Original Article

Effect of *GATAD1* regulating the *SRRM2* gene on recurrence of thyroid tumor and its molecular mechanism

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Background: Thyroid blood vessels and nerves are rich, and their anatomical and physiological structures are complex. Surgery often fails to eradicate the tumor, which has a serious negative impact on the surgical outcomes and patient prognosis. Therefore, it is important to accurately predict the recurrence rate of thyroid cancer.

Methods: Based on bioinformatics analysis, the highly expressed transcription factors and differential genes in thyroid carcinoma (THCA) were obtained. Kaplan-Meier survival analysis was used to analyze the clinical effects of *GATAD1* as well as *SRRM2* on the recurrence of THCA patients. The effect of *GATAD1* on *SRRM2* expression was explored using cell experiments. Other experiments were conducted to reveal the interaction between *SRRM2* and *GATAD1* and their functions in THCA progression, such as cell proliferation and cell cycle.

Results: *GATAD1* was overexpressed in recurrent THCA tissue compared with that in adjacent normal tissue. *GATAD1* and *SRRM2* were identified as the key risk factors for THCA recurrence as well as survival. Knockdown of *GATAD1* and *SRRM2* can inhibit THCA cell proliferation and arrest THCA cells in the G1 phase. Inhibiting *GATAD1* decreased *SRRM2* expression in THCA cells, whereas overexpressing *GATAD1* had the opposite result. *SRRM2* knockdown eliminated *GATAD1*-induced proliferation of THCA cells in vitro, indicating that *GATAD1*-induced THCA cell proliferation was dependent on increased *SRRM2* expression.

Conclusions: We identified *GATAD1* as an underlying diagnostic biomarker in THCA recurrence patients. The *GATAD1*-SRRM2 axis mediates human THCA recurrence progression and is an underlying target for THCA treatment.

Keywords: *GATAD1*; *SRRM2*; thyroid carcinoma (THCA); diagnostic biomarker; potential therapeutic target

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Introduction

Thyroid carcinoma (THCA) mostly originates from thyroid follicular epithelial cells (1,2), and its morbidity is currently showing an annually increasing trend. It has become the main cause of death among head, neck, and endocrine tumors (3). Since the pathological type of tumor is mainly low-grade differentiated thyroid cancer, most patients have a longer survival time after surgery (4,5). However, due to the abundance of thyroid vascular nerves and the complex anatomy and physiological structure of the thyroid, surgery often fails to completely remove cancer tissue, and the

probability of recurrence after surgery is high (6,7). The recurrence of TC usually requires a second operation, which will bring increased complications and can easily lead to death (8). Therefore, it is vitally important to accurately predict the recurrence of THCA after surgery.

Transcription factors (TFs) are the convergence point for various signaling pathways in eukaryotic cells. The deregulation of TFs may lead to a range of diseases such as inflammatory disorders, endocrine disorders, cardiovascular disease, and various cancers (9). To date, more than 1,600 TFs have been found, of which 365 TFs have been identified by tumor cell line histochemistry screening and have been studied in depth. The function of TFs is to regulate the expression of downstream genes (10). Other studies have reported that the target gene network of TF microRNA (miRNA) is associated with survival and recurrence of ovarian cancer (11), and that *FOXM1* can mediate the proliferation of liver cancer cells by regulating *KIF4A* (12).

Reports have shown that TFs play a crucial role in the thyroid. For example, *HOXA9* has been found to regulate *RUNX2* to enhance the ability of papillary thyroid carcinoma (PTC) migration and invasion (13). However, the function of TFs in the recurrence of thyroid cancer has not been reported. In this study, we obtained the highly expressed TFs as well as differentially expressed genes (DEGs) in THCA, based on bioinformatics analysis. Then, we analyzed the clinical effects of TFs and DEGs on the recurrence of THCA patients, and explored the functions of TFs and DEGs in THCA progression. We identified *GATAD1* as a novel potential diagnostic biomarker in THCA recurrence patients. The *GATAD1-SRRM2* axis was shown to mediate human THCA recurrence progression, which can serve as an underlying therapeutic target for THCA treatment. We present the following article in accordance with the MDAR reporting checklist (available at https://gs.amegroups.com/article/view/10.21037/gs-22-666/rc).

Methods

Bioinformatic analysis of gene expression data

The whole genome expression and survival data of recurring and non-recurring THCA samples were downloaded from The Cancer Genome Atlas (TCGA) database, and the genome-wide differences were analyzed using the R language analysis program (The R Foundation of Statistical Computing, Vienna, Austria). Meanwhile, the list of common TFs and their downstream genes were obtained using the Viper data package of R language. The copy number of TFs and survival map of patients with increased copy numbers of TFs were obtained by cBioPortal database (https://www.cbioportal.org/). The activity bar graph of TFs in thyroid cell lines was obtained by DepMap database (https://depmap.org/portal/). The relationship between *GATAD1* and *SRRM2* expression levels was analyzed using R software. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell lines and transfection

The HEK293T, FTC133, and TT human THCA cell lines were purchased from the American Typical Culture Collection Center (ATCC; Manassas, VA, USA). The FTC133 cells were cultured in Dulbecco's modified Eagle medium (DMEM), nutrient mixture F-12 (1:1, by volume) which containing 2% penicillin/streptomycin, 10% fetal bovine serum (FBS), and 1% amphotericin B (14). The TT cells were cultured in F12K medium containing antibiotics, 2 mM glutamine, and 10% FBS (15). All culture reagents were obtained from Gibco (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA). Cells were cultivated at 37 ℃ in an atmosphere with 5% CO_2 (16). No mycoplasma contamination was found in the cell lines.

The sequences of negative control short hairpin shRNA (NC shRNA), sh-*GATAD1*, and sh-SRRM2 were listed as follows: sh-*GATAD1*, 5'-CGGCTG CTGAAAAGAAAGTCTCCAC-3'; sh-SRRM2, 5'-AAUAACUCGGUUUCGGUGCTT-3'; NC shRNA, 5'-UUCUCCGAACGUGUCACGUTT-3'; The *GATAD1* overexpressing recombinant vector pcDNA3.1-*GATAD1* and empty plasmid as negative control (NC) were obtained from GenePharma (Shanghai, China). The THCA cells were transiently transfected using Lipofectamine 3000 (Thermo Fisher, Waltham, MA, USA) and polyetherimide for HEK293T cells.

Cell proliferation assay

FTC133, together with TT cells was cultured in 96 well plate. The cells were cultured for 1, 2, 3, and 4 days after attachment. We then added 0.5% 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma, St. Louis, MO, USA)-containing medium and treated them for 2 hours. The medium was then replaced with 1 mg/mL

Table 1 The primer sequences

Gene	Forward (5'-3')	Reverse (3'-5')
GATAD1	TCTCTAGCCCCAGAGACCAA	AATGGTTGGCAACTGATTCC
GAPDH	CTTCATTGACCTCAACTACATGG	CTCGCTCCTGGAAGATGGTGAT
SRRM2	GAAGAAAGGCCTGCTGTGTC	CCAAAGCTGTTCTCCCTGAG

dimethyl sulfoxide (DMSO; Sigma, USA). The optical density (OD) value was measured at 490 nm.

Colony formation assay

We cultured lentivirus-infected THCA cells in 6-well plates for 7 days. The cells were then stained with 0.5% (w/v) crystal violet, imaged by scanner, and quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot

The whole cell lysates were centrifuged for 10 minutes at 4 ℃, after which the bicinchoninic acid (BCA; Pierce, Rockford, IL, USA) method was used to test protein concentrations. Protein was separated using sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Shanghai, China). The membrane was sealed with 5% skimmed milk powder, and dyed overnight with the primary antibody at 4 ℃. Then, we probed the membranes with a peroxidase conjugated secondary antibody (1:10,000 dilution). We also used enhanced chemiluminescence (ECL) reagent (Pierce, USA) to test the antigen-antibody complexes. Primary antibodies against *SRRM2*, *GATAD1*, and GAPDH were obtained from Sigma-Aldrich (Milan, Italy).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from different cell lines using TRIzol reagent (Invitrogen, USA), and 1 μg extracted RNA was reverse transcribed to complementary DNA (cDNA), using a kit from Promega (Madison, WI, USA), according to the manufacturer's instructions. RT-qPCR was performed by Fast Start universal SYBR Green Master (Roche, Indianapolis, IN, USA). All q-PCR was carried out in triplicate. $2^{-\Delta\Delta CT}$ was used to evaluate the expression levels in comparison to the control. *Table 1* shows the sequences of RT-qPCR primer.

Luciferase assays

We transfected pRL-TK and pGL4.2 basic Luc plasmids into HEK293T cells. The *GATAD1* expression plasmid or empty body were co transfected for 2 days, and the gene activity was analyzed. The experiment was repeated 3 times.

Cell cycle analysis

The lentivirus-infected THCA cells were harvested, washed in phosphate-buffered saline (PBS), and then fixed it for 2 hours at –20 ℃ with cold 70% ethanol. The cells were centrifuged for 3 minutes, washed in PBS 3 times, suspended in 0.5 mL PBS, and added to 100 μg/mL RNase A (Sigma Chemicals, St. Louis, MO, USA) and 50 μg/mL propidium iodide (PI; Sigma Chemicals) for 30 minutes at 4 ℃ in the dark. Flow cytometry was used to analyze the contents of DNA.

Statistical analysis

The software SPSS 23.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The final data were presented as the mean ± SD of 3 separate studies. To compare 2 or 3 groups, the analysis of variance (ANOVA) or Student's *t*-test were utilized, accordingly. A P value less than 0.05 was regarded statistically significant.

Results

Increased GATAD1 activity in THCA

We obtained 365 common TFs from the original gene expression data of recurrent (47 cases) and non-recurrent (65 cases) THCA, using the R language analysis program. The genome-wide differences were analyzed, which

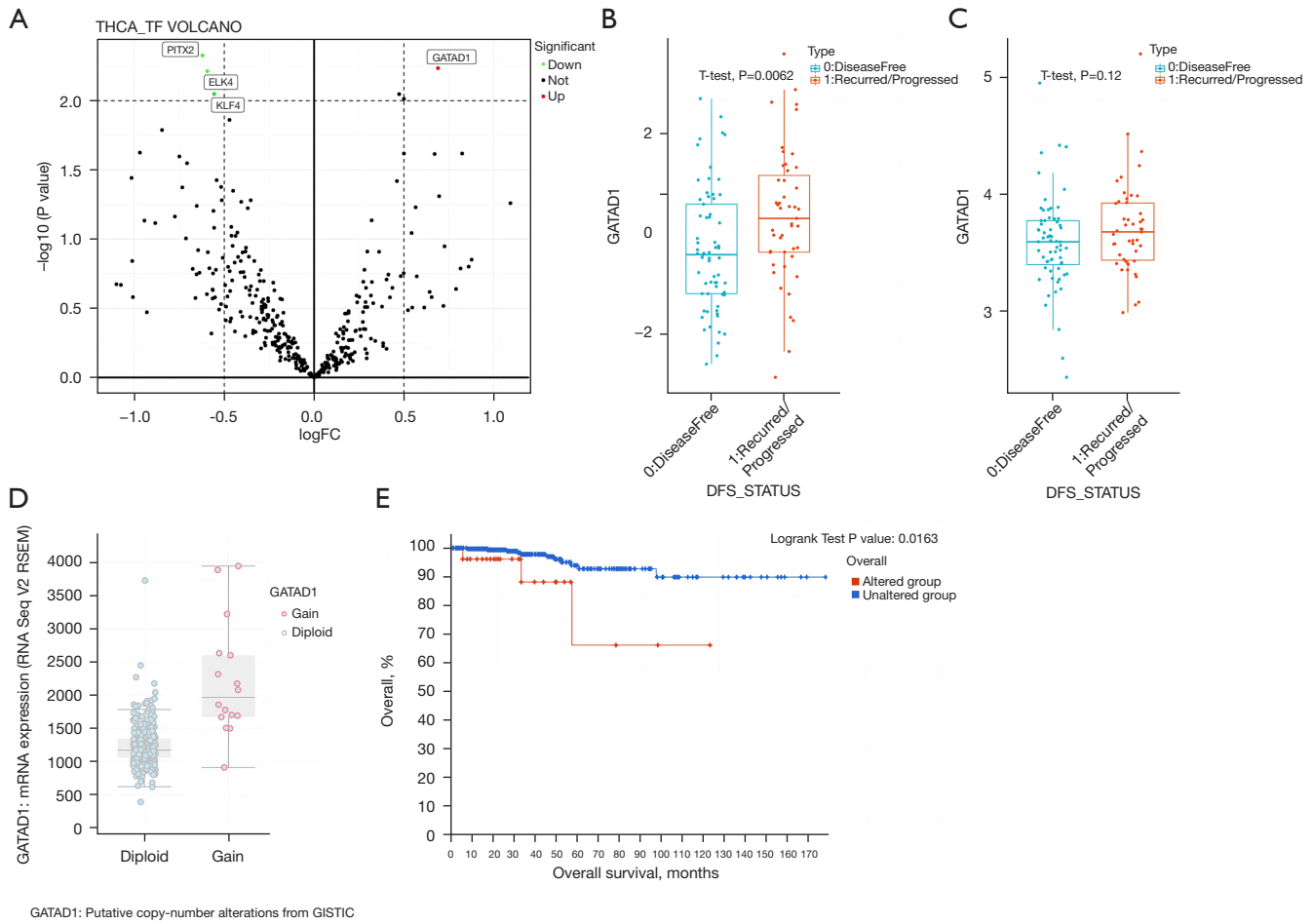

Figure 1 *GATAD1* expression increase in THCA tissues. (A) Volcano plot of 365 TFs. (B) Box plot of activity of the *GATAD1*. (C) Box plot of the *GATAD1* mRNA expression (P=0.12). (D) Compared with non-recurrent sample tissues, the amplification of *GATAD1* gene copy number occurred in recurrent sample tissues. (E) Survival status of patients with thyroid cancer. THCA, thyroid carcinoma; TFs, transcription factors; mRNA, messenger RNA.

showed that *GATAD1* was significantly up regulated (*Figure 1A*). The activity of *GATAD1* was remarkably increased in patients with THCA recurrence (*Figure 1B*), and its expression level was also remarkably increased (*Figure 1C*). The level of *GATAD1* messenger RNA (mRNA) was positively correlated with the number of copies of *GATAD1* (*Figure 1D*). The impact of *GATAD1* on THCA patients was revealed by Kaplan-Meier analysis. In specimens of recurrent (47 cases) and non-recurrent (65 cases) THCA, those with *GATAD1* gene magnification had a shorter survival time (*Figure 1E*). These findings indicated that the amplification of *GATAD1* copy number leads to the high expression of *GATAD1*, indicating a high recurrence rate of THCA.

GATAD1 promotes THCA cell proliferation

We conducted MTT assays and cell flow cytometry experiments to investigate the impact of *GATAD1* on the proliferation of THCA cells. Firstly, the activity bar graph of *GATAD1* in THCA cell lines was obtained through the DepMap database (*Figure 2A*), showing that it inhibited proliferation of FTC133 (*Figure 2B*) and TT (P<0.05) (*Figure 2C*), and arrested FTC133 cells in the G1 phase (*Figure 2D,2E*).

SRRM2 is a key downstream gene of GATAD1

To screen the key downstream genes of *GATAD1*, 112 THCA patients (47 cases of recurrence and 65 cases of

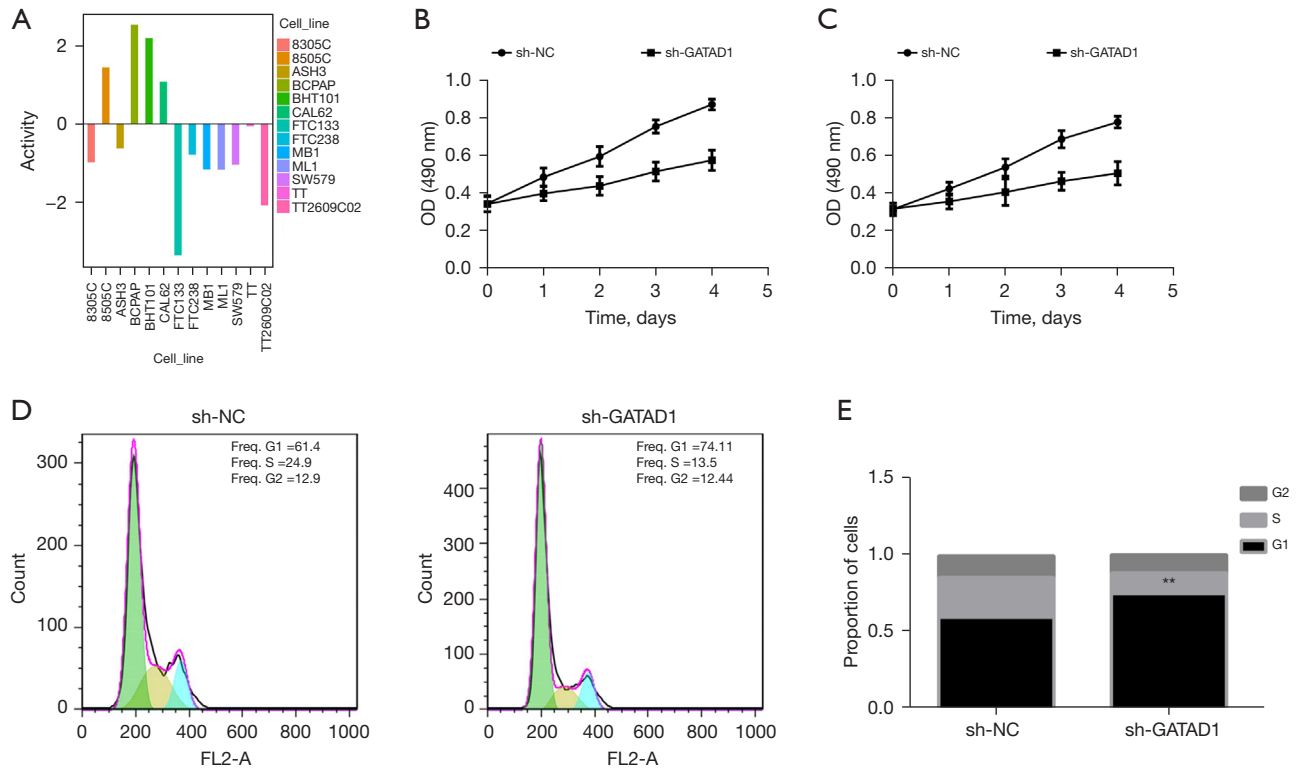

Figure 2 *GATAD1* promotion of THCA cell proliferation. (A) Bar chart of *GATAD1* activity score in THCA cell lines; (B,C) MTT assays showing the proliferation of FTC133 (B) and TT (C) cells stably expressing sh-GATADA1. (D,E) Cell cycle analysis was performed on FTC133 cells stably expressing sh-GATAD1. **, P<0.01. OD, optical density; THCA, thyroid carcinoma; MTT, 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide.

non-recurrence) were divided into different groups. We obtained 4,124 up-regulated genes according to the activity of *GATAD1*. A total of 926 up-regulated genes were found according to *GATAD1* mRNA expression levels. The overall survival analysis of the activity of *GATAD1* yielded 1,601 differential genes. Moreover, there were a total of 26 genes satisfying 3 conditions at the same time (*Figure 3A*). The binding motif prediction of *GATAD1* was obtained by the previous reports (*Figure 3B*). Through the binding motif screening of *GATAD1*, we discovered a total of 16 downstream genes with binding motif. Among them, *SRRM2* was the most highly expressed, and the difference was significantly up-regulated (*Figure 3C*). According to the expression level of *SRRM2* in 112 THCA patients (47 cases of recurrence and 65 cases of non-recurrence), the specimens were divided into low and high expression groups, and it was found that specimens with high *SRRM2* expression had poor survival (P=0.019) (*Figure 3D,3E*).

SRRM2 promotes THCA cell proliferation

We conducted MTT assays, cell clone formation assays, and cell flow cytometry experiments to evaluate the impact of *SRRM2* on the proliferation of THCA cells. The FTC133 and TT cell lines were selected for experiments. It was revealed that *SRRM2* inhibited the proliferation of FTC133 (*Figure 4A*) and TT (P<0.05) (*Figure 4B*), and arrested FTC133 cells in the G1 phase (*Figure 4C,4D*).

GATAD1 positively regulates SRRM2 expression

To determine whether *GATAD1* regulates the expression of *SRRM2* in THCA, the findings of bioinformatics analysis revealed that the expression of *GATAD1* was positively associated with *SRRM2* (*Figure 5A*). Furthermore, we transfected TT cell lines with the FOXM1 overexpression structure, which promoted *SRRM2* protein as well as

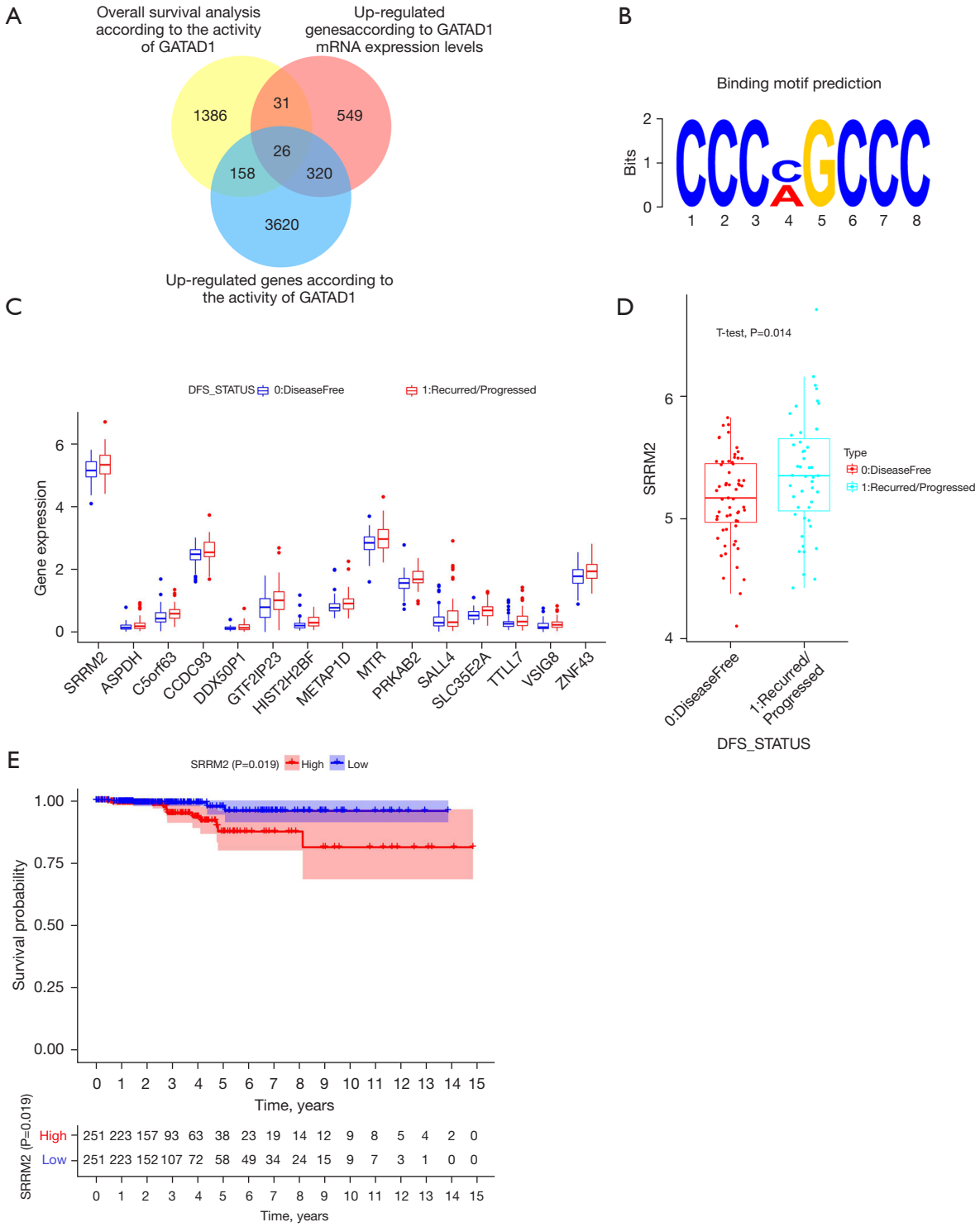

Figure 3 *SRRM2* as a key downstream gene of *GATAD1*. (A) Venn diagram of RNA sequencing of the downstream target gene of *GATAD1*. (B) The binding motif prediction of *GATAD1*. (C) Box plot of 16 downstream gene expression levels with motifs. (D) *SRRM2* expression has a significant impact on THCA patients' survival. (E) KM results of the survival condition of THCA patients samples stratified. DFS, disease-free survival; THCA, thyroid carcinoma; KM, Kaplan-Meier.

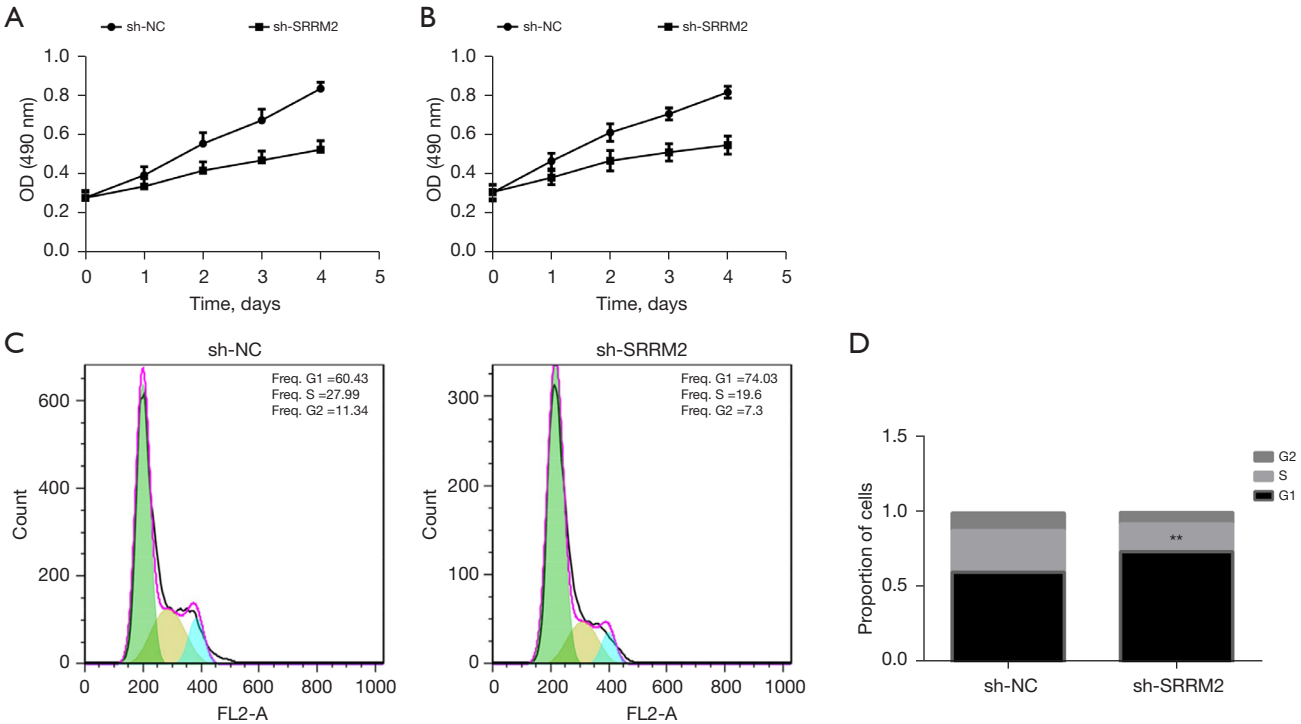

Figure 4 *SRRM2* promotes the proliferation of THCA cells. (A,B) MTT assays showed the proliferation of FTC133 and TT cells stably expressing sh-SRRM2. (C,D) Flow cytometry showed the cell cycle of TT cells stably expressing sh-SRRM2. **, P<0.01. OD, optical density; THCA, thyroid carcinoma; MTT, 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide.

mRNA levels (*Figure 5B-5D*). On the contrary, shRNA-mediated knockdown of *GATAD1* reversed the result (*Figure 5E-5G*), indicating that *GATAD1* regulated *SRRM2* expression.

GATAD1 binds to the SRRM2 promoter

The promoter of the *SRRM2* gene has 3 conserved CCCNNCCC regions (BS1, −840 to −832; BS2, −758 to −750; BS3, −117 to 109), and *GATAD1* can bind to the CCCNNCCC sequence of chromatin. It was speculated that *GATAD1* promoted the transcription of the *SRRM2* gene by recognizing the conserved region of the *SRRM2* gene. To confirm this hypothesis, a different luciferase reporter was transfected into 293T cells with high level *GATAD1*, and the findings revealed that the full-length promoter construction showed increased luciferase activity (SRRM2 Pro Δ BS1 + BS2, BS3), indicating that *GATAD1* directly activated *SRRM2* gene transcription. Several extensions of the TSS core promoter, including BS1, BS2, and BS3, can enhance transcription (*Figure 6*). All the results showed that *GATAD1* enhanced *SRRM2* transcription by binding BS1, BS2, and BS3.

GATAD1 stimulates THCA cell proliferation in a SRRM2-dependent manner

According to the above findings, we inferred that *SRRM2* exerts the effect of *GATAD1* in the progression of THCA. To verify this hypothesis, *SRRM2* was knocked down in *GATAD1*-overexpressing TT cells. The upregulation of *SRRM2* induced with *GATAD1* overexpression was abolished at the protein as well as mRNA level (*Figure 7A-7C*). The cell proliferation experiments also showed similar results. These results indicated that the *GATAD1*-SRRM2 axis promoted THCA progression and that interfering with the expression of *SRRM2* can block *GATAD1*-mediated THCA cell proliferation (*Figure 7D*).

Discussion

Research shows that TFs play a crucial role in cell growth (17). Mounting evidence shows that these proteins are closely related to the genetic level that controls the

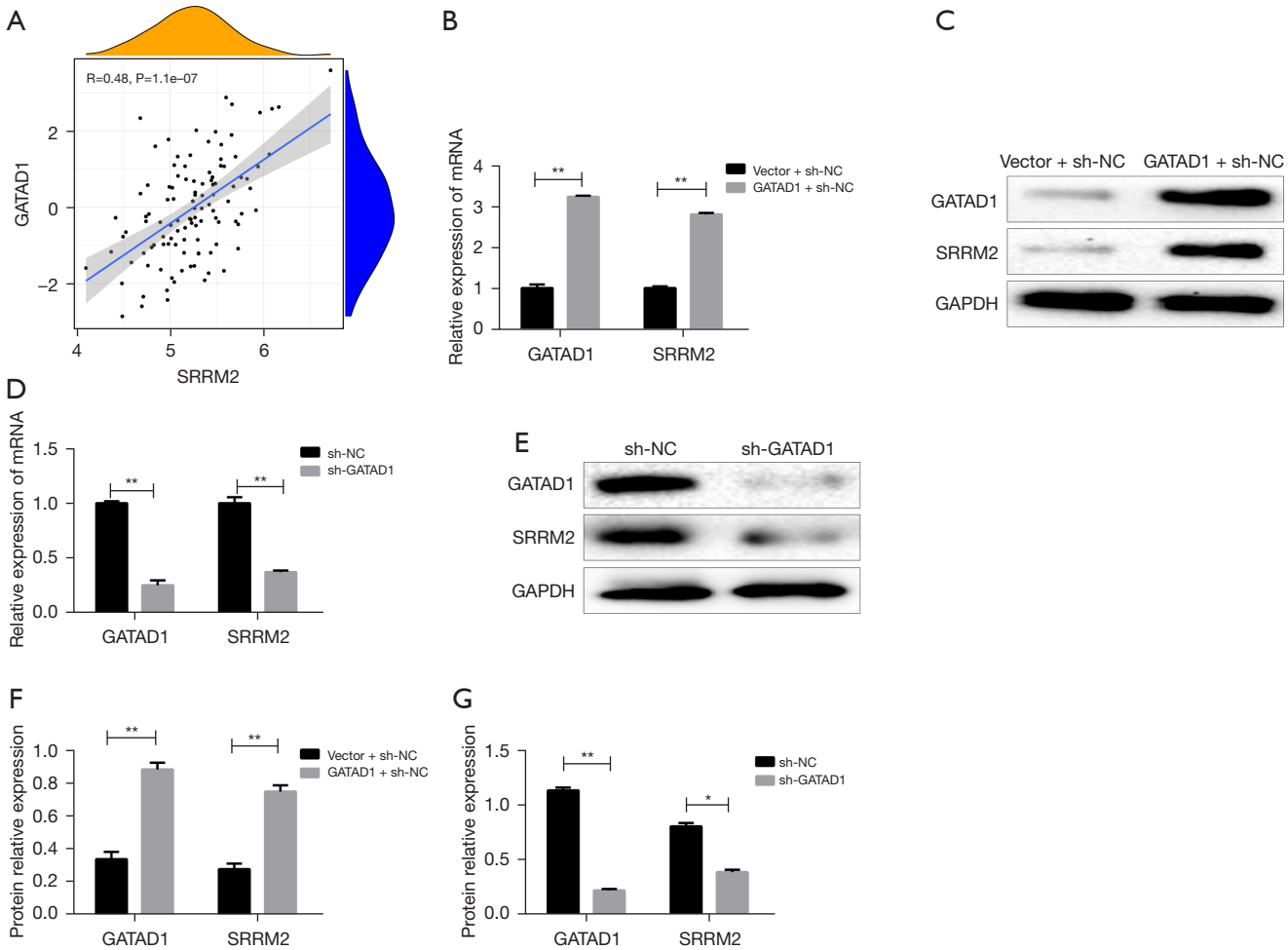

Figure 5 *GATAD1* positively regulates *SRRM2* expression. (A) Correlation between *SRRM2* and *GATAD1* (R=0.48, P less than 0.01). (B-D) RT-PCR and Western blot showed the expression of *SRRM2*. (E-G) RT-PCR and western blot showing the expression of *SRRM2* when transfected with shNC or sh-GATADA1. *, P<0.05; **, P<0.01. RT-PCR, real-time polymerase chain reaction; shNC, short hairpin normal control.

development of cancer (11), and may provide selective targets for new drug interventions (12,18). Recent drug reports have indicated that TFs are easily inhibited by drugs (9). Many currently used anticancer drugs exert their effects by regulating the activity of important TFs (19). In this study, we found that the activity and expression of *GATAD1* were remarkably increased in patients with recurrent THCA. The mRNA of *GATAD1* was positively correlated with the number of copies of *GATAD1*. The survival time of patients with recurrent THCA caused by *GATAD1* gene amplification was shorter. The data showed that the amplification of *GATAD1* copy number leads to the high expression of *GATAD1*, and the recurrence rate of THCA is high. Previous studies have shown that *GATAD1* was significantly up-regulated in hepatocellular carcinoma (HCC) (20) and glioma (21) through gene amplification. A study revealed that *GATAD1* regulated cell cycle via PRL3 (20). We also found that *GATAD1* was overexpressed in recurrent THCA cases, which further suggested that *GATAD1* is crucial in the THCA recurrence process. Knockdown of *GATAD1* reduced the number of THCA cell clones and inhibited the THCA cell proliferation, and arrested THCA cells in the G1 phase.

We found that *SRRM2* could bind to the binding motif of GATDA1, and the expression level of *SRRM2* was remarkably up-regulated. *SRRM2* is a susceptible gene

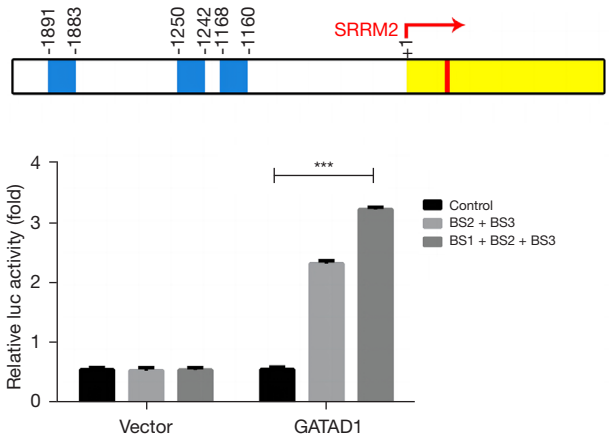

Figure 6 *GATAD1* binds to the SMMR2 promoter and activates gene transcription. SMMR2 promoter was transfected into 293T cells together with *GATAD1* overexpression plasmid or control vector. ***, P<0.001.

of THCA (22,23), and its mutation can accelerate the formation of THCA (24), but its specific mechanism is still unclear. We found that the recurrent THCA patients with high *SRRM2* expression showed worse survival, based on bioinformatics analysis. Knockdown of *SRRM2* also reduced the number of THCA cell clones and inhibited the THCA cell proliferation, and arrested THCA cells in the G1 phase. According to the findings, we speculated that *SRRM2* is also important in the recurrence of THCA.

Bioinformatics findings revealed that the expression of *GATAD1* was positively associated with *SRRM2*. In addition, FOXM1 overexpression was used to construct transfected TT cell lines, which increased *SRRM2* protein and mRNA levels. In contrast, shRNA-mediated *GATAD1* knockout reversed these effects, indicating that *GATAD1* regulates *SRRM2* expression (21). In this study, we discovered 3 conservative CCCNNCCC sequences located

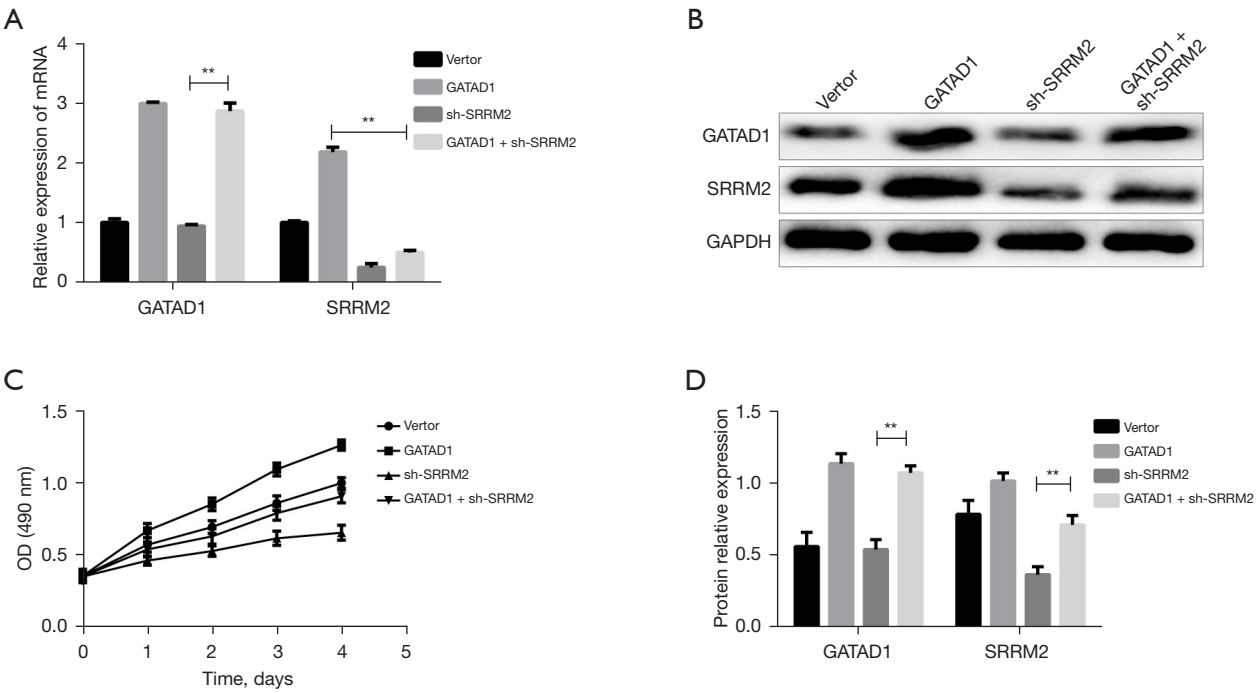

Figure 7 *SRRM2* is required for *GATAD1*-induced THCA cell proliferation *in vitro*. (A-C) RT-PCR and western blot showed the expression of *SRRM2* with *GATAD1* overexpression (*GATAD1*) and *SRRM2* knockdown (sh-SRRM2) lentiviral vectors. (D) Cell growth was used to evaluate the proliferation of TT cells stably expressing *GATAD1* + sh-*SRRM2*. **, P<0.01. OD, optical density; THCA, thyroid carcinoma; RT-PCR, real-time polymerase chain reaction.

in the upstream region of the *SRRM2* promoter. The results of the luciferase reporter indicated that *GATAD1* directly activated *SRRM2* gene transcription. Based on the above observations, we inferred that *SRRM2* exerted the effect of *GATAD1* in the progression of THCA. To verify this hypothesis, *SRRM2* was knocked down in *GATAD1*-overexpressing THCA cells. The upregulation of *SRRM2* induced by *GATAD1* overexpression was abolished at the protein and mRNA levels. Cell cloning and cell proliferation experiments also showed similar results. These results indicated that the *GATAD1*-SRRM2 axis promoted THCA progression and interfered with the expression of *SRRM2*, resulting in the blockade *GATAD1*-mediated THCA cell proliferation.

In summary, based on bioinformatics analysis, we screened out the transcription factor *GATAD1* which is highly expressed in THCA and its key downstream gene SRRM2. To investigate further, we performed a Kaplan-Meier analysis revealed the effect of *GATAD1* on the survival of THCA patients and through cell proliferation and cell cycle experiments, it was proved that *GATAD1* promoted the proliferation of cancer cells and caused the recurrence of THCA by up-regulating its downstream gene *SRRM2*, which confirmed that *GATAD1* could be used as a biomarker for the diagnosis of patients with THCA recurrence and an independent predictor of patient survival. This provides a strong theoretical basis for the diagnosis and treatment of recurrent THCA patients. In addition, it has been shown in the past that transcription factors are susceptible to drug inhibition, which means that *GATAD1* may become a drug therapeutic target for THCA, improving the current situation of high recurrence rate of THCA and extending the survival time of THCA patients.

Conclusions

We found that *GATAD1* was highly expressed in relapsing THCA tissues, and experimentally demonstrated that the *GATAD1-SRRM2* axis mediated THCA recurrence progression through promoting cell proliferation, revealing the possibility of *GATAD1* as a biomarker for the diagnosis of THCA recurrence and a therapeutic target for THCA.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://gs.amegroups.com/article/view/10.21037/gs-22-666/rc

Data Sharing Statement: Available at https://gs.amegroups.com/article/view/10.21037/gs-22-666/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://gs.amegroups.com/article/view/10.21037/gs-22-666/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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