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Original Research

MiR-637 suppresses melanoma progression through directly targeting P-REX2a and inhibiting PTEN/AKT signaling pathway

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Abstract: MicroRNAs (miRNAs) play important roles in melanoma. Although miR-637 has been suggested to be a tumor suppressor in several cancers, its function in melanoma and the molecular mechanism behind that function remain unclear. In this study, we investigated the role of miR-637 in human melanoma and explored its relevant mechanisms. We found that the expression of miR-637 is significantly downregulated in melanoma tissues and cell lines. While overexpression of miR-637 inhibited melanoma cell proliferation and cell cycle G1-S transition, and induced apoptosis. Inhibition of miR-637 promoted cell proliferation and G1-S transition, and suppressed apoptosis. Subsequent investigation revealed that miR-637 expression was inversely correlated with P-REX2a expression in melanoma tissues. P-REX2a was determined to be a direct target of miR-637 by using a luciferase reporter assay. Overexpression of miR-637 decreased P-REX2a expression at both the mRNA and protein levels, and suppression of miR-637 increased P-REX2a expression. Importantly, silencing P-REX2a recapitulated the cellular and molecular effects seen upon miR-637 overexpression, whereas, overexpression of P-REX2a eliminated the effects of miR-637 overexpression on melanoma cells. Furthermore, both enforced expression of miR-637 or silencing of P-REX2a resulted in activation of PTEN, leading to a decline in AKT phosphorylation. Taken together, our study demonstrates that miR-637 inhibites melanoma cell proliferation by activation of AKT signaling pathway and induces apoptosis through regulation of Bcl-2/Bax expression via targeting P-REX2a. These findings suggest that miR-637 plays a crucial role in melanoma progression, and may serve as a potential novel target for melanoma therapy.

Key words: miR-637; Melanoma; P-REX2a; Proliferation; Apoptosis.

Introduction

Melanoma is one of the most aggressive and lethal type of skin cancer that derives from the pigment-containing cells known as melanocytes (1). It is the fifth and seventh most common cancer in United States males and females, respectively (2), and is one of the most common cancers in adolescents and young adults (3). The incidence of melanoma has increased rapidly in the past few decades, such that 3.1 million people were diagnosed and 59,800 died with the active disease in 2015 (4). The major risk factors of affecting melanoma include ultraviolet light and fair feature such as light hair, skin and eye color (5). Unfortunately, despite the great advance in the therapy of melanoma, the five year overall survival of melanoma patients in advanced stage remains poor (6). Tumorigenesis is a polyfactorial and multistep process involving different gene changes, such as the inactivation of tumor suppressor genes, activation of oncogenes and abnormal expression of cancer-related genes (7,8). Therefore, it is critical to discover the molecular mechanisms underlying melanoma development and progression, which could identify novel biomarkers and develop therapeutic strategies for patients with melanoma.

MicroRNAs (miRNAs) is a class of single-stranded, small, endogenous non-coding, highly conserved RNAs, approximately 19-25 nucleotides in length that can regulate gene expression post-transcriptionally by binding to 3′-untranslated regions (UTRs) of target mRNAs, leading to mRNA degradation and/or suppression of translation (9-11). The aberrant expression of miRNAs has been found to play multiple roles in a variety of biological processes, including cell proliferation, differentiation, cycles, metabolism, apoptosis, development and tumorigenesis (12-16). Previous studies have shown that dysregulation of miRNAs plays an important role in melanoma progression. Recently, several studies have shown that miR-637 is clinically significant and acts as a crucial role in carcinogenesis and cancer progression, such as papillary thyroid carcinoma, pancreatic ductal adenocarcinoma, glioma and hepatocellular carcinoma (17-20). However, the role of miR-637 in melanoma and the molecular mechanisms behind its role are still unknown.

In the present study, we investigated the function of miR-637 and the mechanism that regulates this function in human melanoma. We found that the expression of

miR-637 was significantly downregulated in melanoma tissues and correlated with clinicopathological characteristics. Our results also showed that phosphatidylinositol 3,4,5-trisphosphate RAC exchanger 2a (P-REX2a) had an overexpression in melanoma tissues compared with adjacent normal tissues. As predicted, we observed that miR-637 could target P-REX2a by using bioinformatics software (miRanda and TargetScan). P-REX2a is a guanine nucleotide exchange factor for the RAC guanosine triphosphatase, playing a PTEN-interacting protein, which can activate the phosphoinositide 3-kinase (PI3K) signaling pathway by antagonizing PTEN in cancer cells (21). Additionally, our results demonstrated that miR-637 potently inhibited human melanoma cell proliferation, induced G1-S cell cycle arrest and cell apoptosis. More importantly, we provide evidence that P-REX2a is a direct and functional target of miR-637 for the first time. Overall, our data suggest that miR-637 may be a novel therapeutic target in melanoma therapy.

Materials and Methods

Human tissue samples

Human melanoma samples (61) were collected from patients who were diagnosed with the disease at the Department of Dermatology, the First Affiliated Hospital, Xi'an Jiaotong University, China. Informed consents were obtained from each patient before specimen collection. The tissue samples were stored at -80 °C until use. Clinicopathological data were obtained through reviewing their pathology records. The study was approved by the Ethical Committee of Xi'an Jiaotong University, and guidelines of the committee were followed.

Cell culture

Human melanoma cell lines A375, SK-MEL-28, Mel-RM, and normal skin cell line HaCaT were purchased from the Cell Bank (Shanghai Genechem Co., Ltd., Shanghai, China). All cell lines have been detected and authenticated by the Cell Bank. Cells (1×10^5 cells/ml) were cultured in Dublecco's modified essential medium (DMEM; Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL, NY, USA), penicillin (100 U/mL), streptomycin (100 μg/mL) and were incubated at 37 °C in a incubator containing 5% CO_2.

Quantitative real-time PCR

The RNA was extracted from human melanoma tissue samples and cell lines by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). SYBR Premix Ex Taq II Kit (Takara, China) were used for the measurement of miR-637 expression and P-REX2a mRNA expression. Quantitative real-time PCR (qRT-PCR) were conducted through using the iCycler iQ Multicolor qRT-PCR System (Bio-Rad, USA). These results were normalized to RNU6B (U6) gene or β-Actin gene expression. These correlative primer sequences were as follows: miR-637 reverse-transcribed primer, 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACGCAGA-3′; miR-637 forward, 5′- ATCCAGTGCGTGTCGTG-3′; miR-637 reverse, 5′-TGCTACTGGGGGCTTTCGGGC-3′; U6 reverse-transcribed primer, 5′-CGCTTCACGAATTTGCGTGTCAT-3′; U6 forward, 5′-GCTTCGGCAGCACATATACTAAAAT-3′; U6 reverse, 5′-CGCTTCACGAATT TGCGTGTCAT-3′; P-REX2a forward, 5′-AACCATGAGAAGGCACAAAAA-3′; P-REX2a reverse, 5′-CTTGCATATTCTTTGTATTGGTGT-3′; β-Actin forward, 5′-TGGCACCCAGCACAATGAA-3′; β-Actin reverse, 5′- CTAAGTCATAGTCCGC CTAGAAGCA -3′.

Vector construction

Hsa-miR-637 precursor expression vector (named miR-637) and control empty vector (named Control) were constructed with synthetic oligonucleotides and interpolated into pcDNA6.2-GW/EmGFPmiR plasmid according to the manufacturer's instructions. Full-length human P-REX2a complementary DNA was cloned into the pCMV2-GV146 vector (Genechem Co. Ltd). According to the manufacturer's instructions, transfection was fulfilled through using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Anti-miR-637/P-REX2a siRNA synthesis and transfection

Interfering RNA oligonucleotides were used as miR-637 inhibitors (named anti-miR-637), and synthesised from Gene Pharma (Shanghai, China). The sequence of anti-miR-637 was 5′-ACGCAGAGCCCGAAAGCCCCCAGU-3′. Scramble siRNA was used as control (named anti-miR-Control), and this sequence was 5′- CAGUACUUUUGUGUAGUACAA-3′. The RNA oligonucleotides were transfected into human melanoma A375 cells with Lipofectamine 2000. Small interfering RNA (siRNA) was used to silence human P-REX2a gene. Human P-REX2a siRNA (sense 5′-CACUAUGGCCAUCAUUGAUTT-3′, antisense 5′-AUCAAUGAUGGCCAUAGUGTT-3′) and negative siRNA (NC-siRNA, sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGGAGAATT-3′) were compounded by GenePharma Corporation (Shanghai, China). The siRNAs were transfected through using Lipofectamine 2000, and diluted to 60 nM in A375 cells in future experiment.

Dual-luciferase assay

The binding site for miR-637 in the 3′-UTR of P-REX2a was constructed (Beijing AuGCT DNA-SYN Biotechnology Co. Ltd, Beijing, China) and cloned in the pmirGLO Dual-Luciferase expression vector (named P-REX2a-WT). The mutated 3′-UTR sequences of P-REX2a were also cloned and named P-REX2a-MT. The pre-miR-637 expression vector and WT or MT reporter plasmids were cotransfected into HEK293 cells. Then, the cells were collected 24 hours after transfection. Dual-Luciferase Assay System (Promega, Madison, WI, USA) was performed for examining reporter activity according to the manufacturer's protocol.

MTT assay

Human melanoma A375 cells (3,000 cells/well in 200 μl DMEM medium) were planted into 96-well plates and cultivated for 24 hours. The cells were treated with Control, miR-637, anti-miR-Control, anti-miR-637, NC-siRNA (60 nM), P-REX2a siRNA (60 nM), Vector control and P-REX2a expression vector

for 24, 48 and 72 hours, respectively. Cell viability was measured with MTT assay FLUOstar OPTIMA (BMG Labtechnologies, Germany) at a wavelength of 492 nm.

Cell counting assay

To examine cell proliferation, 2.5×10^5 cells were seed into 60 mm diameter plates and cultured for 24 hours. A375 cells were treated with Control, miR-637, anti-miR-Control, anti-miR-637, NC-siRNA (60 nM), P-REX2a siRNA (60 nM), Vector control and P-REX2a expression vector, respectively. The numbers of cells were calculated at 24, 48 and 72 hours after treatment through using the Countess automated cell counter (Life Technologies Corp., Carlsbad, USA).

Cell cycle analysis

The A375 cells were seeded in 6-well plates and treated for 48 hours. The cells were collected and washed in PBS, and fixed in 70% ice-cold ethanol at 4 °C. These fixed cells were washed in PBS and stained through using 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 20 min at room temperature. Next, the cells were examined by fluorescence-activated cell sorting (BD Biosciences, USA). ModFit software was used to analyze the cell-cycle populations.

Apoptosis analysis

A375 cells were cultured into 6-well plates in triplicate and treated for 48 hours. Annexin-V FITC Apoptosis Detection Kit (Invitrogen, USA) was used to measure cell apoptosis according to the manufacturer's instructions. The stained cells were examined by using a flow cytometer (BD Biosciences, USA). ModFit software was used to analyze apoptosis.

Western blot analysis

Human melanoma cells or tissue samples were lysed by using a RIPA lysis buffer (Wolsen, China). Total protein was separated in 10% SDS polyacrylamide gels, and electrophoretically transferred to nitrocellulose membrane (Roche, Switzerland). Primary antibodies were used to incubate the membrane at 4 °C. The primary antibodies were as follows: mouse polyclonal anti-P-Rex2a (1:1,000, Cell Signaling Technology, USA), rabbit monoclonal antibody anti-PTEN (1:1,000, Cell Signaling Technology, USA), rabbit monoclonal anti-AKT (1:2,000, Santa Cruz, CA, USA), rabbit monoclonal anti-p-AKT (1:1,000, Santa Cruz, CA, USA), mouse monoclonal anti-CDK2 (1:1,000, Santa Cruz, CA, USA), mouse monoclonal anti-Cyclin D1 (1:1,000, Santa Cruz, CA, USA), rabbit monoclonal anti-Bcl2 (1:1,000, Santa Cruz, CA, USA), rabbit monoclonal anti-Bax (1:1,000, Santa Cruz, CA, USA), mouse monoclonal anti-β-Actin (1:4,000, Santa Cruz, CA, USA). ECL (Pierce, USA) was used to incubate the membranes for chemiluminescence detection. Then, the blots were scanned and the optical density was calculated with Quantity One imaging software.

Statistical analysis

All experiments were performed in triplicate at least. The data were presented as mean ± SEM of 3 independent experiments. Statistical analysis was performed using SPSS 19.0 software (Abbott Laboratories, Chicago, IL). The statistical significance between the groups was analyzed by using one-way ANOVA or Student's t-test. Chi-square test was employed to analyze the relationships between miR-637 expression and clinicopathologic characteristics. The correlation between miR-637 and P-Rex2a was estimated using Pearson's correlation analysis in melanoma tissues. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MiR-637 is frequently downregulated in human melanoma tissues and cell lines

To explore whether miR-637 was involved in the regulation of tumorigenesis of melanoma, we performed qRT-PCR to examine its expression in clinical samples (61 melanoma specimens and 61 adjacent normal tissues) and tumor cell lines. The qRT-PCR assay showed that miR-637 expression was significantly lower in 80.3% (49/61) of the melanoma tissues than in adjacent normal tissues (Fig. 1A). Further research revealed that the relationship between miR-637 expression and clinicopathologic features of melanoma patients. Low miR-637 expression was associated with lymph node metastasis and TNM stage (Table 1). However, the miR-637 expression was not associated with gender, age and tumor thickness. Additionally, miR-637 expression was remarkably downregulated in human melanoma cell lines (A375, SK-MEL-28 and Mel-RM) as compared with normal skin cell line (HaCaT) (Fig. 1B). The results suggested that miR-637 might be a useful biomarker for malignant status of human melanoma.

MiR-637 inhibites human melanoma cell proliferation, induces G1-S arrest and apoptosis in A375 cells

We investigated the role of miR-637 in human melanoma, and A375 cells were transfected with miR-637 precursor expression vector, empty vector, miR-637 antisense oligonucleotides, or negative control. The expression of miR-637 was measured by qRT-PCR after transfection. The results showed that the miR-637 expression was remarkably increased in miR-637 vector compared with empty vector, whereas there were no significant differences between anti-miR-637 group and anti-miR-Control group (Fig. 2A and B). MTT assay showed that the overexpression of miR-637 significantly inhibited the proliferation of melanoma A375 cells at 48 and 72 hours after transfection (Fig. 2C), but

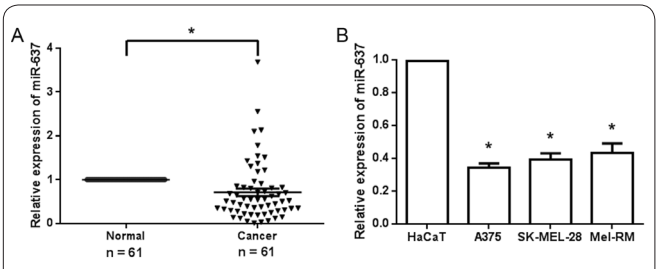

Figure 1. miR-637 is downregulated in human melanoma tissues and cell lines. (A) The qRT-PCR assay revealed that miR-637 expression was significantly decreased in melanoma tissues as compared with adjacent normal tissues. (B) qRT-PCR was used to analyze miR-637 expression in human melanoma cell lines (A375, SK-MEL-28 and Mel-RM), and a normal skin cell line (HaCaT) *P<0.001.

Table 1. The correlation between miR-637 expression and clinicopathologic features of melanoma patients.

Characteristics	Number of cases	miR-637 expression		*P* value
		High (n = 12)	Low (n = 49)	
Gender				0.582
Male	34	7	27	
Female	27	5	22	
Age				0.683
≥60 years	25	5	20	
<60 years	36	7	29	
Tumor thickness				0.329
<1 mm	23	4	19	
≥1 mm	38	8	30	
Lymph node metastasis				0.013
Yes	24	2	22	
No	37	10	27	
TNM stage				0.006
I/ II	28	9	19	
III/IV	33	3	30	

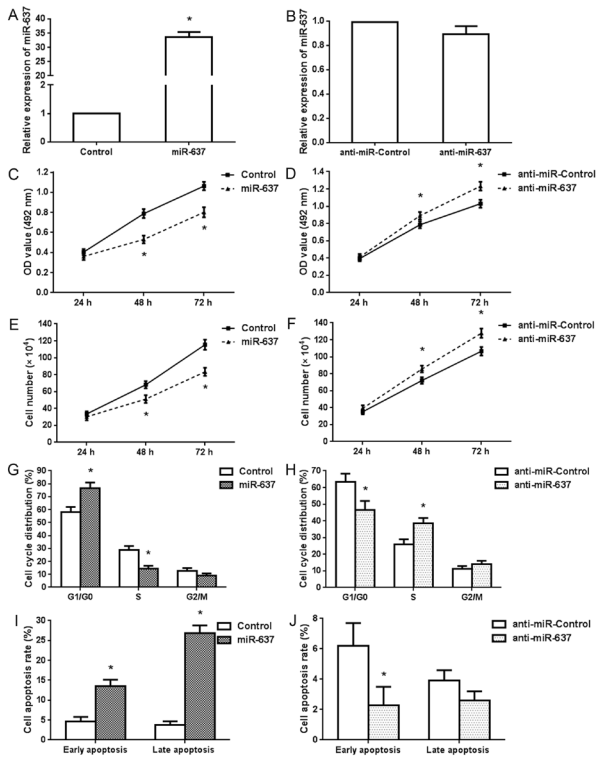

Figure 2. miR-637 inhibits human melanoma A375 cell proliferation and induces G1-S cell cycle arrest. (A) The miR-637 expression was determined in A375 cells after miR-637 overexpression. *P<0.001. (B) The miR-637 expression was measured in A375 cells after anti-miR-637 treatment. (C) MTT assay showed that miR-637 overexpression decreased the cell viability at 48 and 72 hours after transfection. *P<0.001 (D) Anti-miR-637 increased the cell viability at 48 and 72 hours after transfection. *P<0.01. (E) Cell counting assay showed that miR-637 overexpression inhibited A375 cell proliferation. *P<0.001. (F) Anti-miR-637 promoted A375 cell growth. *P<0.01. (G) Flow cytometry analysis of cell cycle visualized via PI staining. Histogram represented the percentage of cells in G1/G0, S, and G2/M phases after miR-637 overexpression. *P<0.01. (H) The percentage of cells in G1/G0, S, and G2/M phases after anti-miR-637 transfection. *P<0.01. (I) Cell apoptosis was visualized using Annexin-V/PI staining. The data showed the percentages of early apoptosis and late apoptosis after miR-637 overexpression. *P<0.01. (J) The data showed the percentages of early apoptosis and late apoptosis after anti-miR-637 transfection. n=3, *P<0.01.

anti-miR-637 promoted A375 cell growth at 48 and 72 hours after transfection (Fig. 2D). At the same time, similar trend were observed in cell counting assay. MiR-637 overexpression suppressed A375 cell proliferation while anti-miR-637 promoted cell growth (Fig. 2E and F). Since cell cycle is involved in controlling cell proliferation, we detected the process with a flow cytometer 48 hours after treatment. Our results showed that miR-637 overexpression resulted in a marked accumulation of G1/G0 phase population and a reduction of S phase population in melanoma A375 cell (Fig. 2G); however, inhibition of miR-637 significantly decreased G1/G0 phase population and increased S phase population (Fig. 2H). Evaluation of cell apoptosis revealed that the proportion of early-apoptotic to late-apoptotic cells increased significantly when miR-637 was overexpressed (Fig. 2I), and remarkably decreased when the cells were transfected with anti-miR-637 carrying plasmids (Fig. 2J). These findings suggested that miR-637 inhibited human melanoma cell proliferation, while inducing G1-S cell cycle arrest and cell apoptosis.

P-REX2a is a direct target of miR-637

Bioinformatic databases (RegRNA and miRanda) were used to identify a large number of potential target genes of miR-637. Of these candidates, P-REX2a was selected for the further analysis. A binding site of miR-637 was discovered in the 3′-UTR of P-REX2a mRNA ranging from dinucleotide 4815 to 4835 bp (Fig. 3A). To verify whether miR-637 directly targets P-REX2a, a dual-luciferase reporter system containing WT and MT 3′-UTR of P-REX2a was used. HEK293 cells were co-transfected with reporter plasmids and pre-miR-637 or pmirGLO empty vector (Control). It was found that pre-miR-637/WT-P-REX2a-UTR transfected cells showed a significant reduction of luciferase activity, but pre-miR-637/MT-P-REX2a-UTR transfected cells failed to decrease the relative luciferase activity (Fig. 3B), suggesting that miR-637 directly targets the 3′-UTR of P-REX2a. Then, we measured P-REX2a expression at the levels of mRNA and protein. These results showed that the expression of P-REX2a remarkably upregulated at both levels in melanoma tissues as compared with adjacent normal tissues (Fig. 3C and D). The effect of miR-

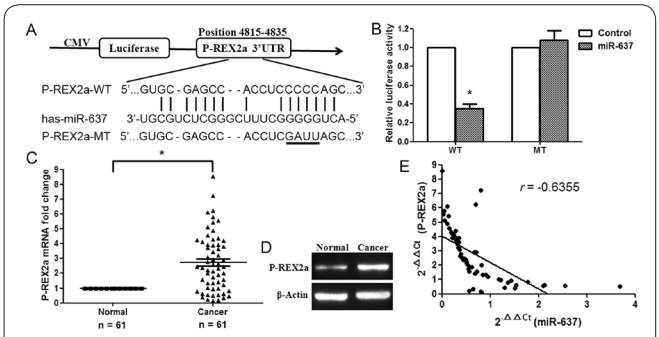

Figure 3. MiR-637 directly targets the P-REX2a gene. (A) Bioinformatics predicted interactions of miR-637 and its binding sites at the 3′-UTR of P-REX2a. (B) The luciferase reporter plasmid containing wild or mutant type P-REX2a-3′-UTR was cotransfected into A375 cells incombination with miR-637 or Control. Luciferase activity decreased in wild type P-REX2a-3′-UTR group. *P<0.01. (C) P-REX2a mRNA expression in human melanoma tissues. *P<0.001. (D) P-REX2a protein level was measured in melanoma tissues by Western blot. (E) miR-637 and P-REX2a levels were inversely correlated. $2^{-\Delta\Delta Ct}$ values of miR-637 and P-REX2a were subjected to a Pearson correlation analysis (n=61, r=-0.6355, P<0.001, Pearson'scorrelation).

637 on P-REX2a was assessed based on the data obtained from qRT-PCR. A significant inverse correlation was identified between P-REX2a and miR-637 (Fig. 3E; n=61, r =-0.6355, P<0.001, Pearson's correlation).

MiR-637 suppresses melanoma cell growth, induces G1-S arrest and apoptosis through PTEN/AKT signaling pathway by targeting P-REX2a

Overexpression of miR-637 significantly downregulated the mRNA expression of P-REX2a in A375 cells, while anti-miR-637 remarkably increased P-REX2a mRNA expression (Fig. 4A and B). Meanwhile, similar trends were observed in protein levels (Fig. 4C and D). To further investigate the potential molecular mechanisms of miR-637-regulated cell proliferation, cell cycle transition and apoptosis, we measured the protein levels of related PTEN/AKT signaling pathway, G1 regulators and Bcl2/Bax by using western blot analysis. The results showed that miR-637 overexpression decreased p-AKT (at serine 473), Cyclin D1 and CDK2 protein expression levels in A375 cells (Fig. 4C). In contrast, anti-miR-637 increased p-AKT, Cyclin D1 and CDK2 protein expression (Fig. 4D) whereas the protein expression of PTEN and total AKT remained unchanged. Moreover, we found that miR-637 could also suppress Bcl2 protein expression and promote Bax protein expression (Fig. 4C and D). These results demonstrated that miR-637 could modulate melanoma cell proliferation, cycle and apoptosis through regulating P-Rex2a/PTEN/AKT signaling pathway.

Knockdown of P-Rex2a inhibits melanoma A375 cell proliferation

Since miR-637 regulated cell proliferation, cycle and apoptosis in melanoma cells, and P-Rex2a was validated as a direct target of miR-637. We knocked down P-Rex2a expression in melanoma cells by RNA interference (RNAi) to confirm its involvement in the tumor suppressor functions of miR-637. We confirmed that P-Rex2a mRNA expression was specifically knocked down in A375 cells by qRT-PCR (Fig. 5A). Silencing

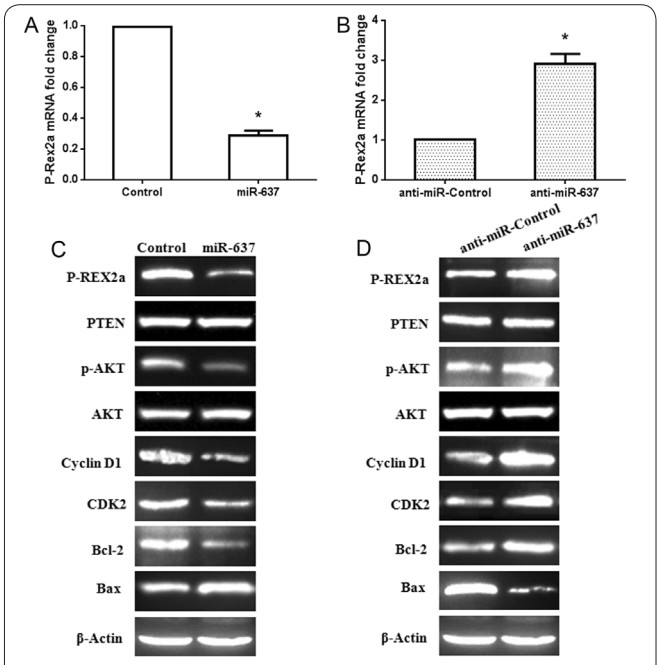

Figure 4. MiR-637 regulates PTEN/AKT signaling pathway in human melanoma cells by targeting P-REX2a. (A) The P-REX2a mRNA was determined in A375 cells after miR-637 overexpression. *P<0.001. (B) The P-REX2a mRNA was examined in A375 cells after anti-miR-637 treatment. *P<0.001. (C) miR-637 overexpression inhibited the expressions of P-REX2a, p-AKT, Cyclin D1, CDK2 and Bcl2, and promoted Bax expressions in A375 cells. (D) Anti-miR-637 upregulated P-REX2a, p-AKT, Cyclin D1, CDK2 and Bcl2 expressions, and downregulated Bax expression.

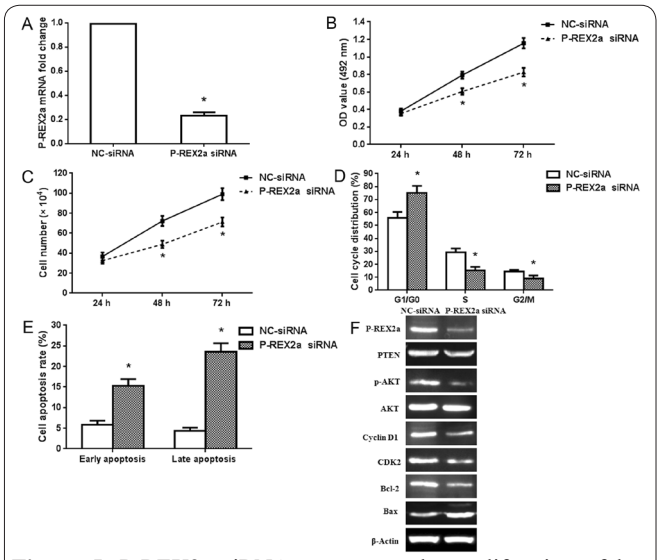

Figure 5. P-REX2a siRNA suppresses the proliferation of human melanoma A375 cells. (A) qRT-PCR results showed the knockdown efficiency of P-REX2a siRNA in A375 cells. (B) MTT assay showed that P-REX2a siRNA decreased the viability of A375 cells at 48 and 72 hours. (C) Cell counting assay showed that P-REX2a siRNA inhibited A375 cell proliferation. (D) Flow-cytometry analysis showed the percentage of A375 cells in the G1/G0, S, and G2/M phases. G1/G0 phase cells increased after P-REX2a siRNA streatment, S and G2/M phase cells decreased. (E) The data showed the percentage of early and late apoptosis after P-REX2a siRNA treatment. *P<0.01. (F) P-REX2a, p-AKT, Cyclin D1, CDK2 and Bcl2 protein expressions decreased after P-REX2a siRNA streatment, while Bax protein expression increased. n=3, *P<0.01.

of P-Rex2a remarkably decreased the cell activity at 48 and 72 hours after transfection (Fig. 5B). Cell counting

assay also showed that silencing of P-Rex2a significantly inhibited A375 cell proliferation (Fig. 5C). Silencing of P-Rex2a increased G1/G0 phase population, decreased S and G2/M phase population in A375 cells (Fig. 5D). Moreover, silencing P-Rex2a induced apoptosis in A375 cells (Fig. 5E). These observations were similar to those of miR-637 overexpression, indicating a similar effect in P-Rex2a knockdown and miR-637 overexpression. In addition, we analyzed knockdown efficiency of P-Rex2a siRNA in protein level. The protein expression of P-Rex2a significantly decreased in siRNA group compared with NC-siRNA group (Fig. 5F). While the p-AKT, Cyclin D1, CDK2 and Bcl2 protein expressions also reduced. Bax protein expression increased in siRNA group (Fig. 5F). However, there were no change in the protein expressions of PTEN and total AKT (Fig. 5F).

Overexpression of P-Rex2a eliminated the effects of miR-637 on melanoma A375 cells

To further demonstrate that miR-637 exhibited tumor suppressor function via P-Rex2a, we constructed P-Rex2a overexpression vector, which was cotransfected with miR-637 into A375 cells. Overexpression of P-Rex2a in A375 cells rescued P-Rex2a expression level reduced by miR-637 (Fig. 6A). After cotransfected with the miR-637 and P-Rex2a vector, we found that the overexpression of P-Rex2a counterbalanced the tumor suppressor effect of miR-637 in melanoma cells at cell proliferation (Fig. 6B and C). The effect of overexpression of P-Rex2a on cell cycle progression was examined by flow cytometry. It was found that overexpression of P-Rex2a was in cline to re-enter S-phase in A375 cells (Fig. 6D). Moreover, overexpression of P-Rex2a eliminated the impact of miR-637 on A375 cell apoptosis (Fig. 6E). Further analysis revealed that overexpression of P-Rex2a upregulated the P-Rex2, p-AKT, Cyclin D1, CDK2 and Bcl2 protein expressions, downregulated the Bax protein expression compared with miR-637 overexpression (Fig. 6F). These results further demonstrated that miR-637 exhibit tumor suppressor role through PTEN/AKT signaling pathway by targeting P-REX2a.

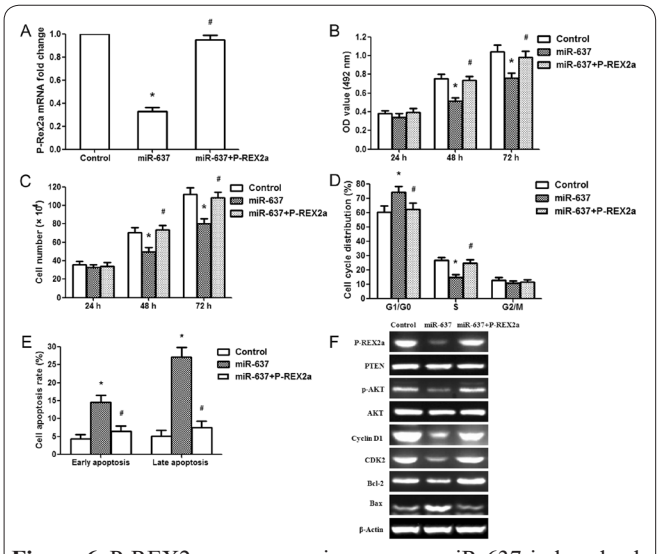

Figure 6. P-REX2a overexpression rescues miR-637-induced cellular phenotypes in melanoma cells. (A) P-REX2a overexpression rescued P-Rex2a mRNA expression level reduced by miR-637. (B) MTT assay was performed to examine the growth of A375 cells after co-transfecting with P-REX2a and miR-637. (C) Cell counting assay was used to measure the A375 cell proliferation following the co-transfection with P-REX2a and miR-637. (D) Cell cycle was determined in A375 cells at 48 hours. (E) Apoptosis was detected in A375 cells at 48 hours. (F) P-REX2a, PTEN, p-AKT, AKT, Cyclin D1, CDK2, Bcl2 and Bax protein expressions were examined after cotransfected with P-REX2a and miR-637. *P<0.01, compared with Vector control group; #P<0.01, compared with miR-637 overexpression group. n=3.

Discussion

According to recent evidence, miRNAs play an crucial role in regulating human cancer cell proliferation, differentiation, survival, apoptosis, migration and invasion (8,22,23). It has been widely researched that abnormal miRNA expression contributes to melanoma carcinogenesis and development by directly downregulating target gene. Although the clinical significance of miRNAs has been well characterized in melanoma, the role and the underlying molecular mechanism of dysregulated miRNAs remain unknown. Identifying miRNAs and elucidating their biological functions in melanoma will help seek for novel targets for the diagnosis and therapy. It is reported that miR-637 was significantly downregulated in glioma tissues and suppressed glioma cell growth, migration and invasion by targeting Akt1 (19). miR-637 inhibited human pancreatic ductal adenocarcinoma cell proliferation and induced apoptosis by suppressing Akt1 expression (18). Moreover, miR-637 dramatically inhibited hepatocellular carcinoma cell growth and induced apoptosis through suppression of Stat3 phosphorylation by restraining leukemia inhibitory factor (20). However, the clinical significance and function of miR-637 in melanoma remain unclear. The clinicopathological significance of miR-637 expression was also analyzed. The results showed that low miR-637 levels were significantly associated with lymph node metastasis and TNM stage in melanoma. The experiment demonstrated that miR-637 remarkably suppressed melanoma cell proliferation by inducing G1-S phase arrest and promoted cell apoptosis. These findings indicate that miR-637 plays an important role in melanoma development and progression.

Furthermore, our miR-637 target analysis identified P-REX2a as a direct target of miR-637. P-REX2a is a guanine nucleotide exchange factor for RAC GTPase, which could encode a protein-suppressing phosphatase activity against phosphatidylinositol 3,4,5-trisphosphate (21,24). In the present study, we found that P-REX2a had an over-expression in melanoma compared with normal tissues, which showed an inverse correlation between P-REX2a expression and miR-637 expression in melanoma tissues. These findings implied that miR-637 might affect the progression of melanoma by targeting P-REX2a. Further bioinformation analysis showed that there was a miR-637-binding site at 4815-4835 nt of the P-REX2a 3'UTR. The dual-luciferase reporter assays demonstrated that miR-637 directly targeted P-REX2a by recognizing the 3'UTR of P-REX2a mRNA and inhibited P-REX2a translation. P-REX2a has shown to involve tumorigenesis and development. Evidences support that P-REX2a stimulated breast cancer cell proliferation (21). In addition, the expression of P-REX2a

was increased in gastric cancer tissues, and silencing of P-Rex2a remarkably inhibited gastric cancer cell proliferation and induced apoptosis (25). Our results also demonstrated that knockdown of P-REX2a suppressed melanoma cell proliferation by inducing G1-S phase arrest and promoted cell apoptosis. In addition, we found that the over-expression of P-Rex2a counterbalanced the tumor suppressor effect of miR-637 in melanoma cells. These results further verify that miR-637 function as at tumor suppressor role by repressing P-REX2a expression.

P-REX2a could regulate AKT phosphorylation in a PTEN-dependent manner by binding to PTEN (21). The PTEN is frequently lost in cancers, and germline PTEN mutations are linked to inherit cancer predisposition syndromes (26). PTEN is a phosphatase that may dephosphorylate PIP3, the lipid product of the class I PI3K (27). PI3K/AKT signaling pathway is one of the most potent prosurvival signaling pathways in cancers (28). The aberration of AKT signaling pathway is involved in tumorigenesis and progression, such as liver, breast, prostate, lung, colorectal and gastric cancers (29). It is reported that the activation of AKT signaling pathway is associated with various clinicopathologic characteristics of cancer (30). AKT affects the function of generous substrates associated with cell cycle progression via direct phosphorylation of target proteins or indirectly controlling protein expression levels (29). AKT downstream regulators Cyclin D1 and CDK2 are crucial transcriptional factors in the G0/G1 phase (31). Cyclin D-CDK4/6 and Cyclin A-CDK2 protein kinase complexes could regulate the cellular progression from G1/G0 phase to S phase o (32). Previous studies found that Cyclin D1 and CDK2 were involved in the human tumorigenesis (33). Our results demonstrated that miR-637 overexpression and P-REX2a siRNA could inhibit the expressions of cyclin D1 and CDK2, induced G1-S phase arrest through suppressing AKT signaling pathway. On the contrary, anti-miR-637 and P-REX2a overexpression promoted the expression of cyclin D1 and CDK2, drove more cells into the S phase by activating AKT signaling pathway. Our findings suggest that miR-637 inhibits G1-S phase transition through suppression of AKT signaling pathway by targeting P-REX2a.

The growth rate of cancer tissues is determined by proliferative and cell death. An imbalance between apoptosis related proteins, such as Bcl-2 and Bax, may induce dysregulation of apoptosis, which lead to oncogenesis and tumour development. Pro-apoptotic members such as Bax induce the release of cytochrome c and cause mitochondrial dysfunction. On the contrary, anti-apoptotic members such as Bcl-2 paly protectors of the outer membrane and preserve its integrity by inhibiting the release of cytochrome (34). Therefore, a critical determinant of the intrinsic apoptosis pathway is the balance between the ratio of Bax and Bcl-2 expression (35). Similar to what has been previously shown (36), we also provide evidence that miR-637-induced PI3K/AKT pathway plays a crucial role in the governance of the Bcl-2 and Bax family.

In conclusion, our study demonstrates that miR-637 functions as a tumor suppressor gene in melanoma. We find that miR-637 is downregulated and associated with clinicopathologic characteristics of melanoma patients. miR-637 inhibits melanoma cell proliferation via suppression of PTEN/PI3K/AKT signaling pathway and induces apoptosis through regulation of Bcl-2/Bax expression by targeting P-REX2a. These findings suggest that miR-637 plays a significant role in melanoma progression, and may serve as a potential novel target for melanoma therapy.

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Conflict of interest

The authors declare no conflicts of interest.

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