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Tiam1 regulates proliferation, invasion, and differentiation in neuroblastoma through the Tiam1/Rac1 signaling pathway

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Neuroblastoma is the most common extracranial solid tumor in children. The purpose of the present study is to detect the prognostic role and potential therapeutic efficacy of the T lymphoma invasion and metastasis 1 (Tiam1) in neuroblastoma. The overexpression of Tiam1 protein is frequently observed in neuroblastoma. Tiam1 expression is closely associated with adverse prognosis of neuroblastoma and risk group classification. Knockdown of TIAM1 by lentivirus expressing short hairpin RNA against TIAM1 (sh-TIAM1) inhibited the proliferation, invasion and cell-cycle progression, and promoted apoptosis of the neuroblastoma cell lines SH-SY5Y and SK-N-AS. Additionally, downregulation of the differentiation-related protein expression and decreased Rac1 expression was observed in the sh-TIAM1-transfected SH-SY5Y cells. *In vivo*, nude mice bearing TIAM1 knockdown SH-SY5Y cells showed improved overall survival and tumor growth suppression. The results demonstrate that inhibition of Tiam1 expression is a potential strategy for targeted therapy in neuroblastoma.

Key words: Tiam1; neuroblastoma; pediatric; differentiation; proliferation

Neuroblastoma is a heterogeneous tumor originating in the sympathetic nervous system and is the most common cause of fatality in pediatric cancer patients. The clinical course and molecular biological characteristics of neuroblastoma are highly heterogeneous [1].

The survival of patients with high-risk neuroblastoma has greatly improved owing to the introduction of intensive multimodality treatment consisting of chemotherapy, surgery, myeloablative therapy, and molecular targeted therapy [2]. However, the outcomes of neuroblastoma remain unsatisfactory with up to 50% of high-risk patients failing to respond to first-line treatment [3]. Molecular targeted therapy has shown promising effects in a variety of malignant tumors; however, appropriate targets remain undiscovered in neuroblastoma.

T lymphoma invasion and metastasis 1 (Tiam1) is a guanine nucleotide exchange factor (GEF). Normally, Tiam1 is expressed at low levels in most adult tissues but can be detected at significantly higher levels in the brain and testis [4]. Tiam1 expression in the brain is limited to a subset of neurons, and its expression during development is closely related to neuronal differentiation and/or migration, suggesting that Tiam1 plays a critical role in these processes [5, 6]. It is also overexpressed in various kinds of malignancies including colorectal, lung, gastric, liver, and ovarian cancers [5, 7, 8]. Tiam1 is involved in various biological processes that are essential for tumor growth and metastasis such as cell-fate decisions, proliferation, migration, and invasion [7, 9]. Tiam1 correlates well with prognosis in various types of malignancies [10–12]. The DH domain of Tiam1 is implicated in neuroblastoma. Its function is to activate downstream Rac1 and regulate the polarity and axons of nerve cells through the Trka/Tiam1/Rac1 signaling pathway. The extension and formation of glial cells can promote positive differentiation, and the absence of Tiam1 leads to neuronal dedifferentiation.

Therefore, clarification is needed on whether Tiam1 promotes invasion, proliferation, and dedifferentiation in neuroblastoma and if its inhibition produces anti-cancer effects. Furthermore, various prognostic markers have been used to predict the overall survival of patients with neuroblastoma [13]. Nevertheless, reliable biomarkers as prognostic indicators are urgently required.

In this study, we aimed to explore whether suppression of Tiam1 expression in neuroblastoma produced therapeutic effects by detecting Tiam1 expression in neuroblastoma tumor tissues and combining specific clinical data to analyze the relationship between Tiam1 and clinicopathological features in patients with neuroblastoma. We then performed a series of *in vitro* and *in vivo* experiments to further explore the mechanism underlying Tiam1 in proliferation, invasion, and de-differentiation of neuroblastoma cells. Our observations suggest that Tiam1 is a cardinal signaling molecule and a potential target for molecular-targeted therapy in neuroblastoma.

Patients and methods

Samples. We retrospectively examined 41 patients with neuroblastoma who underwent biopsy or resection at the Qilu Hospital of Shandong University between 2014 and 2021. All patients were pathologically diagnosed with neuroblastoma, received regular treatment, and had complete follow-up data. None of the patients had received radiotherapy or chemotherapy prior to surgery. The clinical background of the patients is summarized in Table 1. Written informed consent was obtained from the legal guardian of each patient. The study was approved by the Research Ethics Committee of the Qilu Hospital of Shandong University (Approval No. KYLL-2022(ZM)-790).

Patients with neuroblastoma were categorized into four stages (L1, L2, M, and MS) according to the International Neuroblastoma Risk Group (INRG) classification [14].

Immunohistochemistry. To perform immunohistochemical staining, serial sections of representative neuroblastoma specimens were treated in a microwave oven thrice for 5 min in citrate buffer (pH 6.0) at 750 W for antigen retrieval. Next, the slides were immersed in 0.3% H_2O_2 for 15 min and saturated with 20% goat serum in phosphate-buffered saline (PBS) for 1 h at 37 °C. The samples were then incubated overnight at 4 °C in a primary antibody working solution (anti-Tiam1 (1:50, ab11939, Abcam, Cambridge, MA, USA) and anti-Ki-67 (1:200, ab15580, Abcam)). Negative controls were incubated with PBS instead of primary antibodies. Furthermore, the slides were incubated with biotinylated secondary antibody and horse radish peroxidase (HRP)-streptavidin (LSAB2 System-HRP kit, Dako, Glostrup, Denmark) for 30 min at 37 °C.

Evaluation of immunohistochemical staining. The slides were examined by two independent pathologists who specialized in pediatric tumor pathology and were blinded to the patients' clinicopathological background. When different evaluations were obtained, slides were re-evaluated by observation using a double-headed microscope. Tiam1 immunopositivity was categorized based on the percentage of positive cells on the slide (0, no expression; +, 1–30%; ++, 31–60%; +++, >65%). For the survival analysis, Tiam1 expression was categorized as a dichotomous covariate, with Tiam1 low-expression (– to +) versus Tiam1 high-expression (++ to +++).

Ki-67 labeling index (LI) was calculated as the percentage of Ki-67-immunopositive tumor cells [15]. For each slide, 500 tumor cells were counted in five separate areas in which positive nuclear staining was evenly distributed.

Cell culture. The human neuroblastoma SH-SY5Y and SK-N-AS cell lines were obtained from Shanghai Jieluoxuan Bioscience Company (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco, New York, USA) containing 15% fetal bovine serum (FBS) (Gibco) and 0.055 mM 2-mercaptoethanol (Gibco) at 37 °C in a 5% CO_2 atmosphere. Representative images of the cells were obtained using an inverted fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Lentivirus preparation. Lentiviruses harboring short hairpin RNA (shRNA) against TIAM1 mRNA (shTIAM1) or a nonspecific control (NC) shRNA were produced through co-transfection of HEK293 cells with shRNA vector and packaging vectors GV112, pHelper 1.0, and pHelper 2.0 (Shanghai Genechem, Shanghai, China). The following siRNA sequences were used: shTiam1-a 5'-CCGGGGAGATGAGATTCTTGAGACTCGAGTCTCAAGAATCTCATCTCCTTTTTG-3'; shTiam1-b 5'-AATTCAAAAAGGAGATGAGATTCTTGAGACTCGAGTCTCAAGAATCTCATCTCC-3'; shNC-a 5'-CCGGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTG-3'; shNC-b AATTCAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAA-3'. Seventy-two hours later, the virus particles were harvested and purified.

RNA extraction and real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the SH-SY5Y cells and tumor tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized by reverse transcription using the ReverTra Ace qPCR RT Master Mix kit (Toboyo Co., Ltd., Osaka, Japan), in accordance with the manufacturer's instructions. RT-PCR analysis was performed using the ABI Prism 7500 sequence detection system with SYBR green RT-PCR kit (Takara, Shiga, Japan) in 20 µl of the reaction mixture. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min. The primer sequences were as follows: Tiam1: F: 5'-GCCAAGAACATTTAACAAGCAACG-3', R: 5'-TCTGAGCATACAAGTCACCCAAGG-3'; S-100: F: 5'-TGGACAAGGTAATGAAGGAACTG-3', R: 5'-TTGT-TACAAGCCACTGTGAGAG-3'; Neuron-specific enolase (NSE): F: 5'-CCTGATGTTGGAACTGGATGG-3', R: 5'-CTGAGCAATGTGGCGATAGAG-3'; Synaptophysin (Syn): F: 5'-TGATGTGCGTGTCCAGAAGA-3', R: 5'-CTCAAG-CATAGCAGAACCTGTATT-3'; Neurotrophic receptor tyrosine kinase 1 gene (TRKA): F: 5'-GGTGGCTGCTGGTATGGTAT-3', R:

5'-GTGCTGAACTTGCGGTAGAG-3'; Nestin: F: 5'-CCTTAGTCTGGAAGTGGCTACA-3', R: 5'-GGTGCTGGTCCTCTGGTATC-3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control with the following primers: F: 5'-GGCACAGTCAAGGCTGAGAATG-3', R: 5'-ATGGTGGTGAAGACGCCAGTA-3'.

Data were analyzed using Sequence Detection Software 1.4 (Applied Biosystems, Thermo Fisher Scientific). RNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [16].

Western blotting. Western blotting was performed as previously described [17]. Antibodies against Tiam1 (27694-1-AP, 1:500, Proteintech, Rosemont, IL, USA), Rac1 (66122-1-Ig, 1:500, Proteintech), and GAPDH (60004-1-Ig, 1:2000, Proteintech) were used. Chemiluminescence was detected using the ECL Chemiluminescence Detection Kit (Millipore, Temecula, CA, USA) and Image Studio Digits Ver 4.0. GAPDH was used as an internal control.

Mouse models. Mouse models were constructed by Shanghai Jieluoxuan Bioscience Company (Shanghai, China). Briefly, 20 female 4–6-week-old BALB/c nude mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed in a specific pathogen-free environment and fed a standard pellet diet and water *ad libitum*. The neuroblastoma xenograft mouse models were established as follows: On day 1, all mice were administered SH-SY5Y cells (4×10^6/100 µl/mouse) via underarm injection. In the experimental group, SH-SY5Y cells were stably transfected with sh*TIAM1* lentivirus, whereas in the control group, SH-SY5Y cells were transfected with nonspecific lentivirus instead. The length (the longest dimension) and width (the distance perpendicular between the exterior tumor edge and the mouse's body) of each tumor or lobe were measured with vernier calipers every 3 days [17]. Tumor volumes were calculated by the formula (Length × Width^2)/2 [17]. All mice were sacrificed on day 25, and the tumors, peripheral blood, bone marrow, liver, and spleen were removed and stored in liquid nitrogen for pathological analysis. Animal use was approved by the Animal Care and Use Committee of Shandong University.

MTT assay. The proliferation capability was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, cells were cultured in 96-well plates. Twenty-four hours later, MTT (5 mg/ml, Solarbio, Beijing, China) was added to each well. The cells were cultured for 3 h. Next, 100 µl of DMSO was added to the wells to replace the MTT reagent. A microplate reader was used to examine the cell viability at an absorbance of 490 nm. The experiments were performed in triplicates.

Apoptosis assay. Cell apoptosis was analyzed by Annexin V-PE/7-AAD stained flow cytometry (Meilunebio, Dalian, China). Neuroblastoma cells were harvested through trypsinization, washed with PBS, and then centrifuged to collect the pellet. The pellet was resuspended in 1× binding buffer at a density of 1×10^6 cells/ml. One hundred microliters of the binding buffer were incubated with 5 µl of PE-conjugated Annexin V (Meilunebio) and 5 µl of 7-AAD (Meilunebio) and added to samples for 15 min at 25 °C in the dark. Samples were analyzed by flow cytometer (Agilent Technologies, Palo Alto, CA, USA). Early apoptotic (PE positive, 7-AAD negative), late apoptotic, and dead cells (PE positive, 7-AAD positive) can be discriminated on the basis of a double-labeling for Annexin V-PE and 7-AAD. The data were analyzed using FlowJo 10.4 software (TreeStar, Ashland, OR, USA).

Cell cycle assay. About 1×10^6 cells were collected and washed using cold PBS buffer, then fixed with 70% alcohol overnight at 4 °C, cells were washed with PBS twice, and incubated with propidium iodide (50 µg/ml, PI, Sigma-Aldrich, St. Louis, MO, USA) and RNAase A (100 U/ml, Solarbio) for 30 min at 37 °C. Cells were analyzed using a flow cytometer (Agilent Technologies).

Matrigel invasion assay. Transwell migration plates and Matrigel invasion chambers were used to test the invasive capacity of SH-SY5Y cells. Briefly, cells at a concentration of 1×10^6 cells/ml in a serum-free medium were seeded into the upper chamber. Medium with 20% FBS was added to the lower chamber to promote cell invasion through the Matrigel and entry into the lower chamber. After 48 h, the bottom side of the upper chamber membrane was cut off and stained with hematoxylin and eosin. The cells in each high-magnification (×400) area were counted under a microscope. Each experiment was conducted in triplicates.

Neurite length. Neurite growth was observed under an inverted fluorescence microscope (IX71, Olympus) at 6, 12, 24, and 48 h after treatment with lentivirus. Images of the cells were captured in six separate fields. The cells with extended neurite whose length is more than the diameter of the cell body were considered as neurite-bearing. ImageJ software (NIH, Bethesda, MD, USA) was employed to measure the neurite lengths of the cells.

Statistical analysis. All data from the three replicate experiments are presented as the mean ± SD. Statistical analyses were performed using the Student's t-test to compare two groups of continuous variables. Spearman correlation analysis was used to test the correlation between Tiam1 expression and clinicopathological factors such as age, sex, location, and risk group. Differences were considered statistically significant at a probability level less than 0.05 ($p<0.05$). Statistical analyses were performed using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA).

Results

Relationship between Tiam1 expression and clinicopathologic characteristics. The protein expression of Tiam1 in neuroblastoma was detected using immunohistochemistry.

Tiam1 high expression was detected in 51.2% (21/41) of all neuroblastoma specimens (Figure 1, Table 1). Tiam1

low-expression tumors occurred more frequently in the adrenal gland (p=0.042). In addition, Tiam1 expression correlated with age (p=0.042), risk group (p=0.002), and Ki-67 MILB-1 LI (p=0.014; Table 1). No relationship was found between Tiam1 expression and sex (p>0.05; Table 1).

The inhibition of Tiam1 suppresses proliferation and invasion, slows the rate passing through cell the cell cycle, and enhances apoptosis in neuroblastoma cells. The correlation between proliferative marker Ki-67 LI with Tiam1 protein expression indicates a potential role of Tiam1 in proliferation in neuroblastoma. In addition, Tiam1 has been shown to be involved in migration and invasion of a variety of malignancies. To validate the roles of Tiam1 in the proliferation and invasion in neuroblastoma, we suppressed Tiam1 expression in neuroblastoma cells SH-SY5Y and SK-N-AS using lentiviruses harboring shRNA against TIAM1 mRNA.

Western blotting (Figure 2A) and RT-PCR (Figure 2B) results confirmed the successful suppression of Tiam1 protein and mRNA expression. The Transwell invasion assay revealed that downregulated Tiam1 expression inhibited the

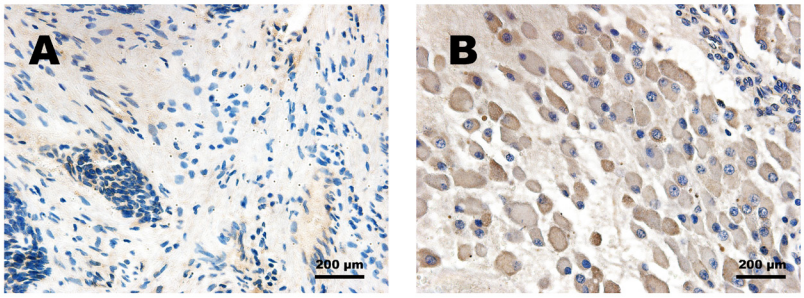

Figure 1. Representative immunohistochemical results of the abnormal expression of Tiam1 in neuroblastoma specimens. A) Low Tiam1 expression. B) High Tiam1 expression. Magnification: 400×.

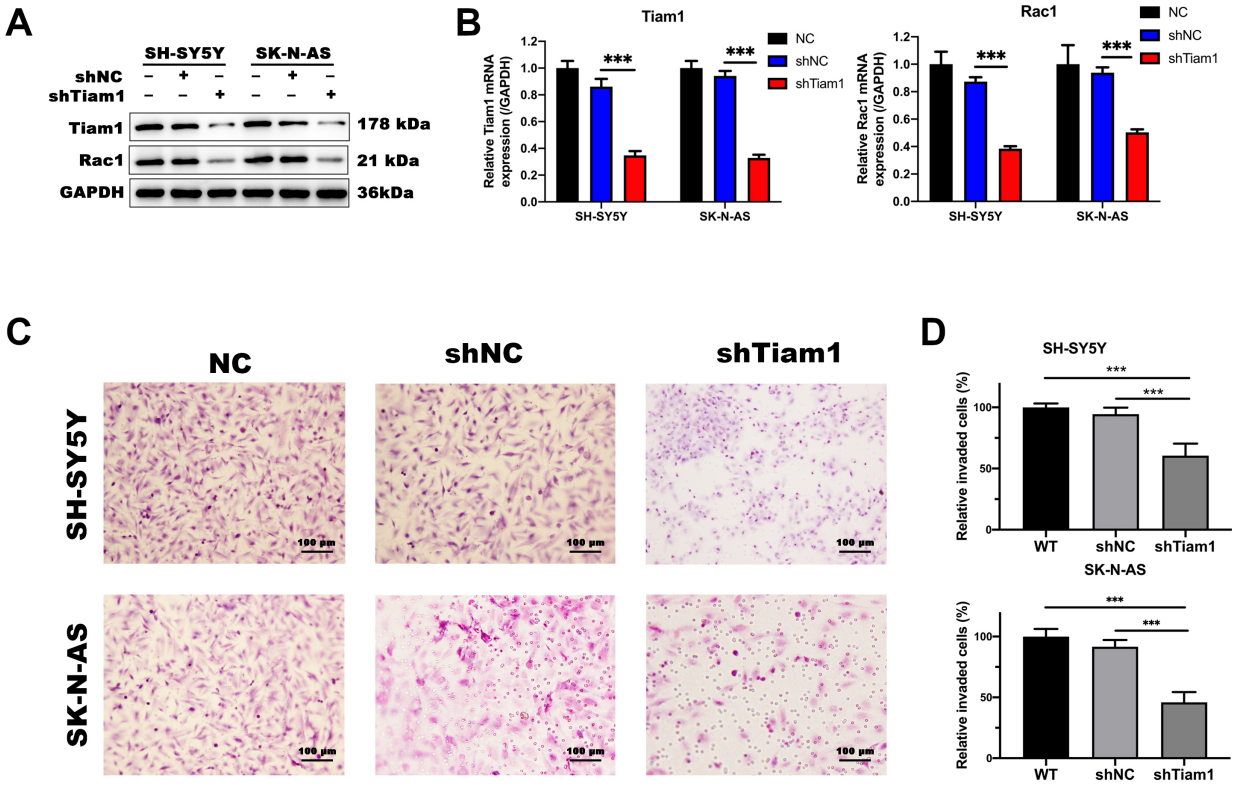

Figure 2. Different Tiam1 and Rac1 expression and invasion assay between sh-Tiam1 and sh-NC groups. A) Western blot confirmed the knockdown of Tiam1 and Rac1 protein expression in the sh-Tiam1 group. B) RT-PCR revealed downregulation of Tiam1 and Rac1 mRNA expression following sh-Tiam1 transfection. C) Relative images of invaded SH-SY5Y and SK-N-AS cells among WT, sh-NC, and shTiam1 groups. D) Representative of Transwell invasion assay cultured for 48 h. ***p<0.001

invasion of SH-SY5Y and SK-N-AS cells (Figures 2C, 2D). To test the potential mechanism underlying the regulation of invasion and proliferation by Tiam1, we explored Rac1 expression following TIAM1 knockdown in SH-SY5Y and SK-N-AS cells. As a cardinal downstream molecule in the Trka/Tiam1/Rac1 pathway, Rac1 protein expression was profoundly downregulated and positively correlated with Tiam1 expression, as shown by the western blot results (Figure 2A).

The MTT proliferation assay revealed that Tiam1 inhibition led to the inhibition of proliferation both in SH-SY5Y and SK-N-AS cells (Figure 3A). To further explore the underlying mechanism of the regulation of Tiam1 on proliferation, we performed flow cytometry to examine the apoptosis and cell cycle change following the knockdown of Tiam1. Inhibition of Tiam1 significantly increased apoptosis in both SH-SY5Y and SK-N-AS cells ($p<0.001$, Figures 3B, 3D). In contrast, flow cytometry results showed that inhibited Tiam1 expression slowed the rate of SH-SY5Y and SK-N-AS cells passing through cell cycle phases, with an increased proportion of non-dividing cells in the G1 phase and a decreased proportion of dividing cells in S and G2 phases (Figures 3C, 3E).

The differential expressions of Tiam1 in neuroblastoma specimens were closely related to proliferation and differentiation *in vivo* and *in vitro*. Three weeks after SH-SY5Y cell administration, the volume and weight of xenograft tumors in mice in the shTiam1 group were smaller than those in the Tiam1 wildtype group (shNC) (Figures 4A–4C). Immunohistochemical analysis confirmed the suppressed expression of Tiam1 in the shTiam1 group. Similarly, immunohistochemistry confirmed that Rac1 protein expression was also downregulated in shTiam1 xenograft tumors compared with those in the shNC group (Figure 4D).

To analyze the differentiation of xenograft neuroblastoma tissues, we examined the mRNA expression levels of differentiation-related markers using RT-PCR. The results showed that, compared with that of the control group, the downregulation of Tiam1 expression led to increased expression levels of the pro-differentiation markers S100, NSE, and Syn and the decreased expression levels of the de-differentiation markers nestin and Trka (Figures 5A–5E). We also observed that treatment of SH-SY5Y cells with shTiam1 significantly enhanced neurite outgrowth at different time points, indicating increased differentiation rates of neuroblastoma cells. (Figures 5F, 5G).

Table 1 The relationship between clinicopathological characteristics and Tiam1 protein expression.

Variables	Number of patients	Low expression (n=20, %)	High expression (n=21, %)	p-value
Age				0.042*
≤18 months	25	9 (45)	16 (76)	
>18 months	16	11 (55)	5 (24)	
Gender				0.455
Male	23	10 (50)	13 (62)	
Female	18	10 (50)	8 (38)	
Tumor location				0.042*
Adrenal gland	25	9 (45)	16 (76)	
No-adrenal gland	16	11 (55)	5 (24)	
Risk group				0.002*
L1	9	7 (35)	2 (10)	
L2	11	8 (40)	3 (14)	
M	12	3 (15)	9 (43)	
MS	9	2 (10)	7 (33)	
Ki-67		33.9±31.1	56.9±25.6	0.014*

Discussion

Our study confirmed for the first time that Tiam1 is pivotal in the regulation of proliferation, invasion, and differentiation in neuroblastoma. We also found that Tiam1 protein expression was closely related to risk group, age, onset site, and proliferative marker Ki-67 LI in patients with neuroblastoma. Knockdown of *TIAM1* showed promising therapeutic effects by inhibiting proliferation and invasion, and promoting differentiation in neuroblastoma cell lines and xenograft mice models.

Prognostic factors and risk assessment are important for patients with neuroblastoma, especially for those in pre-treatment. Currently, based on surgical resection, location, age, *MYCN* amplification, and many other clinicopathological features, patients with neuroblastoma are generally categorized into different risk groups or stages according to the INRG staging system and the International Neuroblastoma Staging System (INSS). Currently, the INRG classification has been proven to be an effective tool in predicting the outcome of patients in pre-treatment for neuroblastoma by categorizing them into four separate cohorts L1, L2, M, and MS [18]. The INRG classification was developed based on the introduction of important biological prognostic factors and excellent overall survival (OS) rate for patients with non-metastatic neuroblastoma [18–20]. The association between Tiam1 expression and risk stratification suggested that Tiam1 expression might serve as a prognostic indicator to supplement current prognostic systems.

Neuroblastoma is one of the most common causes of pediatric mortality and morbidity. Nowadays, treatment for neuroblastoma has evolved into a multimodal therapy consisting of surgical resection, chemotherapy, autologous stem cell transplantation, immunotherapy, and radiotherapy, either individually or in combination. Despite the improvement in the overall survival of patients with neuroblastoma due to the introduction of novel therapeutic modalities, the prognosis of high-risk cases, which accounts for about 40% of all cases, remains dismal and is related to chemotherapy resistance and tumor recurrence [21]. The 5-year survival rate of high-risk cases is approximately 50% [22]. Neuroblastoma is also notably heterogeneous with the clinical

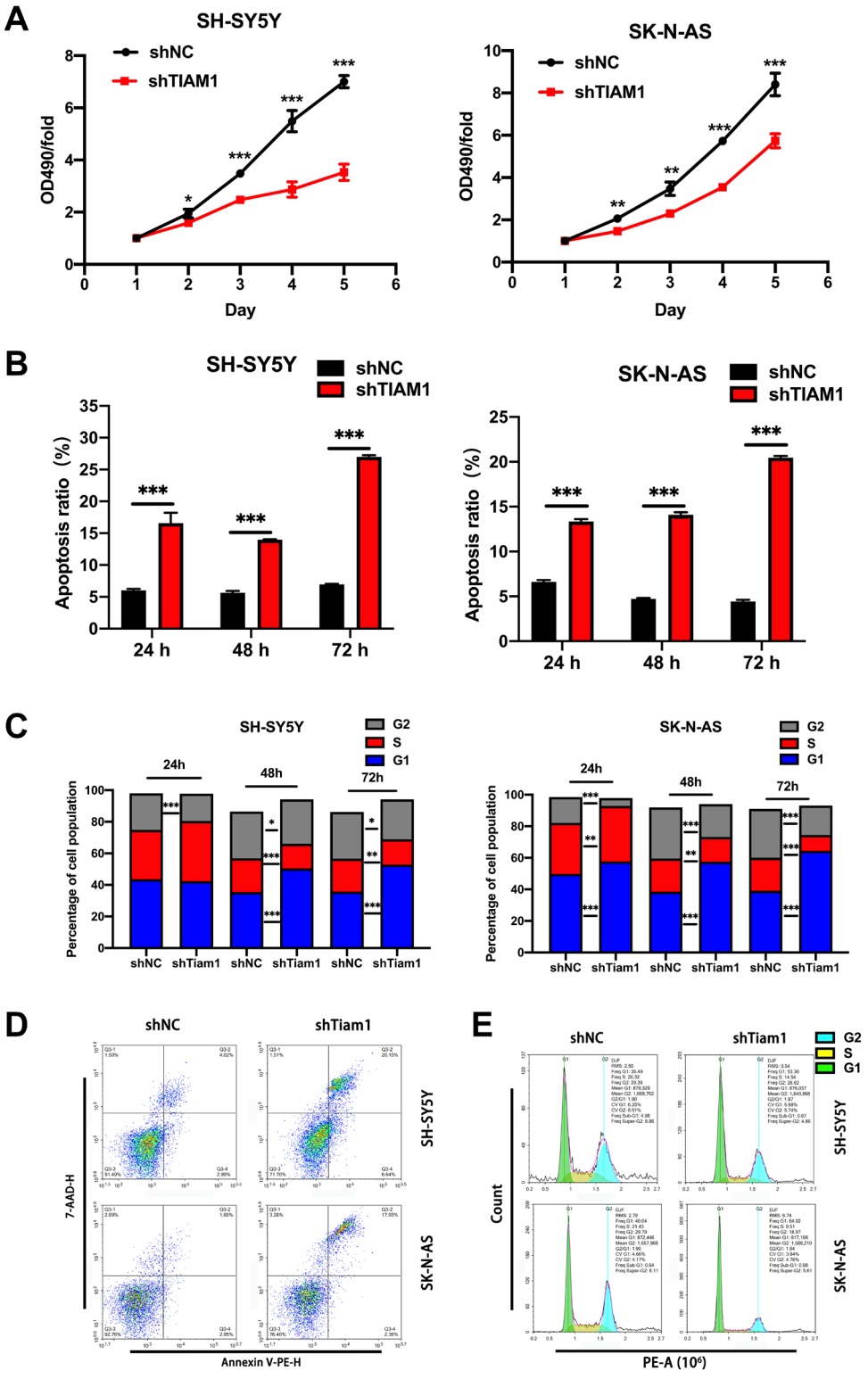

Figure 3. Proliferation, apoptosis, and cell-cycle analysis between sh-Tiam1 and sh-NC groups. A) MTT assay results showed that the proliferation of SH-SY5Y and SK-N-AS cells was inhibited after the knockdown of Tiam1 expression. B) Quantification of the apoptotic cell rates in SH-SY5Y and SK-N-AS cells following knockdown of Tiam1 expression. C) Quantification of the percentage of cells in different cell cycle phases in SH-SY5Y and SK-N-AS cells following the knockdown of Tiam1 expression. D) Representative images of apoptosis assay in SH-SY5Y and SK-N-AS cells at 72 h. (E) Representative images of cell cycle analysis in SH-SY5Y and SK-N-AS cells at 72 h. ***p<0.001, **p<0.01, *p<0.05

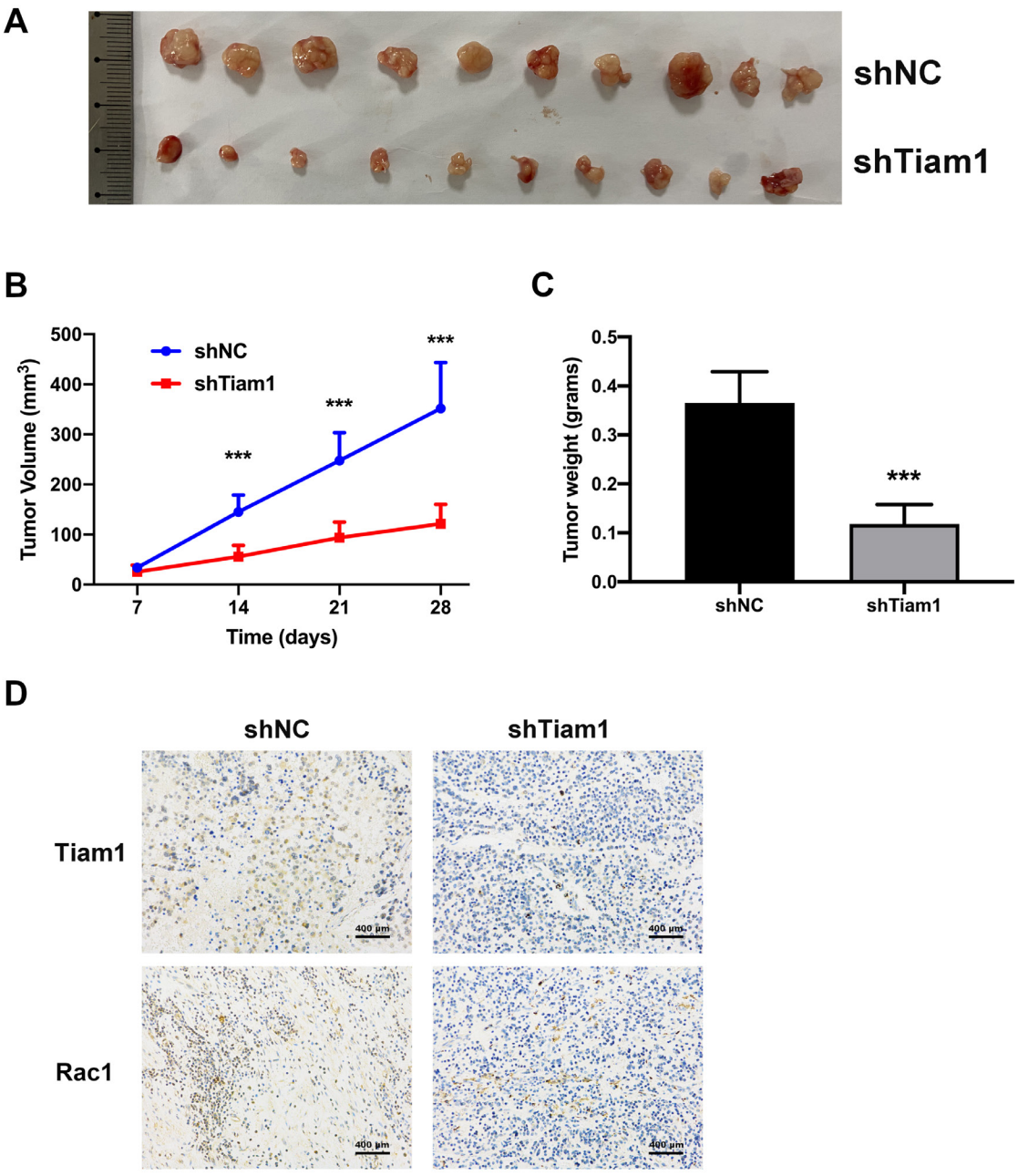

Figure 4. Tiam1 regulates the proliferation and differentiation of neuroblastoma in mice. A) Representative harvested tumor tissues 4 weeks after the injection of SH-SY5Y neuroblastoma cells. B) Comparison of tumor volume between shNC and shTiam1 groups. C) Comparison of the weight of harvested tumor between shNC and shTiam1 groups. D) Representative immunohistochemical images of Tiam1 and Rac1 expression.

course and overall prognosis strongly correlates with their individual genetic or molecular status [23]. Therefore, a novel therapeutic strategy is in urgent need.

The continuous advancement of molecular diagnostic technology has enabled the application of precision therapy in the treatment of malignant tumors, and targeted therapy has become a hotspot in recent years [24, 25]. Since the discovery of the oncogene *N-myc* in neuroblastoma patients in 1983, the application of autologous hematopoietic stem cell transplantation and GD2 antibody under myeloid clearing has gradually improved the survival rate of neuroblastoma patients [26]. However, the identification of appropriate targets for genetic aberrations in neuroblastoma is still underway. Current risk stratification also requires continuous refinement based on advancements in technology and genetic findings in neuroblastoma [27]. In the present study,

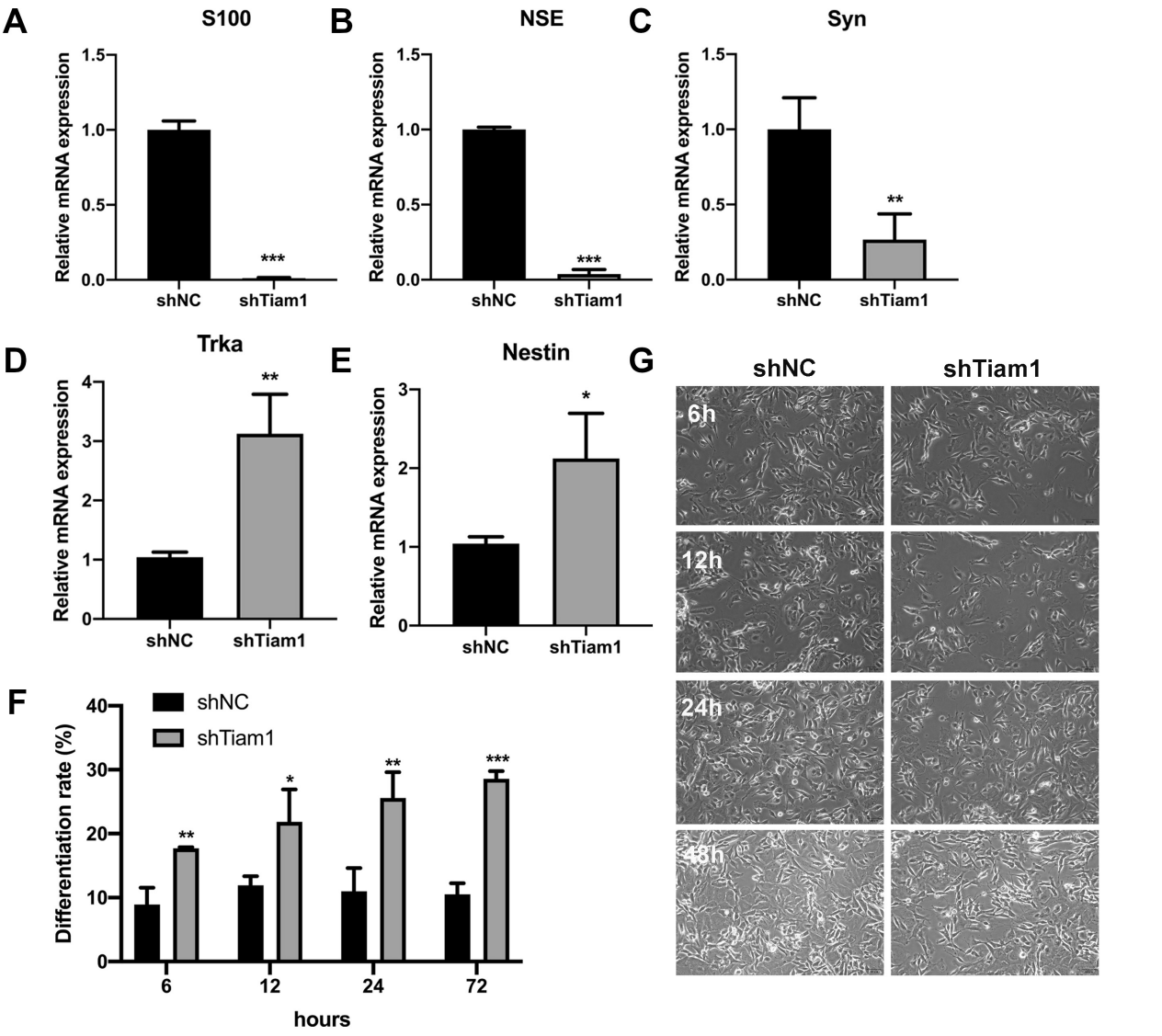

Figure 5. RT-PCR results of differentiation marker expression levels and representative of the morphology of SH-SY5Y cells. A–E) Differentiation markers S100 (A), NSE (B), Syn (C), and de-differentiation markers Trka (D) and Nestin (E) mRNA expression levels examined by RT-PCR. F) Differentiation rate of SH-SY5Y at different time points after lentivirus transfection. G) Representative morphology of SH-SY5Y neuroblastoma cells at different time points after lentivirus transfection. ***p<0.001, **p<0.01, *p<0.05

we found that Tiam1 expression is abnormal in neuroblastoma tissues and is closely related to patient risk stratification, suggesting that it could serve as a potential prognostic marker and a target for targeted therapy.

The role of Tiam1 in the invasion and metastasis of tumor cells has been well documented [27]. However, the oncogenic role of Tiam1 in neuroblastoma remains unclear. A recent study of 240 patients with high-risk neuroblastoma did not identify any mutations in the *TIAM1* gene [28]. One explanation is that while screening for tumor-specific somatic mutations, Tiam1 protective germline variants may have been skipped. In contrast, another study with 87 neuroblastoma patients reported three mutations in the *TIAM1* gene, one with concomitant *MYCN* amplification and poor outcome [29]. Furthermore, Tiam1 plays a cardinal role in the proliferation of various cancers, such as chronic lymphocytic leukemia [30]. In fact, high expression of Tiam1 was found in approximately one-third (13/41, 31.7%) of our neuroblastoma cases. We further analyzed the clinicopathological characteristics of the 41 patients with neuroblastoma and found that the intensity of Tiam1 expression was closely related to age and risk stratification. Moreover, Tiam1 is closely associated with the proliferative marker Ki-67 LI. These results preliminarily suggest that Tiam1 might not only regulate the proliferation and differentiation of neuroblastoma but also participate in the formation of neuroblas-

toma. However, the relatively small sample size limited our conclusions. Thus, we further verified this hypothesis using cell lines and animal experiments.

Tiam1 is a conversion factor between GDP and GTP. It mainly promotes the transition of inactivated Rac1 GDP to activated Rac1 GTP through the Tiam1/Rac signaling pathway. It thereby regulates cytoskeletal reorganization, affects tumor cell morphology polarization, and promotes tumor cell invasion [4]. However, controversial observations have also been reported. Another study found that the introduction of the gene encoding *Tiam1* into human renal cancer cell lines *in vitro* reduced the invasiveness of renal cancer cells, and this decrease was not significantly related to the expression of Rac1. In addition, the anti-metastatic effect of Tiam1 also exists in the Ras-transformed renal carcinoma cell line [31]. Our results confirmed that downregulated Tiam1 expression inhibited invasion in neuroblastoma cells. However, further analysis will be required to better understand the dual role of Tiam1 in invasion across different tumorous cells.

With regards to apoptosis and cell-cycle progression which are closely related to proliferative properties of tumor cells, Tiam1 was also found to play critical roles in these biological processes. It has been reported that Tiam1 depletion leads to lagging chromosomes at anaphase and aneuploidy, potential drivers of malignant progression [32]. Depletion of Tiam1 or Rac1 inhibition leads to small-cell lung cancer cell apoptosis through cytoplasmic translocation of the orphan nuclear receptor Nur77 and pro-apoptotic Bcl2 conformation change [7]. Additionally, Tiam1 overexpression results in increased migration and decreased apoptosis in a variety of cancer cells, such as colorectal carcinoma, gastric cancer, ovarian cancer, leukemia, and T-cell lymphoma [5, 7, 8, 28]. In this study, we also observed that Tiam1 promotes neuroblastoma cell proliferation and differentiation ability through a series of *in vitro* and *in vivo* experiments. In addition, we found that suppressed Tiam1 expression enhanced apoptosis and cell-cycle promotion, which could at least partially explain the effect of Tiam1 on the proliferation of neuroblastoma cells.

Nestin is generally considered as a dedifferentiation molecular marker in neuroblastoma cancer stem cells [33]. Syn, NSE, and S100 are molecular markers of axons, neurons, and Schwann cells, respectively [34]. RT-PCR results showed that inhibition of Tiam1 expression led to significantly decreased Nestin expression and significantly increased Syn, NSE, and S100 expression compared with that of the control group. This indicates that Tiam1 promotes differentiation in neuroblastoma.

The occurrence of embryonic neuroblastoma is attributed mainly to abnormal embryonic development. In other words, differentiation failure causes neural stem cells to fail to differentiate into mature neurons and glial cells [35]. Previous studies have shown that Tiam1/Rac1 regulates the polarity of nerve cells, axon extension, and glial formation and promotes positive differentiation. When Tiam1 is absent, nerve cell dedifferentiation occurs. In contrast, the P75NGFR/RhoA signaling pathway plays a role in negatively regulating TrkA/Tiam1/Rac1, retreating protruding axons, rounding cells, and dedifferentiating neural cells. Tiam1 promotes these two signaling pathways and thus participates in the regulation of neuroblastoma differentiation [36, 37]. In our study, the inhibition of the *TIAM1* gene downregulated Rac 1 expression, proving that Tiam1 inhibits the ability of stem cell-like tumor cells to differentiate in neuroblastomas through the Tiam1/Rac1 signaling pathway.

Notably, we found that Tiam1 correlated well with the adrenal location of neuroblastomas. A great majority of neuroblastomas arise from the abdomen: 40% from the adrenal medulla and 25% from the sympathetic ganglia [38]. However, the etiology of neuroblastoma remains unknown. The different onset site of the tumors and age at diagnosis of patients suggest that these neuroblastomas might bear differing genetic backgrounds.

However, there are a few limitations to this study. First, the sample size of our cohort for clinicopathological analysis was not large enough to yield more reliable statistical results. Second, we did not perform recovery experiments to confirm the biological effect of the downstream molecule Rac1; therefore, further studies are required to test the role of Rac1 in proliferation, invasion, and differentiation. Third, the mechanism of Tiam1 in the regulation of proliferation, invasion, and dedifferentiation is not sufficiently understood. Further analysis will be required to explore the signaling pathway and understand the exact interactions between Tiam1 and its downstream molecules.

In the present study, we confirmed that high Tiam1 protein expression was observed in more than half of the neuroblastoma tissues. Moreover, high expression of Tiam1 correlated well with risk stratification, younger age, adrenal location, and Ki-67 LI. Knockdown of *TIAM1* expression inhibited proliferation, invasion, cell-cycle progression and de-differentiation, and promoted apoptosis both *in vitro* and *in vivo*. The expression of the downstream molecule Rac1 in the Tiam1/Rac1 signaling pathway also decreased, suggesting that Tiam1/Rac1 might be involved in biological behaviors in neuroblastoma. Therefore, Tiam1 could serve as a potential target for molecular-targeted therapy against neuroblastoma.

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