Pakistan J. Zool., pp 1-6, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230601050629

Adenosine Deaminase Acting on RNA1 (ADAR1) Deficiency Promotes Proliferation of Hemangioma-Derived Endothelial Cells via PI3K/Akt/mTOR Pathway

Chaoqi Yin[1]*, Ruyi Tao[1], Kaixi Tan[2] and Jianfei Zhang[3]

[1]Department of Plastic Surgery, The Third Xiangya Hospital, Central South University, Changsha 410013, People's Republic of China
[2]Department of Burns and Plastic Surgery, The Affiliated Nanhua Hospital, University of South China, Hengyang 421001, People's Republic of China
[3]Department of Burns and Plastic Surgery, The Second Affiliated Hospital, University of South China, Hengyang 421001, People's Republic of China

Article Information
Received 01 June 2023
Revised 07 June 2023
Accepted 23 June 2023
Available online 31 August 2023
(early access)

Authors' Contribution
CY, KT, JZ, RT conceptualization, methodology, software. CY, KT data curation, writing-original draft. KT, SW, JC investigation. CY and FT editing.

Key words
Infantile, Hemangioma, ADAR1, Akt/mTOR, HemECs

ABSTRACT

Infantile hemangioma (IH) is one of the most common benign tumours occurs in infancy. The adenosine deaminase acting on RNA1 (ADAR1) activates phosphorylated Akt (p-Akt), but the roles of ADAR1 remain unknown in IH. The objective of this study was to investigate the expression level and functions of ADAR1. Immunohistochemical method and western blotting were used to detect the protein levels of ADAR1 in human IH tissues. ADAR1-targeted siRNAs were transfected into HemECs cells to knockdown the expression of ADAR1. CCK-8 assay and cell tubule formation assay were performed to detect the phenotypic change after knockdown of ADAR1 expression. Western blotting was used to detect the related factors of Akt/mTOR signalling pathway. The expression level of ADAR1 is significantly up-regulated in the IH tissues as compared to the adjacent tissues. A sharp decrease of proliferation and cell tubule formation abilities was observed after knockdown of ADAR1 using ADAR1-targeted siRNAs. The expression levels of p-Akt, Akt, and mTOR were significantly inhibited after knockdown of ADAR1. It was concluded that ADAR1 is highly expressed in IH. Knockdown of ADAR1 inhibits cell proliferation and cell tubule formation abilities through down-regulation of Akt/mTOR signalling pathway.

INTRODUCTION

Infantile hemangioma (IH) is a common soft-tissue tumor in infants with an incidence of 4–5% (Munden *et al*., 2014). The tumor size increases along with the growth and development, which can seriously affect the appearance and psychology of a child (Zweegers and Van der Vleuten, 2019). There are almost 15% of IH need effective treatments to avoid severe complications, such as visual impairment, airway obstruction and tissue distortion (Smithson *et al*., 2017). However, current treatments are not suitable to all the cases and may cause serious side effects (Nagata *et al*., 2022). Therefore, it is urgent to investigate the molecular mechanisms of IH progression and provide more effective therapeutic targets.

Adenosine deaminases acting on RNA1 (ADAR) is a kind of enzyme that deaminate the adenosine to inosine in double-stranded RNAs. Until now, three kinds of ADARs have been found in mammalian cells, including ADAR1, ADAR2 and ADAR3. However, only ADAR1 and ADAR2 have the ability to edit RNA (Yablonovitch *et al*., 2017). Among the ADARs family, ADAR1 are particularly active in most human tissues. The lack of ADAR1 could lead to a series of diseases, for instance, the Aicardi-Goutières syndrome (Rice *et al*., 2012). Meanwhile, ADAR1 is also closely associated with tumorigenesis and the protein level of ADAR1 is usually higher in tumor than in normal tissues (Song *et al*., 2016). Some of the studies indicated that ADAR1 could be a crucial factor to regulate tumor cell proliferation and apoptosis (Nemlich *et al*., 2018; Wang *et al*., 2017), but it still remains unknow if ADAR1 can adjust the proliferation of hemangioma vascular endothelia cells

* Corresponding author: yinchaoqi@163.com
0030-9923/2023/0001-0001 $ 9.00/0

in IH.

The AKT/mTOR pathway often associated with cell proliferation and angiogenesis, has been proved to have an important role in regulating the proliferation of hemangioma derived vascular endothelia cells[3]. ADAR1 regulated endothelial cell survival which was mediated by AKT/mTOR pathway. Silencing of ADAR1 decreased the phosphorylation level of Akt (Jiang *et al*., 2018). This study aimed to investigate the role of ADAR1 in the progression of IH and to explore its mechanism of action.

MATERIALS AND METHODS

Cell lines

Hemogenic endothelial cells (HemECs) and human umbilical vein endothelial cells (HUVECs) were bought from KeyGEN Biological Technology Co. (Jiangsu, China). HemECs and HUVECs were plated in DMEM containing 10% foetal bovine serum (FBS). The cells were cultured in an incubator at 37 ℃ and 5% CO_2.

IH specimens

IH tissues were obtained from surgical specimens of four clinical infantile patients. The diagnosis of IH was made based upon clinical examination and computed tomography in the Third Xiangya Hospital, Central South University. The tumor tissues were placed in 10% neutral formalin buffer, embedded in paraffin, sectioned 5 μm thick, stained with immunohistochemical. The study was approved by the Ethics Committee of Third Xiangya Hospital, Central South University.

Western blot

All groups of cells and specimens were extracted by using the lysis buffer to collect total protein, and the protein concentration was determined by a BCA kit (Biyuntian). Then the target protein was subjected to 10% SDS/polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk powder for 1 h. The primary antibody was added at 4℃ overnight, and the secondary antibody was incubated at room temperature for about 30 min. The two reagents of ECL, A and B, were mixed in equal volumes, and fully contacted with the protein surface of PVDF membrane for 1 min. At last, take photos and use Image J to analyse the gray value of the target band.

Real-time quantitative PCR

Total RNA in the HemECs was extracted with TRIzol reagent. cDNA synthesis and quantitative polymerase chain reaction (PCR) was carried out according to the manufacturer's instructions (Harshitha and Arunraj, 2021). GAPDH was used as the internal reference to detect the relative expression level of ADAR1. The primers were used as follows: ADAR1 F: 5′-TGCTGCTGAATTCAAGTTGG-3′; ADAR1 R: 3′-TCGTTCTCCCCAATCAAGAC-5′; GAPDH F: 5′-TGACTTCAACAGCGACACCCA-3′; GAPDH R: 3′-CACCCTGTTGCTGTAGCCAAA-5′. The $2^{-\Delta\Delta Ct}$ method was performed to evaluate the relative expression level of ADAR1.

Cell transfection

The neonatal hemangioma endothelial cells in logarithmic growth period were randomly divided into 5 groups: Blank group, negative control siRNA (si-NC) group, si-ADAR1-1 group, and si-ADAR1-2 group, Si-ADAR1-3 group. The reaction system was 50 μL Si-NC and si-ADAR1-1 were transfected into infantile hemangioma endothelial cells. The reaction conditions were 95 ℃ for 3 min, 95 ℃ for 15 s, 60 ℃ for 30 s, 68 ℃ for 2 min, 35 cycles; 68 ℃ for 10 min, using GAPDH as an internal reference, the experiment was repeated three times. The data were analyzed by $2^{-\Delta\Delta Ct}$ method and a melting curve was made.

CCK-8 assay

This experiment was divided into 3 groups: blank control group, negative control siRNA (si-NC) group and si-ADAR1-1 group. The three groups of cells were separated and cultured for 3 days, and then seeded into 96-well plates. The 100 μl/well plate was continuously measured for 5 days, and treated with CCK8 for 2 h. The OD450 value and OD450/ fold value of each group were measured using a microplate reader.

Matrigel tube-forming test

Matrigel matrix glue was placed in the precooled 96-well plate, 70 μL per hole, and solidified at 37 ℃ for 30 min for later use. The HemECs re-suspended and transfected with siRNA were 3×10^4 cells/100 μ L, with 96-well plates. After incubating at 37 ℃ (2-4 h), the old solution was discarded, and 50 μ L Calcine AM cell staining reagent and 0.2 μ M culture medium were added to each and left for 8h at a temperature of 37 ℃ and then observed under a microscope to obtain pictures and data.

Statistical analysis

SPSS 21.0 software was used for statistical analysis. T test was used for comparison between the two groups, and Graph Pad Prism 7 was used to draw correlation curves for experimental data. $P < 0.05$ or $P < 0.01$ indicated that the difference was statistically significant.

RESULTS

Human IH specimens were performed to detect the ADAR1 protein using immunohistochemical method, showing ADAR1 is located in the nucleus, the intensity is moderate-strong, and the positive quantity is > 75% in IH tissues, while the intensity is negative-weak and the positive quantity is < 25% in the adjacent tissues (Fig. 1A). Western blotting showing ADAR1 protein levels in IH tissues of proliferating phase was the highest, while, successively, in IH tissues of stationary phase and in adjacent tissues (Fig. 1B).

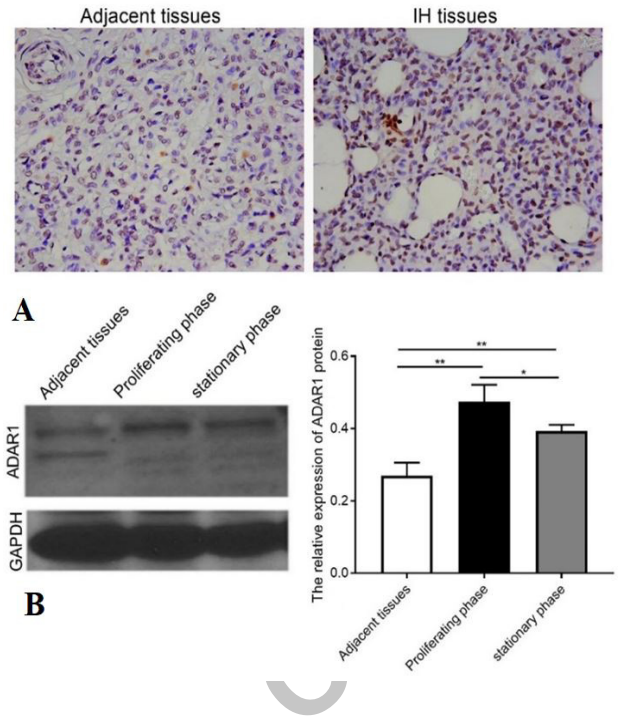

Fig. 1. ADAR1 is up-regulated in IH. (A) Histology characteristics of ADAR1. ADAR1 is located in the nucleus, which has higher positive signal in IH tissues as compared to the adjacent tissues. (B) ADAR1 protein levels in IH tissues of proliferating phase was the highest, while, successively, in IH tissues of stationary phase and in adjacent tissues; *p<0.05.

The protein expression levels of ADAR1 in HemECs and HUVECs were detected using western blotting assay, showing ADAR1 expression was much higher in HemECs than those in HUVECs (Fig. 2A). HemECs were performed to the subsequent experiments. In order to knockdown the ADAR1 expression in HemECs, those cells were transfected with ADAR1-targeted siRNAs (siADAR11, siADAR12, siADAR13), respectively, showing the sequences of siADAR11 had the best knockdown ability as evidenced by qRT-PCR (Fig. 2B). The protein levels of ADAR1 were significantly down-regulated after transfection with siADAR11 as evidenced by the blotting assay (Fig. 2C). Therefore, we choose the sequences of siADAR11 (siADAR1) for the next experiments.

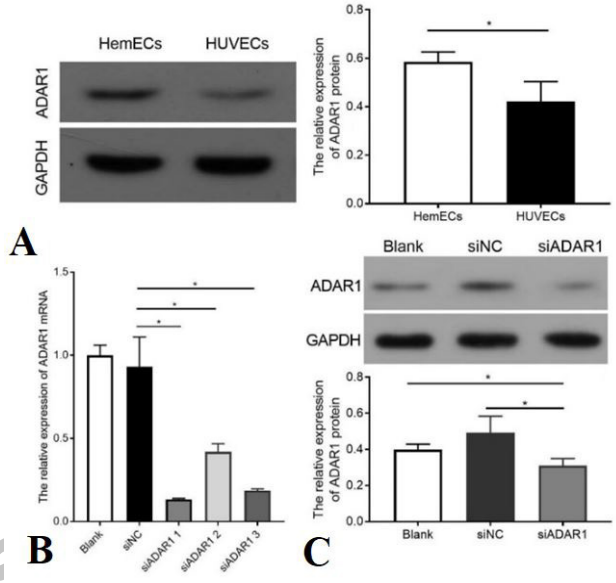

Fig. 2. ADAR1 expression is downregulated after transfection of siADAR1. (A) The protein levels of ADAR1 in HemECs and HUVECs. (B) The mRNA levels of ADAR1 in HemECs after transfection with ADAR1-targeted siRNAs; (C) The protein levels of ADAR1 in HemECs after transfection with siADAR1 1 (siADAR1); *p<0.05.

In order to evaluate the cell proliferation ability after knockdown of ADAR1 expression, the CCK-8 assay was conducted to evaluate the proliferation ability, showing the proliferation ability of ADAR1-knockdowned cells were significantly lower than those of non-treated cells and siNC cells at 48 h and 72 h (Fig. 3A). To assess the cell tubule formation ability, the number of nodes, meshes, blood vessel branches, trunk length were compared between these three groups. Comparing with blank group and siNC group, the number of nodes, meshes, blood vessel branches, trunk length were significantly lower in the siADAR1 group (Fig. 3B).

To explore the mechanisms of ADAR1 in IH, the ADAR1-related Akt/mTOR signalling pathway was assessed using western blotting assay. Comparing with the blank group and siNC group, a significant decrease of the protein levels of p-Akt, Akt and mTOR was observed in those cells after transfection with siADAR1 (Fig. 4).

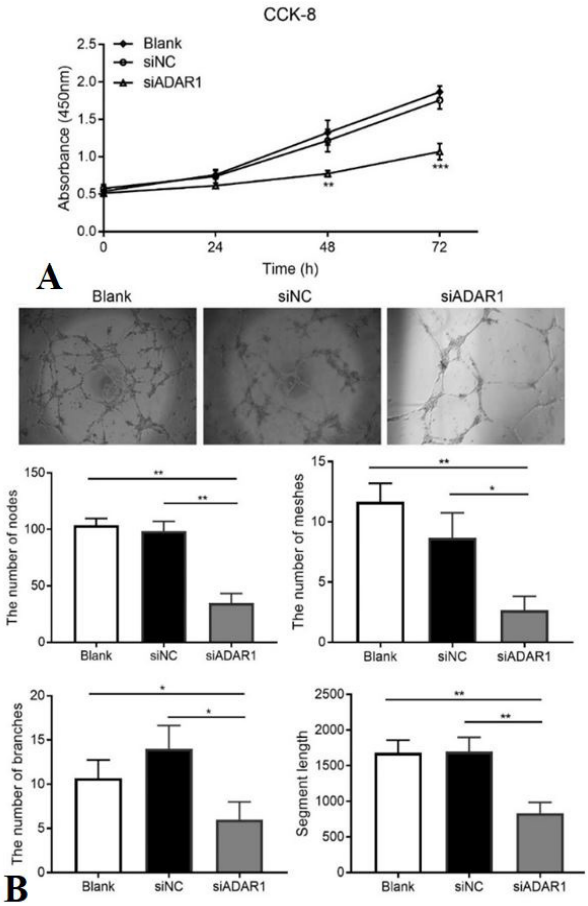

Fig. 3. Knockdown of ADAR1 leads to a decrease of proliferation and cell tubule formation abilities. (A) The proliferation ability of HemECs after transfection with siADAR1. (B) The number of nodes, meshes, blood vessel branches, trunk length were analysed to evaluate the cell tubule formation ability.

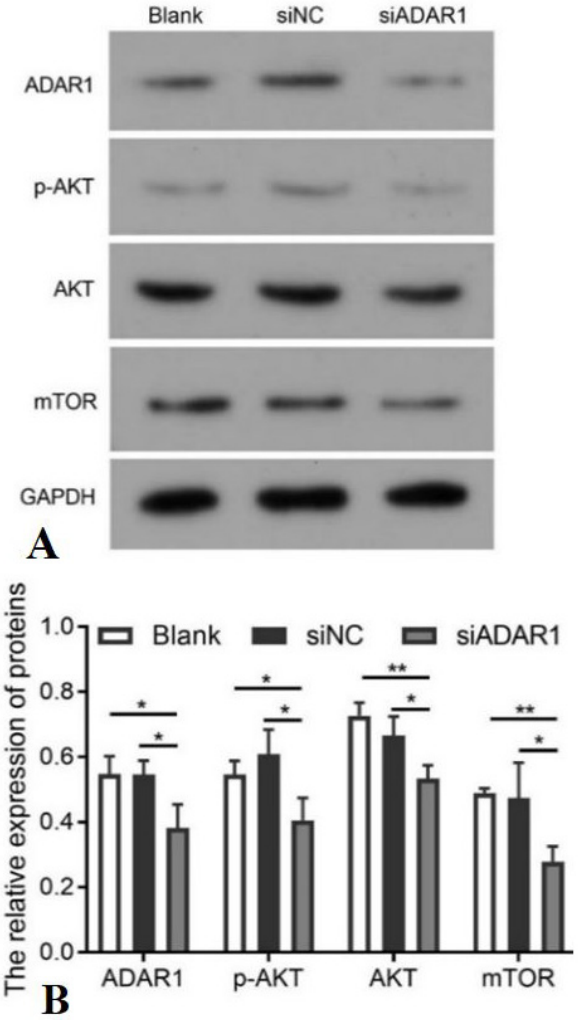

Fig. 4. Knockdown of ADAR1 leads to an inhibition of Akt/mTOR signalling pathway. (A) Comparing with the blank group and siNC group. (B) a significant decrease of the protein levels of p-Akt, Akt and mTOR was observed in those cells after transfection with siADAR1.

DISCUSSION

Infantile hemangioma is the most common infantile tumor. It is characterized by the proliferative phase with a rapid abnormal growth of blood vessels, followed by a slow involuting phase. The pathogenesis mechanism of IH remains poorly understood (Rotter and de Oliveira, 2017). Researchers found that the balance of angiogenic factors is broken in the proliferating phase of IH, for example, the levels of vascular endothelial growth factor, matrix metalloproteinases 2 and 9 are higher than normal tissue. While in the regression phase, a decrease of these factors and an increase of antiangiogenic factors can be detected (Przewratil *et al*., 2010; Pan *et al*., 2015). The change of local microenvironments may be a key factor for the pathogenesis of IH.

ADARs, a kind of enzyme acting on double-strand RNA, have the power to catalyze the deamination of A to I (Goncharov *et al*., 2022). More recent researches have shown that ADAR1 is a key element in the formation of cancers. Jiang et al. (2018) demonstrated that ADAR1 play a key role in regulating tumor cell proliferation using the human umbilical vein endothelial cells. Besides, researchers indicate that ADAR1 can combine with Dicer and form a complex to regulate oncogenic microRNAs (Liu *et al*., 2019; Ota *et al*., 2013). Additionally, ADAR1 has been found to be upregulated in breast, gastric, hepatocellular and other cancers (Kung *et al*., 2021; Li *et al*., 2021; Shi *et al*., 2017).

In our report, for the first time, we identified ADAR1 was significantly highly expressed in the IH tissues compared to the normal tissues. ADAR1 may play an important role in regulation the occurrence and development of IH. Indeed, silence of ADAR1 could significantly induce the proliferation and cell tubule formation abilities of HemECs. IH is characterized by massive proliferation of HemECs (Sun *et al*., 2022). In HemECs, Akt/mTOR signalling pathways trigger multiple downstream signals that promote angiogenesis (Ji *et al*., 2014). Propranolol induces the regression of hemangioma cells by down-regulating Akt/mTOR pathway (Pan *et al*., 2015). We showed that the expression levels of p-Akt, Akt, and mTOR were significantly inhibited after knockdown of ADAR1 in HemECs. These findings suggested that the proliferative effect of ADAR1 in HemECs may be correlated to Akt/mTOR pathway. Additional investigations in vitro and vivo are required to determine detailed molecular targets.

CONCLUSION

This study demonstrates that ADAR1 plays a role in promoting the proliferating process of IH, and the regulatory process may have a probable association with Akt/mTOR signalling pathway. Our finding provides insight into understanding of the cellular and molecular mechanisms of ADAR1 and potential therapeutic target in IH.

ACKNOWLEDGEMENT

The present study was supported by grant from the Hunan Science and Technology Innovation Plan Project (No. 2017SK50125).

Funding

The study received no funding.

IRB approval

All experiments were approved by The IRB of Third Xiangya Hospital.

Ethics statement

All experiments were performed accroding to guidelines of the Ethics Committee of Third Xiangya Hospital.

Statement of conflict of interest

The authors have declared no conflict of interest.

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