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MicroRNA-155 deficiency in CD8+ T cells inhibits its anti-glioma immunity by regulating FoxO3a

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Abstract. – OBJECTIVE: A lot of mammalian micro-RNAs have been identified in regulating immune system function. Here, the aim of this study was to investigate the role of microRNA-155 in promoting anti-glioma ability and its potential mechanism.

MATERIALS AND METHODS: In this study, we constructed microRNA-155 knockout mouse model and glioma mouse model. Subsequently, the progression of glioma and the accumulation of CD8+ T cells were compared between WT and miR-155-/- mice. T cells were transfected with miR-155 mimics and inhibitors, and the proliferative and invasive activities were analyzed. At the same time, we evaluated Akt and Stat5 signaling transduction and the expression level of FoxO3a. Finally, the regulatory ability of FoxO3a to Akt and Stat5 signaling was determined by changing the expression level of FoxO3a in T cells.

RESULTS: We found significantly increased progression of glioma in MicroRNA-155 deficiency mice with reduced accumulation of CD8+ T cells in glioma. The proliferative and invasive abilities of T cells were regulated by MicroRNA-155. Besides, microRNA-155 could induce the activation of Akt and Stat5 signaling by inhibiting its target gene FoxO3a. Furthermore, FoxO3a was a negative regulator of Akt and Stat5 signaling.

CONCLUSIONS: MicroRNA-155 deficiency in CD8+ T cells inhibited anti-tumor activity by suppressing the proliferative and invasive activities of T cells. FoxO3a was a negative regulator of Akt and Stat5 signaling. Besides, microRNA-155 regulated the function of T cells by inhibiting the expression of FoxO3a. Our findings might be a new strategy for the immunotherapy of glioma.

Key Words:
MicroRNA-155, Glioma, CD8+ T cells, FoxO3a, Akt and Stat5 signaling.

Introduction

Surgery combined with radiotherapy and chemotherapy is a common treatment for glioma[1]. In recent years, with the development of surgical techniques and adjuvant therapy, the treatment efficiency of glioma has been greatly improved. However, the recurrence rate of brain tumor remains high due to the high invasiveness of glioma[2]. Therefore, the prognosis of glioma patients is still not optimistic. Developing novel therapies for glioma is of great importance for the improvement of current immunotherapy.

Adoptive immune therapy has been proved to be a promising strategy for glioma treatment[3,4]. The theoretical basis of tumor immunotherapy is that the immune system has the ability to recognize tumor-associated antigens and regulate the immune system to attack tumor cells. The central nervous system not only has complex immune reaction conditions, but also can produce corresponding immune responses. This provides a theoretical premise for immunotherapy of glioma. A few studies have revealed that the accumulation of $CD8^+$ T cells in glioma is a sign for the inhibition of tumor progression, which can repress the migration of glioma[5,6]. However, the number of $CD8^+$ T cells around glioma is very small. The recruitment of more $CD8^+$ T cells is important for enhancing the effects of immunotherapy. The strategies have focused on the generation of more $CD8^+$ T cells to tumor tissues or the improvement of $CD8^+$ T cell function.

More and more studies have suggested that multiple micro-RNAs are involved in regulating the immune system, including the development and differentiation of immune cells, the release

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of inflammatory mediators, the production of B cells antibodies, cell signaling and so on[7-9]. For example, miR-146a/b has been shown to negatively regulate IL-6 and IL-8, all of which are inflammatory mediators in controlling aging and cellular senescence[10]. By evaluating the expression and function of 100 unique miRNAs, Chen et al[11] have found that the thymus-specific miR-181a exhibits its regulatory role in the development of B cells and T cells. Further studies have shown that compared with DN stage T cells and $CD4^+$ or $CD8^+$ T cells, miR-181a is enriched at the $CD4^+CD8^+$DP stage[12]. MiR-155 plays a regulatory role in T cell differentiation and activation, which is indispensable for immune responses. MiR-155 knockout mice show decreased IFN-γ and IL-2 production, which indicates its important role in B and T cell responses[13]. Another team[14] has found that miR-155-null mice generate fewer and smaller germinal centers when compared with control mice in response to antigenic challenge. The strong effect of miR-155 in regulating immune response gives miR-155 the value for immune therapy in the clinic. Studies have demonstrated that miR-155 can enhance $CD8^+$ T antitumor activity by increasing the response to homeostatic gamma cytokines in lymphoreplete[15]. Understanding the correlation between the role of miR-155 in $CD8^+$ T cells and T cells' anti-tumor effect may be of great significance for glioma progression.

In this study, miR-155 showed its anti-cancer activity in glioma, which might be applied in clinical therapy. Then, we focused on its potential underlying mechanism. The results indicated that miR-155 increased the accumulation of $CD8^+$ T cells in glioma by regulating its function. Besides, we also found that miR-155 promoted the proliferative activity of $CD8^+$ T cells by enhancing the activity of Akt and Stat5 signaling. Further experiments showed that FoxO3a, the negative regulator of Akt and Stat5 signaling, was the target gene of miR-155. Altogether, this study identified miR-155 as an immune therapy target for glioma. In addition, the potential underlying mechanism was clarified.

Materials and Methods

Experimental Mice

Mice containing a LoxP-flanked miR-155 and C57BL/6 background B6.C-Tg (CMV-cre)1Cgn/J mice were purchased from Jackson Laboratory. The miR-155 deficient mice were generated by crossbreeding. C57BL/6 mice aged between 8-10 weeks were obtained from Shanghai Laboratory Animal Center, and were used for all the experiments. Real-time PCR was applied to evaluate the expression level of miR-155 in miR-155 knockout and wild-type mice. All experimental protocols were approved by the Institutional Animal Care. Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals. All mice were maintained in pathogen-free housing. This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center.

Cell Lines and Tumor Implantation

Murine GL261 glioma cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), HEPES, penicillin and streptomycin (Gibco, Grand Island, NY, USA). Wild-type and miR-155 knockout C57BL/6 mice were anesthetized for tumor implantation. A total of $3*10^5$ GL261 glioma cells were injected into the right striatum as reported[16]. By using a 10 μL Hamilton syringe, the cells were directly injected to the position of 1 mm posterior to the bregma, 2 mm lateral to the midline and 3 mm deep into the frontal lobes.

Tumor Volume Analysis

Brain tissues of wild-type and miR-155 knockout mice were isolated three weeks after GL261 injection. Before isolation and morphological evaluation, these mice were performed with cardiac perfusion and fixed in 4% buffered formaldehyde. After cutting in a cryostat, the slides of coronal brain were stained with hematoxylin and eosin. All the slides were examined on Olympus. Image J software was used to collect every 1 μm slide for tumor volume analysis. Finally, the total volume of glioma was calculated by the number of slides and the area of each slide.

RNA Isolation and Real Time-Polymerase Chain Reaction (RT-PCR)

After cell transfection for 24 h, T Cells were collected by centrifugation, and the supernatant was discarded for RNA isolation. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the operation instructions. Subsequently, extracted RNA was quantified and purified by ultraviolet spectrophotometer. The in-

tegrity of RNA was identified by 0.8% agarose gel electrophoresis. Then, 1 μg of total RNA was reversed by the ABI reverse transcriptional kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. The reverse transcription reaction procedure was as follows: 25°C for 10 minutes, 37°C for 120 min, and 85°C for 5 minutes. RT-PCR amplification scheme was carried out according to the manufacturer's instructions of ROCH. Primer sequences used are as follows: miR-155 forward, 5'-GTCGTATCCAGTGCAGGGTCCGAGG-3'; reverse, 5'-TATTCGCACTGGATACGACCCCCTA-3'; FoxO3a forward, 5'-GCAAGCACAGAGTTGGATGA-3'; reverse, 5'-CAGGTCGTCCATGAGGTTTT-3';U6forward, 5'-ATTGGAACGATACAGAGAAGATT-3'; reverse, 5'-GGAACGCTTCACGAATTTG-3'; GAPDH forward, 5'- AAGCCTGCCGGTGACTAAC-3'; reverse, 5'-GCGCCCAATACGACCAAATC-3'.

Western Blot

48 h after transfection, T cells were collected by centrifugation, and the supernatant was discarded for protein isolation. The cells were collected by centrifugation and discarded. Cell deposition was re-suspended in cell lysis buffer containing protease inhibitors. After 5 minutes of boiling in sodium dodecyl sulfate (SDS)-loading buffer, the upper cleaning fluid of the cell suspension was separated by SDS-polyacrylamide gel. Then the proteins in the gel were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). To prevent the interactions between nonspecific protein and protein, the PVDF membrane was blocked with Tris-Buffered Saline and Tween 20 (TBST) buffer containing 5% non-fat milk at room temperature. The following antibodies were diluted in TBST buffer, including FoxO3a, 1:4000; Akt, 1:3000; p-Akt, 1:3000; Stat5, 1:4000; p-Stat5, 1:4000; β-Actin, 1:5000. Then the PVDF membranes were incubated with the above primary antibodies at 4°C overnight. After washing with TBST buffer, the membranes were then incubated with the corresponding secondary antibody with peroxidase conjugation at room temperature for 1 hour. Finally, enhanced chemiluminescence (ECL) solution (Thermo Fisher Scientific, Waltham, MA, USA) was prepared in the dark, and the exposure time was measured according to the fluorescence intensity.

Luciferase Reporter Gene Assay

FoxO3a-3'UTR DNA fragment containing putative miR-155 binding sequence was cloned into PGL3 basic vector, namely PGL3/FoxO3a-3'UTR. Besides, mutant plasmids of FoxO3a-3'UTR were constructed by destroying the binding sequence of miR-155, namely PGL3/FoxO3a-3'UTR mutant. Before transfection, T cells were seeded into 24-well plate at a density of 1×10^5 cells per well. Each transfection contained vector DNA and 1/1000 pRL-SV40 plasmid. PGL3-promoter vector was used as a negative control. 48h after transfection, Luciferase reporter gene assay was performed in accordance to the manufacturer's instructions. This experiment was repeated three times.

Cell Viability

After transfection with miR-155 mimics and inhibitors, T cells were seeded into 96-well plates at a density of $5 * 10^5$ cells per well. After normal culture for 24h and 48h, respectively, the cells were harvested in the culture medium. Cell proliferative ability was detected by cell counting kit-8 (CCK-8) Assay Kit (Dojindo, Kumamoto, Japan). All the procedures were performed according to the manufacturer's instruction. The absorbance at the wavelength of 450 nm was detected using a microplate reader.

Transwell Assay

The invasive ability of T cells was detected by Matrigel transmembrane invasion assay. Transwell chambers were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). T cells were first transfected with miR-155 mimics and inhibitors. 24h after transfection, T cells were harvested. The number of cells was calculated, and the cells were added into the upper chamber. Meanwhile, the lower chamber was added with complete medium. After incubation, cells on the upper surface of the filter were removed by scraping with a cotton swab. Invaded cells were fixed with methanol and stained with crystal violet solution. Finally, the number of penetrated cells was counted, and the mean number was calculated. Five fields were randomly selected for each sample.

Statistical Analysis

The experimental results were expressed as mean value ± standard deviation (SD). Student's *t*-test was used to analyze the differences between the two groups. One-way ANOVA test was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). Each experiment was repeated at least three times. *p*-value less than 0.05 was considered statistically significant.

Results

MiR-155 Deficiency Induced Glioma Progression

To evaluate the effect of miR-155 knock-out on the progression of glioma, $3*10^5$ GL261 cells were implanted into the brain of miR-155$^{-/-}$ and wild-type mice (Figure 1A), respectively. The tumor volume was compared between miR-155$^{-/-}$ and wild-type mice after 20 days of injection. As shown in Figure 1B, the tumor volume in miR-155$^{-/-}$ mice was significantly larger when compared with wild-type mice. This indicated that the level of miR-155 in the microenvironment could regulate the progression of glioma. Besides, we also assessed the survival of glioma-bearing mice. The results demonstrated that miR-155 had a significant impact on survival time. As shown in Figure 1C, 1D, miR-155 knockout mice showed a remarkably shorter survival time than wild-type mice. These results clearly indicated that miR-155 deficiency could induce the progression of glioma and reduce the survival time of glioma-bearing mice.

MiR-155 Deficiency Reduced the Accumulation of CD 8+ T Cells in Glioma

MiR-155 possessed the ability to regulate immune response and T cell development. To identify the mechanism of increased progression of glioma in miR-155 knockout mice, we analyzed the immune cells inside and around the glioma. After 20 days of GL261 injection, glioma was harvested for lymphocytes isolation. Flow cytometry demonstrated that most of the lymphocytes were T cells in both GL261-bearing miR-155$^{-/-}$ and wild-type mice. Therefore, we accessed the class of T cells by immunofluorescent staining. Interestingly, the results showed that $CD8^+$ T cells in miR-155$^{-/-}$ were significantly decreased compared with the wild-type mice. However, no marked difference was found in $CD4^+$ T cells between wild-type and miR-155$^{-/-}$ mice. All the results were shown in Figure 2. They suggested a decreased accumulation of $CD8^+$ T cells to glioma induced by miR-155 knockout might contribute to the increased progression of glioma.

MiR-155 Could Regulate T Cell Function by Regulating Proliferative and Invasive Abilities

We then attempted to identify the regulatory role of miR-155 in T cell function. The proliferative abilities of T cells transfected with miR-155 mimics and inhibitors were evaluated by the CCK8 assay. The results showed that the proliferative ability of T cells was remarkably increased by miR-155 mimics. Consistently, the proliferative ability of T cells transfected with miR-155 inhibitors was significantly decreased. These results demonstrated that miR-155 could regulate the proliferative ability of T cells, thereby promoting its accumulation in glioma. All the results were shown in Figure 2. Next, we tried to identify whether miR-155 could regulate the invasive ability of T cells. The overexpression and interference efficiencies were confirmed by RT-PCR, and the invasive ability of T cells was evaluated by transwell assay. The result showed that the invasive ability of T cells was significantly increased by miR-155 overexpression. On the contrary, the invasive ability of T cells was markedly decreased in miR-155 knockout cells. All the results were shown in Figure 3. These results suggested that miR-155could regulate the invasive ability of T cells.

FoxO3a was the Target Gene of miR-155

To investigate the mechanism of the regulatory role of miR-155 in T cell proliferation and invasion, we searched for the target gene of miR-155 by TargetScan. The results predicted that FoxO3a was a candidate target for miR-155. FoxO3a is a member of the FOXO family, which is involved in regulating various biological activities including proliferation. Subsequently, T cells were transfected with miR-155 mimics and inhibitors, and the expression level of FoxO3a was detected. Real-Time PCR results (Figure 4A) found that the expression level of FoxO3a was significantly decreased in the miR-155 mimics group. Meanwhile, the mRNA level of FoxO3a was significantly increased in the miR-155 inhibitors group (Figure 4B). To find the direct evidence for the regulation of FoxO3a by miR-155, we constructed a Luciferase plasmid containing FoxO3a gene 3'UTR (named as PGL3/ FoxO3a-3'UTR). As shown in Figure 4C and 4D, miR-155 mimics transfection significantly suppressed the Luciferase activity of PGL3/FoxO3a-3'UTR, but had no significant effect on PGL3/ FoxO3a-3'UTR mutant plasmid. These results clearly displayed FoxO3a was the direct target gene of miR-155.

FoxO3a was a Negative Regulator of Akt and Stat5 Signaling

In this study, we demonstrated that miR-155 could induce the activation of Akt and Stat5 si-

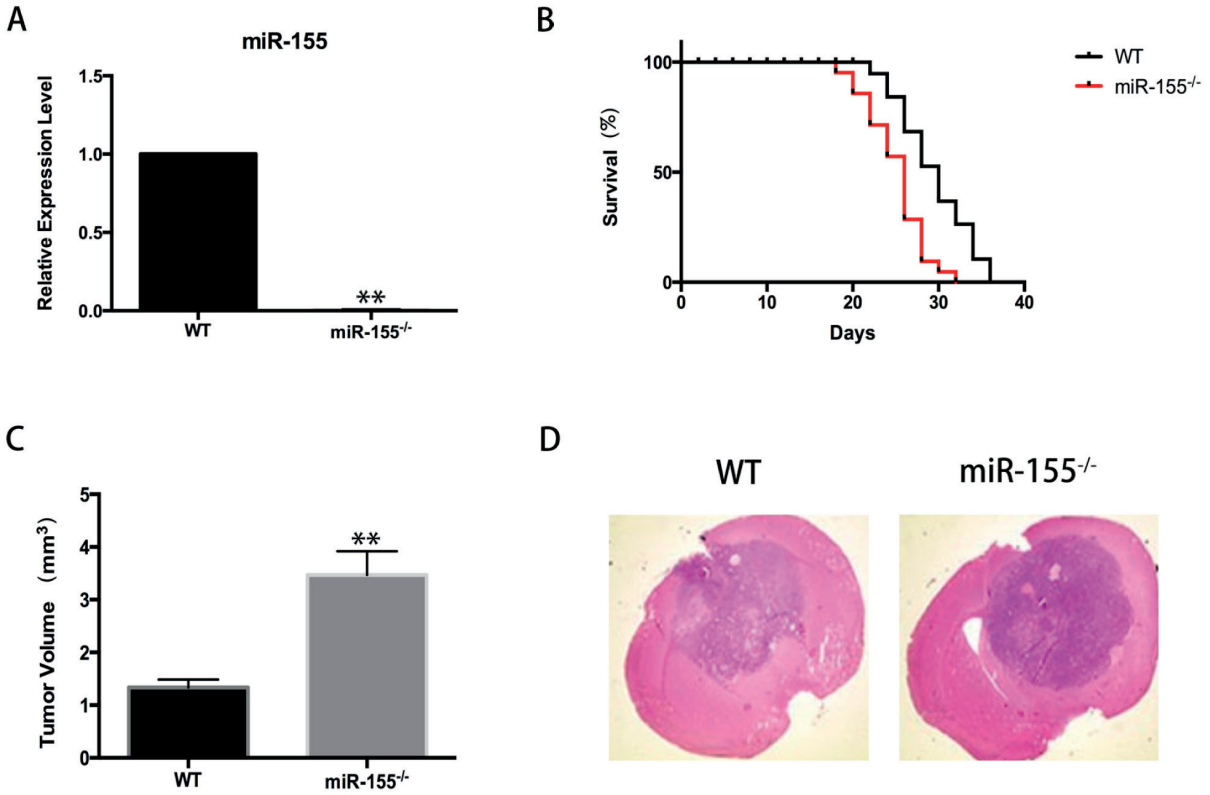

Figure 1. MiR-155 deficiency promoted glioma progression. WT and miR-155$^{-/-}$ mice were implanted with 3*105 GL261 cells. ***A,*** The relative expression level of miR-155 in WT and miR-155$^{-/-}$ mice was evaluated by Real-Time PCR. ***B,*** The survival duration of mice was monitored by Kaplan–Meier survival curve. Survival was analyzed by Kaplan–Meier method and compared by log-rank Mantel–Cox test. Experiments were repeated three times, and a representative repeat was presented. ***C,*** The tumor volume was calculated at Day 21 by Image J software. ***D,*** Representative H&E stained slices from glioma-bearing WT and miR-155$^{-/-}$ mice at Day 21 post injection. Magnification, *40. (**p<0.01).

gnaling. Meanwhile, FoxO3a was the target gene of miR-155. To verify whether FoxO3a regulated the Akt and Stat5 signaling, we transfected T cells with FoxO3a overexpression plasmid and small interference RNAs, respectively. Overexpression and interference efficiencies were evaluated by Western blot. The activities of Akt and Stat5 signaling were also evaluated by Western blot. As shown in Figure 5A-5E, the results suggested that FoxO3a overexpression significantly the Akt and Stat5 signaling. The activation of Akt and Stat5 signaling was markedly enhanced by FoxO3a downregulation (Figure 5F-5J). Altogether, FoxO3a was the negative regulator of Akt and Stat5 signaling, which contributed to regulating the function of T cells.

MiR-155 Induced Akt and Stat5 Signaling Activation in T Cells

MiR-155 has been demonstrated to regulate the expression of FoxO3a. Meanwhile, it negatively regulated the Akt and Stat5 signaling. Subsequently, we tried to investigate the regulatory role of miR-155 on Akt and Stat5 signaling. After transfection with miR-155 mimics and inhibitors in T cells, the activities of Akt and Stat5 signaling were evaluated by Western blot. The results (Figure 6A-6D) suggested that the activation of Akt and Stat5 signaling was significantly enhanced by miR-155 overexpression. Meanwhile, the Akt and Stat5 signaling were inhibited by miR-155 inhibitors (Figure 6E-6H). The above results suggested that the positive regulation of miR-155 on Akt and Stat5 signaling was mediated by FoxO3a.

Overexpression of FoxO3a in T Cells Reversed the Activation of Akt and Stat5 Signaling Caused by MiR-155

MiR-155 played an essential role in regulating the proliferative and invasive abilities of T cells by activating the Akt and Stat5 signaling. At the same time, miR-155 could negatively regulate

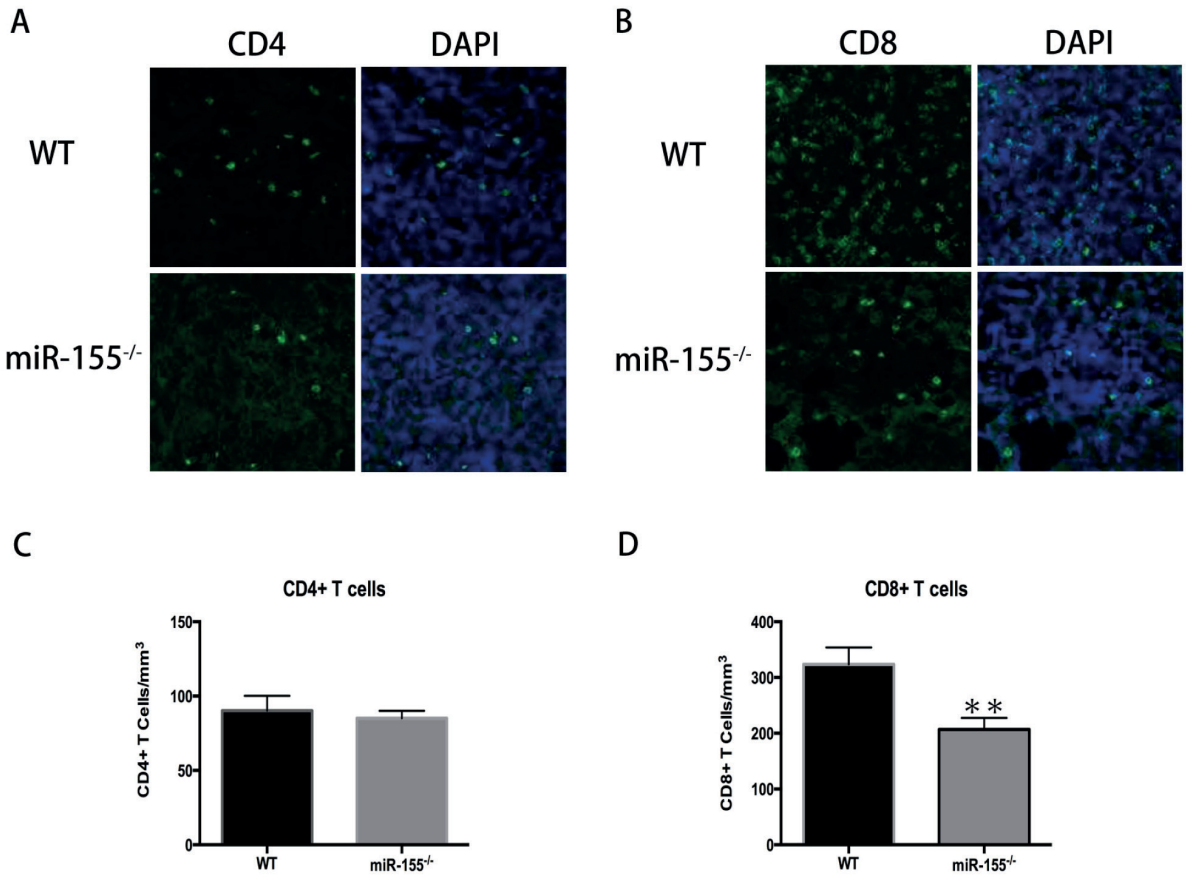

Figure 2. MiR-155 deficiency reduced the accumulation of CD 8$^+$ T cells in glioma. ***A,*** Representative confocal microscopy images of CD4 (green) staining with DAPI (blue) on brain sections in WT and miR-155$^{-/-}$mice. ***B,*** Representative confocal microscopy images of CD8 (green) staining with DAPI (blue) on brain sections in WT and miR-155$^{-/-}$mice. ***C,*** Quantitation of the density of CD4 positive cells in the tumor area was shown. ***D,*** Quantitation of the density of CD8 positive cells in the tumor area was shown. (**p<0.01).

the expression of FoxO3a, which was a negative regulator of Akt and Stat5 signaling. To identify whether miR-155 activated the Akt and Stat5 signaling by downregulating the expression of FoxO3a, T cells were co-transfected with miR-155 mimics and FoxO3a overexpression plasmids. The overexpression of FoxO3a partially restored the increased activity of Akt and Stat5 signaling in T cells by miR-155 (Figure 7). Western blot confirmed that FoxO3a over-expression plasmid worked efficiently (Figure 7). These results suggested that miR-155 activated the Akt and Stat5 signaling by inhibiting the expression FoxO3a.

Discussion

Our study proved that miR-155 deficiency significantly promoted the progression of glioma, suggesting the potential value of miR-155 in immune therapy of glioma. Accordingly, miR-155 deficiency lost the inhibitory role in the growth and proliferation of glioma-bearing tumor growth, suggesting the anti-cancer effect of miR-155. The underlying mechanism was that miR-155 could regulate the accumulation of CD8$^+$ T cells around glioma by regulating the negative regulator of Akt and Stat5 signaling.

MiR-155 is one of the most multifunctional non-coding RNAs, which participates in regulating a lot of biological functions in different cell types[17,18]. MiR-155 has been reported to regulate the immune response in immune cells[19]. In this report, miR-155 knockdown significantly reduced the accumulation of CD8$^+$ T cells in glioma by regulating the proliferative and invasive abilities of CD8$^+$ T cells. In psoriasis, miR-155 could promote the proliferative activity and inhibit apoptosis by

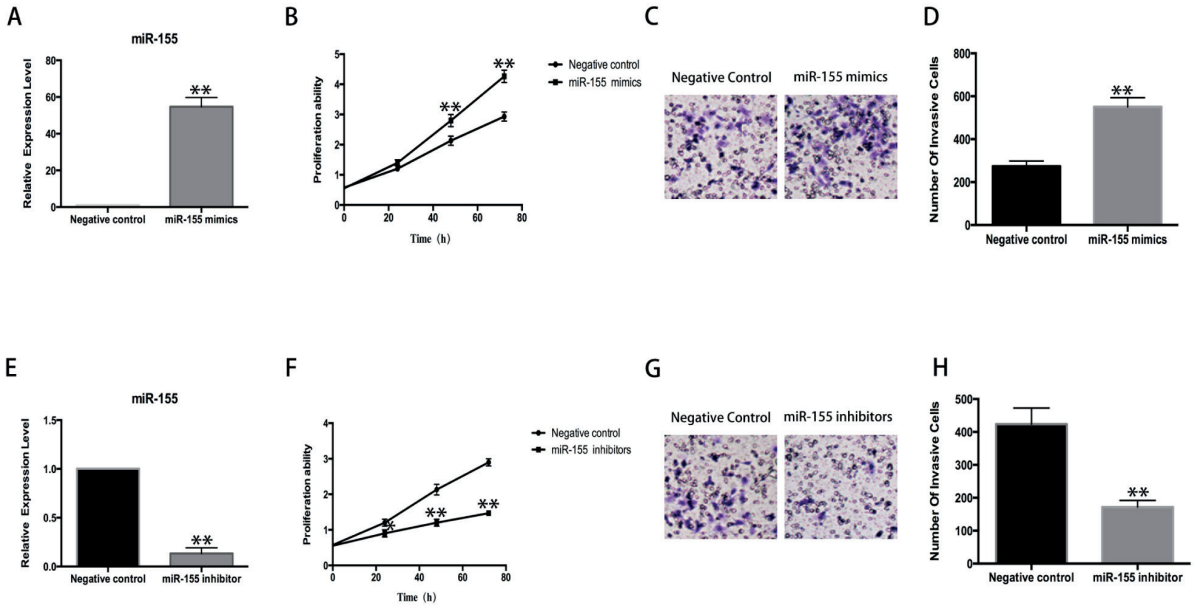

Figure 3. MiR-155 regulated the proliferative and invasive ability of T cells. ***A,*** Transfection efficiency of miR-155 mimics was evaluated by Real-Time PCR after T cells were transfected with miR-155 mimics. ***B,*** CCK-8 assay results showed that cell proliferative ability was increased in the miR-155 mimics group compared with the control group. ***C,*** The invasive ability of T cells transfected with miR-155 mimics was evaluated by transwell assay. ***D,*** The statistical analysis of invasive ability of T cells transfected with miR-155 mimics. ***E,*** Transfection efficiency of miR-155 inhibitors was evaluated by Real-Time PCR after T cells were transfected with miR-155 inhibitors. ***F,*** CCK-8 assay results showed that the proliferation ability of JAR cells was increased in the miR-517-5p inhibitor group compared with the control group. ***G,*** The invasive ability of T cells transfected with miR-155 inhibitors was evaluated by transwell assay. ***H,*** The statistical analysis of invasive ability of T cells transfected with miR-155 inhibitors. (**$p<0.01$).

regulating PTEN signaling[20]. Besides, miR-155 is highly correlated with the progression of IgA tubulointerstitial fibrosis, thereby involving in regulating the Wnt/β-catenin signaling[21]. MiR-155 has also been demonstrated to be regulated by Toll-like receptor by MyD88 or TRIF signaling in innate immunity, eventually promoting the expression of tumor necrosis factor[22]. According to the results in our study, the function of $CD8^+$ T cells was impaired in miR-$155^{-/-}$ $CD8^+$ T cells by negatively regulating Akt and Stat5 signaling. On the one hand, miR-155 might regulate the proliferation and invasive abilities of $CD8^+$ T cells by other mechanisms. On the other hand, the other phenotypes of miR-$155^{-/-}$ $CD8^+$ T cells needed to be identified in further studies.

Forkhead box O transcription factors (FOXO) family participates in regulating cell proliferation, cell cycle and metabolism[23,24]. FoxO3a gene is a member of the FOXO family. Low expression of FoxO3a is highly related to the development and occurrence of various tumors by regulating multiple signal transduction pathways[25]. Studies[26,27] have found that FoxO3a can regulate genes involved in cell cycle arrest, apoptosis and other related pathological processes, which was further involved in these cell activities. In this work, FoxO3a was proved as the negative regulator of Akt and Stat5 signaling. Studies have also found that Akt leads to strand in the cytometry by phosphorylation of FoxO3a. Meanwhile, the expression of FoxO3a is inhibited at both mRNA and protein levels[28]. With the in-depth study of downstream signaling of FoxO3a, researchers have found FoxO3a can regulate various target genes by positive and negative regulatory mechanisms.

Activated Akt and Stat5 signaling significantly conferred the proliferative ability of T cells, which might result in the accumulation of CD8+ T cells in glioma. Matthew *et al*[29] have demonstrated that Stat5 played a vital role in governing T cell homeostasis and B cell development. The activation of Stat5 leads to the proliferation of naïve and memory CD8+ T cells in IL7/IL-15 dependent manner[29]. In addition to the regulating proliferative activity, Stat5 and Akt signaling have been identified in cell apoptosis by regulating the

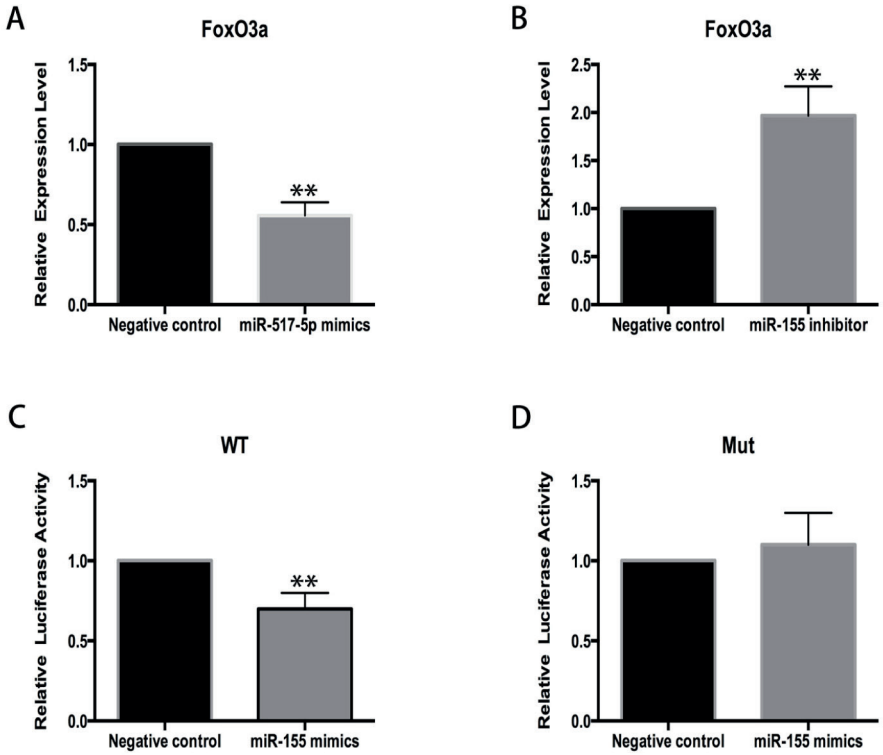

Figure 4. FoxO3a was the target gene of miR-155. T cells were transfected with miR-155 mimics and inhibitor. The expression level of FoxO3a was analyzed by Real-Time PCR. ***A,*** The expression level of FoxO3a was decreased in the miR-155 mimics transfected cells compared with the control group. ***B,*** The expression level of FoxO3a was increased in the miR-155 inhibitor transfected cells compared with the control group. ***C,*** Luciferase plasmid of wild-type PGL3/ FoxO3a -3'UTR was co-transfected with miR-155. The Luciferase activity of wild-type PGL3/ FoxO3a -3'UTR was significantly decreased. ***D,*** Luciferase plasmid of mutant PGL3/ FoxO3a -3'UTR was co-transfected with miR-155. The Luciferase activity of mutant PGL3/ FoxO3a -3'UTR remained unchanged. (**p<0.01).

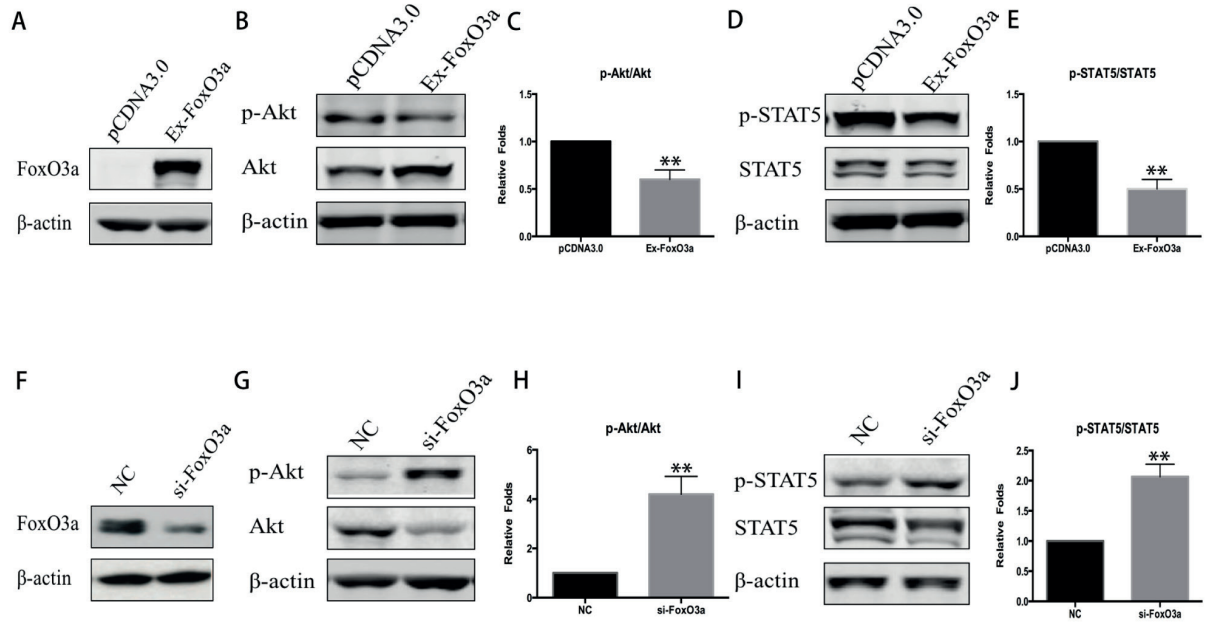

Figure 5. FoxO3a was the negative regulator of Akt and Stat5 signaling. ***A,*** T cells were transfected with FoxO3a overexpression plasmid and control plasmid. The effect of FoxO3a overexpression plasmid was evaluated by Western blot. ***B,*** The protein levels of p-Akt and Akt were evaluated by Western blot in the FoxO3a overexpression group. ***C,*** Gray analysis of the protein levels of p-Akt and Akt. The ratio between p-Akt and Akt was calculated. ***D,*** The protein levels of p-STAT5 and STAT5 were evaluated by Western blot in the FoxO3a overexpression group. ***E,*** Gray analysis of the protein levels of p-STAT5 and STAT5. The ratio between p-STAT5 and STAT5 was calculated. ***F,*** T cells were transfected with FoxO3a interference RNAs and NC RNAs. The effect of FoxO3a interference RNAs was evaluated by Western blot. ***G,*** The protein levels of p-Akt and Akt were evaluated by Western blot in the FoxO3a interference RNAs group. ***H,*** Gray analysis of the protein levels of p-Akt and Akt. The ratio between p-Akt and Akt was calculated. ***I,*** The protein levels of p-STAT5 and STAT5 were evaluated by Western blot in the FoxO3a interference RNAs group. ***J,*** Gray analysis of the protein levels of p-STAT5 and STAT5. The ratio between p-STAT5 and STAT5 was calculated. (**p<0.01).

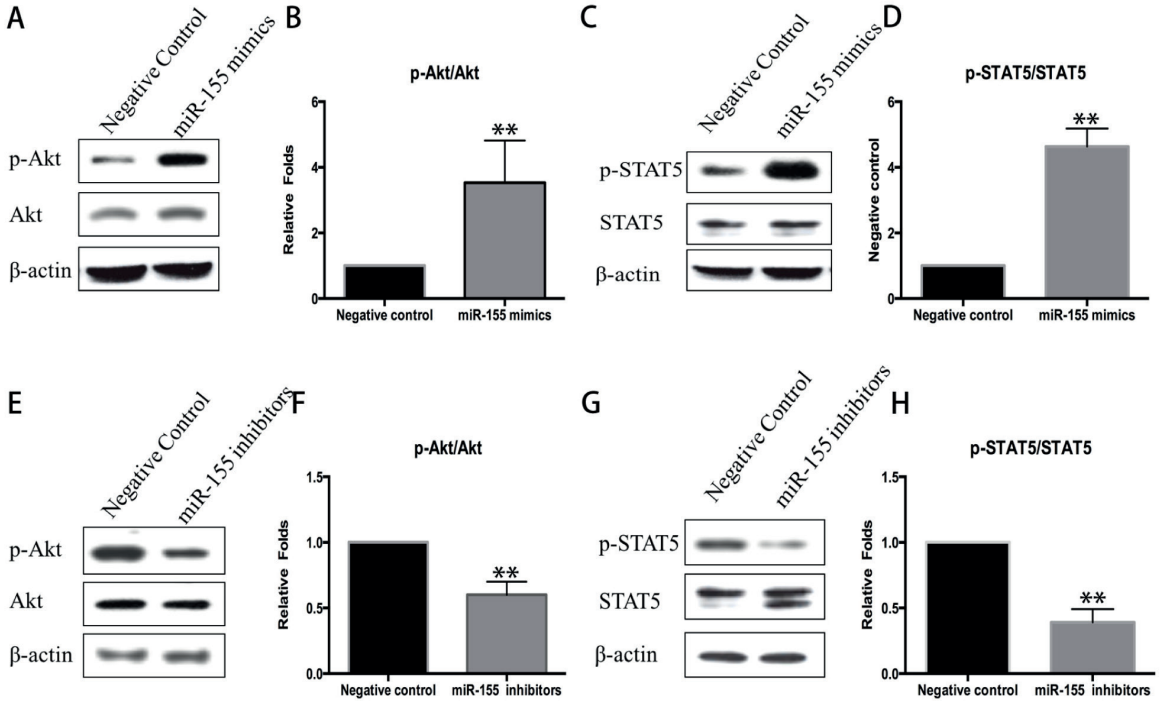

Figure 6. MiR-155 induced Akt and Stat5 signaling activation in T cells. ***A,*** The protein levels of p-Akt and Akt were evaluated by Western blot after T cells were transfected with miR-155 mimics. ***B,*** Gray analysis of the protein levels of p-Akt and Akt. The ratio between p-Akt and Akt was calculated. ***C,*** The protein levels of p-STAT5 and STAT5 were evaluated by Western blot after T cells were transfected with miR-155 mimics. ***D,*** Gray analysis of the protein levels of p-STAT5 and STAT5. The ratio between p-STAT5 and STAT5 was calculated. ***E,*** The protein levels of p-Akt and Akt were evaluated by Western blot after T cells were transfected with miR-155 inhibitors. ***F,*** Gray analysis of the protein levels of p-Akt and Akt. The ratio between p-Akt and Akt was calculated. ***G,*** The protein levels of p-STAT5 and STAT5 were evaluated by Western blot after T cells were transfected with miR-155 inhibitors. ***H,*** Gray analysis of the protein levels of p-STAT5 and STAT5. The ratio between p-STAT5 and STAT5 was calculated. (***p*<0.01).

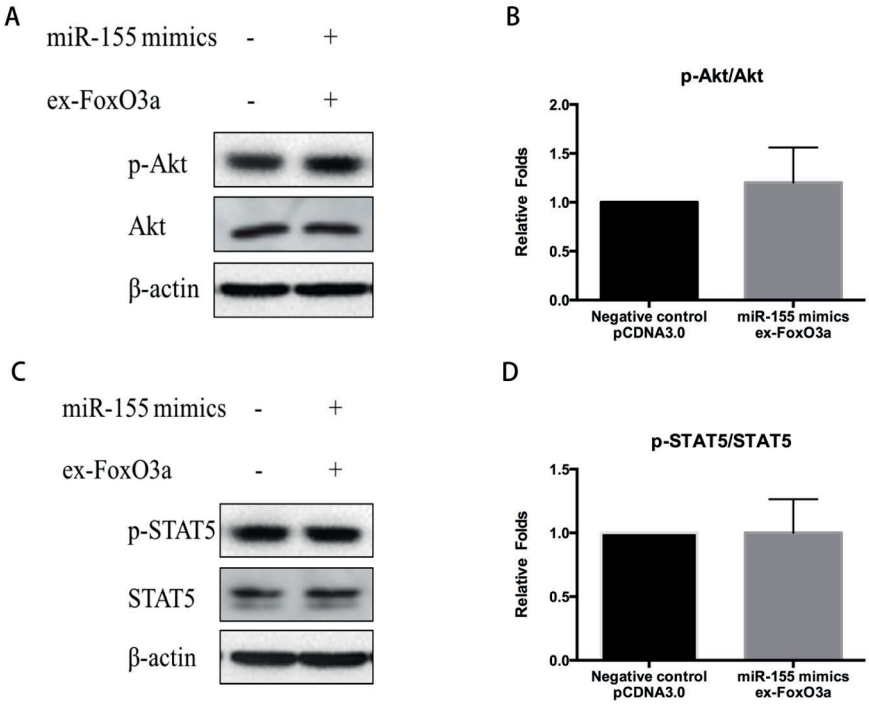

Figure 7. Overexpression of FoxO3a in T cells reversed the activation of Akt and Stat5 signaling caused by miR-155. T cells were co-transfected with FoxO3a overexpression plasmids and miR-155 mimics, or pCDNA3.0 plasmid and negative control. ***A,*** The protein levels of p-Akt and Akt in the two groups were detected by Western blot. ***B,*** Gray analysis of the protein levels of p-Akt and Akt. The ratio between p-Akt and Akt was calculated. ***C,*** The protein levels of p-STAT5 and STAT5 in the two groups were dectected by Western blot. ***D,*** Gray analysis of the protein levels of p-STAT5 and STAT5. The ratio between p-STAT5 and STAT5 was calculated. (***p*<0.01).

expressions of Bcl2 family members, such as Bim and Mcl-1[30]. Furthermore, the complex regulatory mechanisms of Stat5 and Akt signaling in different cellular biological activities have been proved by different studies.

Conclusions

In this work, we demonstrated that miR-155 exerted an anti-cancer effect in glioma by regulating the accumulation of CD 8^+ T cells. FoxO3a was a target gene of miR-155, which was a negative regulator of Akt and Stat5 signaling. Meanwhile, FoxO3a regulated the function of CD 8^+ T cells. Regarding the different functions of miR-155 in regulating the immune response, the development of the mechanism involved in immune regulation was helpful to comprehensively evaluate its anti-tumor value.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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