TRG-AS1 is a potent driver of oncogenicity of tongue squamous cell carcinoma through microRNA-543/Yes-associated protein 1 axis regulation

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ABSTRACT
The long noncoding RNA T cell receptor gamma locus antisense RNA 1 (TRG-AS1) plays an important role in glioblastoma progression. The objective of this study was to determine the expression status of TRG-AS1 in tongue squamous cell carcinoma (TSCC). The regulatory effects of TRG-AS1 depletion on the malignant processes of TSCC cells were illustrated both in vitro and in vivo. Additionally, the precise molecular mechanisms through which TRG-AS promotes TSCC oncogenicity were investigated. TRG-AS1 expression in TSCC tissues and cell lines was detected using reverse transcription–quantitative PCR. Functional experiments including Cell Counting Kit-8 assay, flow cytometric apoptotic assay, migration and invasion assays, and xenograft tumor model analysis were conducted to severally determine the effects of TRG-AS1 on TSCC cell proliferation, apoptosis, migration, and invasion in vitro and tumor growth in vivo. Herein, TRG-AS1 was highly expressed in TSCC and closely associated with advanced TNM stage, high lymph node metastasis, and poor overall survival. Functionally, TRG-AS1 depletion suppressed TSCC cell proliferation, migration, and invasion in vitro; promoted cell apoptosis; and attenuated tumor growth in vivo. Mechanistically, TRG-AS1 served as a molecular sponge for microRNA-543 (miR-543), thereby contributing to the increased expression of Yes-associated protein 1 (YAP1) – a miR-543 target. Rescue experiments confirmed that miR-543 inhibition or YAP1 overexpression abrogated the anticancer effects of TRG-AS1 silencing in TSCC cells. In conclusion, TRG-AS1 aggravates TSCC malignancy by regulating the miR-543/YAP1 axis. Identification of the TRG-AS1/miR-543/YAP1 regulatory pathway may provide novel insights into TSCC diagnosis, prognosis, and therapy.

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Introduction
Tongue squamous cell carcinoma (TSCC) is the most common form of oral cancer and accounts for ~25%–40% of all oral cancer cases [1]. TSCC is characterized by high aggressiveness, uncontrolled growth, and high regional lymph node metastasis and recurrence rates [2]. TSCC usually causes dysfunctions of speech, mastication, and deglutition [3]. Despite considerable developments in diagnostic methods and therapeutic options in recent decades, treatment outcomes of patients with TSCC remain unsatisfactory, with a 5-year survival rate of <50% [4,5]. Treatment failure and death in patients with TSCC are due most often to local or distant metastasis and recurrence [6]. Another important reason for poor prognosis is that the mechanism underlying TSCC oncogenesis and progression are multistep and stringently regulated processes [7,8] that are not fully understood as yet. Hence, clarifying detailed molecular events underlying TSCC pathogenesis may help the development of anticancer treatments.

Currently, noncoding RNAs are a hotspot in the field of cancer biology [9]. MicroRNAs (miRNAs) are a group of highly conserved noncoding RNAs with approximately 17–24 nucleotides [10]. They are involved in regulating gene expression through complete or incomplete binding to the 3ʹ-untranslated regions (3ʹ-UTRs) of their target genes, resulting in mRNA degradation or translational repression [11]. Differently expressed miRNAs have frequently been identified in almost all human cancer types, including TSCC, and play crucial roles in cancer progression [12,13].

Long noncoding RNAs (lncRNAs) are newly identified transcript clusters of over 200 nucleotides in lengths [14]. They do not exhibit protein-coding capacity but are critical players in diverse physiological and pathological processes due to their effects on gene expression at
different levels, including epigenetic modification, transcription, and post-transcriptional modification [15]. A growing body of evidence has shown aberrant lncRNA expression in various human cancers, suggesting that lncRNA dysregulation contributes to the development and progression of cancer [16–18]. In particular, lncRNAs exert tumor-promoting or tumor-inhibiting effects during TSCC onset and development and are involved in the modulation of malignant processes associated with tumorigenesis [19–21]. Additionally, lncRNAs can serve as competing endogenous RNAs (ceRNAs) to adsorb miRNA and thereby negatively regulate miRNA-mediated inhibition of target mRNAs [22]. Therefore, further exploration of the roles of lncRNAs and miRNAs in the genesis and progression of TSCC may contribute to the development of effective therapeutic and diagnostic targets for TSCC.

The lncRNA T cell receptor gamma locus antisense RNA 1 (TRG-AS1) is involved in glioblastoma progression [23]. However, its expression and biological functions in TSCC have not been well studied. Thus, the objective of the present study was to elucidate the expression status of TRG-AS1 in TSCC tissues and cell lines. The regulatory effects of TRG-AS1 depletion on the malignant processes of TSCC cells were illustrated both in vitro and in vivo. Finally, the exact molecular mechanisms through which TRG-AS promotes the oncogenicity of TSCC cells were investigated.

**Material and methods**

**Tissues and cell lines**

In total, 57 TSCC tissues and paired adjacent normal tongue tissues were collected from patients at the First Affiliated Hospital of Zhengzhou University. No participants received radiotherapy, chemotherapy, or other anticancer treatments prior to surgery. All tissue specimens were immediately frozen and stored in liquid nitrogen following surgical excision. The present study was approved of the ethics committee of the First Affiliated Hospital of Zhengzhou University. All participants provided written informed consent for the use of their tissues.

TSCC cell lines (CAL-27 and SCC-15) were supplied by the American Type Culture Collection (Manassas, VA, USA). CAL-27 cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS). SCC-15 cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (Gibco; Thermo Fisher Scientific, Inc) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific) and 400 ng/mL hydrocortisone. Primary normal human oral keratinocytes (NHOKs) were acquired from ScienCell Research Laboratories (San Diego, CA, USA) and incubated in Oral Keratinocyte Medium (ScienCell Research Laboratories). All cells were cultured in a humidified incubator containing 5% CO2 at 37°C.

**Cell transfection**

Small interfering RNAs (siRNAs) targeting TRG-AS1 (si-TRG-AS1), scrambled control siRNA (si-NC), YAP1 overexpression plasmid pcDNA3.1-YAP1, and empty pcDNA3.1 plasmid were synthesized by Guangzhou RiboBio Co, Ltd (Guangzhou, China). The si-TRG-AS1#1 sequence was 5’- AACCTTAATCTCATATTTTTCT-3’, si-TRG-AS1#2 sequence was 5’- TTGTAAAGATTACATAAAATAAT-3’, si-TRG-AS1#3 sequence was 5’- TGCCATATTATAAATATAAATA-3’ and the si-NC sequence was 5’-CACGATAAGACAATGTATTT-3’. miR-543 mimic, miRNA mimic negative control (miR-NC), miR-543 inhibitor (anti-miR-543), and negative control inhibitor (anti-miR-NC) were obtained from GenePharma Co, Ltd (Shanghai, China). The miR-543 mimic sequence was 5’-AAACAUUCGCGUGCACUUCUU-3’ and the miR-NC sequence was 5’- CACGATAAGACAATGTATTTT-3’. miR-543 mimic, miRNA mimic negative control (miR-NC), miR-543 inhibitor (anti-miR-543), and negative control inhibitor (anti-miR-NC) were obtained from GenePharma Co, Ltd (Shanghai, China). The miR-543 mimic sequence was 5’-AAACAUUCGCGUGCACUUCUU-3’ and the miR-NC sequence was 5’- CACGATAAGACAATGTATTTT-3’.
was 5′-ACUACUGAGUGACAGUAGA-3′. Cells were seeded into 6-well plates and transfected with the abovementioned nucleotides or plasmids using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc).

**Subcellular fractionation**

The nuclear and cytoplasmic fractions of TSCC cells were separated using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Belmont, CA, USA). To examine TRG-AS1 localization in TSCC, the resultant nuclear and cytoplasmic fractions were subjected to reverse transcription–quantitative PCR (RT–qPCR).

**RT–qPCR**

TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc) was used for total RNA isolation. To quantify TRG-AS1 and YAP1 expression, first-strand cDNA was synthesized using the PrimeScript RT-Reagent Kit (Takara Bio, Kusatsu, Japan). PCR amplification was performed using the SYBR Premix Ex Taq™ Kit (Takara Bio). GAPDH served as the endogenous control.

miRNA was isolated from tissues and cells using miRcute miRNA Isolation Kit (TIANGEN, Beijing, China). miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN) was used to reverse transcribe miRNA to cDNA. The cDNA samples were used as a template for qPCR with the miRcute Plus miRNA qPCR Kit (TIANGEN). miR-543 expression was normalized to U6 small nuclear RNA expression. Relative gene expression was calculated according to the 2^(-ΔΔCq) method.

**Cell counting Kit-8 (CCK-8) assay**

TSCC cells subjected to different transfections were collected after 24 h of culture and seeded into 96-well plates at a density of 2 × 10^3 cells per well. After incubation for 0, 24, 48, or 72 h, cell proliferation was detected by incubating cells with 10 μL CCK8 solution (Dojindo Laboratories Co, Ltd, Kumamoto, Japan) in 5% CO₂ at 37°C for an additional 2 h. Finally, optical density was measured using a SUNRISE Microplate Reader (Tecan Group, Ltd, Mannedorf, Switzerland) at 450 nm.

**Flow cytometric apoptotic assay**

After 48 h of cultivation, the harvested cells were analyzed using the Annexin V–Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (BioLegend, San Diego, CA, USA) to assess cell apoptosis. Cells were resuspended in 100 μL of flow cytometry binding buffer. Next, cells were double stained with 5 μL Annexin V–FITC and 5 μL propidium iodide solution. The apoptotic rate was determined using flow cytometry (FACScan™, BD Biosciences, Franklin Lakes, NJ, USA).

**Migration and invasion assays**

Transfected cells were suspended in basal medium without FBS. Cell suspension (200 μL) containing 5 × 10^4 transfected cells was added to the upper compartments of transwell chamber (BD Biosciences, San Jose, CA, USA) with an aperture of 8 μm. The lower compartments were filled with 600 μL of basal medium supplemented with 10% FBS. After incubating for 1 day, the cells remaining on the upper surface were removed and the cells that had migrated were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The stained cells were photographed and counted under an inverted light microscope (Olympus Corporation, Tokyo, Japan). To determine the invasive capacity of the cells, the membranes of the transwell chambers were coated with Matrigel solution (BD Diagnostics, Franklin Lakes, NJ). The other experimental conditions were the same as those for the migration assay.

**Xenograft tumor model analysis**

Short hairpin RNA (shRNA) specifically targeting TRG-AS1 (sh-TRG-AS1) and scrambled control shRNA (sh-NC) were synthesized by Shanghai GenePharma Co, Ltd and inserted into the pLKO.1 vector (Biosettia, San Diego, CA, USA). CAL-27 cells were transduced with lentiviruses
stably expressing sh-TRG-AS1 or sh-NC and treated with 5 μg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) to select CAL-27 cells with stable TRG-AS1 knockdown.

CAL-27 cells with stable TRG-AS1 knockdown were harvested and subcutaneously injected into the flanks of BALB/c-nude male mice aged 4–6 weeks (Shanghai SLAC Laboratory Animal Co, Ltd, Shanghai, China). The developed tumors were measured using a caliper once every 4 days, and tumor volume was calculated using the following formula: volume (mm$^3$) = 0.5 × width$^2$ (mm$^2$) × length (mm). Four weeks after tumor cell injection, the mice were euthanized by cervical dislocation and the subcutaneous xenografts were excised and subjected to molecular analysis. All experimental procedures involving animals were approved by the Committee on Ethics of Animal Experiments at the First Affiliated Hospital of Zhengzhou University.

**Bioinformatic analysis**

Two online software databases, starBase 3.0 (http://starbase.sysu.edu.cn/) and miRDB (http://mirdb.org/), were employed to identify potential miRNAs that interact with TRG-AS1. Moreover, starBase 3.0, miRDB, and TargetScan (http://www.targetscan.org/) were used to identify the target genes of miR-543.

**RNA immunoprecipitation (RIP) assay**

RIP assay was performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) to determine the interaction between miR-543 and TRG-AS1. TSCC cells were lysed using equal volume of RIP lysis buffer. The cell supernatants were incubated with magnetic beads conjugated with anti-argonaute 2 (Ago2) or control IgG antibody (Millipore), followed by probing with proteinase K to digest proteins. Finally, RT–qPCR was performed to evaluate the purified RNA for examining miR-543 and TRG-AS1 expression.

**Luciferase reporter assay**

YAP1 3'-UTR containing the wild-type (WT) miR-543-binding site and mutant (MUT) YAP1 3'-UTR were amplified by RT–qPCR and cloned into a pmirGLO vector (Promega, Madison, WI, USA), generating the luciferase reporter vectors WT-YAP1 and MUT-YAP1. The same experimental protocols were applied to prepare WT-TRG-AS1 and MUT-TRG-AS1 reporter plasmids. TSCC cells seeded into 24-well plates were cultured for 24 h and transfected with miR-543 mimic or miR-NC alongside WT or MUT reporter plasmids using Lipofectamine “ 2000. Luciferase activity was tested using dual-luciferase reporter assay (Promega) after 48 h of cultivation.

**Protein extraction and western blotting**

The cultured cells were solubilized with RIPA lysis buffer (Beyotime Institute of Biotechnology; Shanghai, China), followed by centrifugation at 10,000 × g for 10 min at 4°C to collect total proteins. The BCA Protein Assay Kit (Beyotime Institute of Biotechnology) was used for total protein quantification. The same amounts of protein were separated by 10% SDS–PAGE. The isolated proteins were transferred to polyvinylidene difluoride (PVDF) membranes that were subsequently blocked at room temperature for 2 h using 5% nonfat milk. After overnight incubation at 4°C with primary antibodies, the membranes were probed with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat. No. ab205718; Abcam, Cambridge, UK.) at room temperature for 2 h. Immobilon Western Chemilum HRP substrate (Millipore) was used to detect immunoreactive protein bands on the membranes. Primary antibodies against YAP1 (cat. No. ab52771) and GAPDH (cat. No. ab181602) were used in this study and were both acquired from Abcam.

**Statistical analysis**

All results from at least three independent experiments were presented as the mean ± standard deviation (SD). Correlations between patients’ clinicopathological parameters and TRG-AS1
expression in TSCC were examined by Pearson’s chi-square test. Comparisons between two groups were performed using Student’s t-test. One-way analysis of variance followed by Tukey’s post hoc test was used to determine differences among multiple groups. Overall survival of patients with TSCC was determined using the Kaplan–Meier method, and log-rank test was used to compare the Kaplan–Meier survival curves. Pearson’s correlation coefficient was adopted to test correlations between TRG-AS1 and miR-543 expression in TSCC tissues. 
P < 0.05 was considered statistically significant.

Results

**TRG-AS1 is upregulated in TSCC, and its depletion inhibits the malignant characteristics of TSCC cells**

TRG-AS1 expression was first analyzed in 57 pairs of TSCC tissues and paired adjacent normal tongue tissues by use of RT-qPCR. TRG-AS1 was upregulated in the TSCC tissues compared with that in paired adjacent normal tongue tissues (Figure 1(a)). Consistently, TRG-AS1 expression was higher in TSCC cell lines (CAL-27 and SCC-15) than in primary NHOKs (Figure 1(b)). To determine the clinical significance of TRG-AS1, the 57 patients with TSCC were divided into two groups, low-TRG-AS1 (n = 28) and high-TRG-AS1 (n = 29), based on median TRG-AS1 expression in TSCC tissues. High TRG-AS1 expression was significantly correlated with advanced TNM stage (P = 0.012) and high lymph node metastasis (P = 0.033) in all patients (Table 1). Furthermore, Kaplan–Meier analysis revealed that higher TRG-AS1 expression was closely associated with shorter overall survival in patients with TSCC (Figure 1(c); P = 0.0241).

Next, the roles of TRG-AS1 in TSCC progression were investigated. CAL-27 and SCC-15 cells were transfected with si-TRG-AS1 to knock down
endogenous TRG-AS1 expression. Knockdown efficiency was superior with si-TRG-AS1#1 (Figure 1(d)); thus, it was selected for further studies. CCK-8 assay was performed to determine the effect of TRG-AS1 downregulation on TSCC cell proliferation. Depleted TRG-AS1 markedly hindered CAL-27 and SCC-15 cell proliferation compared with control cell proliferation (Figure 1(e)). Furthermore, the apoptotic rate of CAL-27 and SCC-15 cells increased following TRG-AS1 knockdown (Figure 1(F)). Additionally, TRG-AS1 deficiency markedly decreased the migratory (Figure 1(g)) and invasive (Figure 1(h)) capacities of CAL-27 and SCC-15 cells. Collectively, these results suggest that TRG-AS1 is upregulated in TSCC and serves as an oncogenic lncRNA in TSCC.

**TRG-AS1 functions as a miR-543 sponge in TSCC cells**

To understand the molecular mechanisms underlying TRG-AS1-induced TSCC progression, subcellular localization of TRG-AS1 was first examined. IncLocator (http://www.csbio.sjtu.edu.cn/bioinf/IncLocator/) was employed to predict the distribution of TRG-AS1 expression. TRG-AS1 was predicted to mainly localize in the cytoplasm (Figure 2(a)), which was further confirmed by subcellular fractionation (Figure 2(b)). The results implied that TRG-AS1 interacts with cytoplasmic miRNAs to realize its regulatory roles in TSCC cells. Two prediction software programs, Starbase 3.0 and miRDB, were utilized to screen potential miRNAs capable of interacting with TRG-AS1. A total of eight miRNAs were predicted by both programs (Figure 2(c)) and were selected for further experimental identification.

To investigate their interactions with TRG-AS1, expressions of the predicted miRNAs were detected in CAL-27 and SCC-15 cells with TRG-AS1 knockdown. According to RT-qPCR, only miR-543 expression was substantially elevated in CAL-27 and SCC-15 under TRG-AS1 knockdown (Figure 2(d)). In addition, miR-543 expression was markedly reduced in TSCC tissues compared with that in paired adjacent normal tongue tissues (Figure 2(e)). Kaplan–Meier analysis was also performed, and the results manifested none obvious correlation between miR-543 expression and the overall survival among patients with TSCC (Figure 2(F); P = 0.8034). Furthermore, there was an inverse association between TRG-AS1 and miR-543 expression in the 57 TSCC tissues (Figure 2(g); r = −0.6636, P < 0.0001).

To verify the direct binding of miR-543 and TRG-AS1, WT-TRG-AS1 and MUT-TRG-AS1 reporter plasmids were constructed and cotransfected with miR-543 mimic or miR-NC into CAL-27 and SCC-15 cells. Figure 2(h) presents the wild-type and mutant miR-543 binding sites within TRG-AS1. The results of luciferase reporter assay confirmed that the luciferase activity of WT-TRG-AS1 was suppressed in CAL-27 and SCC-15 cells after miR-543 mimic introduction, whereas that of MUT-TRG-AS1 was unaffected by miR-543 upregulation (Figure 2(i)). Additionally, RIP assay showed that miR-543 and TRG-AS1 were markedly enriched in Ago2-containing microribonucleoprotein complexes (Figure 2(j)). Taken together, these results indicate that TRG-AS1 functions as a miR-543 sponge in TSCC cells.

**YAP1 is a direct target of miR-543 in TSCC cells and is positively regulated by TRG-AS1**

To clarify the functions of miR-543 in TSCC cells, miR-543 mimic or miR-NC was transfected into CAL-27 and SCC-15 cells and RT–qPCR was used

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**Table 1.** The correlations between patients’ clinicopathological parameters and TRG-AS1 expression in TSCC.

| Parameters                        | TRG-AS1 | P value |
|-----------------------------------|---------|---------|
| Age (years)                       |         |         |
| <50                               | 14      | 0.792   |
| ≥50                               | 15      |         |
| Sex                               |         | 0.289   |
| Male                              | 15      |         |
| Female                            | 14      |         |
| Tumor size (cm)                   |         | 0.599   |
| <2                                | 12      |         |
| ≥2                                | 17      |         |
| TNM stage                         |         | 0.012   |
| I–II                              | 14      |         |
| III–IV                            | 15      |         |
| Lymph node metastasis             |         | 0.033   |
| Absence                           | 12      |         |
| Presence                          | 17      |         |

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**Table 1.** The correlations between patients’ clinicopathological parameters and TRG-AS1 expression in TSCC.

| Parameters                        | High (n = 29) | Low (n = 28) |
|-----------------------------------|---------------|--------------|
| Age (years)                       |               |              |
| <50                               | 14            | 12           |
| ≥50                               | 15            | 16           |
| Sex                               |               | 0.289        |
| Male                              | 15            |              |
| Female                            | 14            |              |
| Tumor size (cm)                   |               | 0.599        |
| <2                                | 12            |              |
| ≥2                                | 17            |              |
| TNM stage                         |               | 0.012        |
| I–II                              | 14            |              |
| III–IV                            | 15            |              |
| Lymph node metastasis             |               | 0.033        |
| Absence                           | 12            |              |
| Presence                          | 17            |              |
to determine the transfection efficiency. Transfection with miR-543 mimic resulted in dramatic miR-543 overexpression in CAL-27 and SCC-15 cells (Figure 3(a)). CCK-8 and flow cytometric apoptotic assays were used to detect TSCC cell proliferation and apoptosis following miR-543 upregulation. Ectopic miR-543 expression markedly restricted the proliferation (Figure 3(b)) but facilitated the apoptosis (Figure 3(c)) of CAL-27 and SCC-15 cells. In addition, migration (Figure 3(d)) and invasion (Figure 3(e)) of CAL-27 and SCC-15 cells transfected with miR-543 mimic were impaired.

To elucidate the downstream target of miR-543, three publicly available bioinformatic algorithms (starBase 3.0, miRDB, and TargetScan) were applied to find its direct target. The results indicated YAP1 (Figure 3(F)) as a potential target of miR-543. Luciferase reporter assay confirmed that the luciferase activity of WT-YAP1 reporter plasmid (1 and 2) was clearly downregulated in CAL-27 and SCC-15 cells under miR-543 overexpression, whereas no obvious change was identified in the MUT-YAP1 reporter plasmid (1 and 2) cotransfected with miR-543 mimic (Figure 3(g)). Furthermore, sites 1 and 2 have additive effect on the luciferase activity in CAL-27 and SCC-15 cells. Moreover, miR-543 upregulation remarkably decreased YAP1 mRNA (Figure 3(h)) and protein (Figure 3(i)) expression in CAL-27 and SCC-15 cells. Additionally, YAP1 expression was upregulated in TSCC tissues in contrast to that of paired adjacent normal tongue tissues (Figure 3(j)). Kaplan–Meier analysis revealed that patients with high YAP1 expression possessed shorter overall survival relative to those patients with low YAP1 expression (Figure 3(k); P = 0.0161). Besides, YAP1 mRNA expression was inversely correlated...
with miR-543 expression in the 57 TSCC tissues tested (Figure 3(l); r = 0.6348, P < 0.0001).

miRNAs are complementary to their target mRNAs, and lncRNAs sponge miRNAs and consequently decrease the regulatory effects of miRNAs on their targets [24]. To determine whether TRG-AS1 participates in YAP1 regulation, expression levels of YAP1 mRNA and protein in TRG-AS1-deficient CAL-27 and SCC-15 cells were examined. TRG-AS1 loss dramatically suppressed YAP1 expression in CAL-27 and SCC-15 cells at the mRNA (Figure 4(a)) and protein (Figure 4(b)) levels. Notably, there was a positive correlation between TRG-AS1 and YAP1 mRNA expression in the 57 TSCC tissues (Figure 4(c); r = 0.6002, P < 0.0001). Rescue experiments were performed to determine whether the positive regulation of YAP1 expression by TRG-AS1 could be attributed to its miR-543 sponging effect. To this end, miR-543 inhibitor (anti-miR-543) or inhibitor negative control (anti-miR-NC) was cotransfected along with si-TRG-AS1 into CAL-27 and SCC-15 cells, and change in miR-543 and YAP1 expression was subsequently evaluated. TRG-AS1 depletion notably increased miR-543 (Figure 4(d)) but reduced YAP1 mRNA (Figure 4(e)) and protein (Figure 4(f)) levels in CAL-27 and SCC-15 cells; however, these effects were substantially weakened by anti-miR-543 cotransfection. Therefore, TRG-AS1 served as a molecular sponge of miR-
Figure 4. YAP1 is positively regulated by TRG-AS1 in TSCC cells via sponging miR-543.
(a, b) YAP1 mRNA and protein expression in CAL-27 and SCC-15 cells transfected with si-TRG-AS1 or si-NC was examined by RT–qPCR and western blotting, respectively. (c) Pearson’s correlation coefficient showed the positive correlation between TRG-AS1 and YAP1 mRNA in 57 TSCC tissues. (d) Expression of miR-543 was detected in CAL-27 and SCC-15 cells after cotransfection with si-TRG-AS1 and anti-miR-543 or anti-miR-NC. (e, f) CAL-27 and SCC-15 cells were transfected with anti-miR-543 or anti-miR-NC in the presence of si-TRG-AS1. After cotransfection, YAP1 mRNA and protein expression was detected by RT–qPCR and western blotting, respectively. *P < 0.05 and **P < 0.01.

Figure 5. miR-543 inhibition rescues the suppressive effects of si-TRG-AS1 on the malignant phenotype of TSCC cells.
(a) miR-543 expression was examined in CAL-27 and SCC-15 cells after anti-miR-543 or anti-miR-NC transfection. (b, c) cell proliferation and apoptosis was tested using CCK-8 assay and flow cytometric apoptotic assay, respectively, in CAL-27 and SCC-15 cells transfected with anti-miR-543 or anti-miR-NC, along with si-TRG-AS1. (d, e) Migration and invasion assays were performed to determine the migration and invasion of CAL-27 and SCC-15 cells treated as above described. *P < 0.05 and **P < 0.01.
543 and thereby positively regulated YAP1 expression.

**TRG-AS1/miR-543/YAP1 pathway is involved in the oncogenicity of TSCC cells**

Rescue assays were performed to determine whether the TRG-AS1/miR-543/YAP1 pathway was implicated in the oncogenicity of TSCC cells. miR-543 expression in CAL-27 and SCC-15 cells was significantly decreased by anti-miR-543 (Figure 5(a)). si-TRG-AS1 together with anti-miR-543 or anti-miR-NC was cotransfected into CAL-27 and SCC-15 cells, and cell proliferation, apoptosis, migration, and invasion were determined. CAL-27 and SCC-15 cell proliferation was hindered by TRG-AS1 silencing, but this effect was abolished by miR-543 inhibition (Figure 5(b)). In addition, anti-miR-543 cotransfection counteracted the promotive action of TRG-AS1 knockdown on CAL-27 and SCC-15 cell apoptosis (Figure 5(c)). TRG-AS1 downregulation decreased the migratory (Figure 5(d)) and invasive (Figure 5(e)) abilities of CAL-27 and SCC-15 cells, and miR-543 inhibition almost reversed these effects. Meanwhile, rescue assays were conducted by cotransfecting the YAP1 overexpression plasmid pcDNA3.1-YAP1 (Figure 6(a)) or empty pcDNA3.1 plasmid with si-TRG-AS1 into CAL-27 and SCC-15 cells. Restoration of YAP1 expression neutralized the effects of TRG-AS1 depletion on CAL-27 and SCC-15 cell proliferation (Figure 6(b)), apoptosis (Figure 6(c)), migration (Figure 6(d)), and invasion (Figure 6(e)). These results

**Figure 6.** Anticancer effects of TRG-AS1 silencing on the malignant processes of TSCC cells were abrogated by YAP1 overexpression. (a) Western blotting was used to evaluate the transfection efficiency of pcDNA3.1-YAP1 in CAL-27 and SCC-15 cells. (b–e) si-TRG-AS1 alongside pcDNA3.1-YAP1 or pcDNA3.1 was introduced into CAL-27 and SCC-15 cells. Cell proliferation, apoptosis, migration, and invasion were determined by CCK-8, flow cytometric apoptotic, migration, and invasion assays, respectively. *P < 0.05 and **P < 0.01.
suggest that TRG-AS1 executes its regulatory action in TSCC cells by targeting the miR-543/YAP1 axis.

**Discussion**

Many studies have reported that numerous lncRNAs are dysregulated in TSCC and are implicated in its aggressive behavior due to their anti-oncogenic or pro-oncogenic actions [21,25,26]. Therefore, further research on detailed functions of TSCC-related lncRNAs may contribute to the identification of attractive targets for TSCC management. To date, however, many lncRNAs in TSCC have not been studied in detail. In the present study, the expression and regulatory roles of TRG-AS1 in TSCC were explored. In addition, the underlying mechanisms through which TRG-AS1 affects the malignancy of TSCC were thoroughly investigated. Our findings offer new insights into the elements of multifaceted functions of TRG-AS1 in TSCC progression by revealing a novel role of the TRG-AS1/miR-543/YAP1 pathway in the malignant processes of TSCC.

TRG-AS1 was upregulated in glioblastoma tissues and cell lines [23]. Patients with glioblastoma presenting high TRG-AS1 expression showed shorter overall survival than those with low TRG-AS1 expression [23]. In terms of cell function, TRG-AS1 overexpression promoted glioblastoma cell proliferation [23]. However, the expression and roles of TRG-AS1 in TSCC remain
incompletely understood. In the present study, TRG-AS1 was highly expressed in TSCC tissues as opposed to that in normal tongue tissues, and this high expression was positively correlated with poor overall survival. A series of functional assays revealed that TRG-AS1 loss hindered TSCC cell proliferation, migration, and invasion and induced cell apoptosis in vitro. Furthermore, depleted TRG-AS1 expression restricted tumor growth in vivo.

In this study, our results revealed that knockdown of TRG-AS1 decreased TSCC cell proliferation at 72 h, and metastasis in vitro at 24 h, whereas loss of TRG-AS1 promoted cell apoptosis at 48 h post-transfection. The distinct in timing observed for each effect may be ascribed to the different influences of si-TRG-AS1 on function-associated proteins. To a great extent, lncRNAs contribute to the regulation of gene expression via different mechanisms, depending mostly on their cellular location [27]. For instance, lncRNAs located in the nucleus epigenetically decrease mRNA expression at the post-transcriptional level; cytoplasmic lncRNAs are involved in the modulation of gene expression at the post-transcriptional level and operate as ceRNAs of certain miRNAs, consequently increasing the expression of the target mRNAs of those miRNAs [28]. To illustrate the underlying mechanisms through which TRG-AS1 aggravates TSCC progression subcellular localization of TRG-AS1 was first detected in TSCC. With the help of the IncLocator database, TRG-AS1 was predicted to be predominantly distributed in the cytoplasm. Following subcellular fractionation, RT–qPCR revealed that TRG-AS1 was mainly localized in the cytoplasm of TSCC cells, implying the possible implications of TRG-AS1 in the ceRNA network.

Next, putative miRNAs with possible binding sites within TRG-AS1 were predicted using starBase 3.0 and miRDB. Subsequently, RT–qPCR, RIP assay, and luciferase reporter assay collectively confirmed direct binding between TRG-AS1 and miR-543, and that TRG-AS1 down-regulation increased miR-543 expression in TSCC cells. In addition, miR-543 expression was reduced in TSCC relative to that in normal tongue tissues and was inversely correlated to TRG-AS1 expression in the 57 tissue samples. Furthermore, YAP1 was shown to be a direct target of miR-543 in TSCC cells and was positively correlated by TRG-AS1. Depleted TRG-AS1 expression substantially suppressed YAP1 expression in TSCC cells, and miR-543 inhibition partially abrogated this effect. Altogether, TRG-AS1 worked as a ceRNA to compete with YAP1 for miR-543 binding in TSCC cells, thereby inversely modulating miR-543-triggered YAP1 suppression.

Abnormal miR-543 expression has been identified in multiple human cancers [29–33]. To date, however, the expression and roles of miR-543 in TSCC have not been documented. Our results showed that miR-543 was weakly expressed in TSCC, and its upregulation attenuated TSCC cell proliferation, migration, and invasion but facilitated cell apoptosis. Next, we searched for the downstream target gene of miR-543 in TSCC cells. Mechanistic experiments revealed that miR-543 directly targeted YAP1 in TSCC cells. YAP1, a transcriptional coactivator, can specifically interact with oncogenes or tumor suppressors [34]. It is upregulated in TSCC and plays crucial roles in TSCC carcinogenesis and cancer progression [35,36]. In the present study, rescue experiments confirmed that the anticancer effects of TRG-AS1 silencing on the malignant processes of TSCC cells could be abrogated by miR-543 inhibition or YAP1 overexpression. These findings suggest that the cancer-promoting actions of TRG-AS1 in TSCC cells, at least in part, depend on the miR-543/YAP1 axis.

**Conclusion**

In summary, TRG-AS1 is highly expressed in TSCC, and this upregulation is closely related to poor clinical outcomes. Mechanistically, TRG-AS1 acts as a ceRNA and regulates the miR-543/YAP1 axis, thereby playing pro-oncogenic roles in the malignancy of TSCC cells. Identification of the TRG-AS1/miR-543/YAP1 regulatory pathway (Figure 8) may provide novel insights into TSCC diagnosis, prognosis, and therapy.
Disclosure statement
The author reports no conflicts of interest in this work.

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