Hsa_circ_0013401 Accelerates the Growth, Metastasis and Prevents Apoptosis Autophagy of Neuroblastoma Cells by Sponging miR-195 to Release PAK2

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Research

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Abstract

Background: Circular RNA (circRNA) is a newly discovered non-coding RNA with a covalently closed loop structure. Recently, the increased circRNAs have been identified in a variety of cancers. While, the specific functions and mechanisms of some circRNAs in neuroblastoma (NB) are still largely unexplored.

Materials and Methods: The levels of hsa_circ_0045997, hsa_circ_0080307, hsa_circ_0013401, hsa_circ_0077578 and microRNA-195 (miR-195) were confirmed by RT-qPCR assay in NB. Functionally, gain- and loss-of-function assays and the rescued experiment were conducted to determine the influences of hsa_circ_0013401, miR-195 and P21-activated kinase 2 (PAK2) on the proliferation, apoptosis, autophagy, migration and invasion of NB cells. Luciferase reporter gene assay was also applied to examine the relationships between hsa_circ_0013401 and miR-195, miR-195 and PAK2. Hsa_circ_0013401/miR-195/PAK2 axis was also identified by *in vivo* experiment study.

Results: Hsa_circ_0013401 was highly expressed, miR-195 was lowly expressed, and there was a negative correlation between hsa_circ_0013401 and miR-195 in NB. Hsa_circ_0013401 significantly suppressed the proliferation, migration and invasion, and induced apoptosis and autophagy of NB. In mechanism, miR-195 was a direct target of hsa_circ_0013401, and PAK2 was the downstream target gene of miR-195. And the inhibitory effects of hsa_circ_0013401 knockdown on the malignant biological properties of NB can be achieved by targeting miR-195 to upregulated PAK2. Besides, *in vivo* evidences also revealed that hsa_circ_0013401 promotes tumor formation, and regulated miR-195 and PAK2.

Conclusions: Hsa_circ_0013401 induced NB progression through miR-195 to enhance PAK2. Therefore, we might highlight a novel regulatory axis (hsa_circ_0013401/miR-195/PAK2) in NB.

Background

Neuroblastoma (NB) is a malignant tumor commonly discovered in the peripheral nervous system of infants [1]. NB is also a common embryonal solid tumor originating from the neural crests of the sympathetic nervous system [2]. According to statistics, the incidence of NB is second only to leukemia and brain cancer, and the mortality rate is about 15% in children with cancer [3]. Most NB patients are diagnosed in the middle or late stage due to the high malignant degree, rapid development and easy metastasis [4]. Currently, the therapeutic methods of NB mainly contain surgery, radiotherapy, chemotherapy, stem cell transplantation to enhance chemotherapy, and induce differentiation to maintain treatment, etc [5]. Despite combination therapy, the survival rate of patients with advanced NB is still low [6]. Therefore, it is urgent to explore the targeted therapy molecules with strong specificity and sensitivity for the therapy of NB patients.

Circular RNAs (circRNAs) are a class of non-coding RNA molecules without a cap structure at the 5' ends and a poly(A) tail at the 3' ends, which can form a circular structure with covalent bonds [7]. CircRNAs are stable in cells and not easily degraded by exonuclease due to its closed ring structure [8]. Besides, circRNAs have developmental stage specificity and tissue specificity, are abundant and highly

conservative in different species [9]. Recent researches testified that circRNAs have significant effects in regulating gene expression [10]. A growing body of studies also demonstrated that circRNAs play critical roles in multiple cancers, such as circHIPK3 in colorectal cancer [11], circRNA_102171 in thyroid cancer [12], circSLC8A1 in bladder cancer [13], and circCDR1 in gastric cancer [14], etc. Therefore, circRNAs can be used as the diagnostic molecular markers and therapeutic targets for tumors [15]. However, the role of circRNA in NB development has been rarely reported, and the biological functions and potential mechanisms of most circRNAs have not yet been discovered.

MicroRNAs (miRNAs) as crucial post-transcriptional regulator, can directly bind to the non-translational sites of mRNAs through complementary base pairing, thus suppressing protein translation or inducing mRNA degradation [16]. Recent studies also have verified circRNAs are rich in miRNAs reaction elements (MREs), which can reduce the inhibitory effects of miRNAs on target genes by sponging with miRNAs [7, 17]. Compared with other types of competitive endogenous RNA (ceRNAs), circRNA has more binding sites to miRNA and prominent competing endogenous RNA (ceRNA) activity [17]. Previous researches have testified that microRNA-195 (miR-195) presents a trend of low expression in nephroblastoma [18] and other cancers [19–21], indicating that the low expression of miR-195 may be related to the cancer progression to some extent. However, miR-195 has not been reported in NB. Through bioinformation prediction, we also found that there were interaction sites between circRNAs (has_circ_0045997, hsa_circ_0080307, has_circ_0013401, and hsa_circ_0077578) and miR-195. Therefore, further investigation for the potential functions and mechanisms of these circRNAs and miR-195 may be critical for NB treatment.

In our study, we identified the expressions of the predicted 4 circRNAs in NB tissues, and discovered that hsa_circ_0013401 was highly expressed in NB. Besides, we verified the targeted regulatory relationship between hsa_circ_0013401 and miR-195, and investigated the potential biological functions of hsa_circ_0013401 and miR-195 on the proliferation, apoptosis, autophagy, migration and invasion of NB cells. Moreover, we screened and confirmed that P21-activated kinase 2 (PAK2) was a target gene of miR-195, and proved the crucial roles of hsa_circ_0013401/miR-195/PAK2 axis in the biological processes of NB.

Results

Circ_0013401 was identified as highly expressed in NB

To investigate the expression changes of the related circRNAs in NB, we firstly examined circ_0013401, circ_0045997, circ_0077578 and circ_0080307 expressions in a total of 8 GN and NB tissue samples. As displayed in Fig. 1A, only circ_0013401 was significantly upregulated in NB tissues compared to that in GN tissues ($p < 0.01$). Next, we adopted FISH assay to identify the expression of circ_0013401 in GN and NB tissues, the results also uncovered that circ_0013401 was highly expressed in NB tissues relative to GN tissues (Fig. 1B). Additionally, Ki67 and PAK2 expressions were monitored using IHC assay, the results discovered that Ki67 and PAK2 expressions were also markedly elevated in NB tissues with respect to GN

tissues (Fig. 1C and 1D). Consequently, we verified that circ_0013401, Ki67 and PAK2 were highly expressed in NB.

Circ_0013401, as an oncogene, prominently accelerated NB proliferation

Given that circ_0013401 was down-regulated in NB, we further investigated the possible functional roles mediated by circ_0013401 overexpression and knockdown in NB cells. We first determined circ_0013401 expression in different neuroblastoma cells (SK-N-BE, GNP, SH-SY5Y, IMR-32, LAN-1 and SK-N-SH) using RT-qPCR, and the results proved that circ_0013401 was significantly upregulated in SH-SY5Y and SK-N-BE cells compared to other NB cells. And SH-SY5Y and SK-N-BE cells were applied in subsequent experiments ($p < 0.01$, Fig. 2A). RT-qPCR analysis was then conducted to identify the transfection effects of circ_0013401-overexpressed plasmid or circ_0013401 shRNAs in SH-SY5Y and SK-N-BE cells. As presented in Fig. 2B, circ_0013401 was observably upregulated in circ_0013401 overexpression group compared to overexpression-NC group, and circ_0013401 was also signally downregulated in circ_0013401 knockdown group versus sh-NC group in SH-SY5Y and SK-N-BE cells ($p < 0.01$, Fig. 2B). Afterwards, we detected the influences of circ_0013401 overexpression or knockdown on the proliferation of SH-SY5Y and SK-N-BE cells. The CCK-8 results manifested that the viability of SH-SY5Y and SK-N-BE cells was memorably increased in circ_0013401 overexpression group with respect to overexpression-NC group, and dramatically decreased in circ_0013401 knockdown group relative to sh-NC group ($p < 0.05$, $p < 0.01$, Fig. 2C). Consequently, the EdU + number of circ_0013401 overexpressing SH-SY5Y and SK-N-BE cells was significantly more than those of overexpression-NC group, and the Edu + number of circ_0013401 silencing cells was dramatically less than those of sh-NC group ($p < 0.05$, $p < 0.01$, Fig. 2D). Meanwhile, clone formation assay revealed that circ_0013401 overexpression observably induced NB proliferation, and circ_0013401 knockdown prominently suppressed NB proliferation ($p < 0.05$, $p < 0.01$, Fig. 2E). Hence, we testified that circ_0013401 played a significant role in inducing the proliferation of NB cells.

Circ_0013401 markedly facilitated migration and invasion, and prevented apoptosis and autophagy of NB cells

Besides, we further verified the changes of migration, invasion, apoptosis and autophagy in SH-SY5Y and SK-N-BE cells after circ_0013401 overexpression or knockdown. Firstly, the migration and invasion were certified by adopting Transwell assay, and the data disclosed that overexpression of circ_0013401 could result in prominent enhancements, and knockdown of circ_0013401 could lead to remarkable reductions in the migration and invasion capabilities of SH-SY5Y and SK-N-BE cells ($p < 0.05$, $p < 0.01$, Fig. 3A and 3B). The data in flow cytometer detection exhibited that circ_0013401 overexpression notably reduced the apoptosis rate, circ_0013401 knockdown prominently raised the apoptosis rate in SH-SY5Y and SK-N-BE cells ($p < 0.05$, $p < 0.01$, Fig. 3C). Moreover, the TEM results testified that compared with the control group, the autophagosomes in circ_0013401-overexpressed SH-SY5Y and SK-N-BE cells were significantly

reduced, and a large number of autophagosomes were formed in circ_0013401-silenced SH-SY5Y and SK-N-BE cells (Fig. 3D). Thus, we indicated that circ_0013401 could signally induce migration and invasion, and repressed apoptosis and autophagy of SH-SY5Y and SK-N-BE cells.

Circ_0013401 dramatically regulated miR-195/PAK2 axis, autophagy and apoptosis-related proteins in NB cells

Subsequently, we explored the underlying regulatory pathway of circ_0013401 in NB. Through bioinformatics predictions, we discovered that circ_0013401 might be a miRNA response element (MRE) of miR-195, which might bind with circ_0013401; and PAK2 might be the most likely target gene of miR-195. So, miR-195 and PAK2 became our research targets in mechanism. To further confirm the miR-195 expression in NB, the level of miR-195 was analyzed in different neuroblastoma cells using RT-qPCR. As indicated in Fig. 4A, miR-195 was signally downregulated in SH-SY5Y and SK-N-BE cells relative to other NB cells ($p < 0.01$). In addition, we demonstrated that miR-195 could be observably downregulated by circ_0013401 overexpression, and memorably upregulated by circ_0013401 knockdown in SH-SY5Y and SK-N-BE cells ($p < 0.05$, $p < 0.01$, Fig. 4B). While PAK2 could be markedly upregulated by circ_0013401 overexpression, and dramatically downregulated by circ_0013401 knockdown in SH-SY5Y and SK-N-BE cells ($p < 0.05$, $p < 0.01$, Fig. 4C). Meanwhile, the IF results further indicated that circ_0013401 overexpression prominently raised PAK2 expression and reduced LC3B expression; meanwhile, circ_0013401 knockdown notably lowered PAK2 expression and elevated LC3B expression in SH-SY5Y and SK-N-BE cells (Fig. 4D). More importantly, western blotting results showed that circ_0013401 overexpression observably upregulated PAK2, p62 and Bcl-2, and downregulated LC3B II/I, Beclin1, Bax and cleaved Caspase-3, while circ_0013401 knockdown could lead to the opposite effects of its overexpression on these proteins in SH-SY5Y and SK-N-BE cells (Fig. 4E). On the whole, we manifested that circ_0013401 dramatically suppressed autophagy and apoptosis, and regulated miR-195/PAK2 axis in NB cells.

Circ_0013401 sponged miR-195, and PAK2 was a target gene of miR-195

To verify the relationship between miR-195 and circ_0013401 or PAK2, luciferase reporter assay was performed. We proved that miR-195 could significantly lower the luciferase activity of WT-circ_0013401, not Mut-circ_0013401 in SH-SY5Y and SK-N-BE cells ($p < 0.01$, Fig. 5A). Similarly, the luciferase activity of WT-PAK2 was signally repressed by miR-195, while the luciferase activity of Mut-PAK2 was not affected by miR-195 in SH-SY5Y and SK-N-BE cells ($p < 0.01$, Fig. 5B). Thus, we proved that circ_0013401 can significantly regulate miR-195/PAK2 axis through targeted binding.

MiR-195 inhibitor prominently reversed the inhibitory action of circ_0013401 knockdown on the proliferation of NB cells

Next, the rescue assays were conducted to confirm whether circ_0013401 induced NC proliferation by targeting miR-195. MiR-195 inhibitor were transfected into circ_0013401-silenced SH-SY5Y and SK-N-BE cells, and the RT-qPCR results demonstrated that miR-195 inhibitor markedly downregulated miR-195, which was induced by circ_0013401 shRNA in SH-SY5Y and SK-N-BE cells ($p < 0.01$, Fig. 6A). Then EdU staining results uncovered that the proliferation of SH-SY5Y and SK-N-BE cells was memorably increased in the co-transfection group of miR-195 inhibitor and circ_0013401 shRNAs compared with that in the transfection group of circ_0013401 shRNA ($p < 0.01$, Fig. 6B and 6C). Likewise, the results of clone formation assay also revealed that miR-195 inhibitor could prominently facilitate NB cell proliferation mediated by circ_0013401 knockdown ($p < 0.01$, Fig. 6D). Therefore, we testified that the inhibition of cell proliferation mediated by circ_0013401 shRNA could be significantly reversed by miR-195 inhibitor in NB.

MiR-195 was involved in the inhibition of migration and invasion, and induction of apoptosis mediated by circ_0013401 knockdown in NB cells

Additionally, through Transwell assay, we discovered that the migration and invasion abilities of SH-SY5Y and SK-N-BE cells were markedly higher in the co-transfection group of miR-195 inhibitor and circ_0013401 shRNA than that in the transfection group of circ_0013401 shRNA ($p < 0.01$, Fig. 7A and 7B). Flow cytometer results testified that miR-195 inhibitor signally reduced the apoptosis of SH-SY5Y and SK-N-BE cells, which have been induced by miR-491-5p mimics ($p < 0.01$, Fig. 7C). In general, we certified that circ_0013401 knockdown prevented migration and invasion, and accelerated apoptosis of NB cells by miR-195.

MiR-195 inhibitor memorably attenuated the downregulation effects of circ_0013401 knockdown on PAK2, autophagy and apoptosis-related proteins in NB cells

Furthermore, we identified the downstream regulatory molecules of circ_0013401/miR-195 axis in NB cells. SH-SY5Y and SK-N-BE cells were co-transfected circ_0013401 shRNA and miR-195 inhibitor, and RT-qPCR was first conducted to confirm PAK2 expression. And the data indicated that PAK2 expression mediated by circ_0013401 knockdown could be dramatically upregulated by miR-195 inhibitor in SH-SY5Y and SK-N-BE cells ($p < 0.01$, Fig. 8A). Simultaneously, western blot results proved that after transfection with miR-195 inhibitor, PAK2, p62 and Bcl-2 were observably upregulated, LC3BII/I, Beclin1, Bax and cleaved Caspase-3 signally were downregulated in circ_0013401 shRNA-transfected SH-SY5Y and SK-N-BE cells (Fig. 8B). As a result, we suggested that circ_0013401 knockdown could reduce PAK2 expression and induce autophagy and apoptosis-related proteins by miR-195 in NB cells.

Overexpression of PAK2 signally suppressed apoptosis and autophagy mediated by miR-195 in NB cells

Whereafter, we adopted the rescue assays to verify the impacts of miR-195/PAK2 axis on apoptosis and autophagy in NB cells. miR-195 mimics or/and PAK2-overexpressed plasmids were transfected into SH-SY5Y and SK-N-BE cells, and PAK2 expression was assessed using RT-qPCR. As shown in Fig. 9A,

overexpression of PAK2 prominently aggrandize PAK2 expression in miR-195 mimics-transfected SH-SY5Y and SK-N-BE cells ($p < 0.01$). Meanwhile, the data of flow cytometry revealed that the apoptosis rate could be markedly weakened by PAK2 overexpression in SH-SY5Y and SK-N-BE cells after transfection with miR-195 mimics ($p < 0.01$, Fig. 9B). In the mechanism, we disclosed that overexpression of PAK2 also could memorably increase p62 and Bcl-2 expressions, signally decrease LC3BII/I, Beclin1, Bax and cleaved Caspase-3 expressions in miR-195 mimics-transfected SH-SY5Y and SK-N-BE cells (Fig. 9C). Overall, we indicated that PAK2 was significantly involved in the effects of miR-195 on apoptosis and autophagy of NB cells.

Verification of circ_0013401/miR-195/PAK2 axis in vivo experiment

Based on the results of in vitro experiments, we further verified the regulatory effects of circ_0013401 on tumor growth and miR-195/PAK2 expressions in NB. We first established SH-SY5Y cells where circ_0013401 were overexpressed or silenced, then the transfected SH-SY5Y cells were injected into BALB/c nude mice. On the 28th day, the mice were killed and the tumor was removed. As displayed in Fig. 10A and 10B, NB tumor growth was markedly promoted by circ_0013401 overexpression, and suppressed by circ_0013401 knockdown ($p < 0.01$). RT-qPCR results also uncovered that circ_0013401 and PAK2 were significantly upregulated, miR-195 was dramatically downregulated in circ_0013401-overexpressed group relative to the control group; circ_0013401 and PAK2 were notably downregulated, miR-195 was memorably upregulated in circ_0013401-silenced group versus the control group ($p < 0.05$, $p < 0.01$, Fig. 10C). IHC results also testified that circ_0013401 overexpression observably elevated PAK2 and Ki67 expressions, circ_0013401 knockdown dramatically lowered PAK2 and Ki67 expressions in NB tumor tissues (Fig. 10D). Moreover, we further verified that overexpression of circ_0013401 markedly upregulated PAK2, p62 and Bcl-2, and downregulated LC3B II/I, Beclin1, Bax and cleaved Caspase-3 in mice tumor tissues; the influences of circ_0013401 knockdown on the expressions of these proteins were contrary to its overexpression in mice tumor tissues (Fig. 10E). Hence, we further certified that circ_0013401 signally downregulated miR-195 and upregulated PAK2, and inhibited apoptosis and autophagy-related proteins *in vivo*.

Discussion

NB is the most widespread extracranial malignant tumor in children, accounting for 1/6 of all childhood cancer deaths [22]. For NB children with high risk, the therapeutic effect of comprehensive treatment is very limited, and the adverse reactions are obvious [23]. Therefore, the exploration of novel pathogenesis and therapeutic strategy is of great significance for NB treatment. At present, a large number of circRNAs have been reported to be relevant to a number of diseases, especially cancer [24, 25]. Researches have demonstrated that circRNAs could dramatically affect the biological processes of cancers, including differentiation, proliferation, apoptosis, migration, invasion, and autophagy, etc [26, 27]. Due to the specificity and stability, circRNAs has become another clinical hotspot following miRNAs and lncRNAs

[28]. However, the roles of some circRNAs in NB remain largely unclear. In this present study, through the RT-qPCR identification of circ_0013401, circ_0045997, circ_0077578 and circ_0080307, we found that circ_0013401 was highly expressed in NB tissues and cells. *In vitro* and *in vivo* experiments also revealed that hsa_circ_0013401 could prominently induce the proliferation and growth of NB cells. Besides, we proved that hsa_circ_0013401 could markedly facilitate migration and invasion, and prevent apoptosis and autophagy of NB cells.

Based on the theory of ceRNAs, circRNAs could be involved in the tumorigenesis of cancers by functioning as miRNAs spongers to regulate the expressions of corresponding mRNAs [29, 30]. Specific binding of miRNAs has been considered as instinctive function of circRNAs in the circRNA-miRNA-mRNA axis [31]. Therefore, we performed bioinformatics analysis to construct the circRNA-miRNA-mRNA networks of circ_0013401. In our study, we also proved that circ_0013401 was identified to act as miRNAs spongers for miR-195, and miR-195 was verified to directly physical interact with circ_0013401 and PAK2 in NB cells. Moreover, circ_0013401 could indirectly regulate the expression of PAK2 in NB cells by sponging miR-195. Recent studies also testified that miR-195 is closely related to the progression of plentiful cancers, such as glioma [32], acute myeloid leukemia [33], bladder cancer [34], breast cancer [35], and cervical cancer [36]. In our study, we further demonstrated that miR-195 inhibitor could prominently reverse the inhibition of the proliferation, migration and invasion, and induction of apoptosis mediated by circ_0013401 knockdown in NB cells. Moreover, we uncovered that circ_0013401 could upregulate PAK2 by targeting miR-195 in NB.

PAK family belongs to serine/threonine protein kinase, which is conservative [37]. PAK2, as a member of PAK family, is a downstream effector of the GTPase in the Rho family [38]. Multiple studies proved that PAK2 has significant effects in the regulation of cell proliferation, movement and apoptosis [39, 40]. Abnormal function of PAK2 can result in the occurrence of various diseases (including tumors) [38, 41, 42]. Research testified that the combination of Rac and Cdc42 with PAK2 can induce the activation of PAK2 to induce cell proliferation and growth [43]. Meanwhile, PAK2 can be hydrolyzed by aspartic-and cysteine peptidases, and play a role in regulating cell apoptosis [44]. Current researches also certified that PAK2 could be overexpressed in a variety of malignant tumor cells, including lung cancer [45], gastric cancer [46], pancreatic cancer [47], and breast cancer [48], etc. In our study, we further demonstrated that PAK2 was significantly upregulated in NB, and PAK2 expression could also be significantly reduced by miR-195 and elevated by circ_0013401 overexpression in NB. Additionally, we disclosed that overexpression of PAK2 could also dramatically suppress apoptosis and autophagy mediated by miR-195 in NB cells.

Conclusions

Our findings proved that circ_0013401/miR-195/PAK2 axis plays crucial roles in the progression of NB *in vitro* and *in vivo* (**Graphical Abstract**). Therefore, our research production might further enrich our understanding for the pathogenesis of NB, and provide new therapeutic targets for NB patients.

Methods

Tissue samples

8 gangliocytoma (GN) and 8 NB tissues were collected from patients who were diagnosed at Guangzhou Women and Children's Medical Center of Guangzhou Medical University (Guangzhou, China). All histopathology was identified by two professional pathologists through double blind method. We also exhibited the basic information of 8 GN and 8 NB patients (Table 1). Individual use in clinical studies conforms to the Declaration of Helsinki, and written informed consents have been provided by the 8 patients. This study has been approved by the ethics committee of Guangzhou Women and Children's Medical Center of Guangzhou Medical University (Guangzhou, China).

Table 1
The basic information of 8 GN and 8 NB patients.

Patient ID		Age	Gender	Position	NSE
1	GN	7 years	female	adrenal gland	27.98
2	GN	7 years	female	abdomen	14,67
3	GN	16 years	female	pelvic cavity	13.12
4	GN	5 years	male	abdomen	30.38
5	GN	6 years	male	abdomen	16,87
6	GN	4 years	male	abdomen	23.41
7	GN	10 years	male	pelvic cavity	26.43
8	GN	11 years	male	adrenal gland	37.28
9	NB	3 years	male	adrenal gland	≈370
10	NB	1 year	female	adrenal gland	50.36
11	NB	3 years	male	adrenal gland	42.17
12	NB	2 years	male	abdomen	≈370
13	NB	5 years	male	abdomen	58.69
14	NB	3 months	female	adrenal gland	47.5
15	NB	10 months	female	adrenal gland	34.12
16	NB	11 months	female	abdomen	≈370

Cell lines

6 NB cell lines including SK-N-BE, GNP, SH-SY5Y, IMR-32, LAN-1 and SK-N-SH were provided by the type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI-1640 medium (HyClone Laboratories Inc., USA) containing 10% fetal bovine serum (FBS, Life Technologies, cat. No. 10270), 1% penicillin/streptomycin (Gibco) at 37°C in an incubator with 5% CO₂.

Fluorescence in situ hybridization (FISH)

The samples were added with Carnoy's solution for centrifugation (1000 ×g for 6 minutes (min)), and the 100 µL solution was added on clean glass slides. Prehybridization was conducted using the hybridization solution (WAKO, cat. No.#544–01331) for 1 hour (h) at 37°C, and then slides were added 100 nM probes. After denaturing at 74°C for 6 min, the slides were hatched in a humid chamber at 35°C overnights. After washing with 0.4× SSC at 45°C, the slides were then washed using 2× SSC containing 0.05% Tween20 at room temperature (RT) for 1 min. After nuclear staining using DAPI (Life Science), the results were visualized using a fluorescence microscope.

Immunohistochemistry (IHC) assay

GN and NB tissues were soaked in 10% paraformaldehyde (Sigma) at 4°C for 12 h and then dehydrated. After embedding, a rotary microtome (Leica, GER) was applied to cut the samples into 3.5-µm thickness. The slices were treated with water bath at 42.5°C, installed on the adhesion microscope slides (Citoglas, China). After deparaffinization and rehydration, the slides were processed with the SABC kit (Bosterbio, China) based on the instructions, and treated with anti-Ki67 (1:200, Abcam, UK), and anti-PAK2 (1:100, Abcam, UK) at 4°C for 12 h. Then the slides were stained using the DAB Kit (Bosterbio, China), and the results of IHC were obtained using an inverted microscope (Nikon Eclipse TI-SR, Japan).

RNA transfection

miR-195 mimics, miR-195 inhibitor and negative control (NC) were all designed and obtained from the GenePharma (China). Circ_0013401-overexpressed plasmids, circ_0013401 shRNAs, PAK2-overexpressed plasmids were obtained from biovector. All cell transfections were transfected using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions.

Quantitative real-time PCR (RT-PCR)

Total RNAs were extracted from NB tissue samples and the treated NB cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After inspection, the RNAs were applied to synthesize cDNAs using the Bestar™ qPCR RT kit (DBI Bioscience, cat. No. #2220). RT-PCR was conducted through the usage of Bestar™ qPCR MasterMix (DBI Bioscience, cat. No. #2043,). The sequence of primers used in this study was showed in Table 2. Gene expression was analyzed through $2^{-\Delta\Delta C_t}$ method.

Table 2
The sequences of primers in RT-qPCR assay.

Name	Sequence (5'- 3')
GAPDH Forward	TGTTTCGTCATGGGTGTGAAC
GAPDH Reverse	ATGGCATGGACTGTGGTCAT
PAK2 Forward	CACCCGCAGTAGTGACAGAG
PAK2 Reverse	GGGTCAATTACAGACCGTGTG
U6 Forward	CTCGCTTCGGCAGCACA
U6 Reverse	AACGCTTCACGAATTTGCGT
hsa-miR-195-5p Forward	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGCCAATAT
hsa-miR-195-5p Reverse	ACACTCCAGCTGGGTAGCAGCACAGAAATAT
Hsa_circ_0013401 Forward	GTCCTGACTTGTCATGTGCTG
Hsa_circ_0013401 Reverse	CAGACATTCACAAAGGAGCAA
hsa_circ_0080307 Forward	TGCTGCTAAACCTGTCCAAC
hsa_circ_0080307 Reverse	CCACAGCAGCAATACGAACC
hsa_circ_0077578 Forward	TGGATGAGATGCCGGTCAA
hsa_circ_0077578 Reverse	TAAAGCATGCATCTGTGCGT
hsa_circ_0045997 Forward	CTGCGTTTGGAGCCGTT
hsa_circ_0045997 Reverse	CAGACCAGCAGTCAGAGCGT

Cell Counting kit-8 (CCK-8) assay

CCK-8 (Dojindo, Rockville, MD, USA) was adopted to evaluate cell viability of treated NB cells. In brief, the treated NB cells (2×10^4 cells/well) were seeded into 96-well plates and cultured at 37°C for 24 h. Then, cells in each well were added with 10 μ L CCK-8 solution at 37°C for 10 min. The optical density (OD) values were determined at 450 nm.

EdU assay

The treated SH-SY5Y and SK-N-BE cells were fixed using 0.5% paraformaldehyde (Sigma) and addressed with EdU solution (Riobio, Guangzhou, China) for 30 min. Subsequently, a flow cytometer (Becton Dickinson, USA) was utilized to assess cell proliferation.

Colony formation assay

Briefly, the SH-SY5Y and SK-N-BE cells in each group (2000 cells/well) were plated into 6-well plates and cultured at 37°C for 14 days, followed by fixation with 4% paraformaldehyde and dyeing with Giemsa

solution. Under a microscope, the number of colonies was calculated.

Transwell assay

The invasive or migratory abilities of SH-SY5Y and SK-N-BE cells in each group were assessed using the Transwell chambers (8 μm , Corning) coated with or without Matrigel, respectively. Cells (1×10^6 cells) in 500 μL serum-free medium were seeded into the upper chamber, 500 μL 15% FBS-contained culture medium was placed into the lower chamber. After 24 h of incubation, the invaded or migrated cells were fixed using 4% paraformaldehyde (Sigma) and stained using 5% crystal violet (Sigma). The number of invasive or migratory cells was counted under a microscope.

Flow cytometric analysis

The apoptosis rate of SH-SY5Y and SK-N-BE cells in each group was monitored through the application of Annexin-V/FITC double staining (BD Biosciences) in line with the instructions.

Transmission electron microscopy (TEM)

After washing, the treated SH-SY5Y and SK-N-BE cells were fixed in 2% glutaraldehyde for 2 h, treated with 1% osmium tetroxide for 1 h and dehydrated using the graded ethanol for 2 h. TEM observation was conducted with a Leo 912 AB electron microscope.

Immunofluorescence staining

After fixing, the treated SH-SY5Y and SK-N-BE cells were incubated with 5% Tween-20 for 2 h. After blocking in 10% normal goat serum for 1 h, the cells were incubated with anti-LC3 (1:100, Abcam, cat. No. ab62720) and anti-PAK2 (Abcam, cat. No. ab3442) overnight and secondary antibody (Abcam, cat. No. DAR-546) for 2 h. DAPI (Life Science) was applied for the label nuclear for 30 min. The results were photographed using a confocal laser microscope (Zeiss, Germany, LSM710).

Western blot

Total proteins were isolated using RIPA buffer (Beyotime, Shanghai, China), and the concentration was confirmed using BCA kit (Beyotime). The proteins (50 μg) in each group were isolated by SDS-PAGE (10%) and then transferred onto PVDF membranes (Millipore, USA). After blocking with 5% non-fat milk for 2 h. Membranes were treated overnight with primary antibodies, stained using HRP-conjugated secondary antibody for 1.5 h. Signals were monitored using the enhanced chemiluminescence (ECL, Thermo Fisher Scientific) and analyzed by Image J.

In vivo tumor growth assay

Male BALB/c nude mice (eight-weeks old) were provided by the laboratory animal center of Southern Medical University (No.44002100023925). All operating procedures in the animals were approved by Institutional Animal ethics Committee of Guangzhou Medical University (Guangzhou, China) and carried out in the laboratory animal center. Briefly, the left flank of nude mice was subcutaneously injected with the transfected SH-SY5Y cells (200 μL , 1×10^7 cells). Tumor size was monitored every 7 days until 28 day

after injection. After 28 day, the mice were sacrificed by anesthesia with sodium pentobarbital and the xenograft tumors were excised. Then the tumor volumes were counted with the formula (length×width²×0.5).

Dual luciferase reporter assay

To verify the relationship between circ_0013401 and miR-195 in SH-SY5Y and SK-N-BE cells.

circ_0013401-wild type (WT) and circ_0013401-mutant (Mut) plasmids were constructed using the WT and Mut fragments of circ_0013401 including putative miR-195 binding sites and psiCheck-2 vector (Promega, cat. No.C8021). Subsequently, SH-SY5Y and SK-N-BE cells (1×10⁴ cells/well) were maintained in 6-well plates at 37°C overnights, followed by the co-transfection of miR-195 mimics and circ_0013401-WT or circ_0013401-Mut. The firefly and renilla luciferase activities of the SH-SY5Y and SK-N-BE cells in each group were examined using the Dual-Luciferase Assay System (Promega).

Statistical Analysis

Data from three independent experiments was presented as mean ± SEM. and the statistical analysis was conducted using SPSS 20.0 software (SPSS, Chicago, IL, USA) with the Student's t-test or One-way analysis of variance. *P* < 0.05 was considered as significant.

Declarations

Ethics approval and consent to participate

Informed consent was obtained from each patient, and the study protocol and consent procedures were approved by the Ethics Committee of Guangzhou Medical University (Guangzhou, China).

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

None.

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Authors' contributions

Wei Jia and Shibo Zhu: Conceptualization, funding acquisition, writing original draft and Writing - review & editing.

Xiangliang Tang, Xiaofeng Gao and Jingqi Zhang: Resources, Data curation and Formal analysis.

Yanhong Cui and Dian Li: Investigation, Visualization, Methodology and Project administration.

All authors read and approved the final manuscript.

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Not applicable.

Clinical Perspectives

1. Neuroblastoma (NB) is a common malignant embryonal solid tumor in children. Circular RNAs (circRNAs) are characterized by stable structure, high abundance and tissue specific expression. Recent studies revealed that circRNAs have an essential role in gene expression regulation as competitive endogenous RNA (ceRNA).
2. Our current research proves that hsa_circ_0013401 could induce NB progression through miR-195 to enhance PAK2.
3. Therefore, the experimental verification of hsa_circ_0013401/miR-195/PAK2 axis in NB can lay the foundation for the application of hsa_circ_0013401 and related regulatory pathways (miR-195/PAK2) in NB clinical treatment.

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Figures

Figure 1

Circ_0013401 was identified as highly expressed in NB. (A) The levels of circ_0013401, circ_0045997, circ_0077578 and circ_0080307 were confirmed in the collected GN and NB tissue samples (N=8). (B) The expression of circ_0013401 was examined by FISH in GN and NB tissue samples. Magnification, $\times 200$. IHC assay was applied to monitor the expressions of Ki67 (C) and PAK2 (D) in GN and NB tissues. Magnification, $\times 200$; magnification, $\times 400$. $**p < 0.01$.

Figure 2

Circ_0013401, as an oncogene, prominently accelerated NB proliferation. (A) Expression of circ_0013401 was determined by RT-qPCR in different neuroblastoma cells including SK-N-BE, GNP, SH-SY5Y, IMR-32, LAN-1 and SK-N-SH cells. (B) After SH-SY5Y and SK-N-BE cells were transfected with circ_0013401-overexpressed plasmid or circ_0013401 shRNA, the transfection effects were identified using RT-qPCR assay. (C) CCK-8 assay was conducted to confirm the influences of circ_0013401 overexpression or

knockdown on the proliferation of SH-SY5Y and SK-N-BE cells. Cell proliferation was also assessed by Edu staining (D) and clone formation assay (E) in circ_0013401 overexpressed or silenced SH-SY5Y and SK-N-BE cells. *p < 0.05, **p < 0.01.

Figure 3

Circ_0013401 markedly facilitated migration and invasion, and prevented apoptosis and autophagy of NB cells. Transwell assay was performed to identify the effects of circ_0013401 overexpression or

knockdown on the migration (A) and invasion (B) of SH-SY5Y and SK-N-BE cells, and the number of the migratory and invasive cells were counted. (C) Impacts of circ_0013401 overexpression or knockdown on apoptosis of SH-SY5Y and SK-N-BE cells were evaluated by flow cytometer, and the number of the apoptotic cells was calculated in each group. (D) The change of autophagy was examined by TEM in SH-SY5Y and SK-N-BE cells after circ_0013401 overexpression or knockdown. * $p < 0.05$, ** $p < 0.01$.

Figure 4

Circ_0013401 dramatically regulated miR-195, PAK2, autophagy and apoptosis-related proteins in NB cells. (A) RT-qPCR analysis of miR-195 in different NB cells (SK-N-BE, GNP, SH-SY5Y, IMR-32, LAN-1 and SK-N-SH). (B) The effects of circ_0013401 overexpression or knockdown on miR-195 expression were assessed by RT-qPCR. (C) The expression change of PAK2 was evaluated by RT-qPCR in circ_0013401 overexpressed or silenced SH-SY5Y and SK-N-BE cells. (D) After circ_0013401 overexpression or knockdown, PAK2 and LC3B expressions were determined using IF assay in SH-SY5Y and SK-N-BE cells. Magnification, $\times 400$; Scale bar = 20 μm . (E) Western blotting analysis was applied to monitor the levels of

PAK2, LC3B, Beclin1, p62, Bcl-2, Bax and cleaved Caspase-3 in circ_0013401 overexpressed or silenced SH-SY5Y and SK-N-BE cells. *p < 0.05, **p < 0.01.

Figure 5

Circ_0013401 sponged miR-195, and PAK2 was a target gene of miR-195. (A) Dual luciferase reporter assay was adopted to demonstrate the interplay between circ_0013401 and miR-195. (B) Dual luciferase reporter assay proved that PAK2 is a direct target of miR-195.

Figure 6

MiR-195 inhibitor prominently reversed the inhibitory action of circ_0013401 knockdown on the proliferation of NB cells. (A) Circ_0013401-silenced SH-SY5Y and SK-N-BE cells were transfected with miR-195 inhibitor or NC, and RT-qPCR was conducted to confirm miR-195 expression. (B-C) After co-transfection with circ_0013401 shRNA and miR-195 inhibitor, cell proliferation was tested by Edu staining in SH-SY5Y and SK-N-BE cells. And the percentage of Edu⁺ cells was counted. (D) Clone formation assay was also conducted to assess the proliferation of the co-transfected SH-SY5Y and SK-N-BE cells. ** $p < 0.01$.

Figure 7

MiR-195 was involved in the inhibition of migration and invasion, and induction of apoptosis mediated by circ_0013401 knockdown in NB cells. (A-B) The impacts of circ_0013401 shRNA and miR-195 inhibitor on the migration and invasion of SH-SY5Y and SK-N-BE cells were examined by Transwell assay. Magnification, $\times 200$. (C) After co-transfection with circ_0013401 shRNA and miR-195 inhibitor, flow cytometer was adopted to analyze the apoptosis of SH-SY5Y and SK-N-BE cells. ** $p < 0.01$.

Figure 8

MiR-195 inhibitor memorably attenuated the downregulation effects of circ_0013401 knockdown on PAK2, autophagy and apoptosis-related proteins in NB cells. (A) In circ_0013401 shRNA and miR-195 inhibitor-transfected SH-SY5Y and SK-N-BE cells, the level of PAK2 was examined using RT-qPCR in SH-SY5Y and SK-N-BE cells. (B) PAK2, LC3B, Beclin1, p62, Bcl-2, Bax and cleaved Caspase-3 expressions

were certified by western blot in SH-SY5Y and SK-N-BE cells after co-transfection with circ_0013401 shRNA and miR-195 inhibitor. **p < 0.01.

Figure 9

Overexpression of PAK2 signally suppressed apoptosis and autophagy mediated by miR-195 in NB cells. SH-SY5Y and SK-N-BE cells were transfected with miR-195 mimics or/and PAK2-overexpressed plasmids, respectively. (A) PAK2 expression was assessed using RT-qPCR. (B) Apoptosis was assessed by flow cytometry, and the apoptosis rate was also calculated. (C) Western blot results of PAK2, LC3B, Beclin1, p62, Bcl-2, Bax and cleaved Caspase-3 proteins. **p < 0.01.

Figure 10

Verification of circ_0013401/miR-195/PAK2 axis in vivo experiment. (A) The nude mice were injected with circ_0013401-overexpressed or silenced SH-SY5Y cells, and the representative images were presented. (B) Tumor volume was measured at 0, 7, 14, 21, and 28 days. (C) After circ_0013401 overexpression or knockdown, circ_0013401, miR-195 and PAK2 expressions were inspected by RT-qPCR. (D) IHC assay was applied for the detections of PAK2 and Ki67 expressions. (E) Identification of changes in apoptosis and autophagy-related protein expression through Western blot. * $p < 0.05$, ** $p < 0.01$.

Figure 11

The schematic diagram of hsa_circ_0013401 in NB. Increased circ_0013401 causes the upregulation of PAK2 by functioning as a miR-195 sponge, thus promoting growth, proliferation, migration, invasion and preventing apoptosis and autophagy of NB cells.