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Overexpression of SLC25A15 is involved in the proliferation of cutaneous melanoma and leads to poor prognosis

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> Melanoma is a skin tumor with a high degree of malignancy, poor prognosis and few effective therapies. Deprivation of the arginine from cancer cells through transport inhibition and arginine depletion is a novel strategy for cancer therapy. In this study, we have investigated the effect of *SLC25A15*, which encodes the mitochondrial ornithine carrier 1, on melanoma progression. Using bioinformatics methods to screen the data from TCGA and GEO, we found that *SLC25A15* is overexpressed in patients with melanoma and negatively related with the overall and disease-free survival rates. Knockdown the expression of *SLC25A15* by siRNA could effectively inhibit the proliferation of A375 melanoma cells, as detected by CCK8 and colony formation. Furthermore, *SLC25A15* siRNA was able to promote apoptosis of A375 cells, which exhibited decreased expression levels of the anti-apoptotic protein Bcl-2 while showing increased pro-apoptotic protein Bax and cleaved caspase-3. All these results suggest that the overexpression of *SLC25A15* is involved in the progression of melanoma and may predict the prognosis of melanoma. This may shed new lights on the diagnosis and therapy of melanoma in the future. <

Key words: A375 cells, apoptosis, cutaneous melanoma, proliferation, *SLC25A15*.

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Introduction

Melanoma is a skin tumor with a high degree of malignancy and poor prognosis. The incidence of melanoma has increased continuously over the past few decades [1-4]. Although surgery and adjuvant therapy can improve the prognosis of early-stage melanoma patients, the effects of these treatments are unsatisfactory for patients with tumor metastases. Chemotherapy is an important treatment for malignant melanoma; however, its therapeutic effect and indication are limited by its side effects and drug resistance properties [1, 2]. Although the use of monoclonal antibodies directed against immune checkpoints (ICP) has led to major clinical responses in patients with metastatic melanoma in the recent years, only a limited number of them have benefited of this therapeutic breakthrough. Therefore, defining the pathogenesis of melanoma and targeting key molecules involved in this process are the future directions for melanoma therapy.

The metabolic differences between normal cells and tumor cells have been accepted as one of the key marks for cancer progression [5, 6]. Most of the tumor cells need additional nutrients to satisfy the elevated macromolecular biosynthesis and ATP for survival, which has inspired the development of metabolism-based therapies for cancers [7-9]. Among the numerous candidate targets, arginine, a semi-essential amino acid, and its relationship with cancer development has been studied for many years [10-12]. Arginine can generate many metabolites, including nitric oxide, urea, citrulline and ornithine, which are involved in the regulation of key metabolic, immunological, neurological and signaling cellular pathways and affect cell growth, proliferation and survival. Deprivation of arginine from cancer cells through transport inhibition and depletion has been a novel strategy developed for cancer therapy and has shown a promising efficacy against arginine-auxotrophic tumors [13, 14].

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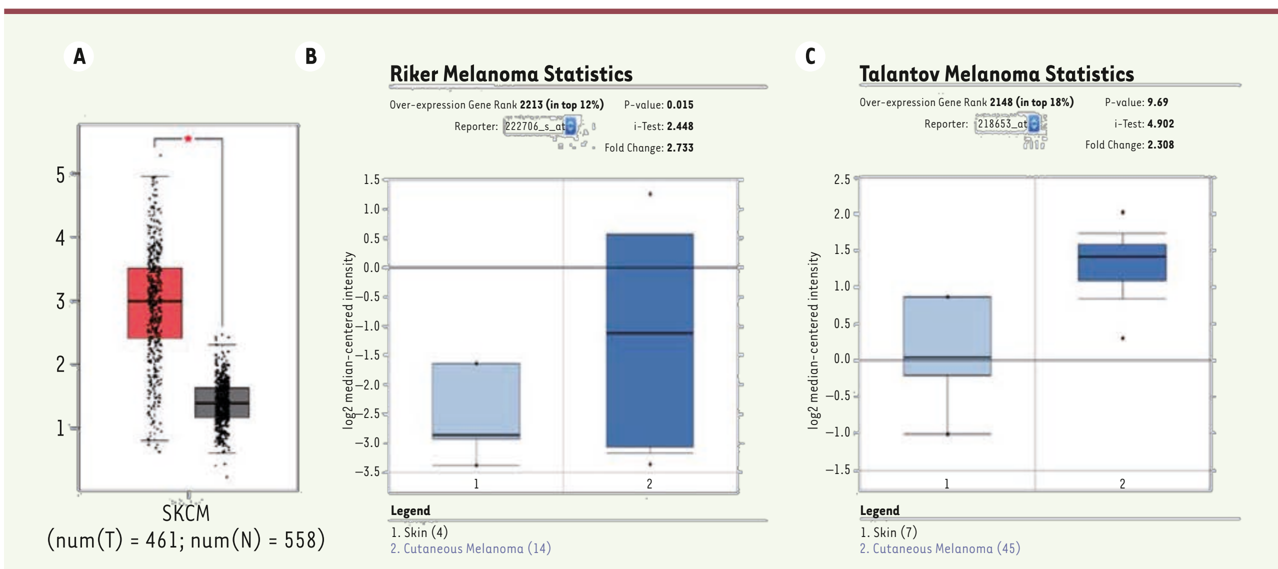

Figure 1. Overexpression of SLC25A15 in melanoma patients from TCGA and Oncomine databases. A. Data from TCGA database. Tumor group: red column; non-tumor group: gray column. ***B-C:*** data from Oncomine database (Riker [21] and Talantov [22]) are plotted. The boxes represent data from the 25th to 75th percentiles. The horizontal lines are the medians. The whiskers represent the 10th and 90th percentiles, respectively.

In addition to imported extracellular arginine, the primary intracellular arginine is de novo synthesized from citrulline through the ornithine cycle [15]. A 301 amino-acid long mitochondrial ornithine carrier 1 (ORC1) is the key transporter for cytosolic ornithine into the mitochondrial matrix in exchange for mitochondrial citrulline [16, 17]. ORC1 is encoded by the solute carrier family 25, member 15 gene (*SLC25A15*), which locates on chromosome 13q14 [16]. Some mutations in the *SLC25A15* gene have been reported to break the ornithine cycle and be related with human hyperornithinemia, hyperammonemia, and homocitrullinuria syndrome [15, 18, 19]. However, whether the *SLC25A15* gene is involved in the progression of cancers, especially melanoma, is still unknown.

In this study, we first detected the differential expression of *SLC25A15* in melanoma patients and normal controls. Second, we analyzed the relationship between the expression of *SLC25A15* and the prognosis of melanoma patients. Finally, we investigated the roles of *SLC25A15* in melanoma cell proliferation and apoptosis. Our study may shed some new lights on the diagnosis and therapy of melanoma in the future.

Materials and Methods

Analysis of TCGA and Oncomine Data

To determine the expression pattern of *SLC25A15* in melanoma, the datasets in The Cancer Genome Atlas (TCGA) and Oncomine database (https://www.oncomine.org) were used. Briefly, we used GEPIA (Gene Expression Profiling Interactive Analysis), a web-based tool to perform the differential gene analysis (Tumor: n=461; Normal: n=558) and survival analysis based on Skin Cutaneous Melanoma (SKCM) data in TCGA [20]. Moreover, *SLC25A15* gene was queried in Oncomine and the results were filtered by selecting melanoma vs. Normal Analysis.

Cell culture

The cell lines used throughout this study included human cutaneous melanoma (A375, MV3) and normal human primary epidermal melanocytes (HEMa), which were all purchased at the ATCC (USA). Cells were routinely cultured in a Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™, USA) at 37 °C in a 5 % CO_2 humid atmosphere, supplemented with 100 U/mL penicillin, 0.1mg/mL streptomycin and 10% heat-inactivated fetal calf serum. Once the adherent cells reached the logarithmic phase of growth, a trypsin solution was added onto the cell layer to get single cell suspensions for further experiments.

Cell transfection

When cell density in the culture plate reached about 80%, they were transfected with small interfering *SLC25A15* RNA (si-*SLC25A15*, F: 5'-GUGGAAAUACGAAUCAAGC-3'), or non-specific control si-RNA (si-con) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After a 48 h culture, the interference effect of si-RNA was evaluated by quantitative real-time reverse transcription-PCR (qRT-PCR) and the cells were used in the subsequent experiments.

qRT-PCR assay

After 48 h, total RNA of transfected cells were extracted by 1 mL Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. For

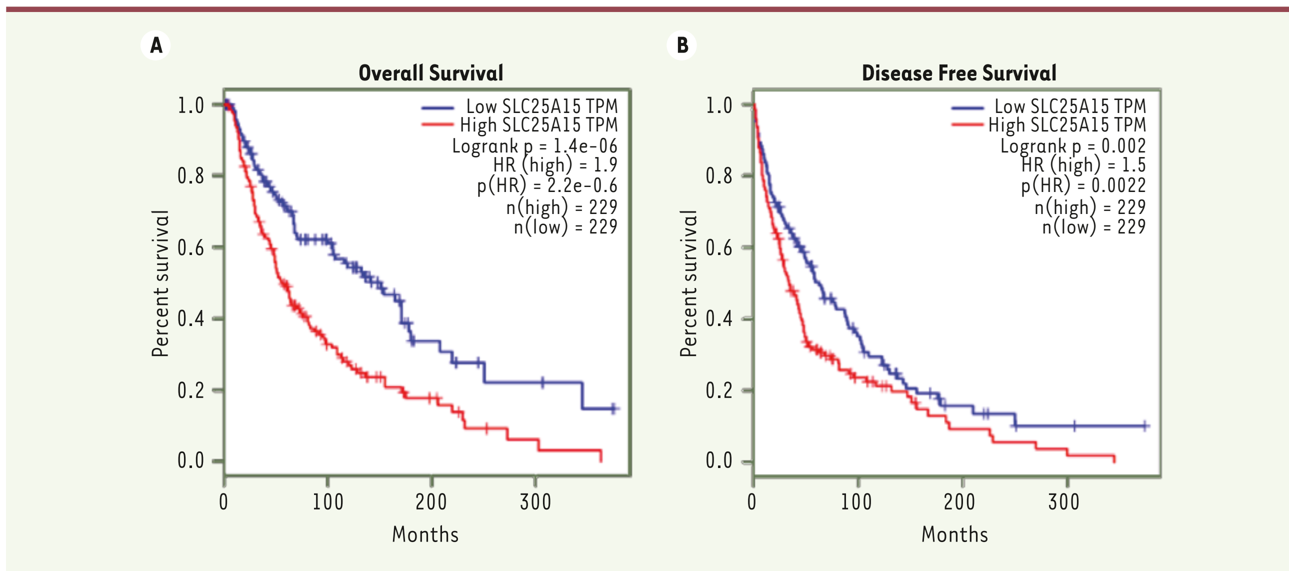

Figure 2. ***Poor prognosis of SLC25A15 overexpression in melanoma patients from TCGA database. A.*** *SLC25A15* high expression is associated with a poor overall survival in melanoma patients. ***B.*** *SLC25A15* high expression is associated with a poor disease-free survival in melanoma patients. Kaplan–Meier plots of survival were generated by the software from Gepia (http://gepia.cancer-pku.cn) using the data from TCGA.

cDNA synthesis, 1 μg of total RNA was used. The expression levels of *SLC25A15* were assessed by qRT-PCR using SYBR Premix Ex Taq™ kit (Applied Biosystems, Foster City, CA, USA). The qRT-PCR reactions started at 95°C for 5 minutes, followed by 95 °C for 30 seconds with 40 cycles, then 60 °C for 45 seconds, finally 72 °C for 30 minutes. GAPDH (Forward, F: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse, R: 5'-GGCTGTTGTCATAC TTCTCATGG-3') was used as an internal control. Specific sequence primers used were *SLC25A15* (F: 5'-CCTGAAGACT-TACTCCCAGGT-3', R: 5'-GCGATGTTGGCGATTAGTGC-3'). All reactions were performed in triplicate. Data analysis was performed according to the $2^{-\Delta\Delta}$Ct method.

Cell proliferation assay

Forty-eight hours after transfection, cells were trypsinized and counted. 100μl cell suspension (1,000 cells/well) were then seeded into 96-well plates and cultured in 5% CO_2 incubator at 37 °C. Cell viability was tested every 24 hours. For each test, 10 μL CCK-8 (Beijing Solarbio science & technology co., ltd.) reagent were added into the wells and incubated for 1.5 h at 37 °C. Proliferation curve was plotted based on optical density (O.D.) values measured using a microplate reader at 450 nm (Bio-Rad, Hercules, CA, USA). The experiments were repeated for three times.

Colony formation assay

Cell suspensions (500 cells/plate) were seeded into 60 mm plate with 5 mL culture medium 48 h post- transfection. The plate was gently shaken to ensure an even distribution of the cells. After 2 weeks, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for another 30 min. Cell colonies were counted under a microscope in 5 random view fields. This experiment was repeated in triplicate.

Western blot analysis

Cells were harvested 48-h post-transfection. Total proteins were extracted by Radio Immunoprecipitation Assay (RIPA) Buffer (Beyotime Inc., Shanghai, China). The protein concentrations were then quantified by the BCA Protein determination method (Beyotime Inc., Shanghai, China). Proteins (20μg / lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking non-specific binding sites with 5% non-fat dried milk for 1 h, the membranes were incubated with primary antibodies (ORC1, 1:1000, Invitrogen; Bcl-2, 1:1000, Bax, 1:1000, caspase-3, 1:1000; β-actin, 1:5000, all from Proteintech) overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibody at room temperature for 1 h. Finally, the protein bands were visualized using ECL reagents (Pierce). β-actin was used as a house-keeping protein and the relative expression was calculated as a target protein / β-actin ratio. Colored bands were scanned and analyzed by QUANTITY ONE software.

Statistical analysis

Kaplan-Meier method and Log-rank test were used for survival analysis. The comparison of two groups was

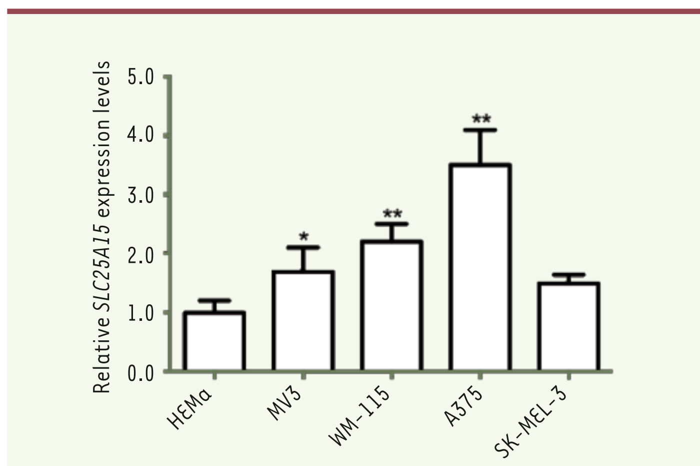

Figure 3. ***Overexpression of SLC25A15 in melanoma cell lines by q-PCR.*** Data are represented as means ± S.D. of three independent experiments. * $P<0.05$, ** $P<0.01$ compared with the HEMa group.

performed by Student's t-test. When more than two groups were compared, the One-way ANOVA analysis was used. All statistical analyses were performed using the SPSS 22.0 (SPSS Inc., Chicago, IL, USA) software. The data were presented as mean ± standard deviation (SD). $P<0.05$ was considered statistically significant.

Results

Up-regulation of *SLC25A15* expression in melanoma

First, we analyzed the SLC25A15 mRNA expression between melanoma and normal skin tissues using the publicly available database TCGA and Oncomine. From the data of TCGA, we found that the expression of *SLC25A15* was up-regulated significantly in the melanoma group as compared with the normal group ($P<0.05$, *Figure 1A*). Moreover, we performed an expression analysis of *SLC25A15* using two datasets from Riker [21] and Talantov [22] melanoma cohorts from Oncomine. In both cohorts, the expression of *SLC25A15* mRNA was significantly increased in melanoma tissues as compared to normal skin tissues *(Figure 1B-C)*. Second, the relationship of *SLC25A15* overexpression with prognosis in all 456 melanoma patients was also analyzed. Based on the median value of *SLC25A15* expression, patients were divided into two groups with high (n = 229) and low (n = 227) *SLC25A15* levels, respectively. The results showed that patients with high *SLC25A15* mRNA expression had shorter overall and disease-free survival times than those with low *SLC25A15* (both $P<0.01$, *Figure 2A-B*). These results suggested that overexpressed *SLC25A15* is correlated with a poor prognosis in melanoma patients.

SLC25A15 is overexpressed in cutaneous melanoma cells

To further characterize the expression of *SLC25A15* in melanoma, we analyzed its mRNA level in normal melanocytes and some cutaneous melanoma cell lines, including A375, MV3, WM-115 and SK-MEL-3, using qRT-PCR. As shown in *Figure 3*, the expression of *SLC25A15* in cells from the melanoma cell lines was higher than in the HEMa normal melanocytes (all $P<0.01$). These results obtained in melanoma cell lines were consistent with the conclusion draw from database analyses. Moreover, the expression of *SLC25A15* in A375 cells was the highest among all tested melanoma cell lines. Therefore, in the next in vitro experiments, A375 cells were used.

Decreased proliferation of *SLC25A15*-knocked down A375 cells

For better evaluating the effect of *SLC25A15* on melanoma progression, a SLC25A15 siRNA (si-*SLC25A15)* was transfected into A375 cells. Forty-eight hours later, the expression of *SLC25A15* RNA and protein in A375 cells was detected by qRT-PCR and western blot to assess the effect of si-*SLC25A15*. Our results showed that *si-SLC25A15* could significantly reduce the RNA and protein expression levels of *SLC25A15* in A375 cells, and the knockdown efficiencies were over 70% (*Figure 4A-C*, $P<0.01$). Then we studied the effect of *SLC25A15* on cutaneous melanoma cells by using the siRNA. Firstly, cell proliferation activity was examined using a CCK-8 assay. Silencing of *SLC25A15* remarkably inhibited the viability of A375 cells as compared to the control at 72 h ($P<0.05$) and 96 h (*Figure 4D*, $P<0.01$). Subsequently, a colony formation assay was also performed. We found that A375 cells transfected with *SLC25A15* siRNA formed fewer clones than the control (*Figure 4E-F*, $P<0.01$). These data suggest that *SLC25A15* may play a promoting role in melanoma proliferation.

Decreased expression of *SLC25A15* promotes the apoptosis of A375 cells

Given the fact that the apoptosis plays an important role in controling cell proliferation, we then tested the expression of apoptosis-related proteins in A375 cells with or without siRNA treatment. The western blot results showed that the expression levels of the anti-apoptotic protein Bcl-2 was decreased; by contrast, the pro-apoptotic protein Bax and cleaved caspase-3 levels were increased (*Figure 5A-B*, all $P<0.01$). These results indicate that *SLC25A15* could inhibit the apoptosis of A375 cells.

Discussion

To our knowledge, there is no study showing the effect of *SLC25A15* in melanoma. In this study, for the first time, we identified an overexpression of *SLC25A15* in melanoma, which was negatively related with the survival rate of patients. Moreover, we also found an up-regulation of *SLC25A15* in some melanoma cells com-

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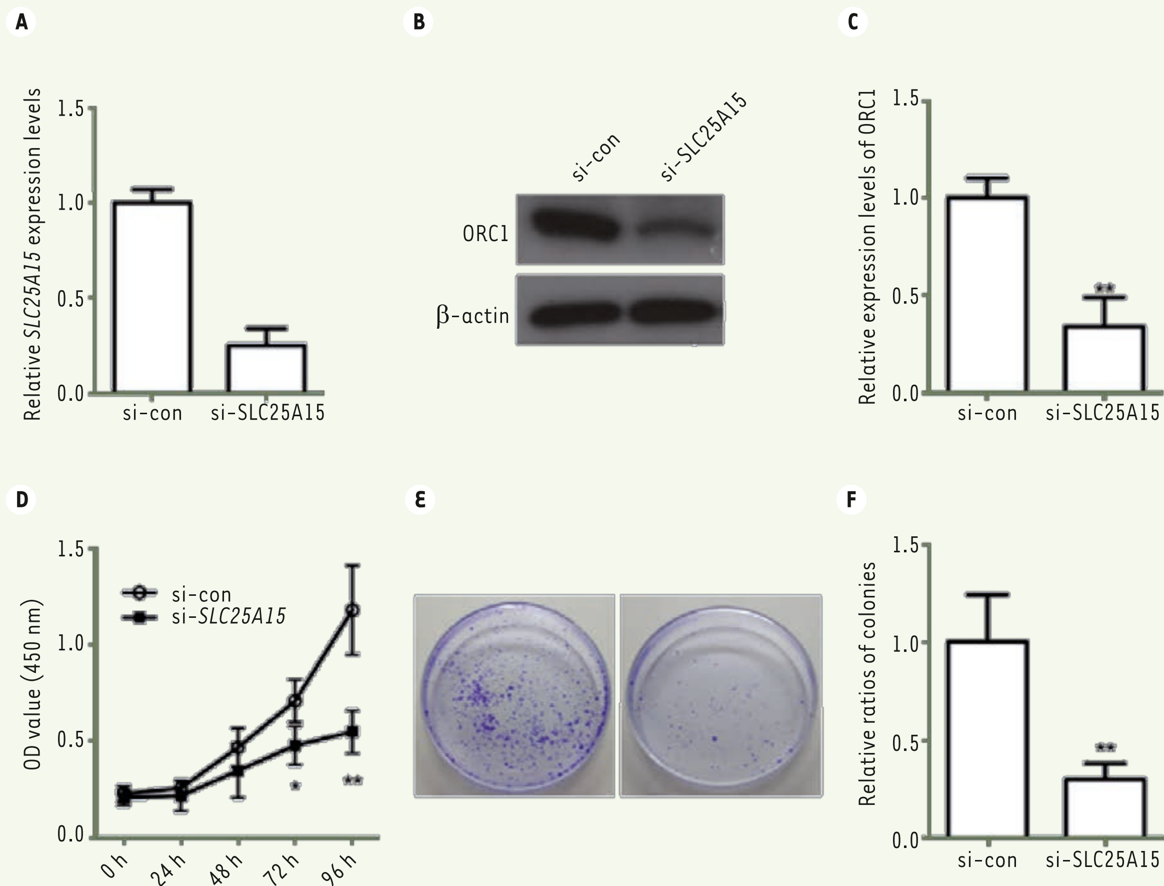

Figure 4. ***SLC25A15 knockdown inhibits the proliferation of A375 cells. A.*** Relative expression levels of *SLC25A15* in A375 cells after treatment with small interfering RNA (q-PCR). ***B-C.*** Relative protein expression levels of ORC1 in A375 cells after treatment with small interfering RNA (Western blot). ***D.*** *SLC25A15* knockdown decreases the viability of A375 cells as shown by a CCK8 assay. E-F. *SLC25A15 k*nockdown inhibits colony formation of A375 cells in a colony formation assay. All values are presented as means ± S.D. * *P*<0.05, ** *P*<0.01 compared with si-con group. n=5 for each group. si-con, scramble control siRNA.

pared with the normal melanocytes. Importantly, *SLC25A15* knockdown inhibited the A375 cell proliferation and promoted their apoptosis.

Arginine is a crucial candidate for various molecular pathways and thus the accessibility of arginine can regulate key metabolic, neurological and immunological pathways of the cells [23]. Because of the rapid growth of cancer cells, their demand for arginine is very high, which causes a metabolic reprogramming in some cancers [24-26]. *SLC25A15* encoded ORC1 is important for the endogenous arginine biosynthesis, and the mutation of *SLC25A15* could lead to the unbalance of arginine metabolism and the appearance of the HHH syndrome [15]. In our study, we found that the overexpression of *SLC25A15* in melanoma is necessary for the proliferation of A375 melanoma cells. Previous reports have indicated that several types of tumors, including melanoma, have abnormalities in arginine metabolism-related enzymes, particularly arginine-succinate synthase (ASS), and depend primarily on extracellular arginine to support necessary biological processes [27-29]. The appearance of *SLC25A15* overexpression in melanoma might accelerate the endogenous synthesis of arginine to compensate for the arginine auxotrophy. Moreover, we also found the overexpression is negatively related with the survival rate of patients. These results suggest that *SLC25A15* is a promising novel diagnostic marker and therapeutic target for human skin melanoma in the future.

Early findings suggest that exogenous arginine deprivation can induce apoptosis and cause some the death of cells from cancer cell lines, including breast cancer, prostate cancer, mesothelioma, and melanoma cell lines [30-33]. Although the signaling pathway responsible for this apoptosis is still unknown, it is believed that the apoptosis caused by arginine elimination could be activated through caspase-dependent and/or independent pathways [11]. In the present study, we also showed that the content in the anti-apoptosis pro-

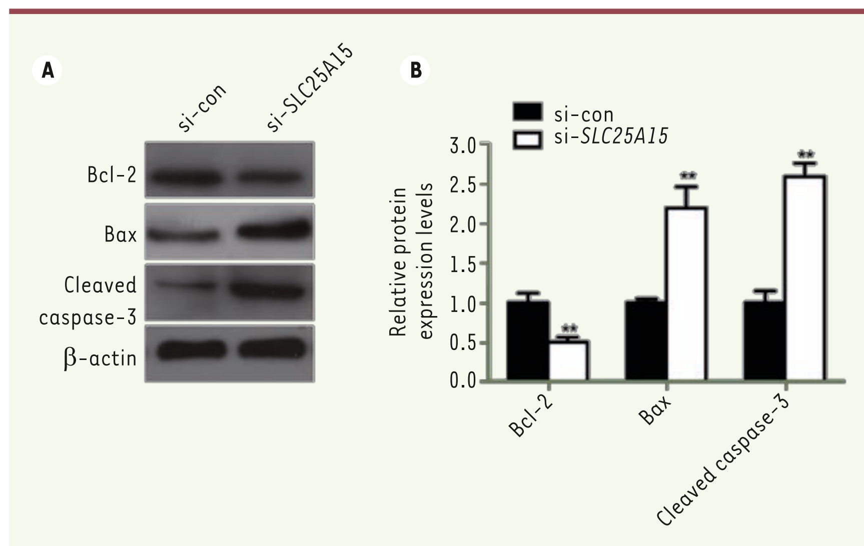

Figure 5. ***Silencing SLC25A15 promotes the apoptosis of A375 cells.*** ***(A)*** Representative images of the expression of Bcl-2, Bax and cleaved caspase-3 by western blot analysis. ***(B)*** Quantification of Bcl-2, Bax and caspase-3 protein expression. All values are presented as means ± S.D. ** *P*<0.01 compared with si-con group. n=5 for each group. si-con, scramble control siRNA.

tein Bcl-2 decreased, while those of the pro-apoptosis protein Bax and cleaved caspase-3 increased after *SLC25A15* knockdown. Our findings are consistent with previous reports and suggest that the targeting of *SLC25A15* may be a novel anti-melanoma method through promoting apoptosis.

In conclusion, our study firstly reported that the overexpression of *SLC25A15* is an important biomarker of melanoma development. The inhibition of the *SLC25A15* expression could effectively block the proliferation of A375 melanoma cells via promoting apoptosis. It might shed new lights on the diagnosis and therapy of cutaneous melanoma. However, our study is a preliminary study that needs more works to be done to clarify the roles and mechanisms of *SLC25A15* in melanoma. ◊

CONFLICT OF INTEREST

The authors state that there are no competing interests in this work.

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