CA* and *TERT* mutation enhanced cell proliferation and invasion. A. CCK-8 analysis revealed that cell viability was significantly enhanced in PIK3CA E545K group compared with that of the PIK3CA E545K-NC group. **B and C**. Transwell invasive assay revealed that cell invasion was significantly enhanced in the PIK3CA E545K group compared with that in the PIK3CA E545K-NC group. The average invasive cell number of PIK3CA E545K group was more than that of PIK3CA E545K-NC group. Microscopic images of migrated C643 cells at 24 hours and 48 hours (magnification 200). ** p < 0.01. *** p < 0.001. OD optical density.

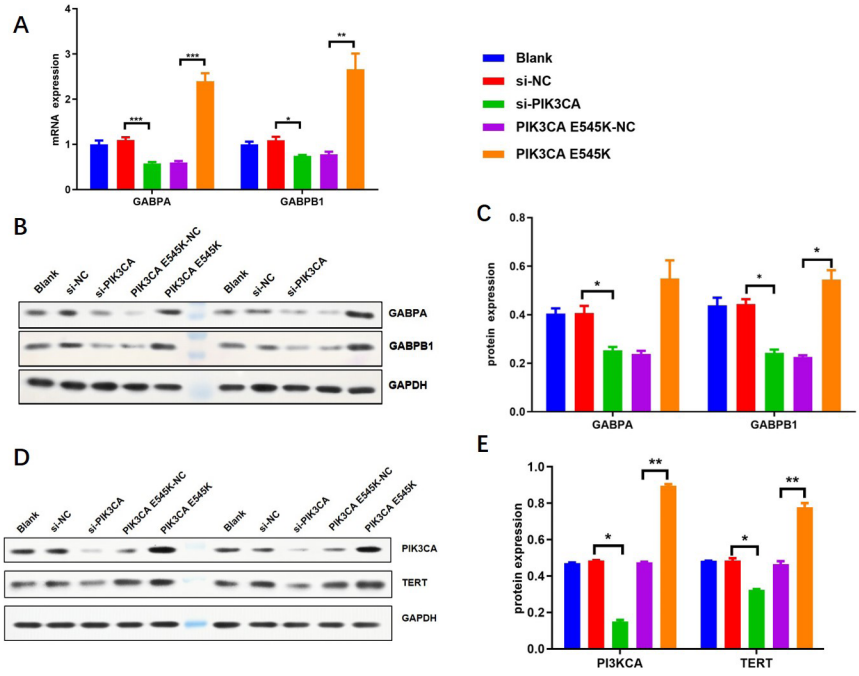

Figure 3. Concurrent *PIK3CA* and *TERT* mutation up-regulated the expression of TERT, GABPA, and GABPB1. Compared with that in PIK3CA E545K-NC cells, PIK3CA E545K cells up-regulated the expression of GABPA/GABPB1 mRNA (**A**), GABPA/GABPB1protein (**B,C**) and TERT protein(**D,E**). * p<0.05.** p<0.01. *** p<0.001.

Concurrent *PIK3CA* and *TERT* mutation promote the proliferation and invasion of thyroid carcinoma cell line

To detect whether PIK3CA E545K group (*PIK3CA* and *TERT* concurrent mutated cells) are more proliferative or invasive, the ability of cell viability and invasion were measured. At 72 hours, cells viability in the PIK3CA E545K group was significantly enhanced than that in the negative control group (PIK3CA E545K-NC group) (**Figure 2A**). Transwell invasive assay revealed that the average cells number in the PIK3CA E545K group were much more than that in the PIK3CA E545K-NC group at 24 hours or 48 hours (**Figures 2B and 2C**). Therefore, concurrent *PIK3CA* and *TERT* mutation may enhance cell proliferation and invasion.

Concurrent *PIK3CA* and *TERT* mutation up-regulates the expression of TERT, GABPA, and GABPB1

GABPA-GABPB1 complex specifically binds and activates the mutant TERT promoter [7]. To explore the role of GABPA/GABPB1 in the molecular mechanism underpinning the synergistic oncogenic operations of the *PIK3CA* and *TERT* promoter mutation, the expression of GABPA/GABPB1, as well as PIK3CA and TERT, were detected by qPCR and western blot, respectively. The results indicated that, compared with si-NC group, si-PIK3CA group cells down-regulated the expression of TERT, GABPA, and GABPB1 protein (**Figures 3B-3E**), as well as the expression of GABPA and GABPB1 mRNA (**Figure 3A**). Meanwhile, compared with that in PIK3CA E545K-NC cells, PIK3CA E545K up-regulated the expression of TERT, GABPA, and GABPB1 protein (**Figures 3B-3E**), as well as the expression of GABPA and GABPB1 mRNA (**Figure 3A**). Therefore, concurrent *PIK3CA* and *TERT* mutation (PIK3CA E545K group) may up-regulates the expression of TERT, GABPA, and GABPB1.

Knocking down of GABPA weakens the ability of cell proliferation

Based on the above research results, we speculate that GABPA and GAPBPB1 may play a key role in regulating cell proliferation and invasion. GABPA siRNA-3 and GABPB1 siRNA-2 were separately transfected with the PIK3CA E545K group cells (**si-GABPA/GABPB1 group**) and its negative control group **(si-NC group**). The mRNA of GABPA/GABPB1 of the si-GABPA/GABPB1 group were significantly reduced than that of the control group (**Figures 4A and 4B**). Compared with that in the negative control, si-GABPA group cells' proliferation ability was significantly decreased at 72h (**Figure 4C).** However, the cell invasive ability and the expression of PIK3CA and TERT mRNA/protein showed no significant change between the above two groups (**Figure 5**).

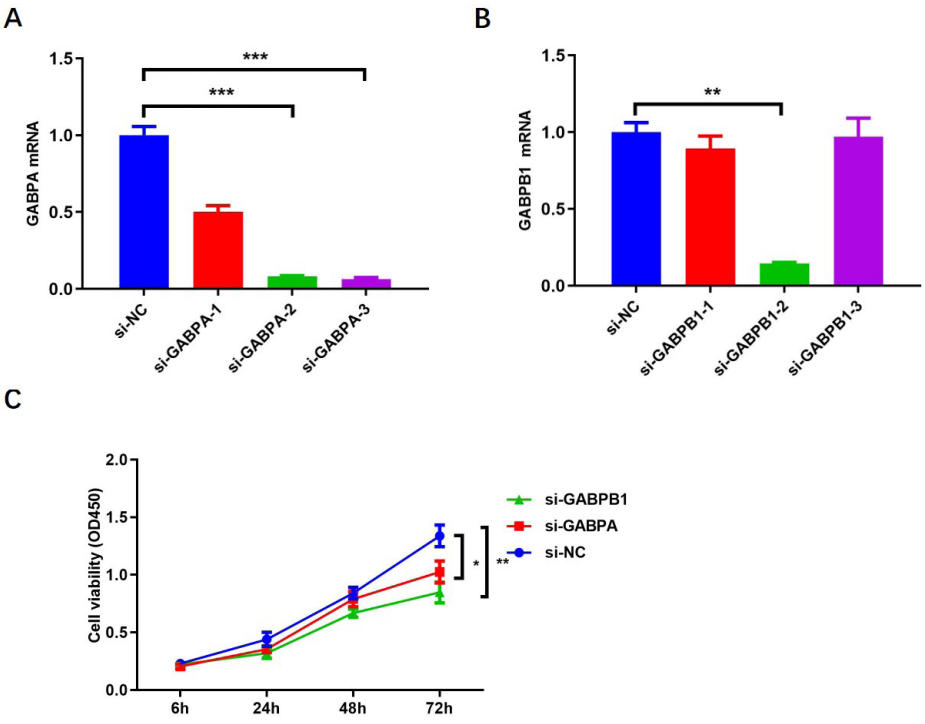

Figure 4. Knocking down of GABPA/GABPB1 weakened the ability of cell proliferation and invasion. A. si-GABPA-2/3 showed the higher transfection efficiency and si-GABPA-3 were used in the further experiment. **B.** si-GABPB1-2 showed the highest transfection efficiency and were used in further experiments. **C**.CCK-8 analysis revealed that cell viability was significantly decreased in the si-GABPA/GBAPB1 cells compared with that in the si-NC cells. * p<0.05.** p<0.01. *** p<0.001. OD: Optical Density.

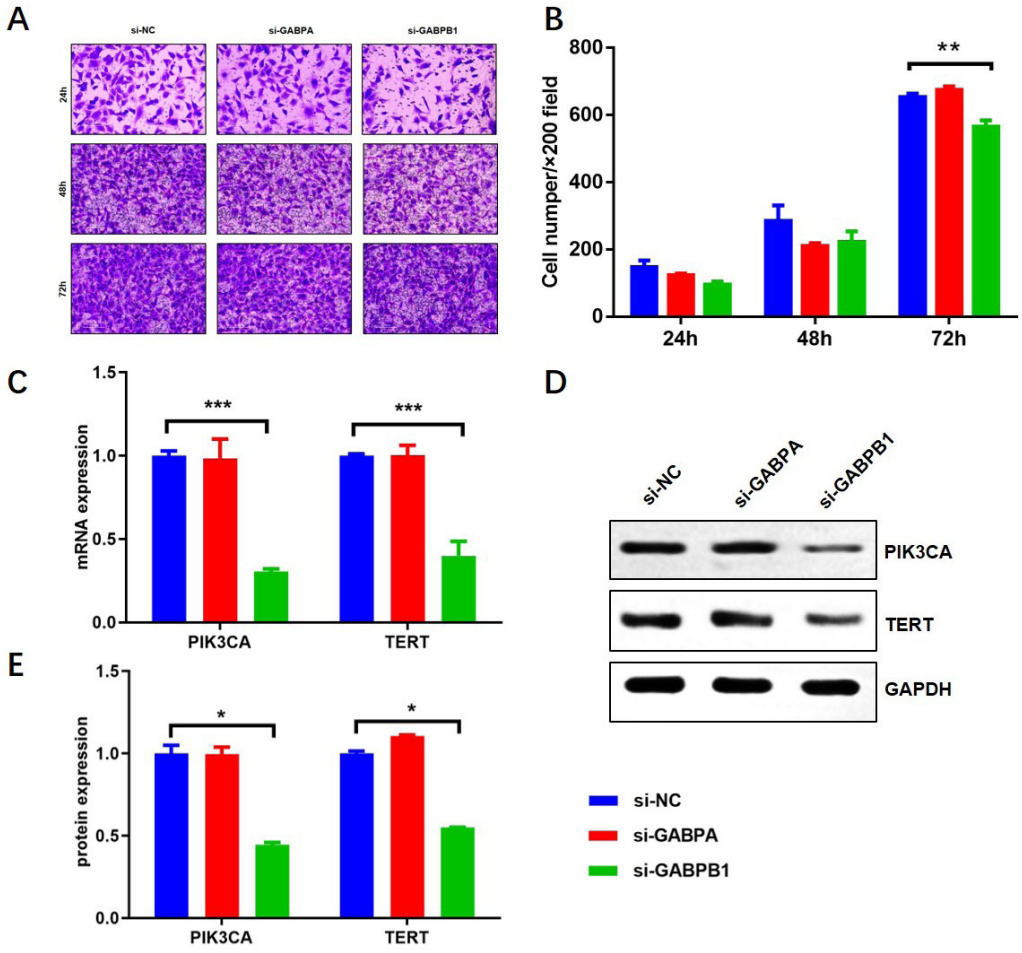

Figure 5. Knocking down of GABPB1 expression weakened the cell invasion ability, and down-regulated the expression of PIK3CA and TERT. A-B. Transwell invasive assay revealed that cell invasion was significantly weakened in the si-GABPB1 cells at 72 hours (magnification 200). **C**. PIK3CA and TERT mRNA were significantly down-regulated in the cells of si-GABPB1 by qPCR. **D-E**. PIK3CA and TERT protein were significantly down-regulated in the cells of si-GABPB1 by western blot. * p<0.05.** p<0.01. *** p<0.001.

Knocking down of GABPB1 expression weakens the ability of cell proliferation and invasion, and down-regulates the expression of PIK3CA and TERT

As shown above, si-GABPB1-2 was transfected with the PIK3CA E545K cells (**si-GABPB1 group**). Compared with that in the negative control (**si-NC group**), the CCK-8 assay, and transwell cell invasion assay indicated that the ability of si-GBAPB1 group cell proliferation ability decreased at 72h (**Figure 4C)**, and the cell invasion ability decreased at 72 hours (**Figures 5A and 5B**). Moreover, the expressions of PIK3CA and TERT were significantly down-regulated in that group by qPCR (**Figure 5C**) and western blot (**Figures 5D and 5E**).

Discussion

Previously, we demonstrated that *TERT* and concurrent *PIK3CA* mutations predict worse overall survival in PDTC/ATC patients [1]. However, the molecular mechanism underpinning the synergistic oncogenic operations of the two oncogenes is unclear. GABPA-GABPB1 complex specifically binds and activates the mutant *TERT* promoter. We aimed to explore the role of GABPA/GABPB1 in regulating the proliferation and invasion of thyroid carcinoma cell line with *TERT* promoter mutation. Our results revealed that *TERT* and *PIK3CA* co-mutations might promote cell proliferation and invasion by up-expressing the GABPA and GABPB1.

Mutant *PIK3CA* is likely to function as an oncogene among ATC and less frequently in well-differentiated thyroid carcinomas [11]. *PIK3CA* E545K plays a role in driving disease progression from a well-differentiated to an undifferentiated thyroid cancer [12]. *PIK3CA* mutation was identified in 14-44% of cases with ATC. *PIK3CA* mutations were significantly associated with poor outcomes [1,2]. Pinto et al. research indicated in culture, *PIK3CA* E545K engineered cell line proliferated faster [12].

No research on the effect of *PIK3CA* mutation on GABPA or GABPB1 was found. It is well established that *TERT* promoter mutation was the independent predictor of OS in thyroid carcinoma. Overexpression of TERT promoted cell proliferation by activating AKT signaling pathway in thyroid cancer cell line [13].

Our results and previous research indicated that C643 cell line carries *TERT* promoter mutation and *HRAS* mutation [14-16]. *HRAS* mutation may promote the activation of PI3K-AKT signaling pathways [17]. TERT over-expressed cells could stimulate PI3K/AKT pathway through inhibiting PTEN expression [13]. That may explain why the C643 cell line showed increased PIK3CA mRNA expression.

The ETS family transcription factors GABPA and GABPB1 have been shown to act as master drivers for the mutant *TERT* promoter activity. Moreover, GABPA or GABPB1 depletion down-regulated the TERT expression in the mutant *TERT* promoter bearing cancer cells [6,18]. This partially agrees with our research results, since GABPB1 depletion led to a significant decrease in TERT expression while GABPA depletion had no influence on TERT expression. With the development of research, GABPA may act as a potent tumor suppressor in several malignancies, including thyroid carcinoma [6,18,19]. Lower GABPA expression was associated with distant metastasis and shorter overall/disease-free survival in PTC patients [18]. However, our study illustrates that GABPA expression was higher in C643 cells with *TERT* promoter mutation and *PIK3CA* E545K mutation, and lower GABPA expression inhibited cell proliferation and invasion. More research is needed to further demonstrate the role of GABPA in thyroid cancer.

The role of GABPB1 in cancer is inconsistent. In *TERT* promoter mutant glioblastoma, GABPB1 was highlighted as having a critical role in enabling immortality [9]. Recently, Xing et al. study suggested that GABPB1 was required for TERT expression and telomerase activation, but itself served as a tumor suppressor to inhibit TC progression [20]. Similarly, we demonstrated that lower GABPB1 inhibited TERT expression. Lower GABPB1 expression was significantly associated with weaker cell proliferation and invasion in our study. More research is needed to investigate the role of GABPB1 in thyroid cancer.

The oncogenic mechanism of the *TERT* and *PIK3CA* co-mutation on anaplastic cancer cells may be correlated with elevated expression of GABPA and GABPB1, which promotes TERT expression, promoting cell proliferation and invasion. On the other hand, the gene mutation rate impacted survival in poorly differentiated thyroid carcinoma [1]. TP53 mutation was considered the definite markers of PTC transition from differentiated to undifferentiated carcinoma. Our previous research indicated poorly differentiated thyroid carcinoma with *TERT* and *PIK3CA* mutations were more correlated with *TP53* mutations, which may, to some extent, explain why concurrent *TERT* and *PIK3CA* had more aggressive behavior [1].

There are several limitations in our study. One, in this study, cell proliferation was measured by cell viability instead of direct cell proliferation. The decreased cell number could be caused by more cell death. On the other hand, *TERT* or *PI3KCA* mutation alone is significantly associated with aggressive tumor behavior. It would be great to add experiments to measure proliferation and invasion using cells with single mutation compared to each corresponding wild-type cell.

To sum up, our research indicates that *PIK3CA* and *TERT* co-mutations promoted cell proliferation and invasion and may be caused by up-expression of GABPA and GABPB1 in thyroid cancer. Molecular marker-based risk stratification of thyroid cancer may be proposed to better predict the cancer risk.

Conflict of Interest

The authors declare no competing interests.

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Author Contributions Statement

LH Teng designed the study. HL Duan and Q Ma performed the experiment and data collection. HL Duan, Q Ma, LM Wang, SN Wang and YL Xiong contributed to the date analysis and interpretation. HL Duan and Q Ma contributed to the draft of the manuscript. All authors read and revised the manuscript, and then approved the final manuscript.

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