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Functional involvement of ADRA1D in cutaneous melanoma progression and angiogenesis

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ABSTRACT

Cutaneous melanoma is a highly aggressive and malignant skin cancer, and its high recurrence rate and drug resistance increase the difficulty of treating advanced-stage patients. Studies have revealed that treatment via stimulation of alpha-1 adrenergic receptor (ADRA1) subtypes inhibits melanoma growth in mice. However, the associations between alpha-1D adrenergic receptor (ADRA1D) and cutaneous melanoma are poorly understood. In this study, we collected tissue specimens from 16 pairs of patients with pigmented nevus and cutaneous melanoma were analyzed for ADRA1D expression using immunohistochemical staining. Western blotting and RT-qPCR were carried out in order to detect ADRA1D expression levels in melanoma cells and human epidermal melanocytes (HEMs), hypoxia-inducible factor-1α (HIF-1α), and vascular endothelial growth factor (VEGF) levels in HUVECS. A375 cells were transfected with a lentivirus overexpressing ADRA1D. Wound-healing, Transwell, and cell proliferation assays were utilized to identify the ADRA1D effect on the migration, invasion, and proliferation of the two groups of A375 cells in vitro. In order to evaluate the function of ADRA1D in vivo, a melanoma xenograft model was developed in immunodeficient mice. Results showed that ADRA1D was low expressed in cutaneous melanoma tissues. Overexpression of ADRA1D inhibited the tubulation and migration of HUVECs in vitro, inhibited the invasion and proliferation of A375 melanoma cells and reduced its angiogenesis in vivo. It also significantly decreased the HIF-1α and VEGF expression. It is concluded that ADRA1D inhibits cutaneous melanoma growth and angiogenesis. It attenuates melanoma cell proliferation and invasion. Meanwhile, its anti-angiogenic effect is achieved by negatively regulating the HIF-1α/VEGF axis in melanoma tissue, thereby attenuating the growth of cutaneous melanoma and reducing the potential of metastasis.

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Introduction

The most aggressive and malignant form of skin cancer worldwide is cutaneous melanoma. According to the most recent global cancer burden study, cutaneous melanoma is the primary reason for death among skin cancer patients (1). The predicted percentage of cutaneous melanoma mortality in the United States in 2022 (except basal cell and squamous cell carcinomas of the skin) was 57% (2). Skin health has become a subject of concern, which has led to the detection of an increasing number of cutaneous melanomas at an early stage. Under current treatment standards, including surgical resection, radiotherapy, and chemotherapy, most early localized cutaneous melanomas can be cured (3). With the emergence of new therapeutic methods, such as targeted therapy and immunotherapy, additional treatment options have been developed for patients with advanced skin melanoma, and they have greatly improved the prognosis of these patients (4). However, the high recurrence rates and drug resistance of cutaneous melanoma increase the difficulty of treating patients in the advanced stage. Therefore, a better understanding of the molecular mechanisms leading to the evolution of cutaneous melanoma is essential for the development of more effective treatments relative to the currently available therapies.

Previous studies have confirmed that the oxygen diffusion limit equals 100–200 μm, and when cells grow beyond this limit, they must recruit new blood vessels by angiogenesis (5,6). This phenomenon is frequently observed in malignant cases because neoplastic cells must develop the ability to generate angiogenesis in order to meet their rising needs for nutrients and oxygen in order to continue to proliferate. More than 100 years ago, researchers observed angiogenesis around tumors (7). Moreover, Gullino showed that cells in precancerous tissue become capable of angiogenesis during their progression toward cancerous cells (8). Angiogenesis is known to have a vital role in forming and developing all cancer types (9). Studies have also shown that tumor cells can recruit endothelial cells by secreting angiogenic factors and other active substances (10).

Studies on the involvement of adrenergic receptors (ADRs) in melanoma have shown that upregulated levels of circulating catecholamines promote melanoma growth (11,12). Several mechanisms are involved, and excess tumor cell secretion of vascular endothelial growth factor (VEGF) is the most significant (13). ADRs are G protein-

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coupled receptors (GPCRs) on the membrane surface, and they are ubiquitous in human tissues and can be activated by catecholamines to mediate vasoconstriction. Three subclasses of ADRs have been identified: alpha-1, alpha-2, and β. ADRA1s exist as three molecular subtypes (ADRA1A, ADRA1B, and ADRA1D). Alpha-1D adrenergic receptor (ADRA1D) is a key regulator of sympathetic nervous system function, and it is mainly distributed on blood vessels and nerves and mediates vasoconstriction. It also offers opportunities to modulate leukocyte and cancer cell trafficking during disease progression (14). The functions of ADRA1s vary from disease to disease. An observational cohort study suggested that ADRA1 antagonists significantly decrease the incidence of prostate cancer (15). However, another animal experiment showed that stimulating ADR subtypes negatively affects melanoma growth, with ADRA1 stimulation inhibiting melanoma growth by approximately 70% without altering tumor perfusion (16). Studies have revealed that ADRA1D may have a regulatory effect on vascular osmotic function and angiogenesis (17). And the inhibition of PI3K/Akt/HIF-1α/VEGF signal pathway has an anti-tumor effect on B16 melanoma mice (18).

Based on the above studies, we hypothesized that ADRA1D may attenuate melanoma growth and has anti-angiogenic effects in melanoma. And it works by inhibiting HIF-1α/VEGF signal pathway. Thereby, reducing hematogenous metastasis potential in advanced cutaneous melanomas. We studied the ADRA1D expression in cutaneous melanoma tissues and its function in vivo and in vitro in this study. The findings provide preliminary evidence of a new therapeutic target for cutaneous melanoma.

Materials and Methods

Tissue specimens and cell culture

The Department of Pathology of Nanchang University's First Affiliated Hospital provided paraffin tissue specimens of 16 pairs of pigmented nevus and cutaneous melanoma that had not undergone radiotherapy or chemotherapy prior to the procedure. All subjects in the study provided written consent, and all experiments using participant specimens were approved by the Ethics Committee of Nanchang University's First Affiliated Hospital. The human melanoma cell lines A375 and M14 and the human epidermal melanocytes (HEMs) were obtained from Pricella (Wuhan, China). The cell line Sk-mel28 was obtained from Fuxiang Biotechnology (Shanghai, China). Human umbilical vein endothelial cells (HUVEC) were collected from the gastrointestinal surgery team at Nanchang University's First Affiliated Hospital. Solarbio (10000 IU/mL, Beijing, China) provided Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin. Gibco supplied the fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., USA). The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 incubator (Thermo Fisher Scientific Inc., USA) in DMEM containing 100-U/mL penicillin, 10% FBS, and 100-μg/mL streptomycin.

Immunohistochemistry (IHC)

Briefly, the sections of cutaneous melanoma specimens, pigmented nevus specimens, and xenograft tumor samples were baked for 30 min at 80°C, deparaffinized, and finally rehydrated. The sections were incubated with rabbit anti-human ADRA1D antibody (Biogot, Nanjing, China, BS61380, 1:500) or mouse anti-human CD34 antibody (Proteintech, Wuhan, China, KHC0023, 1:500) after heat-mediated antigen retrieval with EDTA buffer (PH 8.0) and sealing, followed by a mouse/rabbit polymer detection system (ZSGD-BIO, Beijing, China, #PV-6000). After immunostaining, two experienced pathologists reviewed and assessed the sections blindly using the immunoreactive score (IRS) system (19). The IRS value was based on the percentage of the staining intensity (scored as 0: negative; 1: weak; 2: moderate; 3: strong) and positive cells (scored as 1: 0–10%; 2: 11–50%; 3: 51–80%; 4: 81–100%). The final scores of IRS (ranging from 0 to 12) were calculated by multiplying the two values. Finally, we classified IHC results of all the sections into three types: ISR 0 was negative, ISR range 1–12 was positive (1–7 were regarded as weakly positive; 8–12 were regarded as strongly positive).

Western blotting

Western blotting was utilized to identify expression levels of ADRA1D protein in melanoma cells (A375, Sk-mel28, M14) and HEMs, hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) levels in HUVECS. Protein lysates from cultured cells were separated employing 10% SDS-PAGE and moved into polyvinylidene difluoride membranes (Millipore, USA). The membranes were incubated overnight at 4°C with primary antibodies after blocking for 2 h with 5% skim milk. The primary antibody dilution ratios were: ADRA1D (dilution, 1:800), VEGF, HIF-1α, and β-actin (dilution, 1:1000). Blots were washed with PBS and incubated with secondary antibodies (dilution, 1:1000) at room temperature for 1 h. Membrane visualization was done by enhanced chemiluminescence (Millipore, USA), and then they were photographed by G-BOX (Gene Company Ltd., Beijing, China). ImageJ V1.5 was used to analyze the results.

Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA TRIzol reagent (TransGen Biotech, Beijing, China) was used to extract the RNA of melanoma cells (A375, Sk-mel28, M14) and HEMs. Reverse transcription was carried out following the manufacturer's instructions; the cDNA products were then utilized as templates for subsequent PCR amplification. The forward and reverse primers for *ADRA1D* were 5′-CTCCAGCCTGTCGCACAAG-3′ and 5′- TGTAGTCGGCCAATTCGTAGG-3′, and the forward and reverse primers for *GAPDH* were 5′-AGATCCCTCCAAAATCAAGTGG-3′ and 5′- GGCAGAGATGATGACCCTTTT-3′. The forward and reverse primers for *VEGF* were 5′-TGCTGTCTTGGGTGCATTGG-3′ and 5′-AGGTCTCGATTGGATGGCAG -3′. The forward and reverse primers for *HIF*-1α and 5′-AGTGTACCCTAACTAGCCG-3′ and 5′- CACAAATCAGCACCAAGC-3′ (Sangon, Shanghai, China), respectively. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression levels.

Lentivirus infection

For further research, the ADRA1D effect on the progression of melanoma in vitro and vivo, we overexpressed ADRA1D in A375 cells, which were the most studied in melanoma cells. Lentivirus vectors overexpressing

ADRA1D and the negative control were obtained from Genechem (Shanghai, China). The recombinant lentiviral vector contained the target genes ADRA1D, green fluorescent protein (GFP), and anti-purinomycin. A375 cells were infected with the two lentiviral vectors. Fluorescence efficiency was observed through fluorescence microscopy after 3 days. The effect of the infection was further validated using RT-qPCR and western blotting.

Conditioned medium and endothelial tube formation assay

In a special medium (DMEM+10%FBS+1%P/S, Pricell, Wuhan, China, CM-0014), the two groups of A375 cells were cultured overnight at a density of 1×10^6 cells per well in 6-well plates, and the medium was then changed to a fresh one in each well. After 24 h, the conditioned medium was collected and utilized to culture HUVECs. HUVECs were positioned in 96-well plates coated with 50 μL Matrigel (200 μg/mL, Corning, New York, USA, 356234) at a density of 1×10^4 cells per well; then, they were positioned in an incubator for 4 to 8 h at 37°C with 5% CO2. The tubule formation was observed and photographed using a microscope and then assessed utilizing Image-Pro Plus software.

Cell proliferation assay

A CCK-8 kit (Cell Counting Kit-8, GlpBio, USA, GK10001) was utilized to identify the ADRA1D effect on the proliferation of A375 melanoma cells. Two groups of A375 cells in the logarithmic growth phase were harvested and seeded at a concentration of 8000 cells per well in 96-well plates, cultured in 10% FBS-DMEM medium, and put in a 5% CO2 incubator at 37°C. 10 μL of CCK-8 reagent was then added 2 h prior to every detection time point (2, 24, 28, and 72 h) and incubated for 2 h in the dark. The optical density (OD) was calculated at a wavelength of 450 nm. A higher OD value indicates a stronger cell proliferation ability.

Wound healing assay

The vector group and ADRA1D overexpression (OE) group cells in the logarithmic growth phase (A375 and HUVEC) were seeded at a density of 1×10^6 cells per well in 6-well plates and cultured in DMEM containing 10% FBS at 37°C incubator overnight. A scratch was made in the center of each dish while the cells were grown to 90–100% confluence. Once wounded with a pipette tip, the cells were washed smoothly with phosphate-buffered saline to take out loose cells and cultured in DMEM comprising 10% FBS. For 48 hours, the cells were permitted to migrate. Wound widths were measured at scheduled time points (0, 12, 24, & 48 h). Using an inverted microscope, images of cells migrating into the wounded area were recorded at 0 and 48 hours (IX71, Olympus, Tokyo, Japan), and the wound area was analyzed using ImageJ V1.5.

Transwell invasion assay

First, the upper chambers (8 μm pore size, Corning, New York, USA) with 100 μL diluted Matrigel (200 μg/mL, Corning, New York, USA, 356234) were placed for 30 min in an incubator. The vector group and ADRA1D OE group A375 cells in the logarithmic growth phase were harvested; then, in the upper chamber, they were seeded in 200 μL serum-free medium at a density of 4×10^4 cells per well. The lower chambers were filled with 200 μL DMEM comprising 10% FBS. The cells were kept in the incubator at 37°C. After 16 hours of incubation for migration experiments or 24 hours for invasion assays, the cells were treated with 4% paraformaldehyde for 15 minutes and stained for 15 minutes with 1% crystal violet staining solution (Beyotime, Shanghai, China, #C0121) for 15 minutes. Finally, employing an inverted microscope, cells on the lower chamber membrane were photographed and calculated.

Establishment of the tumor-bearing nude mouse model

Vital River Laboratories in China provided BALB/c male nude mice (4 weeks old, 13 g). All animals were maintained at the Animal Experiment Center at Nanchang University's First Affiliated Hospital. All experiments (including euthanasia) were carried out in accordance with the guidelines and procedures of Nanchang University's First Affiliated Hospital Institutional Animal Care, as well as the AAALAC and IACUC procedures. The Ethics Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China) approved the study (no. 2018MEC106).

The logarithmic growth phase A375 cells of the two groups were harvested and resuspended then in saline at a density of 1×10^7/mL; then, they subcutaneously were inoculated into nude mice's left forelimb at a volume of 200 μL/mouse. 20 male nude mice were grouped into two randomly (n = 10), the negative control group inoculated with A375 cells, and the transfection group inoculated with A375 ADRA1D OE cells. The experiment continued for 4 weeks. The animals' diet, defecation, and conditions were monitored every day. When the tumor volume reached 1000 mm3, the experiment was terminated, and the nude mice were euthanized; the tumors were then taken out for immunohistochemistry.

Statistical analysis

SPSS 20.0 software was used for statistical analysis. Data are expressed as the mean ± SD. Multi-group comparison of means was carried out using ANOVA, LSD-*t*-test was used to assess pairwise comparison, and a *t*-test was used to assess intergroup comparisons of means. The statistical significance level was $P < 0.05$.

Results

ADRA1D expression was decreased in melanoma tissues

To clarify the expression of ADRA1D in cutaneous melanoma, the staining of 16 melanoma tissues and 16 pigmented nevus specimens was analyzed using immunohistochemical techniques. For melanoma, the positivity rate was 25% (4/16), and for pigmented nevus, the positivity rate was 87.5% (14/16) ($P < 0.001$) (Figure 1A). The staining intensity of melanoma was considerably lower compared to that of the pigmented nevus (Figure 1B). In order to clarify these data at the protein and mRNA level, we assessed the expression of ADRA1D in different melanoma cell lines (A375, Sk-mel28, and M14) and HEMs employing RT-qPCR and western blotting analysis. The RT-qPCR analysis showed that ADRA1D mRNA was lower in melanoma cells ($P < 0.05$) (Figure 1C). And western blotting analysis revealed that ADRA1D protein

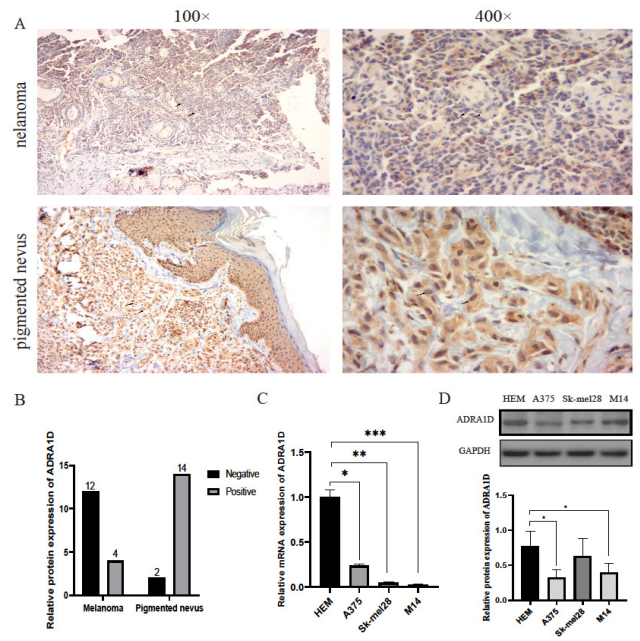

Figure 1. ADRA1D expression was decreased in malignant melanoma (MM) tissues. (A) ADRA1D immunohistochemical staining in MM tissue and pigmented nevus tissue. (B) Differential distribution of staining intensity of ADRA1D in MM tissue and pigmented nevus tissue. Expression of ADRA1D at the cellular level: (C) mRNA expression difference of ADRA1D in HEM and melanoma cells (A375, Sk-mel28, and M14); (D) difference of ADRA1D protein expression between HEM and the melanoma cell groups (*$P < 0.05$, **$P < 0.01$).

was especially lower in A375 melanoma cells ($P < 0.05$) (Figure 1D). Taken together, these data indicated that the ADRA1D expression level in the three types of melanoma cells was significantly lower than in HEMs.

Lentivirus overexpression and verification

A375 cells are the most common cells studied in melanoma, to further investigate the function of ADRA1D in cutaneous melanoma, ADRA1D was overexpressed in A375 cells using lentivirus transfection technology. After transfection, green fluorescence was observed in both groups of cells under a fluorescence microscope. The results suggested that fluorescent proteins were expressed in more than 80% of the cells (Figure 2A). Western blotting and RT-qPCR further verified that the ADRA1D level in the overexpression group was considerably higher compared to that in the vector group (*$P < 0.05$, ***$P < 0.001$) (Figure 2B, C). Thus, transfection was successful.

Overexpression of ADRA1D inhibits the tubulation and migration of HUVEC in vitro

In order to study the ADRA1D effect on angiogenesis, A375 cells were transfected with lentivirus to increase the levels of ADRA1D. The ADRA1D OE and vector groups were compared and cocultured with HUVEC cells. According to the results, the number of vascular rings in the ADRA1D OE group was considerably lower compared to that in the vector group ($P < 0.01$, Figure 3A), indicating that ADRA1D overexpression inhibits HUVEC growth in vitro. The protein extracted from cocultured endothelial cells was used for western blotting. Based on the results, overexpression of ADRA1D significantly decreased the HIF-1α and VEGF expression ($P < 0.05$, Figure 3B, C). In the scratch wound assay of cocultured endothelial cells, there were no significant differences in the migration area of endothelial cells between the two groups ($P > 0.05$, Figure 3D)

Overexpression of ADRA1D inhibits the proliferation and invasion of melanoma cells in vitro

A CCK-8 proliferation assay was employed in order to analyze the ADRA1D effect on A375 cell proliferation. The results showed that the OD value of the ADRA1D OE group was lower than that of the vector group after 24 h, and the gap increased significantly with time, suggesting that overexpression of ADRA1D inhibited A375 cell proliferation (Figure 4A). The ADRA1D effects on the A375 cell's migration and invasion were analyzed using the wound healing and Transwell invasion assays, respec-

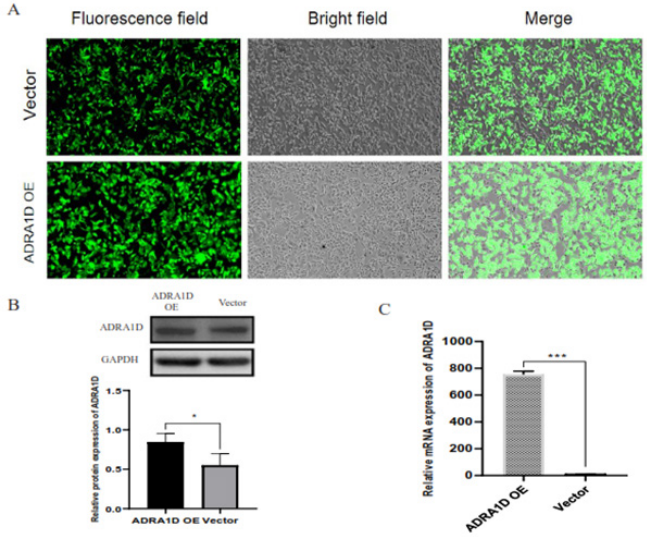

Figure 2. Overexpression and verification. (A) Green fluorescence was observed under the fluorescence microscope. (B) The protein expression level of ADRA1D in the ADRA1D OE group was significantly higher than that in the vector group (*$P < 0.05$). (C) the mRNA expression level of ADRA1D in the ADRA1D OE group was significantly higher than that in the vector group (***$P < 0.001$).

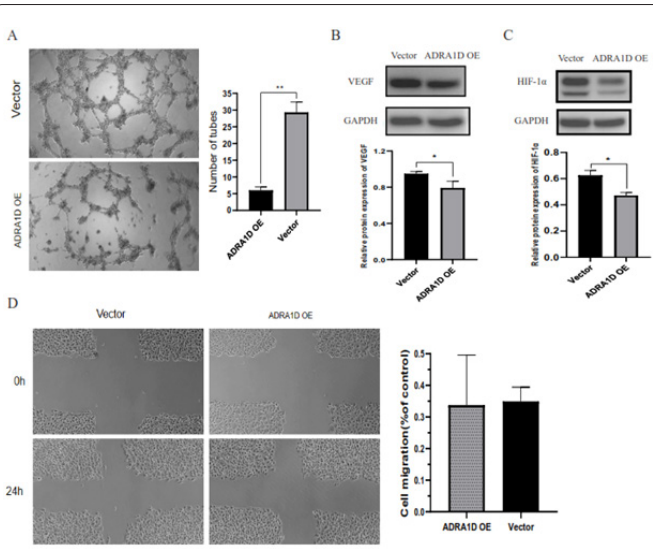

Figure 3. Overexpression of ADRA1D inhibits the tubulation and migration of HUVEC in vitro. (A) Overexpression of ADRA1D decreased the tube formation of endothelial cells in vitro (**$P < 0.01$). (B, C) Overexpression of ADRA1D decreased VEGF and HIF-1α expression in endothelial cells (*$P < 0.05$). (D) Overexpression of ADRA1D could not affect endothelial cell migration.

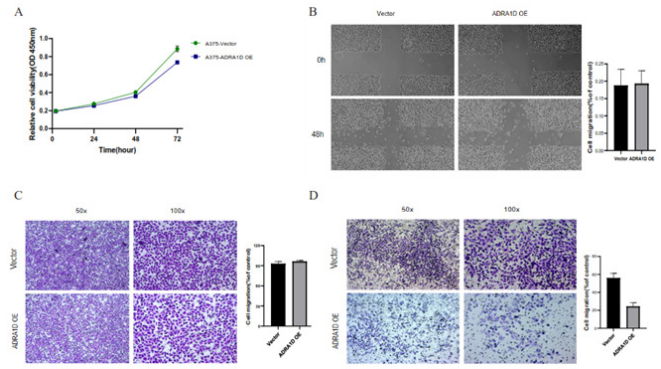

Figure 4. Effects of overexpression of ADRA1D on proliferation, migration and invasion of melanoma cells in vitro. (A) Overexpression of ADRA1D inhibited A375 cells proliferation (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$). (B, C) Overexpression of ADRA1D had no effect on A375 cell migration. (D) Overexpression of ADRA1D inhibited A375 cells invasion (**$P < 0.01$).

tively. Transwell invasion assay showed that the number of cells passing through the chamber in the ADRA1D OE group was lower compared to that in the vector group ($P < 0.05$), with a statistically significant difference. The results of the wound healing assay showed no significant difference in the scratch area of A375 cells between the groups (Figure 4B, C). This suggests that ADRA1D overexpression inhibits the invasion of A375 cells in vitro but does not have a significant inhibitory effect on migration.

Overexpression of ADRA1D reduces angiogenesis *in vivo*

A375 cells from the OE and vector groups were injected subcutaneously into BALB/C nude mice to validate the role of ADRA1D in regulating angiogenesis in vivo. The tumor volumes and weights of the two groups were compared, and the tumors were removed for CD34 immunohistochemical staining. The results revealed that the tumor volume and weight were considerably lower in the ADRA1D OE group compared to the vector group (Figure 5A, B, C); the number of CD34 positive vessels was also lower in the OE group compared to the vector group (Figure 5D), suggesting that ADRA1D overexpression inhibited angiogenesis *in vivo*. This was consistent with the results obtained *in vitro*.

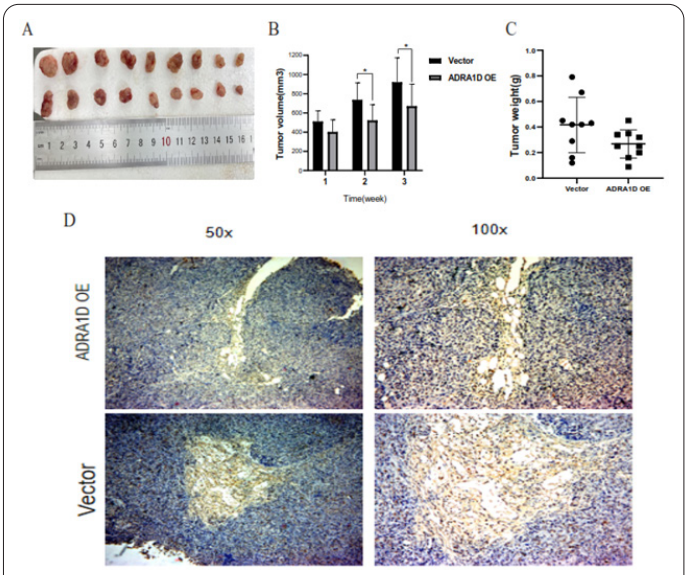

Figure 5. Overexpression of ADRA1D reduces angiogenesis in vivo. (A) Schematic diagram of a subcutaneous tumor in nude mice in the two groups. (B, C) Overexpression of ADRA1D decreased the volume and weight of subcutaneous tumors in nude mice (*$P < 0.05$). (D) Overexpression of ADRA1D decreased the microvessel density in tumors in nude mice.

Discussion

Cutaneous melanoma is the most fatal skin cancer; its 5-year survival rate is related to its clinical stage. Owing to the high recurrence rate and drug resistance, the treatment of patients with advanced metastasis remains a challenge; thus, new molecular targets must be identified for the treatment of advanced patients. Survival analysis demonstrated that low expression levels of ADRA1s (ADRA1A, ADRA1B, and ADRA1D) were associated with favorable overall survival (OS) in patients with gastric carcinomas, thus revealing that ADRA1D promotes gastric carcinoma growth (20). However, the association between ADRA1D expression and the prognosis of cutaneous melanoma is poorly understood. The study has shown that ADRA1D can regulate the inflammatory response in the neurotransmission of vascular smooth muscle cells by interacting with other genes (21). Other studies have confirmed that ADRA1D can improve high-altitude pulmonary edema by regulating vascular homeostasis (17), suggesting that ADRA1D may have a regulatory effect on vascular osmotic function and angiogenesis. Therefore, this study aimed to determine the role of ADRA1D in melanoma growth and its impacts on angiogenesis.

The degree of angiogenesis is associated with tumor aggressiveness and clinical outcomes, and findings have shown tumor angiogenesis to be closely associated with the hematogenous metastasis development in human gastric carcinomas and the OS of patients with esophageal squamous cell carcinoma (22,23). Warren BA discovered that angiogenesis has a vital role in melanoma development in 1966 (24,25). In most human tumors, VEGF mRNA is overexpressed and correlates with invasiveness, metastasis, recurrence, vascular density, and prognosis (26). For example, in primary esophageal squamous cell carcinoma, angiogenesis is mediated mainly by VEGF (27), which has a key role in tumor progression and increases malignancy via angiogenesis. Many studies have explored the role of VEGF in tumors, such as gastric carcinoma, ovarian cancer, papillary thyroid carcinoma, breast cancer, non-small cell lung cancer, and prostate cancer, and the results have shown that VEGF promotes tumor growth (28–33). And studies found that inhibition of PI3K/Akt/HIF-1α/VEGF signal pathway has anti-tumor effect on B16 melanoma mice (18). Therefore, while exploring the anti-angiogenic effect of ADRA1D, we hypothesized that it may work through the PI3K/Akt/HIF-1α/VEGF signal pathway.

First, we analyzed the difference in the expression of ADRA1D between 16 cutaneous melanoma and 16 pigmented nevus specimens using immunohistochemistry. Notably, the ADRA1D expression levels were higher in pigmented nevus compared to in cutaneous melanoma tissues in the current study, and we found that the positive rates of ADRA1D in cutaneous melanoma and pigmented nevus were 25% and 87.5%, respectively. In addition to the lower expression of ADRA1D in cutaneous melanoma, our study demonstrated that ADRA1D could downregulate the VEGF and hypoxia-inducible factor-1α

(HIF-1α) expression, which may suppress angiogenesis in melanoma. This result is different from that of a previous study on ADRA1 subtype expression in primary gastric tumors, which showed no marked differences in the ADRA1D expression levels compared to normal gastric tissues and revealed that ADRA1A could promote cancer metastasis (20,34). Findings suggest that ADRA1 subtypes play different roles in different tumors. In melanoma, the expression of ADRA1D is significantly low in tumor tissue, which suggests that it has an inhibitory impact on melanoma progression, which has not been reported in other tumors.

To verify our hypothesis, we cocultured A375 cells that overexpressed ADRA1D with HUVEC cells and found a significant decrease in HUVEC cell growth and the number of microvessels as compared to that in the control group. This revealed that ADRA1D could inhibit angiogenesis in vitro. Furthermore, studies have shown that under hypoxic conditions, HIF-1α enables tumor cells to better adapt to the hypoxic environment, which facilitates tumor progression (35,36). we detected lower expression of VEGF and HIF-1α by RT-qPCR and western blotting, regardless of the mRNA or protein level. These findings suggest that ADRA1D may attenuate melanoma angiogenesis by inhibiting the VEGF/HIF-1α signal pathway, thereby reducing the potential of hematogenous metastasis in advanced cutaneous melanoma.

CD34 immunohistochemical staining in normal tissues and tumors is usually used as a marker of angiogenesis. To further verify the inhibitory effect of ADRA1D on angiogenesis in melanoma in vivo, a melanoma xenograft model was established in immunodeficient mice and found that the overexpression of ADRA1D considerably inhibited tumor growth in animals as compared to the control group by measuring the tumor volume and growth. Next, the CD34 expression in tumor tissue was assessed by immunohistochemical staining and found that the ADRA1D overexpressing group had a higher microvessel density than the vector group, suggesting that ADRA1D can inhibit the angiogenesis of melanoma in vivo. In addition, according to the Transwell invasion, wound healing, and cell proliferation assays, ADRA1D inhibited the invasion and proliferation of A375 cells in vitro but had no significant effect on migration.

Overall, these results indicate that ADRA1D negatively affects melanoma proliferation and invasion, and the anti-angiogenic effect is achieved through the HIF-1α/VEGF signal pathway inhibition in melanoma tissue, thereby attenuating its growth and reducing the potential of metastasis. Therefore, the development of targeted drugs for the selective activation of ADRA1D may be of great significance in preventing the growth and metastasis of melanoma.

In conclusion, ADRA1D has a key role in melanoma progression by inhibiting the proliferation and invasion of melanoma cells and negatively regulating the HIF-1α/VEGF axis, which inhibits angiogenesis. Therefore, upregulation of ADRA1D can inhibit melanoma growth and reduce microvessel density in vivo. To the best of the authors' knowledge, the present research is the first to disclose a relationship between ADRA1D protein expression and cutaneous melanoma progression. In the future, the specific mechanism of action must be further investigated to develop new targets for antitumor therapy.

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Interest conflict

The authors declare that they have no conflict of interest.

Author's contribution

JW, DN, CW : have made the proposal and research design, data collection; DX, XCh,: did the data analysis and drafted the paper; XC, CW: revising and rewriting the manuscript. All the authors have approved the final version of the manuscript.

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