Original Article
Protective effects of SS-31 against SDHB suppression-mitochondrial dysfunction-EndMT axis-modulated CBT sclerosis and progression

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Abstract: Sclerosis variant in carotid body tumor (CBT) is characterized by extensive stromal sclerosis, which results in an uncommon pattern of growth that closely resembles that of an invasive malignant neoplasm. However, the clinical significance and the mechanism remains unclear. In this study, we provide evidence that SS-31 exerts protective effects against SDHB suppression-mitochondrial dysfunction-EndMT axis-modulated CBT sclerosis and progression. In human CBT specimens, sclerosis extent was consistently related to decreased recurrence-, death-, systematic metastasis-, and major adverse event-free survival, decreased SDHB expression, and aggravated EndMT. In human umbilical vein endothelial cells (HUVECs), SDHB KD aggravated hypoxia-induced EndMT, mitochondrial dysfunction and metabolic switch, while SS-31 treatment could significantly attenuate these changes caused by SDHB KD and hypoxia. In patient-derived xenograft (PDX) mice models of CBT, we also observed increased tumor growth speed and extent of EndMT, mitochondrial dysfunction, and metabolic switch in sclerosing carotid body tumor (SCBT) group than in conventional carotid body tumor (CCBT) group. And treating with SS-31 could significantly retard SCBT progression by rescuing the mitochondrial dysfunction-induced EndMT. Altogether, these results show that SDHB suppression-mitochondrial dysfunction-EndMT axis is a critical part of the CBT sclerosis and progression, while mitochondria-targeted drug SS-31 exerts an inhibitive effect on the above-mentioned axis, which opens new strategies to prevent and treat malignancies of CBT.

Keywords: Carotid body tumor, endothelial-mesenchymal transition, mitochondrial dysfunction, sclerosis, SS-31

Introduction

Carotid body tumors (CBTs) are rare, usually benign, tumors arising from glomus cells of the carotid body [1]. CBTs usually display a characteristic histological “Zellballen” pattern composed of nests of uniform cells separated by numerous blood capillaries [2].

The presence of extensive stromal sclerosis of these tumors has occasionally been described, including clear cells, spindling of cells, and angiomatoid features [3], which resulted in an uncommon pattern of growth that closely resembled that of an invasive malignant neoplasm [4]. However, the clinical significance and the mechanism of sclerosis variants in CBT has never been investigated.

Tumor-associated fibroblasts (TAFs) are one of the most important cells associated with tumor malignancy [5]. TAFs derive from cells of different origin, like endothelium and epithelium, and are regarded as key components of tumor stroma which induces cancer growth and invasiveness through the extracellular matrix (ECM) structure modulation and tumor metabolism [6]. This cell transdifferentiation process involving endothelial cells or epithelial cells is known as endothelial-to-mesenchymal transition (EndMT) or epithelial-to-mesenchymal transition (EMT), both of which play significant roles in various cardiovascular diseases, tissue fibrosis and, tumor progression [5, 7, 8]. Therefore, EndMT or/and EMT might be responsible for the progression and sclerosis variants of CBT.
The SDH complex is composed of four subunits (SDHA, SDHB, SDHC, and SDHD), of which SDHB mutations result in more aggressive CBTs with higher rates of metastasis compared with mutations in the other three subunits [9]. Consequences of SDHB deficiency include mitochondrial dysfunction, rewired metabolism, and pseudohypoxic signaling [10-12], all of which play important role in promoting tumors progression [12, 13]. Therefore, therapeutic strategies that target mitochondrial metabolism might be of importance in inhibiting CBT progression.

SS-31, a family of mitochondrion-targeted peptide antioxidants, can cross the cell membrane freely and accumulate in the mitochondrial inner membrane independently of the mitochondrial transmembrane electric potential [14]. It is revealed that SS-31 improves ATP production and prevents mitochondrial dysfunction [15, 16]. However, the effect of SS-31 on CBT progression has not been elucidated. In the present study, we investigated the clinical importance and mechanism of the CBT sclerosis variant, and whether the treatment of SS-31 could protect CBT from sclerosis variant and progression.

Materials and methods

Ethics statement

Studies involving human samples were approved and supervised by the Ethics Committee of Zhongshan Hospital (Shanghai, China). All protocols were conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and written informed consent was obtained from every patient.

Definitions

The resected carotid body tumors were classified into conventional carotid body tumor (CCBT) group, sclerosing carotid body tumor (SCBT) I group, and SCBT II group according to the histological assessment.

CCBT was defined as previously described. In brief, chief cells uniformly have a zellballen or nest-like growth pattern with sustentacular cells at their periphery and may be quite uniform or pronounced nuclear pleomorphism, and collagen area accounted for less than 20% of the total area. SCBT was defined as previously described [3]. In brief, SCBT was characterized as carotid body tumor with extensive stromal fibrosis and nested large polyhedral cells. SCBT I was characterized as collagen accounting for 20%-50% of the total area, while SCBT II was characterized as collagen accounting for more than 50% of the total area.

Cell culture and treatments

Control and SDHB knockdown (SDHB KD) human umbilical vein endothelial cell (HUVECs) (Sciencell Research Laboratories, San Diego, USA) and cultured using an Endothelial Cell Medium (ECM, 1001, ScienCell) as in our previous report [17]. The Xvivo Closed Incubation System (Xvivo system 300 C, BioSpherix, Lacona, New York, USA) was used in order to accurately maintain different oxygen tensions in different chambers. Cells were cultured at 37°C in an atmosphere of 5% CO2. After 24 hours of cultivation in ECM with 1% FBS to synchronize cells, the cells were cultured in hypoxia (1% O2) or in normoxia for 48 hours in the presence or absence of SS-31 (30 nM, China Peptides Co., Ltd., Shanghai, China), a new and innovative mitochondrion-targeted antioxidant.

SDHB knockdown, transduction and validation

HUVEC SDHB knockdown model was established as previously described [7]. To stably knockdown SDHB in HUVECs, a short hairpin RNA (shRNA) was designed and inserted into the SMARTvector (SDHB KD) (Dharmacon, USA), and scrambled shRNA was used as a negative control (Control). Then HUVECs were transsected using Polybrene Infection/Transfection Reagent (EMD Millipore, USA). The final knockdown efficiency was assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Cell morphology analysis

To analyze cell morphological changes, the cobblestone-like cells, and spindle-like cells were visualized by phase-contrast microscopy (Zeiss LSM 510 META, Oberkochen, Germany) and counted by a hemocytometer following gentle trypsinization. Spindle-shaped cells, which have presumably undergone EndMT, were defined as endothelial cells (ECs) that maintained their spindle shape upon trypsinization in the non-adherent state.
Protective effects of SS-31 against CBT sclerosis and progression

Table 1. Primer sequences for the genes targeted in quantitative reverse transcription PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>F: GTCTCCTGTGAATTCAAGGCG&lt;br&gt;R: ACCACCACTGGCTTGAGCCAA</td>
</tr>
<tr>
<td>VIMENTIN</td>
<td>F: GACGCCATCAACACCGAGTG&lt;br&gt;R: CTTTGCTGTTGTTACGCTTG</td>
</tr>
<tr>
<td>S100A4</td>
<td>F: GATGACCACTTTGACACCAA&lt;br&gt;R: CTGGGCTGTATCTCGGGAAG</td>
</tr>
<tr>
<td>CDH5</td>
<td>F: AACCAGATGCATCTTGAAGAG&lt;br&gt;R: ATTCTTGGAGCTACGCTTGA</td>
</tr>
<tr>
<td>EPAS1</td>
<td>F: CGGAGGTTGTTCTAGACCTGG&lt;br&gt;R: AGCTTGTGTTGCTCGGAA</td>
</tr>
<tr>
<td>SLC2A1</td>
<td>F: TCTGGCATCAGCAGCTCTTC&lt;br&gt;R: CGATACCCGAGCCAAATG</td>
</tr>
<tr>
<td>LDHA</td>
<td>F: TGACATCTGCTGCTTGGAAG&lt;br&gt;R: GGAACGGAATCGGCTGAAT</td>
</tr>
<tr>
<td>EDN1</td>
<td>F: AAGGAAACAGAGCCGAAAT&lt;br&gt;R: CGACCTGTTTTTGCTTAGG</td>
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Patient-derived xenograft (PDX)

Eight weeks old NPI. NOD-Prkdc<sup>scid</sup> - Il2rg<sup>emlIDMO</sup> (NSG) male mice were used as the host of PDX. The animals were randomly assigned to four groups with six animals in each group: CCBT + saline group, SCBT + saline group, CCBT + SS-31, and SCBT + SS-31 group. Fresh carotid body tumor tissues were cut into 3 × 3 mm cubes under sterile condition, and were then transplanted subcutaneously within 24 h from surgery under the neck skinfold(s). For drug administration, SS-31 (3 mg/kg/day) or saline alone (same volume as SS-31) was administered intraperitoneally until harvest after transplantation. All mice were maintained in temperature-controlled cages docked on a ventilated rack with HEPA filters. Eight months post-transplantation, all mice were euthanized by CO<sub>2</sub> inhalation, and were dissected to identify putative growths in transplant areas, that were measured, photographed, and harvested.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

TRizol (Thermo Fisher Scientific, Waltham, MA, USA) was used to isolate total RNA from HUVECs. An Omniscript RT kit (Qiagen) was used to synthesize first-strand complementary DNA. qRT-PCR using SYBR Green was used to quantify changes in mRNA. Expression levels were calculated using the ΔΔCT method [18]. All primers are listed in Table 1.

Immunofluorescence (IF) and confocal microscopy

Carotid body tumor immunostaining was performed using pre-existing tissue blocks derived from human carotid body tumor resection samples and PDX model. The samples were formalin-fixed and paraffin-embedded. Blocks were sectioned at 5-μm intervals using a microtome. Slides were deparaffinized in xylene, followed by washes in 100, 90, 80, 70, and 50% ethanol, followed by rinsing in phosphate-buffered saline (PBS). Antigen retrieval was performed in citrate buffer (10 mM, pH 6.0) at 95°C. After cooling, tissue slides were blocked for 1 h in 5% bovine serum albumin (BSA) and 20% donkey serum in PBS, and then incubated with primary antibodies in 3% BSA overnight at 4°C in a humidified chamber. Primary antibodies used for IF were anti-Vimentin (ab8978; Abcam), anti-CD31 (550274; BD Biosciences), anti-E-cadherin (sc-59778, Santa Cruz Biotechnology) and anti-S100A4 (ab27957; Abcam). Slides were then washed with Tris-buffered saline and incubated with Cyanine3 (Cy3)- or Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 1 h in 5% BSA overnight at 4°C in a humidified chamber. Primary antibodies used for IF were anti-Vimentin (ab8978; Abcam), anti-CD31 (550274; BD Biosciences), anti-E-cadherin (sc-59778, Santa Cruz Biotechnology) and anti-S100A4 (ab27957; Abcam). Slides were then washed with Tris-buffered saline and incubated with Cyanine3 (Cy3)- or Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 1 h at 20°C. All immunofluorescence micrographs were acquired using an Axiolab 200M microscopy system (Carl Zeiss, Jena, Germany). Images quantifications were performed using ImageJ software.

Histology staining

Histological staining of CBT from human CBT resection samples and PDX model was performed as previously reported [19]. In brief, the samples were formalin-fixed and paraffin-embedded. The blocks were sectioned at 5 μm intervals using a microtome. Then, the slides were deparaffinized in xylene and rehydrated with graded ethanol. Then, the sections were stained with hematoxylin and eosin (H&E) and Masson’s trichrome stain.

Subcellular fractionation

The carotid body tumor from PDX model or HUVECs was rinsed with ice-chilled PBS and then homogenized in 500 μL of homogenizing buffer with a 2 mL-glass-Teflon homogenizer on ice. The homogenate was centrifuged at 600 ×
g for 5 min to remove nuclear fraction. Finally, the obtained supernatant was further centrifuged at 4500 × g for 10 min and at 20,000 × g for 20 min to precipitate the mitochondrial. The obtained supernatant and mitochondria were used in following experiments. All experimental procedures were carried out at 4°C unless mentioned.

**Determination of mitochondrial membrane potential (MMP) level**

MMP level was determined using a mitochondrial membrane potential detection kit (C2006, Beyotime Institute of Biotechnology, China), as previously described [20]. Briefly, the isolated mitochondria were incubated with JC-1 staining solution immediately, then fluorescence intensity of both mitochondrial JC-1 aggregates (λex 550 nm, λem 600 nm) and monomers (λex 485 nm, λem 535 nm) was detected using a Multi-Mode Microplate Reader (Syn-ergy2, BioTek, USA). The MMP of mitochondria in each group was calculated as the fluorescence ratio of red (JC-1 aggregates) to green (JC-1 monomers).

**ATP level assays**

ATP level of CBT from PDX model or HUVECs was measured using an ATP assay kit (S0026, Beyotime Institute of Biotechnology, China) according to the manufacturer’s instructions, as previously described [21]. Briefly, after collection of the cell supernatant, an aliquot (100 μL) of ATP detection working solution was added to each well of a black 96-well plate. After 3 min at room temperature, 50-μL cell supernatant was added to the wells, and the luminescence was measured immediately using GloMax® 96 Microplate Luminometer (Promega, China). A fresh standard curve was prepared each time and ATP content was estimated according to the curve. Results were normalized to sample protein concentration, which was determined by an Enhanced BCA Protein Assay kit (Beyotime, China).

**Electron transport chain (ETC) enzymatic activities**

The NADH dehydrogenase activity of isolated mitochondria was measured using the complex I enzyme activity microplate assay kit (Abcam). The procedure was adapted from the previous report [10, 21]. The complex II assay was performed with the Succinate-coenzyme Q reductase activity assay kit (Comin Biotechnology Co., Suzhou, China) following the manufacturer’s instructions. The activity was calculated by measuring the reduction of the absorbance of 2,6-dichlorindole at 605 nm. The Mitochondrial complex III activity was measured, based on the reduction of cytochrome c through the activity of complex III following the manufacturer’s instructions (Biovision Inc., Milpitas, CA, USA).

**Statistical analysis**

The data were calculated and expressed as the mean ± standard deviation (SD). All experiments were conducted at least three times. Differences between groups were evaluated using one-way analysis of variance combined with Bonferroni’s post hoc test, and those between two groups were evaluated using Student’s t-test. p-values were considered significant at P < 0.05.

**Results**

**Clinicopathologic characteristics**

We studied a total of 238 unilateral CBT samples, from 37 SCBT I, 16 from SCBT II, and 185 CCBT patients (Figure 1A-C). No significant differences were observed among CCBT, SCBT I, and SCBT II group in age, sex, smoking, hypertension, diabetes, hyperlipidaemia, cardiovascular disease, tumor volume, or Shamblin grade I and II (Supplementary Table 1). And there were significantly more Shamblin grade III CBT in the SCBT II group (43.75%) than in the CCBT group (18.38%).

**Follow-up results**

The 1-, 5-, and 10-year recurrence-free survival was 100%, 100%, and 100%, respectively, in the CCBT group; 100%, 95.83%, and 95.83%, respectively, in the SCBT I group; and 100%, 100%, and 80.00%, respectively, in the SCBT II group. Recurrence-free survival at 10 years was significantly lower in the SCBT I and II group than in the CCBT group (P < 0.05, Figure 1D).

The 1-, 5-, and 10-year all-cause mortality-free survival was 100%, 100%, and 100%, respectively, in the CCBT group; 100%, 100%, and 100%, respectively, in the SCBT I group; and
Protective effects of SS-31 against CBT sclerosis and progression
Protective effects of SS-31 against CBT sclerosis and progression

100%, 90.91%, and 90.91%, respectively, in the SCBT II group. All-cause mortality-free survival at 10 years was significantly lower in the SCBT II group than in CCBT group (P < 0.01, Figure 1E), but similar in SCBT I group and CCBT group (P > 0.05, Figure 1E).

The 1-, 5-, and 10-year systemic metastasis-free survival was 100%, 100%, and 100%, respectively, in the CCBT group; 100%, 100%, and 100%, respectively, in the SCBT I group; and 100%, 92.31%, and 92.31%, respectively, in the SCBT II group. Systemic metastasis-free survival at 10 years was significantly lower in the SCBT II group than in CCBT group (P < 0.01, Figure 1F), but similar in SCBT I group and CCBT group (P > 0.05, Figure 1F).

The 1-, 5-, and 10-year major adverse event (MAE)-free survival in the CCBT group was 100%, 100%, and 100%, respectively; was 100%, 95.65%, and 95.65% in the SCBT I group, respectively; and was 100%, 92.31%, and 73.85% in the SCBT II group, respectively. MAE-free survival at 10 years was significantly lower in the SCBT I and II group than in CCBT group (P < 0.05, Figure 1G).

SDHB level in CBT

The IHC results revealed that SDHB expression was significantly decreased in SCBT groups than in CCBT group, and the SDHB expression was decreased significantly with the increased sclerosis extent (P < 0.05, Figure 1C and 1H).

Consistent with the IHC results, the mRNA level of SDHB was significantly decreased in SCBT groups than in CCBT group, and decreased significantly with the increased sclerosis extent (P < 0.05, Figure 1I).

EndMT and HIF-2α related gene involved in carotid body tumor sclerosis

Immunostaining demonstrated that the S100A4 and VIMENTIN expression in SCBT group was significantly higher than in CCBT group, while the CD31 expression in SCBT group was significantly lower than in CCBT group (Figure 2A-G). In addition, double immunostaining further confirmed there were significantly more cells co-expressing CD31 and VIMENTIN in SCBT group than in CCBT group (P < 0.01, Figure 2A, 2B, 2H). Similarly, double immunostaining also demonstrated that there were significantly more cells co-expressing CD31 and S100A4 in SCBT group than in CCBT group (P < 0.05, Figure 2C, 2D, 2I). These clinical results suggest the presence and participation of EndMT in carotid body tumor sclerosis.

The mRNA level of EPAS1, LDHA, EDN1, SLC2A1 was significantly increased in SCBT groups than in CCBT group (P < 0.01, Figure 2J-M). As for EMT, the E-cadherin expression was similar in CCBT and SCBT group (P > 0.05, Figure 3A-E), and there was no significant difference between SCBT group and CCBT group regarding the proportion of VIMENTIN+ or S100A4+ epithelial cells (P > 0.05, Figure 3A-D, 3F, 3G).

SDHB KD aggravated hypoxia-induced EndMT

The number of elongated and spindle-like HUVECs significantly increased under the hypoxia,
Protective effects of SS-31 against CBT sclerosis and progression

A
CCBT
CD31
VIMENTIN
MERGE

B
SCBT
CD31
VIMENTIN
MERGE

C
CCBT
CD31
S100A4
MERGE

D
SCBT
CD31
S100A4
MERGE

E
Relative IF intensity of CD31
P < 0.05

F
Relative IF intensity of S100A4
P < 0.01

G
Relative IF intensity of S100A4
P < 0.01

H
Relative proportion of VIMENTIN
P < 0.01

I
Relative proportion of S100A4-expressing cells
P < 0.05

J
Relative gene expression of EPAS1
P < 0.01

K
Relative gene expression
P < 0.01

L
Relative gene expression
P < 0.01

M
Relative gene expression
P < 0.01
Protective effects of SS-31 against CBT sclerosis and progression

Figure 2. EndMT and HIF-2α related gene involved in carotid body tumor fibrosis. A. Immunofluorescence staining for CD31 (green) and VIMENTIN (red) in CCBT samples. Nuclei were stained with DAPI (blue). B. Immunofluorescence staining for CD31 (green) and VIMENTIN (red) in SCBT samples. C. Immunofluorescence staining for CD31 (green) and S100A4 (red) in CCBT samples. D. Immunofluorescence staining for CD31 (green) and S100A4 (red) in SCBT samples. E. Immunofluorescence intensity of CD31 in SCBT and CCBT samples. F. Immunofluorescence intensity of VIMENTIN in SCBT and CCBT samples. G. Immunofluorescence intensity of S100A4 in SCBT and CCBT samples. H. Immunocytochemical analysis of the percentages of VIMENTIN+ endothelial cells in SCBT and CCBT samples. I. Immunocytochemical analysis of the percentages of S100A4+ endothelial cells in SCBT and CCBT samples. J-M. The mRNA expression levels of EPAS1, LDHA, EDN1, SLC2A1 were detected by qRT-PCR. Values represent the mean ± SD. *P < 0.05, **P < 0.01.

Figure 3. EMT did not involve in carotid body tumor fibrosis. A. Immunofluorescence staining for E-CADHERIN (green) and VIMENTIN (red) in CCBT samples. Nuclei were stained with DAPI (blue). B. Immunofluorescence staining for E-CADHERIN (green) and VIMENTIN (red) in SCBT samples. C. Immunofluorescence staining for E-CADHERIN (green) and S100A4 (red) in CCBT samples. D. Immunofluorescence staining for E-CADHERIN (green) and S100A4 (red) in SCBT samples. E. Immunofluorescence intensity of E-CADHERIN in SCBT and CCBT samples. F. Immunocytochemical analysis of the percentages of VIMENTIN+ endothelial cells in SCBT and CCBT samples.
cal analysis of the percentages of VIMENTIN* epithelial cells in SCBT and CCBT samples. G. Immunocytochemical analysis of the percentages of S100A4* epithelial cells in SCBT and CCBT samples. Values represent the mean ± SD. *P < 0.05. **P < 0.01.

Figure 4. Effect of SDHB-knockdown and SS-31 on EndMT and mitochondrial function of HUVECs under hypoxia condition. HUVECs were treated with vehicle + normoxia, vehicle + hypoxia, SDHB-knockdown + hypoxia, or SS-31 + hypoxia + SDHB-knockdown for 48 h respectively. A. The number of spindle-like cells was counted using a hemocy-
and SDHB KD of HUVECs further aggravated the morphological change of HUVECs caused by hypoxia (P < 0.01, Figure 4A). In accordance with the morphology changes, expression of the endothelial marker CDH5 also decreased significantly, while the mesenchymal marker VIMENTIN and S100A4 increased significantly under hypoxia condition (P < 0.01, Figure 4B-D). And SDHB KD significantly aggravated these gene changes caused by hypoxia (P < 0.01, Figure 4B-D).

SDHB KD aggravated hypoxia-induced mitochondrial dysfunction and metabolic switch

We investigated the mRNA levels of EPAS1, SLC2A1, and LDHA to access metabolic switch in HUVECs. The results showed that the mRNA levels of EPAS1, SLC2A1, and LDHA were significantly elevated under hypoxia condition (P < 0.01, Figure 4E-G), which indicated the increased aerobic glycolysis in HUVECs under hypoxia condition. And SDHB KD significantly aggravated these mRNA level changes caused by hypoxia (P < 0.01, Figure 4E-G).

To investigate the impact of hypoxia and SDHB KD on mitochondrial dysfunction, the levels of ATP production, MMP, and the activities of respiration complexes I, II, and III were measured. The results showed that hypoxia lead to decreased activities of respiration complexes I, II, and III, and decreased level of MMP and ATP production (P < 0.01, Figure 4H-L). And SDHB KD significantly aggravated the decrease in MMP, ATP production, and activities of respiration complexes I, II, and III caused by hypoxia (P < 0.05, Figure 4H-L).

SS-31 inhibits SDHB KD-aggravated EndMT by restoring mitochondrial function

To investigate the effect of SS-31 on SDHB KD-aggravated EndMT, we treated HUVECs with SS-31 under hypoxia condition. In vitro study demonstrated that, compared with vehicle treatment, SS-31 treatment significantly reduced VIMENTIN and S100A4 mRNA levels and the number of elongated and spindle-like HUVECs but increased CDH5 mRNA level of SDHB KD HUVECs under hypoxia (P < 0.01, Figure 4A-D). As SS-31 is a synthetic tetrapeptide that selectively targets mitochondria to restore mitochondrial bioenergetics, we also investigated the effect of SS-31 on mitochondrial dysfunction and aerobic glycolysis. In vitro study demonstrated that, compared with vehicle treatment, SS-31 treatment not only significantly rescued the reduced level of ATP production, and reduced activities of respiration complexes I, II, and III, but also alleviated the increased level of the mRNA expression of EPAS1, SLC2A1, and LDHA caused by SDHB knockdown under hypoxia condition (P < 0.05, Figure 4E-K).

SCBT PDXs show increased level of EndMT and progression speed

At harvest, 8 months post-transplantation, the PDXs of SCBT group is increased to 5.96±0.38 mm in diameter, while PDXs of CCBT group is increased to 4.05±0.24 mm in diameter, which showed significantly faster growth rate in SCBT group (P < 0.01, Figure 5A).

Double immunostaining demonstrated that, however, there was no significant difference in CD31 expression between groups (P > 0.05, Figure 5B, 5E-H), the VIMENTIN expression and the proportion of VIMENTIN+ ECs significantly increased in SCBT group (P < 0.01, Figure 5C-H). Furthermore, the S100A4 expression and the proportion of S100A4+ ECs were also increased significantly in SCBT PDX models than in CCBT PDX models (P < 0.01, Figure 6A-F). In addition, qRT-PCR further confirmed the elevated mRNA levels of VIMTNIN and S100A4 and decreased mRNA level of CDH5 in SCBT PDX models than in CCBT PDX models (P < 0.01, Figure 7A-C).

SCBT PDXs show aggravated mitochondrial dysfunction and aerobic glycolysis

We investigated the mRNA levels of EPAS1, SLC2A1, EDN1, and LDHA in SCBT PDX model and CCBT PDX model. The results showed that the mRNA levels of EPAS1, SLC2A1, EDN1, and LDHA were significantly elevated in SCBT PDX model compared with CCBT PDX model (P < 0.01, Figure 7D-G).
Figure 5. Effect of SS-31 on VIMENTIN expression and progression of SCBT and CCBT PDX models. SCBT and CCBT PDX models were treated with vehicle or SS-31 (3 mg/kg/day) for eight months. A. The maximum diameter of PDX models in different groups. B. Immunofluorescence intensity of CD31 in different PDX models. C. Immunofluorescence intensity of VIMENTIN in different PDX models. D. Immunocytochemical analysis of the percentages of VIMENTIN+ endothelial cells in different PDX models. E. Immunofluorescence staining for CD31 (green) and VIMENTIN (red) in CCBT treated with saline PDX models. Nuclei were stained with DAPI (blue). F. Immunofluorescence staining for CD31 (green) and VIMENTIN (red) in SCBT treated with saline PDX models. Nuclei were stained with DAPI (blue). G. Immuno-
Protective effects of SS-31 against CBT sclerosis and progression

fluorescence staining for CD31 (green) and VIMENTIN (red) in CCBT treated with SS-31 PDX models. Nuclei were stained with DAPI (blue). H. Immunofluorescence staining for CD31 (green) and VIMENTIN (red) in SCBT treated with SS-31 PDX models. Nuclei were stained with DAPI (blue). Values represent the mean ± SD. *P < 0.05, **P < 0.01.

Figure 6. Effect of SS-31 on S100A4 expression of SCBT and CCBT PDX models. SCBT and CCBT PDX models were treated with vehicle or SS-31 (3 mg/kg/day) for eight months. A. Immunofluorescence intensity of S100A4 in different PDX models. B. Immunocytochemical analysis of the percentages of S100A4+ endothelial cells in different PDX models. C. Immunofluorescence staining for CD31 (green) and S100A4 (red) in CCBT treated with saline PDX models. Nuclei were stained with DAPI (blue). D. Immunofluorescence staining for CD31 (green) and S100A4 (red) in SCBT treated with saline PDX models. Nuclei were stained with DAPI (blue). E. Immunofluorescence staining for CD31 (green) and S100A4 (red) in CCBT treated with SS-31 PDX models. Nuclei were stained with DAPI (blue). F. Immunofluorescence staining for CD31 (green) and S100A4 (red) in SCBT treated with SS-31 PDX models. Nuclei were stained with DAPI (blue). Values represent the mean ± SD. *P < 0.05, **P < 0.01.
The results also showed decreased activities of respiration complexes I, II, and III, and decreased level of MMP and ATP production in SCBT PDX model compared with CCBT PDX model \((P < 0.01, \text{Figure 7H-L})\).

SS-31 inhibits CBT progression by inhibiting mitochondrial dysfunction-aggravated EndMT

After treated with SS-31, the maximum diameter of the PDXs of SCBT and CCBT group...
decreased to 4.59±0.41 mm and 3.27±0.37 mm, respectively, which were significantly smaller than those treated with vehicle (P < 0.01, Figure 5A).

Double immunostaining demonstrated that compared with vehicle treatment, SS-31 treatment significantly reduced the VIMENTIN expression and the proportion of VIMENTIN+ ECs in SCBT PDX model (P < 0.01, Figure 5C-H). Likely, compared with vehicle treatment, SS-31 treatment significantly reduced the S100A4 expression and the proportion of S100A4+ ECs in SCBT group (P < 0.01, Figure 6A-F). However, SS-31 treatment failed to increase the CD31 expression in SCBT model (P > 0.05, Figure 5B, 5E-H). Likely, compared with vehicle treatment, SS-31 treatment significantly reduced the S100A4 expression and the proportion of S100A4+ ECs in SCBT group (P < 0.01, Figure 6A-F). However, SS-31 treatment failed to rescue the aforementioned index in CCBT PDX model. These in vivo results suggest that SS-31 treatment can retard SCBT progression by inhibition of EndMT.

qRT-PCR demonstrated that SS-31 treatment failed to increase CDH5 expression in both CCBT and SCBT PDX model (Figure 7A), but significantly reduced VIMENTIN, S100A4, EPAS1, EDN1, SLC2A1 and LDHA expression in SCBT group, and reduced VIMENTIN expression only in CCBT group (P < 0.01, Figure 7B-G).

Furthermore, SS-31 treatment also increased activities of respiration complexes I, II, and III, and the level of MMP and ATP production in both PDX model, however, the significance was observed only in the activity of respiration complexes II, and the level of ATP production in SCBT PDX model (P < 0.05, Figure 7H-L).

Discussion

This study supports that attenuation of SDHB represents an inherent element and driver mechanism of carotid body tumors sclerosing. Through comprehensive cellular analyses, we characterized regulatory and functional aspects of the relationship between sclerosis and SDHB, and further found the EndMT program to involve distinct changes in mitochondrial function and tumor sclerosis.

Thorough analyses in CBT patient cohorts revealed a relatively consistent association between sclerosis extent and decreased event-free survival. In another word, higher sclerosis extent is associated with a worse outcome in CBT. Consistently, we also found that SCBT showed significantly increased growth speed than CCBT in NSG mice PDX model. It is reported that EMT and EndMT take an active part in tissue sclerosis, tumor progression, and TAF formation in tumors [22, 23]. Therefore, we hypothesized that enhanced EMT and EndMT may lead to CBT progression and sclerosis. Indeed, through IF, we found significantly increased S100A4 and VIMENTIN expression, and increased S100A4 and VIMENTIN positive ECs in SCBT than in CCBT of human specimen and NSG mice PDX model, which indicates the contribution of EndMT to CBT sclerosing. In addition, these results also imply that increased EndMT might be associated with sclerosis and worse prognoses in CBT.

Thorough IHC analyses, we found significantly decreased SDHB expression in human SCBT than in CCBT. Based on previous reports suggesting that mitochondrial dysfunction and SDHB mutations promote EMT [24-26], we hypothesized that altered enzyme function of SDHB may be a determining factor and possibly an integral part of EndMT in CBT, which could be linked to mitochondrial dysfunction. Upon mitochondrial function analysis also showed decreased ATP, MMP, and complex I, II, III activities in SCBT PDX models compared with CCBT PDX models. Furthermore, EndMT and mitochondrial dysfunction is induced by SDHB KD in HUVECs. Mitochondrial dysfunction underlies the etiology of a broad spectrum of diseases including heart disease, cancer, neurodegenerative diseases, and the general aging process [27]. Carotid body type I cells engage in oxygen sensing, and a loss in mitochondrial function is one of the prime factors affecting carotid body aging processes [28, 29]. It has also been reported that EMT involved inhibition of SDH enzyme activity, as the ability to utilize succinate as respiratory fuel was significantly reduced [11]. Therefore, we hypotheses that mitochondrial dysfunction might also play a central role in this SDHB suppression-mitochondrial dysfunction-EndMT axis, and rescuing the mitochondrial function might be the critical step in SDHB KD-induced EndMT.

In order to verify the hypothesis above, SS-31, a synthetic tetrapeptide that selectively targets mitochondria to restore mitochondrial bioenergetics [30], was employed. It is reported that SS-31 improves mitochondrial function by inte-
Protective effects of SS-31 against CBT sclerosis and progression

In conclusion, EndMT is recognized as a driver mechanism of recurrence, metastasis, and therapy resistance, this study provides mechanistic support for further investigations of mitochondrial features as potential therapeutic targets. The presented findings pointed out that SDHB suppression-mitochondrial dysfunction-EndMT axis is a critical part of the CBT sclerosis and progression, while mitochondria-targeted drug SS-31 exerts an inhibitive effect on the above-mentioned axis, which opens new strategies to prevent and treat malignancies of CBT.

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Disclosure of conflict of interest

None.

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References


Protective effects of SS-31 against CBT sclerosis and progression


Protective effects of SS-31 against CBT sclerosis and progression


Supplementary Table 1. Summary demographic and descriptive data of carotid body tumor patients

<table>
<thead>
<tr>
<th></th>
<th>CCBT (n=185)a</th>
<th>SCBT I (n=37)b</th>
<th>SCBT II (n=16)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>42.75±12.32</td>
<td>42.07±13.28</td>
<td>40.50±14.40</td>
</tr>
<tr>
<td>Male</td>
<td>70 (37.84%)</td>
<td>15 (40.54%)</td>
<td>7 (43.75%)</td>
</tr>
<tr>
<td>Comorbidities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>15 (8.11%)</td>
<td>3 (8.11%)</td>
<td>2 (12.5%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6 (3.24%)</td>
<td>0</td>
<td>2 (12.5%)*</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>6 (3.24%)</td>
<td>0</td>
<td>1 (6.25%)</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>2 (1.08%)</td>
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<td>0</td>
</tr>
<tr>
<td>Smoker</td>
<td>14 (7.57%)</td>
<td>4 (10.81%)</td>
<td>1 (6.25%)</td>
</tr>
<tr>
<td>Shamblin grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>37 (20.00%)</td>
<td>6 (16.22%)</td>
<td>3 (18.75%)</td>
</tr>
<tr>
<td>II</td>
<td>114 (61.62%)</td>
<td>23 (62.16%)</td>
<td>6 (37.5%)</td>
</tr>
<tr>
<td>III</td>
<td>34 (18.38%)</td>
<td>8 (21.62%)</td>
<td>7 (43.75%)*</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>17.09±15.31</td>
<td>21.36±14.69</td>
<td>19.20±11.65</td>
</tr>
</tbody>
</table>

Data are presented as n (%) or mean ± standard deviation (SD). *conventional carotid body tumor; *sclerosing carotid body tumor. *P < 0.05 vs. CCBT; *P < 0.05 vs. SCBT I.