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RLIP76 silencing inhibits cell proliferation and invasion in melanoma cell line A375

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Abstract. – OBJECTIVE: RLIP76 is a stress-responsive membrane protein regulating multiple cellular signaling pathways and is overexpressed in a number of malignant tumor cells. The aim of this study is to test whether the RLIP76 plays a critical role in cellular growth, apoptosis, cell cycle distribution and chemoresistance in melanoma.

MATERIALS AND METHODS: In this study, human melanoma cell line A375 was stably transfected with an RLIP76-targeted shRNA-containing vector to investigate the role of RLIP76 in cellular function. Quantitative RT-PCR and Western blot revealed significant suppression of RLIP76 in A375 cells after transfection with shRNA-containing vector. Cell proliferation was determined by MTT assay. Wound scratch ad transwell assays were used for cell migration and invasion.

RESULTS: The results showed that shRNA decreased the expression of RLIP76 in human melanoma cell line A375, and the knockdown of RLIP76 gene significantly inhibited A375 cell growth, resulted in G1 phase arrest and apoptosis increase of A375. Moreover, by testing the cell migratory and invasive abilities by wound scratch and transwell assays, it determined that the RLIP76 also suppressed cell migration and invasion in A375 cells and P13K/Akt signalling

CONCLUSIONS: Overall, this study suggests that RLIP76 is a potential therapeutic target against melanoma.

Key Words:
Melanoma, RLIP76, Apoptosis, Invasion.

Introduction

Malignant melanoma is an aggressive and deadly skin cancer accounting for a majority of skin cancer-related deaths and keeps a stable mortality rate[1]. The increased occurrence of malignant melanoma has been rising worldwide, especially in the past decades particularly in young people[2,3]. The high mortality and treatment resistance pose an enormous challenge[4]. Most of the advanced melanomas respond poorly to radiotherapy and chemotherapy. Effective therapies against the metastatic spread of melanoma are unavailable[5]. Therapy aims at the novel molecular targets, which potentially reduces the growth and progression of melanoma, may facilitate the development of an effective preventive or therapeutic strategy.

RLIP76 (DNP-SG ATPase) is a multi-functional protein involved in the ATP-dependent transport of glutathione conjugates and chemotherapy drugs[6-8]. The majority of early studies focused on the transporter activity of RLIP76. Accumulating evidences suggest that RLIP76 is involved in cell proliferation, metastasis and ligand-dependent receptor endocytosis[9-11]. Furthermore, RLIP76 is a stress-inducible non-ABC transporter that is overexpressed in most of the cancer cell lines and various human cancers[12]. RLIP76-targeted therapy using antibody, shRNA or antisense leads to durable and complete remission in xenografts of human lung and colon cancer[13] as well as prostate and kidney cancer[14, 15]. An early research by Singhal et al[16] showed that the administration of RLIP76 antibodies, siRNA, or antisense oligonucleotides of B16 melanoma cells in syngeneic mouse caused complete tumor regression within 10 days. However, the role of RLIP76 in the cellular activity of melanoma is not fully understood.

In this study, we tested the hypothesis that RLIP76 plays a critical role in cellular growth, apoptosis, cell cycle distribution and chemoresistance in melanoma. Our findings showed that the RLIP76 silencing by shRNA inhibited proliferation, induced apoptosis, blocked cell cycle progression and promoted the invasion of melanoma cells.

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Materials and Methods

Cell Lines and Cultures

The human melanoma A375 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 4 mM/L glutamine, 3.7 g/L sodium bicarbonate, 4.5 g/L glucose and 10% fetal bovine serum. Cells were maintained in a 5% CO_2 humidified incubator at 37ºC.

Interference Vector Construction and Transfection

The shRNA oligos for *RLIP76* gene knockdown were designed and synthesized as previously described[16]. Three different shRNA sequences and one scramble sequence as control were inserted into the plasmid vector pGCsi-H1. The resulting plasmids were designated as pGCsi-H1-RLIP76-1, -2, -3 and -control, respectively. The A375 cells in logarithmic growth phase were seeded in a 6-well plate and transfected with plasmids as previously described[17]. The resulting cell lines were designated as A375/pGCsi-H1-RLIP76-1, -2, -3 and -control, respectively. After 48-72 h post-transfection, the transfection efficiency was examined under florescence microscopy. RT-PCR and Western blot were used to determine the inhibitory activity. The vector containing shRNA sequences with greater than 70% inhibitory rate (pGCsi-H1-RLIP76) and the vector containing control sequence (pGCsi-H1-control) were selected for the subsequent experiments.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the cells using RNAsimple Total RNA Kit (TIANGEN Co., Beijing, China). The primer was synthesized based on published sequence[17]. Quantitative RT-PCR was performed as previously described [19] by using a SYBR Green reaction kit (TaKaRa, Dalian, Shenyang, China) and an Exicycler 96 RT-PCR machine (BIONEER co., Daejeon, Korea). Briefly, the first-strand cDNA was reversely transcribed from 1 μg total RNA by using the super M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (BioTeke Co., Beijing, China). For each PCR reaction, a master mix was prepared that included SYBR GREEN mastermix (Solarbio Co., Beijing, China), forward primer, reverse primer, and 10 ng template cDNA. The PCR conditions were 5 min at 95ºC followed by 40 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s. The products were stored at 4ºC.

Western Blot

Cells were washed with ice-cold PBS (phosphate-buffered saline) and harvested in 100 μL of cell lysis buffer (Cell Signaling, Danvers, MA, USA) containing protease inhibitors (Sigma-Aldrich, St. Louis, MI, USA). Extracted proteins were separated on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Proteins were probed with specific antibodies following standard protocol. The secondary antibodies used in this work were goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP and donkey anti-goat IgG-HRP (Beyotime Institute of Biotechnology, Shanghai, China).

Cell Proliferation Assay

Cell proliferation was determined by methyl thiazolyl tetrazolium (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, cells were plated at a density of 5×10^3 cells/well in 96-well culture plates. After treatment, 20 μL of MTT (5 mg/mL in PBS) was added to each well and incubated for 2 h. MTT formazan was dissolved in 150 μL of isopropanol, and the absorbance was measured at 595 nm with an ELISA reader (Tecan Group Ltd, Männedorf, Switzerland).

Cell Cycle Analysis

Cell cycle analysis was conducted using Cell Cycle and Apoptosis Analysis Kit (Beyotime, Shanghai, China) following manufacturer's instructions at 48 h after transfection. Briefly, cells were collected and fixed with 70% cold ethanol at 4ºC overnight. DNA was stained with propidium iodide (0.05 mg/mL) and RNase (2 mg/mL) for 30 min at room temperature. A FACScan cytometry (Beckman Coulter Inc., Miami, FL, USA) was used to analyze cells. The percentage of cells in G_0/G_1, S, and G_2/M phases of the cell cycle was assessed with Cell Lab Quanta SC Software.

Cellular Apoptosis

The effect of RLIP76 knockdown on apoptosis was evaluated using flow cytometry and Hoechst staining as previously described. 1) Flow cytometry analysis: Cellular apoptosis was determined using an FITC Annexin V apoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to manufacturer's instructions. In brief, cells were

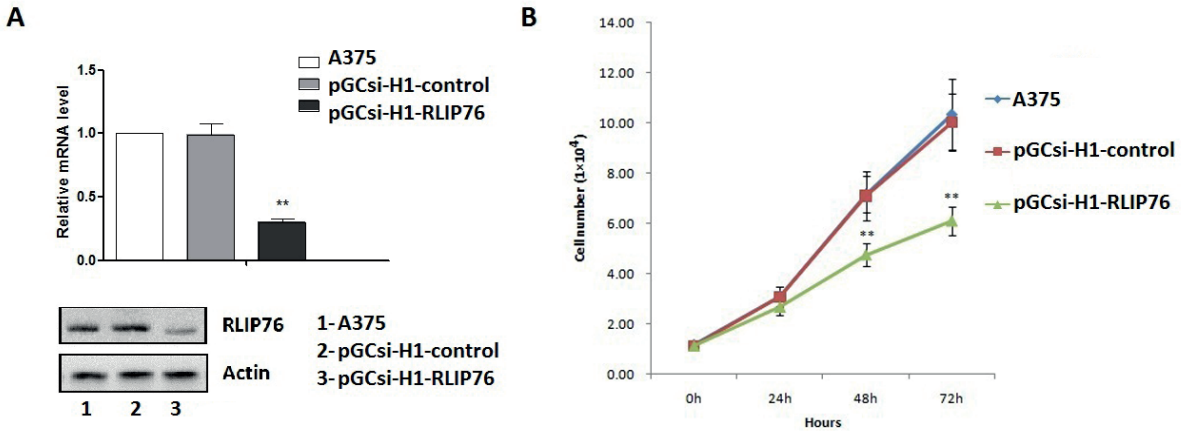

Figure 1. Effect of RLIP76 knockdown on cell growth (**p<0.01 *vs.* control).

washed with ice-cold phosphate-buffered saline and re-suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. Cells were stained with annexin V-FITC and propidium iodide (PI) for 15 min in the dark before flow cytometry (Beckman Coulter Inc., Miami, FL, USA).

Wound Scratch Assay

Stably transfected cells were seeded into 6-well plates and cultured to 90% confluence. The confluent cell monolayer was wounded using a sterile 200 µL pipette tip. The suspended cells were washed using normal growth medium. The scratch wound was captured after 24 h using an OLYMPUS IX81 microscope (Tokyo, Japan) in three fields of view at 100× magnification. The area of the open wound was quantified using Photoshop (Adobe, San Jose, CA, USA). The cellular migration was determined by the ratios of decreased open area after 24 h relative to the open area at 0 h. Three independent assays were performed.

Transwell Invasion Assay

24-well Transwells coated with Matrigel (8 µm pore size; BD Biosciences, San Jose, CA, USA) were used for cell invasion assays. Equal numbers (1×10^5) of non-transfected cells, as well as stably transfected cells, were plated in separate wells. Cells were cultured overnight in serum-free medium before trypsinization and re-suspended at a density of 2×10^5 cells/mL in DMEM containing 1% FBS. The cells were loaded into the upper chamber, with MEM (Modified Eagle Medium) containing 10% FBS as chemo-attractant in the lower chamber. The medium containing 1% FBS in the lower chamber was used as a control. The Matrigel and the cells remaining in the upper chamber were removed by cotton swabs following 24-h incubation. The cells in the lower surface of the membrane were stained with hematoxylin after the cells were fixed with formaldehyde solution. The cells in at least five random microscopic fields (×200) were counted and photographed.

Statistical Analysis

Three independent experiments were performed with the cells in independent cultures at three different times. All the data were expressed as mean ± SD. Results were evaluated by one-way ANOVA using SPSS13.0 software (SPSS Inc., Chicago, IL, USA) and followed by Dunnett's *t*-test. A value of p<0.05 was considered as statistically significant.

Results

shRNA Decreased the Expression of RLIP76 in Human Melanoma Cell Line A375

RLIP76 was knocked down with shRNA, using a GFP-containing vector pGCsi-H1. After transfection, more than 70% cells in each group were GFP-positive, indicating high transfection efficiency. The qRT-PCR and Western blot were used to confirm RLIP76 silencing. qRT-PCR analysis revealed a significant decrease in *RLIP76* transcription in cells transfected with pGCsi-H1-RLIP76 while no significant difference was found between pGCsi-H1-control and parental A375 cells. Western blot also revealed a significant decrease in *RLIP76* expression in the transfected cell lines pGCsi-H1-RLIP76 (Figure 1A).

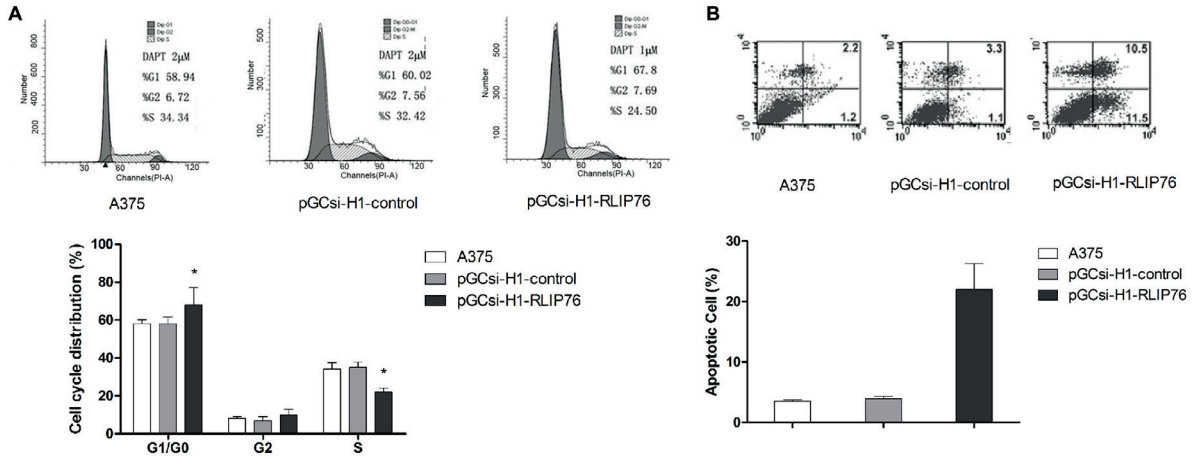

Figure 2. Effect of RLIP76 knockdown on cell cycle distribution and apoptosis (*p<0.05 *vs.* control).

Knockdown of RLIP76 Suppresses A375 Cell Growth

The effect of *RLIP76* knockdown on *in vitro* A375 cell growth was assessed by MTT assay at 0, 24, 48 and 72 h post-transfection. Compared with controls, the growth of shRNA-transfected cells was significantly inhibited in a time-dependent manner with the highest inhibitory rate of 41.0±3.5% observed at 72 h in A375/pGCsi-H1-RLIP76 cell lines, respectively (Figure 1B).

Knockdown of RLIP76 Arrested A375 Cells in the G1 Phase

To investigate whether *RLIP76* knockdown affected cell cycle regulation, flow cytometry was performed at 48 h post-transfection. As shown in Figure 2A, interference with *RLIP76* expression led to cell cycle arrest at G1 phase compared with controls. Also, the increase in G1 phase cell population was accompanied by a concomitant decrease in cell number during S-phase in both cell lines with *RLIP76* knockdown without altering the cell population in G2 phase. These results suggested that RLIP76 knockdown inhibited cell cycle at G1 phase and increased the A375 cell population in the proliferative S phase.

Knockdown of RLIP76 Increases A375 Cell Apoptosis

Our results showed that *RLIP76* knockdown inhibited cell growth and affected cell cycle distribution in A375 cells. Therefore, we examined whether knockdown of *RLIP76* enhanced apoptosis in A375 cell line using flow cytometry at 72 h after transfection. We found that the apoptosis rate was significantly higher in shRNA-transfected A375 cells compared with untransfected parental control or control-transfected cells (Figure 2B).

Knockdown of RLIP76 Suppresses Cell Migration and Invasion in A375 Cells

We tested the cell migratory and invasive abilities using the wound scratch and transwell assays to determine the role of *RLIP76* knockdown in migration and invasion *in vitro*. The results of wound scratch migration assay (Figure 3A) showed that the A375 cells with RLIP76 knockdown migrated significantly slower than the control cells at 24 h. Furthermore, the transwell invasion assay showed that *RLIP76* knockdown significantly impaired the invasive abilities of A375 cells compared with the control cells (Figure 3B).

Knockdown of RLIP76 Suppresses PI3K/Akt Signaling

Next, we examined the downstream signaling modulated by RLIP76 silencing. Our results showed that RLIP76 knockdown was associated with the suppression of PI3K-AKT pathway in A375 cells, suggesting that RLIP76 exerted anti-tumor effect in vitro in A375 cells by inhibiting PI3K/Akt signaling (Figure 4).

Discussion

Some papers[12-15] have reported that RLIP76 overexpression is a common feature of malignant cancer cells. Previously, *in vitro* studies demonstrated RLIP76 expression in melanoma cell lines.

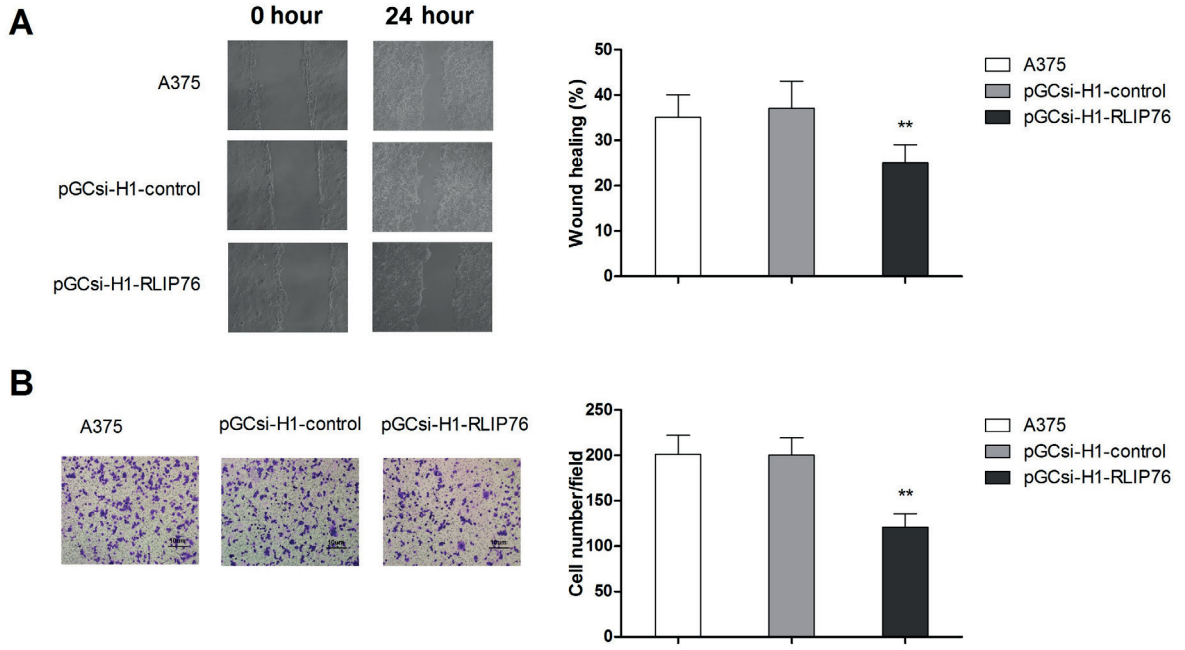

Figure 3. Effect of RLIP76 knockdown on cell migration and invasion ($^{**}p<0.01$ *vs.* control).

In vivo researches showed that the administration of RLIP76 antibodies, siRNA, or antisense oligonucleotides to mice bearing the syngeneic B16 mouse melanoma cells caused complete tumor regression within 10 days[16]. Therefore, we designed this work to investigate the cellular function of RLIP76 to elucidate the mechanism of melanoma formation.

In the present study, shRNA was used to silence *RLIP76* in human melanoma A375 cell lines. Results from the qRT-PCR and Western blot assays confirmed the efficient suppression was due to the selected shRNA-containing vector. The effect of RLIP76 knockdown on cell growth of A375 cells was explored. MTT assay results showed that transfection with RLIP76-targeted shRNA significantly inhibited cell growth in a time-dependent manner. Singhal et al[13] reported that RLIP76 suppresses tumor growth via Ral or Ras-R signaling pathway or through the regulation of the expression of heat shock proteins. Subsequently, Leake et al[20] also demonstrated that RLIP76 regulates P13/Akt signaling pathway, which is important in signal transduction via upstream growth factor receptors. Therefore, cell growth inhibition induced by *RLIP76* knockdown may be a result of crosstalk in multiple signaling.

Accumulating evidences suggest that RLIP76 regulates cell cycle progression. Wang et al[17] reported that the inhibition of RLIP76 expression arrests glioma cells in the G1 phase. Yao et al[21] also found that the reduced RLIP76 expression by shRNA results in cell cycle arrest at G1 phase in leukemia cells. According to previous investigations, our results showed that the RLIP76 inhibition in A375 cells resulted in G1 phase arrest and decreased the percentage of cells in S phase, further supported the functional role of RLIP76 in cell cycle progression.

Previous studies demonstrated the role of RLIP76 as an anti-apoptotic GS-E and drug transporter in solid tumors[16]. The anti-apoptotic role of RLIP76 was reported in a number of human cancer cell lines such as glioma and leukemia cell lines[17,21]. Further, Wang et al[12] suggested that RLIP76 may suppress apoptosis and promote the proliferation of glioma cells by direct ATP-dependent xenobiotic transport and activation of Rac1-JNK signaling pathway. In this paper, we also found that the RLIP76 knockdown in A375 cells significantly increased apoptotic cells compared with controls, which was consistent with the previous findings.

The role of RLIP76 in metastasis has been studied in a variety of *in vitro* and *in vivo* models. *In vitro* studies in colon cancer and glioma cells showed that RLIP76 knockdown suppressed cell invasion[17,22]. Lee et al[23] observed that RLIP76

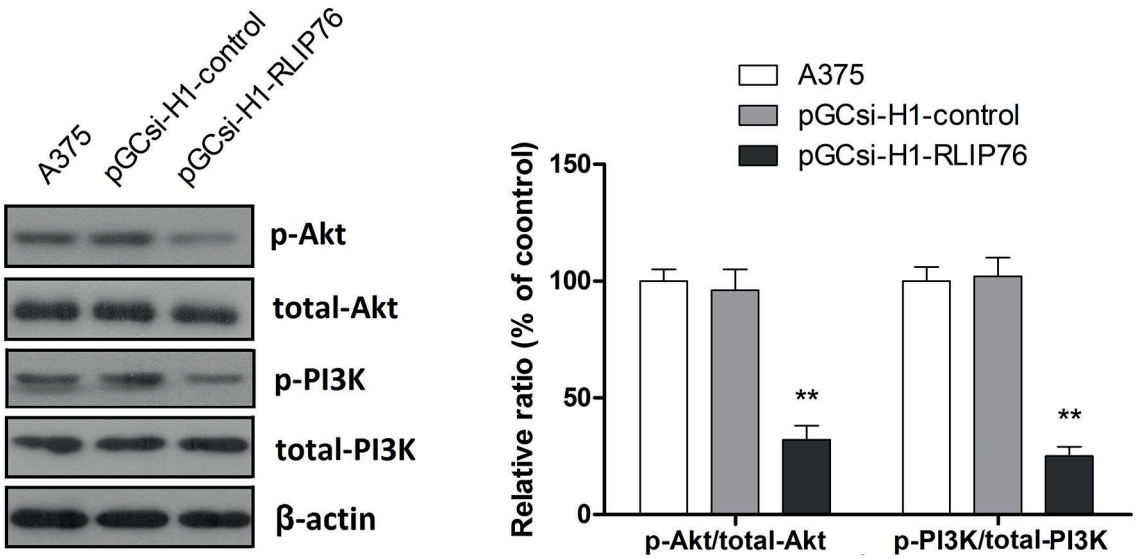

Figure 4. Effect of RLIP76 knockdown on PI3K/Akt signaling (**p<0.01 *vs.* control).

regulates tumor cell transactivation of endothelial cells via control of VEGF expression and secretion, suggesting induction of angiogenesis. RLIP76 depletion inhibited tumor neovascularization in a mouse model of melanoma[24]. A recent study[25] releaved that ARNO (a guanine nucleotide exchange factor for Arf6) interacts with RLIP76 N-terminus and regulates cell spread and motility via PI3K and Arf6, independent of RLIP76 control of Rac.

Conclusions

We demonstrated that the RLIP76 knockdown with shRNA in human melanoma A375 cells resulted in anti-proliferative effect, induced apoptosis, caused cell cycle arrest, and suppressed cell migration and invasion. Results suggested that the RLIP76 knockdown might represent potential gene therapy for melanoma in conjunction with conventional chemotherapy.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Maddodi N, Setaluri V. Role of UV in cutaneous melanoma. Photochem Photobiol 2008; 84: 528-536.
2) Bottoni U, Paolino G, Didona D, Corsetti P, Clerico R, Cantisani C, Richetta AG, Arcidiacono V, Scali E, Pranteda G, Calvieri S. Improvement of survival in patients with melanoma and non-melanoma skin cancers compared to patients without double cutaneous malignancies. Eur Rev Med Pharmacol Sci 2015; 19: 1640-1644.
3) Strouse JJ, Fears TR, Tucker MA, Wayne AS. Pediatric melanoma: risk factor and survival analysis of the surveillance, epidemiology and end results database. J Clin Oncol 2005; 23: 4735-4741.
4) Eberle J, Kurbanov BM, Hossini AM, Trefzer U, Fecker LF. Overcoming apoptosis deficiency of melanoma-hope for new therapeutic approaches. Drug Resist Updat 2007; 10: 218-234.
5) Gogas HJ, Kirkwood JM, Sondak VK. Chemotherapy for metastatic melanoma: time for a change? Cancer 2007; 109: 455-464.
6) Singhal SS, Singhal J, Nair MP, Lacko AG, Awasthi YC, Awasthi S. Doxorubicin transport by RALBP1 and ABCG2 in lung and breast cancer. Int J Oncol 2007; 30: 717-725.
7) Singhal SS, Sehrawat A, Sahu M, Singhal P, Vatsyayan R, Rao Lelsani PC, Yadav S, Awasthi S. Rlip76 transports sunitinib and sorafenib and mediates drug resistance in kidney cancer. Int J Cancer 2010; 126: 1327-1338.
8) Singhal SS, Sehrawat A, Mehta A, Sahu M, Awasthi S. Functional reconstitution of RLIP76 catalyzing ATP-dependent transport of glutathione-conjugates. Int J Oncol 2009; 34: 191-199.
9) Yadav S, Zajac E, Singhal SS, Singhal J, Drake K, Awasthi YC, Awasthi S. POB1 over-expression inhibits RLIP76-mediated transport of glutathione-conjugates, drugs and promotes apoptosis. Biochem Biophys Res Commun 2005; 328: 1003-1009.

10) Singhal SS, Yadav S, Vatsyayan R, Chaudhary P, Borvak J, Singhal J, Awasthi S. Increased expression of cdc2 inhibits transport function of RLIP76 and promotes apoptosis. Cancer Lett 2009; 283: 152-158.

11) Hu Y, Mivechi NF. HSF-1 interacts with Ral-binding protein 1 in a stress-responsive, multiprotein complex with HSP90 *in vivo*. J Biol Chem 2003; 278: 17299-17306.

12) Wang Q, Wang JY, Zhang XP, Lv ZW, Fu D, Lu YC, Hu GH, Luo C, Chen JX. RLIP76 is overexpressed in human glioblastomas and is required for proliferation, tumorigenesis and suppression of apoptosis. Carcinogenesis 2013; 34: 916-926.

13) Singhal SS, Singhal J, Yadav S, Dwivedi S, Boor PJ, Awasthi YC, Awasthi S. Regression of lung and colon cancer xenografts by depleting or inhibiting RLIP76 (Ral-binding protein 1). Cancer Res 2007; 67: 4382-4389.

14) Singhal SS, Roth C, Leake K, Singhal J, Yadav S, Awasthi S. Regression of prostate cancer xenografts by RLIP76 depletion. Biochem Pharmacol 2009; 77: 1074-1083.

15) Singhal SS, Singhal J, Yadav S, Sahu M, Awasthi YC, Awasthi S. RLIP76: a target for kidney cancer therapy. Cancer Res 2009; 69: 4244-4251.

16) Singhal SS, Awasthi YC, Awasthi S. Regression of melanoma in a murine model by RLIP76 depletion. Cancer Res 2006; 66: 2354-2360.

17) Wang Q, Qian J, Wang J, Luo C, Chen J, Hu G, Lu Y. Knockdown of RLIP76 expression by RNA interference inhibits invasion, induces cell cycle arrest, and increases chemosensitivity to the anticancer drug temozolomide in glioma cells. J Neurooncol 2013; 112: 73-82.

18) Qin FX, Shao HY, Chen XC, Tan S, Zhang HJ, Miao ZY, Wang L, Hui C, Zhang L. Knockdown of NPM1 by RNA interference inhibits cells proliferation and induces apoptosis in leukemic cell line. Int J Med Sci 2011; 8: 287-294.

19) Wang J, Wang Q, Cui Y, Liu ZY, Zhao W, Wang CL, Dong Y, Hou L, Hu G, Luo C, Chen J, Lu Y. Knockdown of cyclin D1 inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human glioblastoma cells. J Neurooncol 2012; 106: 473-484.

20) Leake K, Singhal J, Nagaprashantha LD, Awasthi S, Singhal SS. RLIP76 regulates PI3K/Akt signaling and chemo-radiotherapy resistance in pancreatic cancer. PLoS One 2012; 7: e34582.

21) Yao K, Xing H, Yang W, Liao A, Wu B, Li Y, Zhang R, Liu Z. Knockdown of RLIP76 expression by RNA interference inhibits proliferation, enhances apoptosis, and increases chemosensitivity to daunorubicin in U937 leukemia cells. Tumour Biol 2014; 35: 8023-8031.

22) Zhang Y, Song X, Gong W, Zhu Z, Liu X, Hou Q, Sun Y, Chai J, Zou L, Guan J. RLIP76 blockade by siRNA inhibits proliferation, enhances apoptosis, and suppresses invasion in HT29 colon cancer cells. Cell Biochem Biophys 2015; 71: 579-585.

23) Lee S, Goldfinger LE. RLIP76 regulates HIF-1 activity, VEGF expression and secretion in tumor cells, and secretome transactivation of endothelial cells. FASEB J 2014; 28: 4158-4168.

24) Lee S, Wurtzel JG, Singhal SS, Awasthi S, Goldfinger LE. RALBP1/RLIP76 depletion in mice suppresses tumor growth by inhibiting tumor neovascularization. Cancer Res 2012; 72: 5165-5173.

25) Lee S, Wurtzel JG, Goldfinger LE. The RLIP76 N-terminus binds ARNO to regulate PI 3-kinase, Arf6 and Rac signaling, cell spreading and migration. Biochem Biophys Res Commun 2014; 454: 560-565.