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SHORT COMMUNICATION

CIZ1 Expression Is Upregulated in Hemangioma of the Tongue

Yue Wang[1] · Xiaorui Li[2,3] · Jiahao Zhang[1] · Qiang Liu[2] · Peng Gao[2] · Di Li[4] · Shijie Zhang[5] · Ju Liu[2]

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Abstract
Hemangioma is a vascular neoplasm and one of the most common benign tumors. The pathogenesis of hemangioma has not been fully understood. CIZ1 (Cip1-interacting zinc finger protein 1) is a nuclear protein and the binding partner of p21. Dysregulation of CIZ1 expression has been reported in various types of cancerous tissues. In this study, we examined CIZ1 expression in hemangioma of the tongue and explored its function in vascular endothelial cells, the proliferative cell type in hemangioma. Immunohistochemistry showed that CIZ1 was highly expressed in hemangioma of the tongue while its expression is minimal in the normal tongue tissues. In vitro, knockdown of CIZ1 expression by shRNA transfection significantly reduced the proliferation and migration of human umbilical vein endothelium cells (HUVECs), suggesting a positive role of CIZ1 in endothelial cell proliferation and migration. Therefore, CIZ1 might involve in pathogenesis of hemangioma of the tongue by regulation of endothelial cell functions.

Keywords CIZ1 · Hemangioma · Cell proliferation · Migration

Introduction

Hemangioma is one of the most common benign neoplasms, occurring on the surface of skin and oral mucosa [1]. The hemangioma of tongue severely affects the health, as it is vulnerable to trauma as a result of the motion of the tongue. Consequent bleeding, ulceration, infection may occur after trauma [2]. Hemangiomas show a rapid development during proliferative phase due to excessive proliferation of capillary endothelial cells, which usually remain stable and rarely divide under normal conditions [3]. The hyper-proliferation of endothelial cells in hemangioma is mainly stimulated by estrogen, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [4]. However, the precise mechanism underlying the pathogenesis of hemangiomas has not been fully understood.

Cip1 interacting zinc finger protein 1(CIZ1), a binding protein of p21Cip1/Waf1, plays an important role in the initiation of DNA replication and replication fork organization. During DNA replication, CIZ1 interacts with cyclins (cyclin E and cyclin A), and promotes the formation of CDK2/cyclin A complex. Upregulation of CIZ1 altered the distribution of cell cycle by increasing the S phase, while knockdown of CIZ1 expression inhibited the distribution of S phase [5].

Dys-regulation of CIZ1 has been observed in several types of cancers including breast cancer, medulloblastoma, Ewing sarcoma, hepatocellular carcinoma, colon cancer, gallbladder cancer, prostate carcinoma and lung cancer [5, 6]. Our recent study showed that CIZ1 was specifically up-regulated in the endothelial cells of newly-formed capillaries in lung squamous cell carcinoma [7], suggesting that CIZ1 might be an regulator of angiogenesis.

To date, the expression pattern and the biological function of CIZ1 in hemangiomas tissue remain unknown. The aim of this study is to examine the expression of CIZ1 in hemangioma of

Yue Wang and Xiaorui Li contributed equally to the work.

✉ Shijie Zhang
meijie0909@sina.com

✉ Ju Liu
ju.liu@sdu.edu.cn

[1] Department of Orthodontics, School of Dentistry, Shandong University, Jinan, China

[2] Medical Research Center, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, China

[3] Taishan Medical College, Tai'an, China

[4] Eberly College of Sciences, Pennsylvania State University, University Park, PA 16801, USA

[5] Department of Stomatology, Qilu Hospital of Shandong University, Jinan, China

the tongue and the effects of CIZ1 on the function of hemangioma endothelial cells. Our study helps explore the molecular mechanisms of pathogenesis of hemangioma of the tongue.

Materials and Methods

Immunohistochemistry

A total of 16 samples of hemangioma of the tongue and 3 healthy tongue samples were collected between January 2012 and February 2017 in Department of Pathology of QiLu Hospital of Shandong University (Jinan, China), in accordance with the code established by the Ethical Committee of Shandong University. The samples were all fixed with formalin and embedded with paraffin. All the patients did not receive treatments until surgical excision.

Tissues were cut into 6-μm thick slices. Then the slices were deparaffinized using xylene for 20 min, and rehydrated using a graded ethanol series of alcohol. To expose antigenic epitopes, the slices were placed in 0.01 M citrate buffer, boiled and left to cool naturally. To quench endogenous peroxidase activity, the slices were incubated with 3% H_2O_2 for 10 min. After washing, the sections were incubated with the anti-CIZ1 antibody (dilution, 1:500; rabbit, polyclonal. no. ab102013; Cambridge, MA, USA), anti-CD31 (dilution, 1:800; rabbit, polyclonal.no.11265–1-AP; Rosemont, IL, USA), and anti-HIF-1α (dilution, 1:50; rabbit, polyclonal.no. 20960–1-AP; Rosemont, IL, USA) at 4 °C overnight. The sections were then washed with PBS three times, and incubated with HRP-Polymeranti-Mouse/Rabbit antibody (KIT-5020; MaxVision; Fuzhou, China) for 20 min. DAB (3,3-diaminobenzidine solutions, ZSGBBIO, Beijing, China) was used to the sections and then all the sections were counterstained with hematoxylin. The stained slides were photographed with an inverted microscope. Image Pro Plus5.0 (Media Cybernetics, Silver Spring, MD) were used to analyze the immunoreactivity of CIZ1.We acquired the mean integrated optical density (IOD) and mean stained area (MSA), which were averaged from 6 × 6 images (6 images each section and 6 sections each patient).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were maintained in DMEM(Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO2 under 37 °C. The HUVECs were cultured in 1% oxygen for 24 h to induce hypoxia.

ShRNA Transfection

To establish a stable-transfection cell line with CIZ1 knocked down, RNAi lentivirus particles (containing a puromycin resistant gene, ShCon and ShCIZ1) transfected in HUVECs for 24 h-incubation. Then the medium containing lentiviral particles was replaced with fresh medium, and the cells were incubated for 2 days. Three days after infection, 1 μg/ml puromycin (Sigma,USA) was used to select the stable-transfection cell line. The stable transfection cell lines were obtained and maintained with media containing 0.1 μg/ml puromycin.

Quantitative Real-Time PCR (qRT-PCR)

The total RNA was isolated from HUVECs using the E.Z.N.A. Total RNA Kit II (OMEGA Bio-tek, Inc., Norcross, GA, USA) and stored at −80 °C. Complementary cDNA was synthesized using the RevertAid First strand cDNA Synthesis kit (Thermo Fisher, Grand Island, NY, USA). Diluted cDNA (4.7 μL; 50 ng cDNA) and 5.3 μL of primer and supermix mixture (TaKaRa Biotechnology, Shiga, Japan) were used in each qRT-PCR reaction. The qRT-PCR process was performed on the CFX96 Real-Time System (Bio-Rad, Hercules, California, USA). All PCR reactions were repeated in triplicate. Relative quantitative values were obtained using the ΔΔCt method. The human CIZ1 PCR primers were 5′-taccagcactccctctaagca-3′ (sense) and 5′-ctcctcatcctctgtctccag-3′ (antisense).

Western Blotting

Cells were washed twice with PBS and lysed in RIPA buffer at 4 °C. The cell lysates were mixed with loading buffer, boiled for 5 min, separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with the 5% defatted-milk for an hour, and incubated with the primary antibody against CIZ1 (dilution, 1:1500; rabbit, polyclonal. no. ab102013; Cambridge, MA, USA) and GAPDH (14C10, CellSignaling) at 4 °C overnight. After washing with TBS-T, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase for 1.5 h at room temperature and then washed with TBS-T. The Immune complexes attached on membranes were visualized by using ECL kit (Pierce).

Proliferation Assays

MTT assay was performed with the MTT solution (Solarbio, Beijing, China) and the absorbance was determined at 570 and 670 nm on SpectraMax M2 (Molecular Devices LLC, Sunnyvale, CA, USA). 5-Ethynyl-20-deoxyuridine (EdU) incorporation assay was performed with a commercial kit (Cell Light EdU DNA imaging Kit, Guangzhou RiboBio, China)

following the manufacturer's instructions. The images were taken and analyzed using High Content Imaging Pathway 550(BD, USA).

Wound Healing Assay

Cell migration was evaluated by wound healing assay. The ShNC and ShCIZ1cells (1×10^6/well) were seeded in 6-well plates. When cells reached confluency, each well was scratched with a 100 μl pipette tip. After 0, 12 and 24 h of incubation, the wound healing areas were captured with a microscope. The migration area was analyzed by ImageJ software. Wound closure area was equal to the initial area minus the existing area.

Statistical Data Analysis

All the data were analyzed using GraphPad Prism 5.0 (Graphpad Software, La Jolla, USA). The data were expressed as means ± S.D. Groups were compared by Student's *t* test and analysis of variance for repeated measures. A statistical difference of $P < 0.05$ was considered significant.

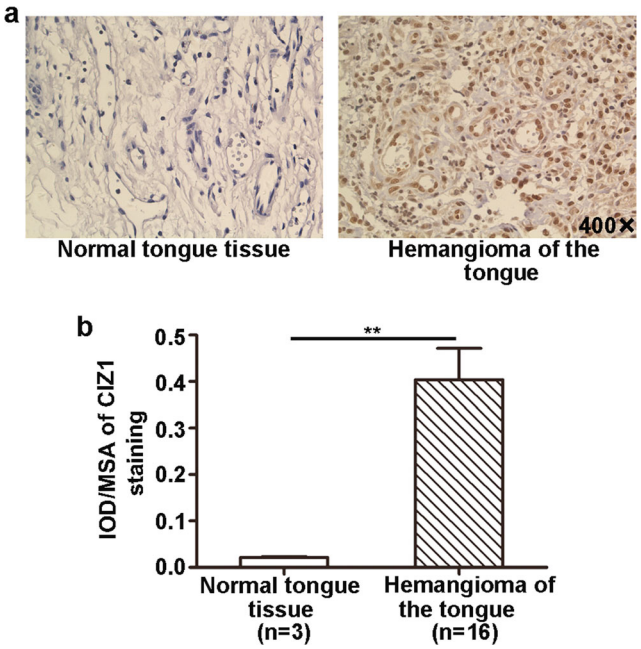

Fig. 1 The expression of CIZ1 in hemangioma of the tongue. **a** Immunohistochemistry of CIZ1 on normal tongue tissue and hemangioma of the tongue (Original magnification: 400×). **b** The bar graph of the strength of immunoreactivity of CIZ1 staining (**$P < 0.01$). IOD, integrated optical density. MSA mean stained area

Results

The Expression of CIZ1 Is Upregulated in Human Hemangioma of the Tongue Samples

To investigate the expression of CIZ1 hemangioma of the tongue, we collected human samples of hemangioma of the tongue and performed immunohistochemistry with a CIZ1 polyclonal antibody. The results showed that CIZ1 expression has hardly been detected in tissues from 3 normal tongue tissues (Fig. 1a). However, strong staining of CIZ1 has been detected in endothelial cells of all of the 16 hemangioma of the tongue tissues (Fig. 1a).The positive staining was restricted in the nucleus of the cells, which was consistent with previous reports [5]. Quantification of the staining intensity showed that CIZ1 expression was up-regulated by 16.83 times in tissues of tongue hemangioma than normal tongue by analyzing the average optical density ($P < 0.0001$) (Fig. 1b). Our results demonstrated that CIZ1 expression was significantly increased in hemangioma of the tongue.

Knockdown of CIZ1 Inhibits HUVECs Proliferation and Migration In Vitro

In vitro, the expression of CIZ1 was knocked down in HUVECs utilizing lentiviral particles, and the knockdown efficiency of CIZ1 protein in HUVECs was validated by western blot (Fig. 2a). MTT assay showed that knockdown of CIZ1 decreased the proliferation rate by 39.60% in HUVECs compared with the control ($P = 0.0057$) (Fig. 2b). In addition, we performed EdU staining to detect the cells undergoing proliferation. The EdU staining demonstrated that knockdown of CIZ1 in HUVEC cells reduced proliferative rate by 18.96% ($P = 0.0184$) (Fig. 2c, d). As endothelial cell migration is also necessary in the development of hemangioma, we performed the wound healing assay for HUVECs with knockdown of CIZ1. Fig. 2e and f showed that the healing area was significantly reduced for the group of CIZ1 knockdown (ShNC vs ShCIZ1) ($P = 0.0246$). These results suggest that CIZ1 may participate in pathogenesis of hemangioma of the tongue by promoting proliferation and migration of endothelial cells.

Hypoxia Decreases the Expression of CIZ1 in HUVECs

We explored the effects of hypoxia on expression of CIZ1 in HUVECs. By RT-PCR and western blot, we examined expression of *CIZ1* genes and protein in HUVECs 24 h after 1% oxygen exposure. We found that hypoxia down-regulated the transcription of *CIZ1* in HUVECs compared with the control ($p = 0.03$) (Fig. 3a). Upon hypoxia treatment, the level of CIZ1 protein was decreased as well (Fig. 3b). In addition, endothelial cells in both hemangioma of the tongue and normal tongue tissues were stained positive by the immunohistochemical localization of CD31 (Fig. 3c). As shown in Fig. 3d, positive staining of HIF-1α were similar in hemangioma of

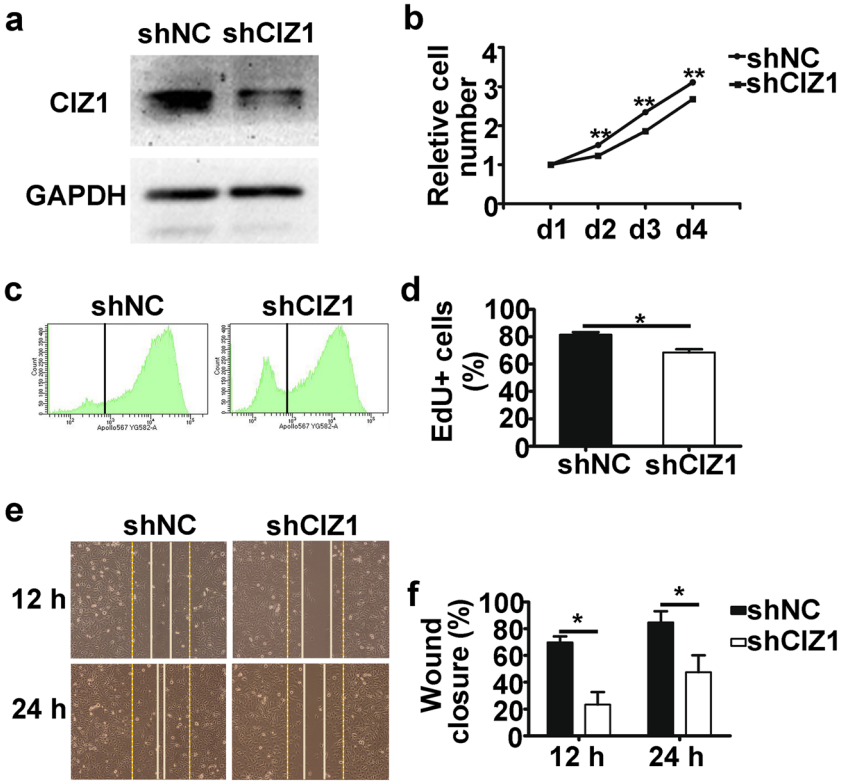

Fig. 2 Knockdown of *CIZ1* inhibits HUVEC proliferation and migration in vitro. **a** Western blot of CIZ1 protein from HUVECs transfected with CIZ1-shRNA. **b**, **c**, **d** The proliferation of CIZ1-shRNA-targeted HUVECs measured by MTT (**b**) and EDU (**c**, **d**).*$P < 0.05$, **$P < 0.01$. **e**, **f** Scratch wound healing assay of HUVECs transfected with CIZ1-shRNA. $n = 3$, *$P < 0.05$

the tongue and the normal tongue tissues. Taken together, our results suggest that hypoxia does not induce overexpression of CIZ1 in hemangioma.

Discussion

In the current study, we found that CIZ1 was significantly upregulated in human samples of hemangioma of the tongue by immunohistochemistry staining (Fig.1). In addition, we found that knockdown of CIZ1expression in HUVECs inhibited cell proliferation and migration. Our study uncovered a role of CIZ1 in the development of hemangioma.

Previous reports have shown that CIZ1 is closely related to the pathogenesis of tumors. Overexpression of CIZ1 has been found in brain tumor, hepatocellular carcinoma, human colorectal cancer, non-small cell lung cancer, prostate carcinoma, gallbladder cancer [5]. Increased expression of CIZ1 was proven to promote hepatocellular carcinoma and gallbladder carcinoma cell proliferation [5, 8]. Contrarily, CIZ1 downregulation inhibited cell growth and migration in cell lines of colon cancer and lung cancer [5, 6]. Previously we found that CIZ1 is overexpressed in vascular endothelial cells of lung squamous cell carcinoma rather than normal tissues, which implied that CIZ1 may contribute the development of lung squamous cell carcinoma by promoting tumor angiogenesis [7]. Hemangioma is a typical proliferative disease and mainly caused by hyper-proliferation of endothelial cells [3]. We speculated that CIZ1 might also be involved in the pathogenesis of hemangioma. Our results demonstrated that CIZ1 was overexpressed in hemangioma of the tongue. To our knowledge, this is the first demonstration of the expression profile of CIZ1in hemangioma tissue of tongue.

The molecular mechanisms of CIZ1 in the pathogenesis of hemangioma remain to be elusive. However, the functional roles of CIZ1 in hemangioma may be mediated through estrogen-estrogen receptor pathway. Hemangioma patients displayed higher plasmatic estrogen concentration and estrogen receptor expression [9]. Estrogen treatment remarkably promotes endothelial cell proliferation, while the antagonist of estrogen tamoxifen showed contrary effects [4]. CIZ1 had been demonstrated to be a novel interacting protein of estrogen receptor [5, 10]. Binding to estrogen receptor with the second glutamine-rich region of CIZ1 significantly increase the transcriptional activity of estrogen receptor and its downstream target genes, such as *cyclin D1*. Overexpression of CIZ1 induced tumorigenic phenotype of breast cancer cells and tumor formation in nude mice through estrogen receptor pathway, characterized by increased cell growth, colony formation and tumor volume [10]. Moreover, CIZ1 is a transcriptional target of estrogen receptor [10]. Overexpression of CIZ1 in endothelial cell might also enhance the transcriptional activity of estrogen receptor in endothelial cells to promote the development of hemangioma.

Our study showed that positive staining of CIZ1 appeared in the nuclear of the vascular endothelial cells, which is

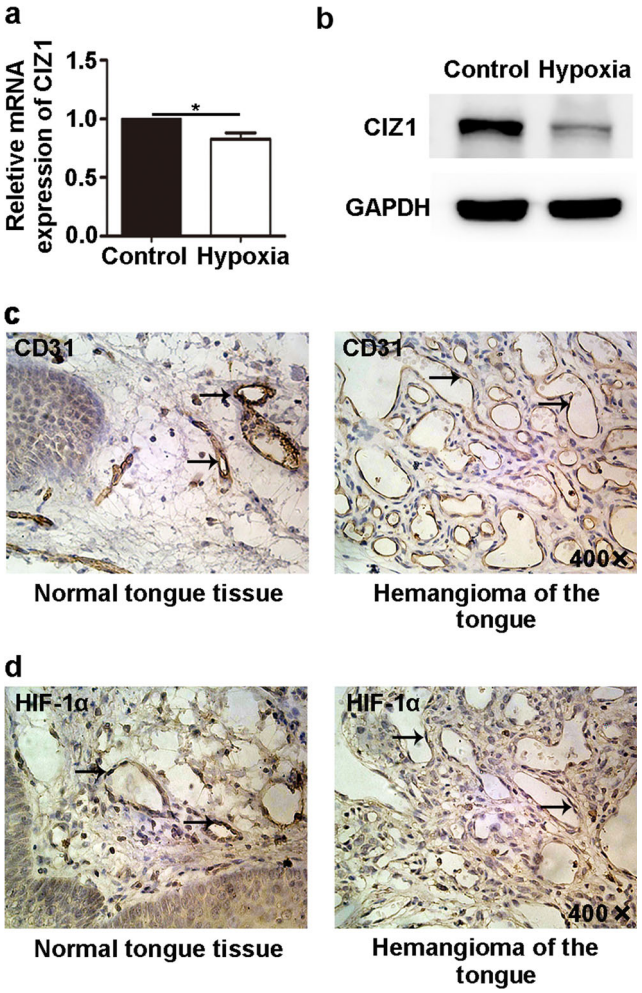

Fig. 3 Hypoxia decreases the expression of CIZ1 in HUVECs. **a** Relative CIZ1 mRNA expression measured by RT-PCR in HUVECs after 1% oxygen exposure for 24 h. n = 3; * $p < 0.05$. **b** Immunoblots of CIZ1 from protein samples of HUVECs exposed to 1% oxygen for 24 h. GAPDH was used as the loading control. **c** Immunohistochemistry of CD31 on normal tongue tissue and hemangioma of the tongue (Original magnification: 400×). **d** Immunohistochemistry of HIF-1α on normal tongue tissue and hemangioma of the tongue (Original magnification: 400×)

consistent with other reports. As a nuclear matrix protein CIZ1promotes DNA replication initiation by interacting with a cohort of cell cycle regulators including p21, cyclin A, cyclin E, CDK2, CDC6 and PCNA to facilitate the formation of DNA pre-replication complex and replisome. Our results demonstrated that knockdown of CIZ1 markedly attenuate endothelial cell proliferation and migration rate based on current evidences, CIZ1 might form a positive feedback loop with estrogen receptor to promote the development of hemangioma.

During angiogenesis, hypoxia induces proliferation of ECs. However, our data suggested that hypoxia decreases the expression of CIZ1 in HUVECs, therefore CIZ1 overexpression in hemangioma of the tongue is not induced by hypoxia. Unlike angiogenesis, in which normal endothelial cells proliferate in response to the hypoxic microenvironment, proliferation of ECs of hemanigoma is driven by their intrinsic properties [11]. Compared with normal ECs, hemECs express a significantly high level of mRNA angiogenic cytokines in vivo, including VEGF, bFGF, matrix metalloproteinase-1 (MMP-1), and angiopoietin-2 (Ang2) [11, 12]. Additionally, vascular endothelial growth factor receptor-2 (VEGFR-2) is higher in ECs of hemangiomas [13]. The autocrine loop of VEGF/VEGFR-2 of ECs in hemangioma is not associated with hypoxic microenvironment [14, 15]. Therefore, overexpression of CIZ1may regulate proliferation of ECs through a mechanism independent of hypoxia.

Conclusions

We demonstrated that CIZ1 was overexpressed in hemangiomas of the tongue, and knockdown of CIZ1 inhibited HUVECs proliferation and migration in vitro. Our study suggests that CIZ1 is involved in the pathogenesis of hemangioma of the tongue, and might have the potential as therapeutic target of hemangioma.

Compliance with Ethical Standards

Conflicts of Interest The Authors have no conflicts of interest to declare.

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