TRIM21-a potential biomarker for the prognosis of thyroid cancer

ZHEN WU1,2, YONGKUN WANG3, ZHENGYA YU1, ZHEN MENG3, WENCHAO DUAN4, WEI ZHANG2 and JUGAO FANG1

1Department of Otorhinolaryngology Head and Neck Surgery and Department of Thyroid Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing 100730; 2Department of Thyroid and Breast Surgery, Liaocheng People's Hospital; 3Department of Stomatology, Medical School of Liaocheng University, Liaocheng, Shandong 252000; 4Department of Otorhinolaryngology, Liaocheng People's Hospital, Liaocheng, Shandong 252000, P.R. China

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Abstract. Thyroid cancer (THCA) is one of the commonest malignancies associated with increased recurrence. Therefore, identifying the putative molecular markers and therapeutic targets to improve the treatment of THCA is essential. The present study analyzed the potential role of tripartite motif-containing 21 (TRIM21), a member of the TRIM family belonging to the subfamily of E3 ubiquitin ligases, in the progression of THCA. Using bioinformatics analysis and immunohistochemistry of THCA tissues, it was observed that TRIM21 is overexpressed in THCA tissues. The present study also found that TRIM21 is associated with lymph node metastasis and high-risk recurrence of THCA. Furthermore, it identified a promotional role of TRIM21 in THCA cell migration and invasion. In addition, the present study analyzed TRIM21-enriched pathways and co-expressed genes in THCA. The present study suggested that TRIM21 may serve as a potential biomarker for THCA prognosis.

Introduction

Thyroid cancer (THCA) is one of the commonest malignancies associated with increased recurrence (1). Generally, THCA shows a good overall prognosis and low fatality rate in most cases; however, due to its aggressive characteristics and metastasis, and poor prognosis can be seen in some patients with THCA (2). Identifying available molecular markers and therapeutic targets is urgently required to improve the treatment outcome.

The tripartite motif (TRIM) family of proteins belongs to the subfamily of E3 ubiquitin ligases and participates in various biological and pathophysiological processes, including tumor progression (3‑5). TRIMs share similar domains in their protein structure, including the N-terminal RING domain with E3 ubiquitin ligase activity, the B‑box domain, and the coiled-coil domain (6). Several members of the TRIM family are associated with tumorigenesis and disease progression of THCA. TRIM14 has been reported as an oncogene in THCA (7). TRIM44 knockdown suppresses the tumor progression of THCA by inhibiting the Wnt/β‑catenin signaling pathway (8). TRIM8 serves as a target for miR‑182 in promoting tumor growth and increasing chemoresistance in human THCA (9). However, the roles of other TRIMs in THCA remain to be elucidated. The authors of the present study aim to investigate the roles of other TRIMs in THCA and so far, TRIM21 is the one which has been elucidated.

The present study aimed to identify the role of TRIM21 in THCA and to analyze the functional networks related to TRIM21 using public databases such as The Cancer Genome Atlas (TCGA) database. The function of TRIM21 in the proliferation, migration and invasion of THCA cells was evaluated.
Materials and methods

Clinical sample collection. Paraffin tumor tissue samples and paraffin para-tumor normal tissues 1 cm away from the tumor tissues were collected from 120 patients diagnosed with papillary thyroid carcinoma and who underwent surgical resection in Liaocheng People's Hospital between 2018 and 2020. The patients were aged from 21-70. The clinical information of all cases was collected and is given in Table I. All patients were free of other malignancies or a history of chemoradiotherapy. Written informed consent was obtained from all the participants. The experiment was approved by the Ethics committee of Liaocheng People's Hospital (approval no. LC2021059).

Immunohistochemical staining and scoring. Immunohistochemical staining (IHC) was performed to detect the expression of TRIM21 in papillary thyroid carcinoma tissues. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (cat. no. 88597; Merck KGaA) after routine dewaxing, hydration, and antigen retrieval. Permeabilization of samples was performed using 0.1% Triton X-100 (cat. no. ST797; Beyotime Institute of Biotechnology) and blocked with 5% bovine serum albumin (BSA) (cat. no. ST025; Beyotime Institute of Biotechnology). Tissue sections (10 µm thick) were incubated with TRIM21 antibodies (1:200 dilution; ProteinTech; cat. no. 121081-1-AP) at 4˚C for 12 h. After washing with phosphate-buffered saline (PBS), the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1,000 dilution; Jackson Immuno Research Laboratories, USA) at 20˚C. TRIM21 expression was visualized using 3,3'-diaminobenzidine (DAB; cat. no. P0202; Beyotime Institute of Biotechnology). Tissue sections were counterstained witha hematoxylin. Brown-yellow staining indicated a positive expression. Staining score = staining intensity score x staining-positive area score. The staining intensity was scored as 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). The score of the staining-positive area was recorded according to the proportion of positive cells: 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 points (>75%). A staining score of <3 was classified as low TRIM21 expression, and those ≥3 were classified as high TRIM21 expression.

Reverse-transcription quantitative (RT-q) PCR. TRIzol® reagent (cat. no. 15596-026; Thermo Fisher Scientific, Inc.) was used for total RNA isolation according to the manufacturer's protocols. To quantify TRIM21 expression, the total RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent (cat. no. 15596-026; Thermo Fisher Scientific, Inc) containing 10% fetal bovine serum (FBS, cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at 20˚C. TRIM21 expression was visualized using 3',3'-diaminobenzidine (DAB; cat. no. P0202; Beyotime Institute of Biotechnology) staining at 20˚C for 1 min. A blind evaluation was performed by two pathologists. Brown-yellow staining indicated a positive expression. Staining score = staining intensity score x staining-positive area score. The staining intensity was scored as 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). The score of the staining-positive area was recorded according to the proportion of positive cells: 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 points (>75%). A staining score of <3 was classified as low TRIM21 expression, and those ≥3 were classified as high TRIM21 expression.

Public database data sources. THCA transcriptome data were downloaded from The Cancer Genome Atlas (TCGA) database using the UCSC Xena tool (https://xena.ucsc.edu/). TRIM21 expression levels in all types of cancer obtained from the cBioPortal database (https://www.cbioportal.org/) were analyzed using R software version 3.6.1 (http://www.R-project.org/). An unpaired Student's t-test was applied to compare TRIM21 expression in various cancers with that in normal tissues.

Gene set enrichment analysis (GSEA) of TRIM21-related cancer pathways. THCA transcriptome expression profiles were obtained from the TCGA database (https://gdc-portal.nci.nih.gov/). A total of 510 THCA samples and 59 normal tissues derived from healthy individuals were included in the present study. The correlations between the Kyoto Encyclopedia Of Genes And Genomes (KEGG) signaling pathways of TRIM21 and co-expressed genes were explored using GSEA (http://software.broadinstitute.org/gsea/index.jsp) (20). The latter was performed using three enrichment statistics: Enrichment scores, normalized enrichment scores and nominal P-values. The enrichment score indicates the degree of enrichment of a functional gene set before or after a given sequence. The normalized enrichment score is the major parameter in enrichment analyses of functional gene sets. The nominal P-value indicates the statistical significance of the enrichment score of a given functional gene subset with lower P-values. KEGG enrichment was reported at a significance threshold of P<0.05.

Cell culture and lentivirus infection. FTC-133 cell line (cat. no. 1101HUM-PUMC000687) was purchased from the National Infrastructure of Cell Line Resource of China. The cells were cultured in Dulbecco's modified eagle medium F-12 (DMEM-F12, cat. no. 11320-033, Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetow serum (FBS, cat. no. 10100147, Gibco; Thermo Fisher Scientific, Inc.) in an incubator with 5% CO2 at 37°C.

Cultured cells were seeded into 24-well plates at 30,000 cells/well. Once the cells reached 90% confluence, lentivirus (Lv-shCon or Lv-shTRIM21) was added to the wells at a multiplicity of infection (MOI) of 10. At 48 h later, infected cells were selected using 10 µg/ml puromycin (cat. no. ST551; Beyotime Institute of Biotechnology). Virally infected cells were observed under a fluorescence microscope (cat. no. IX73; Olympus Corporation). TRIM21 expression was determined using RT-qPCR analysis. Lv-shCon and Lv-shTRIM21 were designed and constructed at Shanghai GeneChem Co., Ltd. The targeting sequence of Lv-shCon was: 5'-AAC AAG ATG AAG AGC ACC AAC -3'. The targeting sequence of Lv-shTRIM21 was: 5'-GGA AGT CAC TTC ACC ATC ACT CGA GTGA TGG TGA AGT GAC TTC CTT TTT T-3'. The targeting sequence of Lv-shTRIM21 was: 5'-AAC AAG ATG AAG AGC ACC AAC -3'.

Transwell assay. Migration: Cells were seeded into the upper chambers of a Transwell plate. The upper chambers contained DMEM media without FBS, whereas the lower
chambers contained DMEM media with 10% FBS. The cells were cultured for 24 h, and then the remaining cells in the upper chamber were wiped away with a swab. The cells passed through the membrane were stained with crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology). The cells in three randomly selected visual fields were

Table I. Relationship between TRIM21 expression and clinicopathologic features of patients with thyroid papillary carcinoma.

| Pathological clinical data          | Expression of TRIM21 protein in cancer tissue |
|------------------------------------|---------------------------------------------|
|                                    | High-expression (89 cases) | Low-expression (31 cases) | Statistical quantity | P-value |
| Sex                                |                             |                            |                        |         |
| Male                               | 35                          | 10                         | $\chi^2 = 0.23$        | 0.62    |
| Female                             | 54                          | 21                         |                         |         |
| Age (years)                        |                             |                            |                        |         |
| <55                                | 46                          | 19                         | $\chi^2 = 0.51$        | 0.47    |
| $\geq 55$                          | 43                          | 12                         |                         |         |
| Tumor diameter (cm)                |                             |                            |                        |         |
| ~1.0-2.0                           | 36                          | 11                         | $\chi^2 = 0.55$        | 0.75    |
| ~2.1-3.0                           | 28                          | 12                         |                         |         |
| ~3.1-4.0                           | 25                          | 8                          |                         |         |
| Extragranular invasion             |                             |                            |                        |         |
| Yes                                | 60                          | 11                         | $\chi^2 = 2.78$        | 0.09    |
| No                                 | 29                          | 21                         |                         |         |
| Lymph node metastasis (pieces)     |                             |                            |                        |         |
| No                                 | 11                          | 10                         | $\chi^2 = 12.63$       | 0.002   |
| ~1-3                               | 30                          | 15                         |                         |         |
| $\geq 4$                           | 48                          | 6                          |                         |         |

Figure 1. TRIM21 expression levels in different types of tumors. *P<0.05; **P<0.01; ***P<0.001. ACC, adrenocortical carcinoma; BLCA, bladder carcinoma; BRCA, breast carcinoma; CESC, cervical squamous cell carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal Papillary carcinoma; LAML, acute myeloid leukemia; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCGP, pheochromocytoma and paraganglioma; PRAD, rectum adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testis germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.
counted under a fluorescence microscope (cat. no. IX73; Olympus Corporation).

Invasion: The invasion assay was performed with the similar procedures as the migration assay, excepting that the wells were

Table II. Expression levels of TRIM in various cancers vs. normal tissue included in TCGA.

| Tumor     | Normal     | P-value   |
|-----------|------------|-----------|
| KIRC.Tumor (n=533) | KIRC.Normal (n=72) | 2.08x10⁻²⁰ |
| LUSC.Tumor (n=501)  | LUSC.Normal (n=51)  | 1.22x10⁻¹⁸ |
| HNSC.Tumor (n=520)  | HNSC.Normal (n=44)  | 2.37x10⁻¹¹ |
| LUAD.Tumor (n=515)  | LUAD.Normal (n=59)  | 8.63x10⁻⁹  |
| CHOL.Tumor (n=36)   | CHOL.Normal (n=9)   | 6.77x10⁻⁸  |
| LIHC.Tumor (n=371)  | LIHC.Normal (n=50)  | 8.37x10⁻⁶  |
| UCEC.Tumor (n=545)  | UCEC.Normal (n=35)  | 1.55x10⁻⁵  |
| ESCA.Tumor (n=184)  | ESCA.Normal (n=11)  | 6.16x10⁻⁴  |
| GBM.Tumor (n=153)   | GBM.Normal (n=5)    | 1.96x10⁻⁴  |
| STAD.Tumor (n=415)  | STAD.Normal (n=35)  | 2.54x10⁻³  |
| KICH.Tumor (n=66)   | KICH.Normal (n=25)  | 2.94x10⁻³  |
| THCA.Tumor (n=501)  | THCA.Normal (n=59)  | 1.55x10⁻²  |
| COAD.Tumor (n=457)  | COAD.Normal (n=41)  | 4.65x10⁻²  |
| PRAD.Tumor (n=497)  | PRAD.Normal (n=52)  | 9.42x10⁻²  |
| PAAD.Tumor (n=178)  | PAAD.Normal (n=4)   | 9.59x10⁻²  |
| BRCA.Tumor (n=1093) | BRCA.Normal (n=112) | 1.59x10⁻¹  |
| KIRP.Tumor (n=290)  | KIRP.Normal (n=32)  | 3.10x10⁻¹  |
| READ.Tumor (n=166)  | READ.Normal (n=10)  | 3.69x10⁻¹  |
| BLCA.Tumor (n=408)  | BLCA.Normal (n=19)  | 5.75x10⁻¹  |
| CESC.Tumor (n=304)  | CESC.Normal (n=3)   | 6.83x10⁻¹  |
| PCPG.Tumor (n=179)  | PCPG.Normal (n=3)   | 9.38x10⁻¹  |

Figure 2. The Kaplan-Meier curve of prognosis is related TRIM21 expression level in different tumor samples. The blue and red curves represent the high and low TRIM21 expression sample groups, respectively. TRIM21, tripartite motif-containing 21; THCA, thyroid carcinoma; SKCM, skin cutaneous melanoma; SARC, sarcoma; MESO, mesothelioma; LGG, Low-grade glioma; KIRC, kidney renal clear cell carcinoma.
pre-coated with 20 µg Matrigel at 37°C for 2 h (MilliporeSigma).

**CCK-8 assay.** Cells were seeded into a 96 well plate at 5,000 cell/well. After the cells were cultured for 0, 24 and 48 h in an incubator with 5% CO₂ at 37°C, 10 µl of CCK-8 reagent (cat. no. C0037; Beyotime Institute of Biotechnology) was added. The cells were then incubated for 1 h at 37°C and the absorbance at 450 nm wavelength was measured using a Multiskan GO microplate reader (Thermo Fisher Scientific, Inc.).

**Statistical analysis.** All pathological and experimental data were analyzed using SPSS software (version 25.0; IBM Corp.). The measurement data conformed to a normal distribution and are presented as mean ± standard deviation (SD). The χ² test was performed to analyze the association between TRIM21 expression and clinicopathologic features of patients with thyroid papillary carcinoma. Considering the normal tissues derived from different patients to those who donated the cancer tissues, the expression of TRIM21 in 14 different types of tumors was evaluated using an unpaired Student's t-test. TRIM21 expression in 120 pairs THCA and the corresponding adjacent normal tissues was evaluated using paired Student's t-test. P-value was obtained from a two-tailed Student's t-test. The survival time of the patients was calculated using the Kaplan-Meier method. Log-rank test was performed to analyze the Kaplan-Meier survival curves. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**TRIM21 expression in multiple tumor sites.** TRIM21 expression was assessed in various cancers based on TCGA and GTEx databases. TRIM21 expression was dysregulated in the 14 tumor types compared with the corresponding normal tissues, including kidney renal clear cell carcinoma (KIRC), lung squamous cell carcinoma (LUSC), head and neck squamous carcinoma (HNSC), lung adenocarcinoma (LUAD), cholangiocarcinoma (CHOL), liver hepatocellular carcinoma (LIHC), uterine corpus endometrial carcinoma (UCEC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), stomach adenocarcinoma (STAD), kidney chromophobe (KICH), skin cutaneous melanoma (SKCM) tumor and THCA. (Fig. 1 and Table II).

**Association of TRIM21 with the survival of patients with THCA.** In the TCGA data set, according to TRIM21 expression, tumors were divided into Low TRIM21 group and High TRIM21 group. Fig. 2 and Table I show the survival analysis of the two groups. In low grade gliomas, the low TRIM21 group showed a better prognosis than that in the High TRIM21 group. In contrast, in SKCM, mesothelioma (MESO), sarcoma (SARC), KIRC, and THCA, the patients in the high TRIM21 group showed a better prognosis compared with that in the low TRIM21 group.

**TRIM21 expression in THCA and normal tissues.** The expression of TRIM21 in 120 THCA and corresponding para-cancer normal tissues was evaluated using IHC. As shown in Fig. 3A, the staining intensity of TRIM21 in THCA tissues was significantly higher than that in para-cancer normal tissues. Simultaneously, the staining score of TRIM21 protein was calculated, and the score in THCA tissues was significantly higher than that in para-cancer normal tissues (Fig. 3B). These results indicate that TRIM21 is overexpressed in THCA.

**Figure 3. Expression of TRIM21 in THCA.** (A) IHC staining of TRIM21 in THCA and para-cancer normal tissues. Magnification: Left, x40; Right, x200. (B) The IHC staining score of TRIM21 protein in THCA and para-cancer normal tissues. *P<0.05. TRIM21, tripartite motif-containing 21; THCA, thyroid carcinoma; IHC, immunohistochemistry.

**Figure 4. Expression of TRIM21 mRNA in high- and low recurrence-risk group.** *P<0.05. TRIM21, tripartite motif-containing 21.
Table I, the expression of TRIM21 showed no relevance to patients' gender, age, tumor diameter, and extra-granular invasion; however, TRIM21 was significantly associated with lymph node metastasis. According to the recurrence risk based on the American Thyroid Association (ATA) guidelines 2021 (21), 120 patients were divided into high- and low recurrence-risk groups. As shown in Fig. 4, the expression of TRIM21 was measured using RT-qPCR and the expression of TRIM21 in the high recurrence-risk group was 1.69 folds of that in the low recurrence-risk group (P=0.0242).

Knockdown of TRIM21 induced inhibition of cell proliferation, migration and invasion of THCA cells. As TRIM21 was overexpressed in THCA and was associated with lymph node metastasis, the role of TRIM21 in THCA cell migration and invasion was further examined. TRIM21 was knocked down in lentivirus-infected FTC-133 cells. As shown in Fig. 5A, green fluorescent labeling indicated that the cells were infected with lentivirus. Fig. 5B demonstrated that TRIM21 expression was reduced ~62.88% in Lv-shTRIM21 infected cells, indicating that the efficiency of TRIM21 knockdown was 37.12%. Cell migration and invasion capacities were measured using Transwell assays. The migration and invasion capacities of Lv-shTRIM21-infected cells were inhibited compared to those infected with Lv-shCon (Fig. 5C, D and E). These results indicate that TRIM21 knockdown inhibits proliferation, migration and invasion of THCA cells.

**KEGG analysis of TRIM21 and TRIM21 co-expression genes in THCA.** The potential biological functions of TRIM21 with high or low expression in THCA were investigated using GSEA, and the genes were significantly enriched in 38 KEGG pathways, including ‘butanoate metabolism’, ‘oxidative phosphorylation’, and ‘valine leucine and isoleucine degradation’ (Fig. 6).

After excluding the genes that met the condition of false discovery rate <0.05 and with a correlation coefficient >0.6 were included. A total of 252 TRIM21 co-expression genes were identified, including SP110, APOL2, and UBE2L6. Furthermore, the KEGG database was used to screen for TRIM21 co-expression gene enrichment pathways in thyroid carcinoma. As shown in Fig. 7A, the genes were significantly enriched in seven KEGG pathways. The top three were ‘antigen processing and presentation’, ‘autoimmune thyroid disease’, and ‘cell adhesion molecules’. The enrichment of co-expression genes in the transcription factor (TF) and kinase datasets were further investigated. As shown in Fig. 7B, a total of 14 TFs of TRIM21 co-expressed genes were identified, including NFKAPPAB, NFKB, and IRF2.

**Discussion**

THCA is one of the most common endocrine malignancies worldwide. Dysregulation of TRIM21 is responsible for the progression of various diseases, including tumors. However,
limited information is available regarding the potential contribution of TRIM21 to THCA.

The current study found, using bioinformatics analysis, that TRIM21 was upregulated in THCA. In addition, the
results of the bioinformatics analyses were verified by measuring TRIM21 expression in THCA and matched adjacent normal tissues. Higher TRIM21 expression was observed in THCA tissues compared with matched adjacent normal tissues. Furthermore, a high TRIM21 level was associated with a high risk of recurrence and lymph node metastasis. The results indicated that TRIM21 may be a potential biological marker to distinguish tumor recurrence rates.

TRIM21 expression and its role in various cancers have been previously investigated. The effects of TRIM21 on tumor progression differed in different types of cancer. Zhao et al (14) observed TRIM21 upregulation in gliomas and confirmed its role in tumor proliferation, migration, and drug resistance. By contrast, TRIM21 is downregulated in breast cancer, associated with tumor size and clinical stage, and is considered an important factor for overall survival (22). In patients with colitis-associated colorectal cancer, decreased TRIM21 expression causes dysregulation of epithelial cell proliferation, angiogenesis and pro-inflammatory responses, resulting in intestinal epithelial carcinogenesis (23). The present study investigated the role of TRIM21 in THCA progression in vitro. It was observed that TRIM21 knockdown inhibited THCA cell proliferation, migration and invasion. This may be one of the biological involvements of TRIM21 in the high recurrence risk and lymph node metastasis of THCA.

TRIM21 may destabilize the tumor suppressor protein p53, the disruption of which often leads to cancer development (22). TRIM21 can degrade p27 and enable cells to enter the S phase, leading to tumor progression (24). By contrast, TRIM21 negatively regulates anti-apoptotic proteins and inactivates the glycogen synthase kinase-3β (GSK3β)-NF-κB pathway to initiate cell apoptosis (25). In addition, increased TRIM21 expression increases the activation of caspase-8 and enhances the death receptor-mediated apoptosis (26). In the present study, 252 TRIM21 co-expressed genes, including SP110, APO12, and UBE2L6, were identified. These significantly enriched genes were associated with the ‘antigen processing and presentation’, ‘autoimmune thyroid disease’ and ‘cell adhesion molecules’ pathways. The expression of TRIM21 co-expression genes in THCA may be influenced by 14 TFs, including NFKAPPAB, NF-κB, and IRF2. However, the mechanism by which TRIM21 regulates THCA progression remains to be elucidated.

There are several members of the TRIM family. TRIM14 has been reported as an oncogene in THCA (7). TRIM44 knockdown suppresses the tumor progression of THCA by inhibiting the Wnt/β-catenin signaling pathway (8). Although others are also of concern, the current study focused on TRIM21. Further studies on the expression and function of other members of the TRIM family in THCA progression are required to establish the regulation network of the TRIM family in THCA.

In conclusion, using bioinformatics analysis and an in vitro study, the present study revealed that TRIM21 promoted tumor progression, indicating that TRIM21 may be a potential biomarker and therapeutic target for THCA. In the future, the mechanism by which TRIM21 regulates THCA progression will be further investigated.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Figshare repository, https://figshare.com/articles/article/Untitled_Item/19501369.

Authors' contributions

ZW and JF designed the study and wrote the manuscript. ZW and YW participated in performing the experiments. ZM was responsible for data acquisition and the interpretation of data. ZY was responsible for statistical analysis and the literature search. WD participated in collecting the tissue samples, performing the RT-qPCR experiments and revising the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study has been checked and approved by the Ethics committee of Liaocheng People’s Hospital (approval no. LC2021059).

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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