HIF-1 α and HIF-2 α Mediated PARVB Expression Promotes Tumor Growth and Metastasis in Malignant melanoma

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Research

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Abstract

Background

Malignant melanoma is the leading cause of skin cancer-related death. The role of PARVB in malignant melanoma remains unclear. Hypoxia is a hallmark of solid tumors including melanoma. But the regulation role of hypoxia in PARVB expression has not been reported.

Methods

Human malignant melanoma tissues, cell lines and their controls were collected. IHC staining, qRT-PCR and Western blot were performed to reveal the differential PARVB expression. The role of PARVB in tumor growth and metastasis of malignant melanoma was evaluated in vitro and in vivo. The regulation role and mechanism of hypoxia and HIFs in PARVB expression was validated by qRT-PCR, Western blot, ChIP-PCR and Luciferase reporter assays.

Results

PARVB was upregulated in malignant melanoma and correlated with patient survival. Overexpression of PARVB promoted tumor growth and metastasis of malignant melanoma. Furthermore, hypoxia induced HIF-1 α and HIF-2 α expression activated PARVB transcription and expression through binding to the specific hypoxia-responsive element (HRE) in the promoter region of PARVB.

Conclusions

In malignant melanoma, Hypoxia induced HIF-1 α and HIF-2 α expression could directly activate PARVB expression, which further promoted tumor growth and metastasis, inducing poor prognosis. These results indicated that PARVB might be a potential therapeutic target for malignant melanoma.

Background

Malignant melanoma is the leading cause of skin cancer-related death, the incidence of which is on the rise over the past 20 years [1]. The prognosis of melanoma remains poor due to the aggressive clinical behavior, metastatic potential and high resistance to chemotherapy or radiotherapy [2]. The long-term survival of patients with advanced and metastatic melanoma is low [3], and therefore it is urgent to decipher the molecular mechanisms underlying the progression and metastasis of melanoma.

The low-oxygen condition or hypoxia was found to have significant impact on the proliferation, differentiation and survival of tumor cells [4]. Hypoxic microenvironments existing in many pathological conditions including atherosclerosis, stroke and solid tumors were shown to enhance the invasiveness and metastatic capacity of tumor cells [5]. Hypoxia-inducible factor (HIF) is a master regulator of the cellular and physiological response to hypoxia [6]. It is a heterodimeric transcription factor composed of an α and a β subunit [7]. HIF activation can transcriptionally regulate genes involved in epithelial-to-

mesenchymal transition (EMT) [8]. Breakdown of the cell-extracellular matrix (ECM) and metastases thus plays a pivotal role in these processes [9]. The role of HIF-1 α and HIF-2 α in promoting tumorigenesis of melanoma has also been reported. Both of them were shown to be overexpressed in melanoma [10]. Elevated HIF-1 α expression was found to be correlated with melanoma drug resistance and poor prognosis [11]. Besides, studies also demonstrated that HIF-1 α played a crucial role in regulating the proliferative-to-invasive cell change, leading to regional and distal disease progression in vivo [12]. However, more investigations are required to further elucidate the downstream molecular mechanism of HIF-1 α and HIF-2 α in regulating the progression and metastases of malignant melanoma.

ECM adhesion plays a crucial role in various biological processes such as cell proliferation, migration and invasion [13]. PARVB was also reported to participate in facilitating cell-ECM adhesion as a key component and regulating cell survival and morphology [14, 15]. Increasing evidence suggests that PARVB may be a potential biomarker for cancer and involved in the regulation of several types of cancers. For example, PARVB was found to be differentially expressed in ovarian serous carcinoma, and its overexpression increased the cell migration capability and metastasis in tongue squamous cell carcinoma [15]. Besides, it could be a potential prognostic factor for patients with urothelial cell carcinoma of the upper urinary tract [16]. However, the molecular mechanisms underlying its regulatory role are only now beginning to emerge. So far, no study has reported its role in melanoma. In addition, whether hypoxic conditions have a significant impact on the expression of PARVB remains unclear.

Here, PARVB was identified as an oncogene, up-regulated in human malignant melanoma, and promoted the proliferation and metastasis of melanoma cells. Both human and mouse melanoma models were constructed to confirm the role of PARVB in malignant melanoma growth. Hypoxia trans-activated hypoxia-responsive elements (HREs) in the promoter region of PARVB through HIF-1 α and HIF-2 α . Our findings suggest that transcriptional regulation of PARVB could be the downstream mechanism of HIF-1 α and HIF-2 α on mediating cell growth and invasion and may prove to be a potential therapeutic target for malignant melanoma.

Materials And Methods

Patient tissues

This study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center, and written informed consent was obtained from all patients. Human normal skin tissue (n = 39), nevus (n = 32), basal cell carcinoma (n = 32) and malignant melanoma tissues (n = 213) were collected during surgical resection of malignant melanoma at Fudan University Shanghai Cancer Center between 2014 and 2017. Seven of the normal skin tissues were obtained from malignant melanoma patients. The specimens were immediately frozen in liquid nitrogen after surgery. Patient features were seen in Supplementary Table 1.

Immunohistochemistry (IHC) assay

The tissue sections were stained with hematoxylineosin and by IHC using indirect immunoperoxidase technique. The antibody of human PARVB (sc-374581) was purchased from Santa Cruz (USA). The IHC stains were assessed independently by three observers who had no knowledge about the specimen characteristics.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted with Trizol reagent (Invitrogen, USA). Then, first-strand cDNA was generated by Prime Script™ RT Master Mix (Takara, Japan). Gene transcripts were quantitated on 7900HT Fast Real-Time PCR System (Life Technologies Corporation, USA) using SYBR® Premix Ex Taq™ II (Takara, Japan) and normalized with GAPDH. The primers were listed in Supplementary Table 2.

Western Blot

Total protein was resolved on SDS-PAGE gel. Western blot assays for human and mouse PARVB were performed using the antibodies described in IHC staining (sc-374581). Human and mouse β -actin proteins were determined using the specific antibody (sc-8432, Santa) as a loading control.

Cell lines

Human melanoma cell line A375 and mouse melanoma cell line B16 were purchased from the Cell Bank of the Type Culture Collection Committee of the Chinese Academy of Science (Shanghai, China). Human melanoma cell line SK-MEL-28 were purchased from the American Type Culture Collection (ATCC, USA). A375 was cultured in DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen). SK-MEL-28 and B16 were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen). All cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂.

Plasmids construction and cell transfection

The code sequence of human HIF-1 α , HIF-2 α and PARVB were synthesized (Genewiz Co., Suzhou, china) and inserted into pcDNA3.1 vector (Invitrogen, USA). The shRNA plasmids of PARVB and control plasmids were purchased from Genomeditech Co. (Shanghai, China). GV248 was used as the plasmid vector. Sh-PARVB-2 plasmid and the control plasmid were packaged into lentivirus (Genomeditech Co.).

The possible promoter region (-1706bp ~ 264 bp from transcription initiation site) of PARVB containing the potential HRE sites (5'-G/ACGTG-3') were synthesized (Genewiz Co.) and inserted into pGL3-reporter Luciferase Reporter Vector (Promega, USA). The fragments containing partial potential HRE sites (b: -1109bp ~ 264 bp; c: -593bp ~ 264 bp; d: -116bp ~ 264 bp; e: 48bp ~ 264 bp from transcription initiation site) were obtained through PCR methods (primers seen in Supplementary Table 2) and cloned into pGL3-reporter Luciferase Reporter Vector using a Quick-Fusion cloning kit (Bimake, USA) according to the manufacturer's protocol. VEGF promoter (-1080bp~-874 bp from transcription initiation site) luciferase reporter vector was obtained as reported previously [17]. The mutant HRE site 7 and 8 promoter plasmids were constructed based on PARVB promoter plasmid (a: -1706bp ~ 264 bp) using a MutanBEST Kit (Takara) following the manufacturer's instructions.

Plasmids were transiently transfected into cells with DNA Transfection Reagent (Bimake) when they grew to 50 ~ 70% confluence according to the manufacturer's instructions.

Cell Counting Kit 8 (CCK8) assay

Cells were seeded in 96-well plates at a density of 5×10^3 and cultured for 72 h and analyzed with the Cell Counting Kit 8 (Bimake, USA). Results were measured by absorbance at 450 nm using an ELx800 microplate reader (BioTek Instruments Inc., USA).

Cell cycle assay

Cycle distribution was analyzed by flow cytometry. Briefly, Cells were collected and fixed with ice-cold 70% ethanol, washed with PBS, and resuspended in 0.5 mL PBS containing propidium iodide and RNase A. After final incubation at 37 °C for 30 min, cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, USA). Data were analyzed using FlowJo software (Tree Star, USA).

PARVB knockout B16 cell line construction

CRISPR-Cas9 target sites were designed (Supplementary Table 2), synthesized (Genewiz Co.) and cloned into pCas9/gRNA3 plasmid (Inovogen Tech. Co., Beijing, China) using a Quick-Fusion cloning kit (Bimake). B16 cells were transfected with the constructed plasmids and selected with Ampicillin. Selected cells were cloned using limiting dilution and the targeted sequence was confirmed by DNA sequencing.

Xenograft mouse models

Eight-week-old female BALB/c nude mice were purchased from JRDun Biotechnology (Shanghai, China), and were maintained under specific pathogen-free, 12-h light–dark cycle (room temperature 22.5 ± 0.2 °C, humidity 40–60%) conditions and the animals had free access to feedstuff and water. Mice were euthanized by injecting excessive dose of Pentobarbital Sodium at the end of the experiments to ameliorate the suffering of mice. Animal care and experiments were performed in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Ethics Committee of Fudan University Shanghai Cancer Center.

For human melanoma animal models, fourteen mice were equally randomized into a normal control group and a shRNA group. After transfection with control lentivirus or sh-PARVB-2 lentivirus. A375 cells were injected subcutaneously to the right upper flank of the nude mice. Tumor volumes were counted every 4 days in accordance with the equation: $0.5 \times \text{length} \times \text{width}^2$. 28 days after tumor cell injection, all mice were dislocated to death and the excised tumor tissues were weighed. Ki-67 staining were performed to detect tumor cell proliferation.

For mouse melanoma animal models, ten mice were equally randomized into a normal control group and a knockout group. Wild type or PARVB knockout B16 cells were injected subcutaneously to the right upper flank of the nude mice. Tumor volumes were counted every 5 days. 30 days after tumor cell injection, all mice were dislocated to death and the excised tumor tissues were weighed.

Wound healing assay

Cells were cultured in 12-well plates, transfected as indicated, and cultured to confluency. Cells were scraped with a 200 µl pipette tip (time 0 h), washed twice with PBS, and cultured in serum-free medium. Cell migration progress was photographed at 0 h, 24 h and 48 h following injury.

Transwell assay

The invasion capability of melanoma cells was measured 24-well-cell permeable supports 8 µm pore size transwell chamber with matrigel (354480, Corning, USA). 1×10^5 cells in serum-free medium were seeded in the upper chamber, and 500 µl medium supplemented with 10% FBS was added to cover the bottom chamber as chemoattractant. After 24-h incubation, cotton swabs were used to gently remove cells remaining on the upper membrane. Cells on the bottom surface were then fixed with 4% paraformaldehyde and stained with 1% crystal violet for 15 min. Cell invasion was assessed by counting stained cells under a microscope on five random fields.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed by using a ChIP assay kit (Millipore, USA) following the manufacturer's instructions. Briefly, cells cultured under the hypoxic or normoxic conditions were subjected to ChIP assays with anti-HIF-1α or anti-HIF-2α antibody. Normal IgG was used as a control for nonspecific binding of genomic DNA. DNA fragments pulled down by the antibodies were then recovered and subjected to qRT-PCR with PCR primer sets (Supplementary Table 2).

Luciferase reporter assay

HIF-1α or HIF-2α overexpression vector and the constructed luciferase reporter plasmids were co-transfected into A375 cells by DNA Transfection Reagent (Bimake). The luciferase activity was measured by using the Dual Luciferase assay kit (Promega) and normalized with the internal standard.

Statistical analysis

Statistical analysis was conducted by SPSS 21.0 software (IBM, USA). Data are presented as Mean ± Standard Deviation (SD). Chi-square test or Fisher's exact test (two-tailed) was used for the comparison of count data. Independent Student t-test (two-tailed) was used for the comparison of measurement data. The Kaplan-Meier method was used to establish survival curves, and log-rank test was applied for comparative analysis of differences in patient survival. Factors measuring $p \leq 0.1$ from log-rank tests were subjected to the cox proportional hazard analysis and calculation of the hazard ratio and 95% confidence interval. $P \leq 0.05$ was considered statistically significant. All experiments were repeated at least three times.

Results

PARVB is elevated in malignant melanoma and correlated with tumor progression

The expression of PARVB in human normal skin tissue (n = 39), nevus (n = 32), basal cell carcinoma (n = 32) and malignant melanoma tissues (n = 213) was detected by IHC staining (Fig. 1a). And the mRNA level of PARVB was examined in thirty-two of each normal skin, nevus, basal cell carcinoma and malignant melanoma tissues by using qRT-PCR (Fig. 1b). The results showed that PARVB was up-regulated in the malignant melanoma tissues compared with that in other groups. In addition, the level of PARVB protein was confirmed to be upregulated in malignant melanoma compared to that in paired normal skin tissues in Western blot assay (Fig. 1c). qRT-PCR and Western blot assays also showed that PARVB was overexpressed in malignant melanoma cell lines (A375, SK-MEL-2, M14, SK-MEL-28 and SK-MEL-5) compared to human epidermal melanocytes HEMa-LP (Fig. 1D). Besides, the statistical results showed that the expression level of PARVB was correlated with the appearance of ulceration, T grade and AJCC grade (Table 1). Upregulation of PARVB predicted significantly low survival rates in malignant melanoma patients (Fig. 1E). Multivariate COX analysis of clinical factors additionally identified PARVB expression as an independent prognostic factor (Supplementary Table 3). Meanwhile, data obtained from the GEO database shows that the expression of PARVB in malignant melanoma is higher than normal skin and benign nevi (Supplementary Fig. 1a, GSE 3189). and further upregulated in melanoma metastasis compared to that in primary melanoma (Supplementary Fig. 1b, GSE8401). Furthermore, the prognostic data of melanoma in TCGA were also analyzed, confirming that PARVB overexpression was correlated with poor overall survival and progression-free survival of melanoma patients (Supplementary Fig. 1c, d). .

Table 1
Correlation of PARVB expression and patients' clinical features

Characteristics	PARVB expression (Number of patients)			<i>p</i> value
	Low	High	Very high	
Age				0.138
<60 years	23	64	30	
≥60 years	28	52	16	
Sex				0.938
Male	27	63	26	
Female	24	53	20	
Site				0.184
Limbs	23	54	22	
Trunk	22	59	20	
Head or neck	6	3	4	
Ulceration				0.015
Absent	45	86	29	
Present	6	30	17	
T grade				0.001
T1-T2	39	80	20	
T3-T4	12	36	26	
N grade				0.214
N0	23	58	16	
N1-3	28	58	30	
M grade				0.067
M0	45	94	32	
M1	6	22	14	
AJCC grade				0.047
I-II	22	55	16	
III-IV	29	61	30	

PARVB regulates melanoma cell proliferation and tumor growth

Then, the impact of PARVB knockdown on melanoma tumor growth was investigated by using two different PARVB shRNAs (sh-PARVB - 1, -2). As shown in Fig. 2A, sh-PARVB constructs effectively abolished the PARVB expression in SK-MEL-28 and A375 cells. PARVB knockdown decreased the proliferation of the two melanoma cell lines concurrently (Fig. 2b). Flow cytometry assay showed that PARVB knockdown triggered cell cycle arrest in G0/G1 in SK-MEL-28 and A375 cells (Fig. 2c). In consistent with these results, PARVB overexpression was found to promote the proliferation and cell cycle in SK-MEL-28 and A375 cells (Supplementary Fig. 2).

To determine whether PARVB knockdown could affect tumorigenesis in vivo, A375 cells transduced with sh-PARVB-2 lentivirus or control lentivirus were transplanted into nude mice. It was found that tumor growth in the PARVB knockdown group was significantly slower than that in the control group. After 28 days, both tumor volume and weight were decreased markedly in the PARVB knockdown group compared with the control group (Fig. 2d). In addition, the expression of Ki-67 was significantly reduced after PARVB knockdown compared with the control group, indicated that inhibition of PARVB suppressed the proliferation ability of melanoma cells in vivo (Fig. 2e).

To further confirm the impact of PARVB on melanoma growth, we constructed PARVB knockout mouse melanoma cell line B16 by using CRISPR-Cas9 System (Supplementary Fig. 3a). Western blot assay confirmed the knockout of PARVB in B16 cells (Supplementary Fig. 3b). Similar with the role of PARVB in human melanoma cells, knockout of PARVB in B16 cells obviously inhibited the cell proliferation in vitro (Supplementary Fig. 3c) and significantly suppressed tumor growth in vivo (Supplementary Fig. 3d, e). All these results suggested that PARVB could regulate melanoma cell proliferation and tumor growth both in vitro and in vivo.

PARVB regulates melanoma cell migration and invasion

We further explored the impact of PARVB on melanoma cell migration and invasion, due to that PARVB was overexpressed in melanoma metastasis compared with in primary melanoma (Supplementary Fig. 1B). We found that the migration ability was suppressed after the transfection of PARVB shRNAs in both SK-MEL-28 and A375 cells (Fig. 3a and Supplementary Fig. 4). Transwell assay also showed that knockdown of PARVB suppressed the invasion ability (Fig. 3B), while overexpression of PARVB promoted the invasion ability in SK-MEL-28 and A375 cells (Fig. 3C). These results indicated that PARVB might promote melanoma metastasis through the regulation of cell migration and invasion ability.

Hypoxia induces PARVB overexpression in human melanoma cells

Hypoxia is a hallmark of solid tumors including melanoma [18]. Interestingly, we found that the levels of PARVB mRNA and protein were identified to be up-regulated in both SK-MEL-28 and A375 cells cultured under the hypoxic condition (Fig. 4a). To investigate whether transcriptional induction of PARVB by hypoxia was mediated by HIFs, both HIF-1 α and HIF-2 α were overexpressed in SK-MEL-28 and A375 cells. It was found that HIF-1 α induced PARVB overexpression in both melanoma cells (Fig. 4b). Similarly, HIF-2 α also induced PARVB overexpression in both melanoma cells significantly (Fig. 4c). These results showed that hypoxia could induce PARVB overexpression in melanoma cells via HIF-1 α and HIF-2 α mediation.

HIF-1 α and HIF-2 α bind to the specific HRE in the promoter region of PARVB under the hypoxic condition

HIFs were reported to bind to DNA containing HREs (5' -G/ACGTG-3') dependent on the subunit HIF-1 α and HIF-2 α [19]. Thus, we searched for the HRE consensus sequence in the possible promoter region of PARVB (-1706 bp to 264 bp from transcription initiation site). Eight putative HRE sites (site 1–8) were identified in the promoter region (Fig. 5A). Then, ChIP-PCR assay was carried out to determine whether HIF-1 α or HIF-2 α could bind to HRE in the PARVB promoter region physically. The chromatin fragments containing HRE site-6, 7 and 8 were pulled down by the antibody against HIF-1 α or HIF-2 α in A375 cells under the hypoxic condition but not the normoxic condition (Fig. 5b-c). To investigate whether these putative HRE sites accounted for the hypoxia-mediated induction of PARVB expression, the DNA fragments containing HRE sites 1–8 (a), 6–8 (b), 7–8(c) and 8 (d) or none HRE sites (e) were inserted into a pGL3 luciferase reporter plasmid. The pGL3 luciferase reporter plasmid containing the HRE in the VEGF promoter was served as a positive control. A375 cells were then transiently transfected with these plasmids respectively, combined with HIF-1 α or HIF-2 α overexpression vectors. The luciferase activity of the reporter plasmids containing the HRE sites 1–8 (a), 6–8 (b) and 7–8(c) were obviously enhanced, while the plasmid of HRE site 8 (d) was slightly enhanced after HIF-1 α and HIF-2 α overexpression compared with the control group (Fig. 5e-f). Furthermore, we mutated HRE site 7 and site 8 sequences (Fig. 5g) and found that the luciferase activity was significant decreased when site 7 was mutated while mutation on site 8 had only a slight impact on luciferase activity (Fig. 5h). Taken together, these results indicate that the hypoxia-induced PARVB expression mainly depended on the promoter containing functional HRE site 7 (-336 bp).

PARVB mediated HIF-1 α induced cell proliferation and invasion

HIF-1 α has been confirmed to activate melanoma cell proliferation and invasion [20]. To investigate whether PARVB mediated HIF-1 α induced cell proliferation and invasion, SK-MEL-28 and A375 cells were co-transfected with sh-control + OE-control, sh-PARVB + OE-control, sh-control + OE-HIF-1 α and sh-PARVB + OE-HIF-1 α plasmids. Sh-PARVB-2 plasmid was used in this part. As shown in Fig. 6a, HIF-1 α overexpression promoted cell proliferation of the two melanoma cell lines, while PARVB knockdown greatly undermined this effect. Meanwhile, the invasion of SK-MEL-28 and A375 cells was distinctly activated in HIF-1 α overexpression group compared with that in the control group, while PARVB

knockdown abolished the effect of HIF-1 α (Fig. 6b). These results indicate that PARVB possibly mediated HIF-1 α induced cell proliferation and invasion in melanoma cells.

Discussion

Malignant melanoma is the most lethal form of skin cancer. Once this disease metastasizes to other parts of the body, only about 50% patients can survive over one year after diagnosis [21]. The process of progression and metastasis of malignant melanoma involves complex regulatory changes in multiple genes and signaling pathways [22]. Uncovering genes associated with this malignancy and its underlying molecular mechanisms will be of great significance to explore novel therapeutic agents.

PARVB is highly expressed in the spleen, kidneys, heart and skeletal muscles and its regulatory role in healthy cells has been well established¹⁵. However, few studies have discussed the molecular mechanism of PARVB in cancers. The role of PARVB in tumor progression remains controversial. Some studies reported that PARVB was down-regulated in breast cancer and could inhibit tumorigenicity [23, 24], while others argued that PARVB was overexpressed in colorectal carcinoma and seemed to be implicated in cancer progression [25]. Still others reported that it also increased the cell migration capability in tongue squamous cell carcinoma [15]. However, the role of PARVB in malignant melanoma remains unreported. It was found in our study that PARVB was elevated in human malignant melanoma tissues and negatively correlated with the prognosis of melanoma patients. PARVB knockdown suppressed the tumor growth and retarded the metastasis of melanoma. To the best of our knowledge, this is the first research showing a relationship between PARVB overexpression and malignant melanoma and elucidating a regulatory role whereby PARVB affected cell proliferation and metastasis. Although PARVB has been reported to be overexpressed in several tumors, the mechanism underlying the overexpression of PARVB remains unclear.

To explore the mechanism of PARVB upregulation in malignant melanoma, we analyzed the promoter region of PARVB gene and found that the promoter of PARVB contained eight possible HREs, indicating that hypoxia in the tumor tissue might be the possible reason for PARVB overexpression.

Hypoxia has been reported to be associated with metastasis in many cancers, including melanoma [26]. As master regulators of the cellular and physiological responses to hypoxia, HIF-1 α and HIF-2 α contribute to hypoxia-mediated progression and metastatic spread of melanoma through diverse pathways¹⁰. For example, HIF-1 α and HIF-2 α could promote melanoma metastasis through activating SRC independently [7]. PDGFRA and FAK were also identified to be the targets of HIF-1 α or HIF-2 α and participate in melanomagenesis [27, 28]. Besides, HIF1-mediated suppression of melanoma growth could be rescued by MITF overexpression [29]. In addition, previous studies have revealed that there is a correlation between the expression of HIFs and the expression of PARVB [30]. In this study, we demonstrated that PARVB expression in melanoma cells was induced by hypoxia and HIFs. Our study further revealed that both HIF-1 α and HIF-2 α could bind to the specific HRE in the promoter region PARVB, thus regulating its expression. Besides, HIFs play active roles in the metastatic progression of melanoma through mediating

PARVB. To the best of our knowledge, our work for the first time identified PARVB as the target gene of HIFs. Our finding about the role of HIFs in melanomagenesis is also in line with the results of previous studies. It is therefore plausible to speculate that HIFs might also play a role in tumorigenesis of various cancer types by regulating PARVB, though this speculation needs to be verified in future studies. The specific downstream mechanism whereby PARVB regulated cell proliferation and invasion still requires further elucidation.

Conclusions

In conclusion, our data demonstrate that PARVB was overexpressed in malignant melanoma. and promoted tumor growth and metastasis. Furthermore, the expression of PARVB was induced by both HIF-1 α and HIF-2 α through binding to the specific HRE on its promoter. All these findings provide novel insights into the mechanism underlying melanoma malignancy and suggest that PARVB may prove to a therapeutic target for this devastating disease.

List Of Abbreviations

B-parvin (PARVB)

hypoxia-responsive elements (HREs)

Hypoxia-inducible factors (HIF)

epithelial-to-mesenchymal transition (EMT)

cell-extracellular matrix (ECM)

Chromatin Immunoprecipitation (ChIP)

Cell Counting Kit 8 (CCK8)

Immunohistochemistry (IHC)

room temperature (RT)Dulbecco's modified Eagle's medium (DMEM)

American Type Culture Collection (ATCC)

fetal bovine serum (FBS)

optical density (OD)

propidium iodide (PI)

phosphate-buffered saline (PBS)

polyvinylidene fluoride (PVDF)

real-time quantitative PCR (RT-qPCR)

Diamino benzidine chromogen (DAB)

standard deviation (SD)

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center, and written informed consent was obtained from all patients. Animal care and experiments were performed in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Ethics Committee of Fudan University Shanghai Cancer Center. The study was conducted in accordance with the ethical principles that have their origins in the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

W.C., W.H., and Y.W. conceived the idea. W.T., W.Z., B.Y., S.H., W.Z., and W.Y. performed the experiments. All authors analysed and interpreted the data. W.T., W.Z., B.Y. drafted the paper. All authors have agreed with the final version of the paper and provided their consent for publication.

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Not applicable

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Tables

Table 1
Correlation of PARVB expression and patients' clinical features

Characteristics	PARVB expression (Number of patients)			<i>p</i> value
	Low	High	Very high	
Age				0.138
<60 years	23	64	30	
≥60 years	28	52	16	
Sex				0.938
Male	27	63	26	
Female	24	53	20	
Site				0.184
Limbs	23	54	22	
Trunk	22	59	20	
Head or neck	6	3	4	
Ulceration				0.015
Absent	45	86	29	
Present	6	30	17	
T grade				0.001
T1-T2	39	80	20	
T3-T4	12	36	26	
N grade				0.214
N0	23	58	16	
N1-3	28	58	30	
M grade				0.067
M0	45	94	32	
M1	6	22	14	
AJCC grade				0.047
I-II	22	55	16	
III-IV	29	61	30	

Figures

Figure 1

PARVB is overexpressed in human malignant melanoma tissues and cells. (A) IHC detection of PARVB in human normal skin tissue (n=39), nevus (n=32), basal cell carcinoma (n=32) and malignant melanoma (n=213). (B) PARVB mRNA expression in human normal skin tissue (n=32), nevus (n=32), basal cell carcinoma (n=32) and malignant melanoma (n=32) by qRT-PCR. (C) PARVB protein level in seven paired

human normal skin tissues and malignant melanoma tissues by Western blot. (D) PARVB mRNA and protein expression were detected in five different melanoma cell lines (A375, SK-MEL-2, M14, SK-MEL-28 and SK-MEL-5) and human epidermal melanocytes HEMA-LP through qRT-PCR and Western blot assays. (E) The correlation between PARVB expression and the survival rate in patients with malignant melanoma. *** $p \leq 0.001$.

Figure 2

PARVB knockdown inhibits melanoma cell proliferation and tumor growth. (A) qRT-PCR and Western blot assays were used to verify the knockdown efficiency of PARVB shRNAs in SK-MEL-28 and A375 cells. (B) Melanoma cell proliferation was detected by CCK8 assay after PARVB knockdown in SK-MEL-28 and A375 cells. (C) Cycle distribution of SK-MEL-28 and A375 cells after detection of PARVB knockdown by flow cytometry. (D) The progression of tumor generation was assessed by tumor xenografts in nude mice after PARVB knockdown in A375 cells. The tumor volume and tumor weight were assessed. (E) IHC detection of Ki-67 in the tumors with or without PARVB knockdown. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 3

PARVB knockdown inhibits melanoma cell migration and invasion. (A) Wound healing assay for the evaluation of PARVB knockdown on SK-MEL-28 and A375 cells migration ability.(B) Transwell assay for the evaluation of PARVB knockdown on SK-MEL-28 cell invasive capacity. (C) Transwell assay for the evaluation of PARVB knockdown on A375 cell invasive capacity. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 4

Hypoxia induces PARVB expression in melanoma cells. (A) RNA and protein expressions of PARVB were detected at 24 and 36 h of hypoxia vs. normoxia in SK-MEL-28 and A375 cells via qRT-PCR and Western blot assays. (B) RNA and protein expressions of PARVB were detected at 24 and 36 h after HIF-1α overexpression vector or control vector transfected in SK-MEL-28 and A375 cells. (C) RNA and protein expressions of PARVB were detected at 24 and 36 h after HIF-1α overexpression vector or control vector transfected in SK-MEL-28 and A375 cells. *** $p \leq 0.001$.

Figure 5

Hypoxia trans-activates HREs in the promoter region PARVB through HIF-1α and HIF-2α mediation. (A) The sketch map of eight putative HREs in the promoter region of human PARVB. (B-C) ChIP-PCR assay examined the possible HRE sites bound to HIF-1α (B) or HIF-2α (C) under the hypoxic condition in A375 cells. The HRE site in the VEGF promoter served as a positive control. (D) The sketch map of the constructed luciferase reporter vectors containing different HRE sites in the PARVB promoter. (E-F) Luciferase activities were measured after transfection of A375 cells with the luciferase reporter vectors as well as HIF-1α (E) or HIF-2α (F) overexpression vector. Reporter vector containing the HRE site in the VEGF promoter was constructed as a positive control. (G) The sketch map of the mutant HRE site 7 and 8. (H) Luciferase activity were measured after HRE site 7 and 8 mutation. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 6

PARVB mediated HIF-1α induced cell proliferation and invasion. SK-MEL-28 and A375 cells were transfected with sh-control + OE-control, sh-PARVB + OE-control, sh-control + OE- HIF-1α and sh-PARVB + OE- HIF-1α. (A) Melanoma cell proliferation was detected by CCK8 assay in SK-MEL-28 and A375 cells at 24, 48, and 72 h after transfection. (B) The invasive capacity of melanoma cells 48 h after transfection was assessed by Transwell assay. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

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