RESEARCH COMMUNICATION

Inhibition of the NEDD8 Conjugation Pathway by shRNA to UBA3, the Subunit of the NEDD8-Activating Enzyme, Suppresses the Growth of Melanoma Cells

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Abstract

Neural precursor cell-expressed developmentally down-regulated 8 (NEDD8), a ubiquitin-like protein, mainly functions through covalent ligation to cullin proteins. Conjugation of NEDD8 with cullins can promote ubiquitination, which plays a critical role in the degradation of many proteins. UBA3 is the subunit of NEDD8-activating enzyme which is one of the keys for NEDD8 linkage to cullin proteins. Previous research showed NEDD8 conjugation to be up-regulated in highly proliferative cell lines. In the present study, up-regulated NEDD8 conjugation was observed in melanoma cell lines by Western blot analysis. After down-regulation with a RNAi to UBA3, proliferation of M14 was suppressed *in vitro* and *in vivo*. In conclusion, up-regulated NEDD8 conjugation may be involved in the development of melanoma. Interference in this pathway might offera promising method for melanoma therapy.

Keywords: Melanoma - growth - NEDD8 - ubiquitin - shRNA - conjugation

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Introduction

Ubiquitin (Ub) is responsible for labeling proteins which are degraded by the 26s proteasome (Hershko, 2005; Hjerpe et al., 2008). The process of poly-Ub chain linking to substrate is named ubiquitination. The process of ubiquitination requires the consecutive cascade action of three enzymes, Ub-E1, Ub-E2 and Ub-E3. Many proteins relating closely to apoptosis (Allende-Vega et al., 2010; Tsvetkov et al., 2010), cell-cycle regulation (Yu et al., 1998) and receptor regulation (Dace et al., 2000) can be labeled by Ub.

Neural precursor cell-expressed developmentally down-regulated 8 (NEDD8), which is 60% identical and 80% homologous to ubiquitin, is one member of ubiquitin-like protein superfamily (Wada et al., 2000). NEDD8 expressed conservatively in most eukaryotes or most, if not all, tissues (Rabut and Peter, 2008). 6-kDa NEDD8 monomer and a series of NEDD8-conjugated proteins could be detected (Kamitani et al.,1997; Gong and Yeh, 1999; Huang et al., 2009).

Regulating ubiquitination is the major function of NEDD8 conjugation. NEDD8 links to target proteins with the help of series of enzymes. First, NEDD8 is activated by APPBP1/UBA3 dimer (NEDD8-Activating enzyme), to form the thioester bond with cysteine residue of UBA3. Second, NEDD8 is transferred to UBC12. Finally, with the help of NEDD8-E3, NEDD8 loaded by UBC12 attach to the substrates by its carboxy-terminal glycine (Chairatvit and Ngamkitidechakul, 2007).

The major substrates of NEDD8 are members of cullin family. Cullin proteins act as the scaffold of the Skp/Cul/F-box protein (SCF) complex, which are the core of subunit of Ub-E3. Covalent conjugation of NEDD8 with cullins makes the structure of Ub-E3 change, which plays a critical role in promoting the process of ubiquitination (Wu et al., 2000).

For it could promote the process of ubiquitination which is correlative closely with apoptosis, cell-cycle regulation, the conjugation of NEDD8 to cullins may be involved in the development of tumors. Researchers studied the NEDD8 conjugation in some cancer cells. The results revealed that NEDD8 conjugation increased in highly proliferative cell lines (Brownell et al., 2010). Melanoma like other malignant tumors grows and metastasizes rapidly. In this report, we observed the NEDD8 conjugation in melanoma cells and studied whether inhibition of NEDD8 conjugation pathway would have effect on the proliferation of melanoma cells. To cut off the NEDD8 conjugation pathway, but did not influence the function of free NEDD8 as far as possible, we chose UBA3, the subunit of NEDD8-Activating enzyme, not NEDD8, as the target to interfere.

Materials and Methods

Cell lines and culture conditions

Human melanoma cell line A375 was kindly provided

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by XIJING hospital (Shanxi, China), and M14 and MV3 were purchased from KeyGEN (KeyGen Biotech 3F, Nanjing, China). The three cell lines were cultured in the DMEM medium (Gibco, Eggenstein, Germany) with 10% fetal bovine serum (Gibco, Eggenstein, Germany) at 37°C with 5% CO2. The normal melanocytes were separated from prepuce and cultured with M254 medium (Gibco, Eggenstein, Germany) containing with Human Melanocytes Growth Supplement (HMGS). The culture condition was same with the melanoma cell lines.

Western blot assay

After washed twice with PBS, total proteins were extracted from cells using lysis buffer (50 mM pH 8.0 Tris–HCl, 150 mM NaCl, 0.02% NaN3, 0.1% SDS, 1% nonidet P-40, 0.5% sodium deoxycholate, 100 mg/ml of phenylmethylsulfonyl fluoride, 1 mg/ml of aprotinin, 1 mg/ml of leupeptin, 1 mg/ml of pepstatin A). The concentration of protein was determined by spectrophotometer. Seventy micrograms of protein separated by 10 % SDS polyacrylamide gel electrophoresis (Bio-Rad, California, USA), and subsequently transferred onto PVDF membrane by electroblotting. PVDF membrane was stained with polyclonal anti-goat NEDD8 antibody, polyclonal anti-rabbit UBA3 antibody, anti-rabbit p53 antibody (Santa Cruz Biotechnology, California, USA), and β-actin antibody worked as loading control. Peroxidase-conjugated anti-rabbit IgG was used as secondary antibody and visualized by enhanced chemiluminescence method. The relative quantity of protein was quantified with Glyco Band-Scan software 4.5 (PROZYME, San Leandro, CA, USA).

Semiquantitative Real-time PCR

According to the manufacturer's protocol, total RNA was extracted respectively from A375, M14, MV3 and melanocytes, and reversely transcripted into cDNA with Reverse Transcription System (Promega, America). The purity and concentration of RNA were regulated by the radio of optical density (OD) at 260 nm and 280 nm. The specific primers for each gene were synthesized by GenScript (GenScript Inc., USA) and the sequences were as follows: NEDD8-Forward 5'-ACAGTGGCAAGCAGATGAATGA-3', NEDD8-Reverse 5'-ATGAGCGACAGGGTAAAGAGGT-3'; UBA3-Forward 5'-GCGAGGAGCCGGAGAAGAAAAG-3' UBA3-Reverse 5'-TCGAAATCAGGGTGTGTGAAGG-3'; β-actin-Forward 5'-GCAGAAGGAGATCACAGCCCT-3', β-actin-Reverse 5'-GCTGATCCACATCTGCTGGAA-3'. Reaction system: 10 μl 2 × real-time PCR Mix (TOYOBO, Japan), 10 pmol upstream and downstream primers, 1 μl DNA template were brought up to 20 μl with purified water. Reaction conditions: 95°C 5 min, 1 cycle; 95°C 15 s, 60°C 30 s, 72°C 30 s for 40 cycles. The size and quantity of amplified products were confirmed by 2% agarose gel electrophoresis. The thermal cycler's software package was used to calculate the DCt value to analysis the fluorescent quantity. The 2-DDCt was presented as the relative expression of the gene expression.

siRNA transfection

Three shRNA fragments against UBA3 were designed using Ambion and Qiagen software and were subcloned into pRNAT-U6.2/Lenti siRNA expression vector (Invitrogen, Amarica). The recombinant plasmid were identified by PCR and sequencing. The transfection was performed by Fugene HD (Roche, Switzerland) in Opti-DMEM medium without antibiotics. The final conditions were determined: 2 μg DNA mixed with 30 μl Fugen HD in a six-well plate with 2 lm culture medium. After 2 weeks of selection with 1000 μg/ml G418 (Invitrogen, America), G418-risistant clones were chosen. The clones, expressing the bright green fluorescent under fluorescent microscope, were picked out for further analysis.

Cell proliferation analysis

Cell proliferation was evaluated by the Cell Counting Kit-CCK8 assay (Dojindo, Japan). Nontransfected M14, M14/shRNAT-U6.2, M14/shRNA-UBA3 were seeded in 96-well plate separately with the concentration of 3 × 10^3/ml. After incubation for 24, 48 and 72 h, the cells were treated with fresh medium containing 10% volume of CCK-8 and incubated at 37°C for 4 h. The OD values at 450 nm were quantified by spectrophotometer. Assays were performed in three replicated wells and each experiment was repeated three times independently.

Subcutaneous tumor formation in nude mice

The animal care and usage protocol was carried out with approval from our Institution's Animal Care and Ethics Committee. Nontransfected M14, M14/shRNAT-U6.2, M14/shRNA-UBA3 were subcutaneously injected into nude mice (5 weeks of age) in the right axillae with 2 × 10^6 tumor cells. The animals were then monitored for tumor formation and growth. when the subcutaneous tumor xenografts were touchable, measured the size of xenografts with slide gauge every other day. At the end of the experiments, the tumor xenografts were excised for wet-weight determination.

Statistical analysis

Data were expressed as mean ± standard deviation (M ± SD). SPSS 13.0 statistic software package (SPSS Ins., USA) was used to perform independent samples t-test in CCK8 assay, real-time PCR analysis, and tumor volume and weight.

Results

NEDD8 conjugation in melanoma cells

According to previous report (Kamitani et al., 1997; Huang et al., 2009), NEDD8 conjugation showed a series of bands in a ladder-like pattern in which the band of 90 kDa, consistent with the molecular mass of NEDD8-cullins conjugation, was detected in all mammalian cells. In this test, western blot was performed to analyze the amount of NEDD8 conjugation in melanoma cells A375, M14, MV3 and melanocytes. As shown in Figure 1, the density of 90-kDa band in melanocytes was weaker than in the other three melanoma cell lines.

Expression of NEDD8 and UBA3 gene in melanoma cell

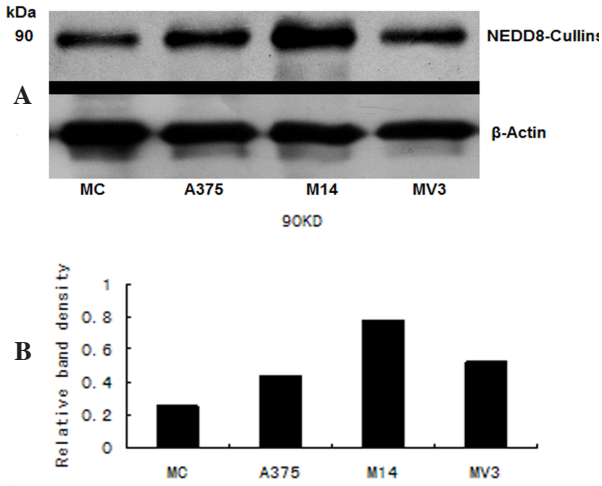

Figure 1. The NEDD8 Conjugation Levels in Cell Lines were Analyzed by Western Blot Assay, and the β-actin Level Worked as Loading Controls. (A) The relative density of NEDD8 at 90 kDa: 0.26 for melanocytes, 0.44 for A375, 0.79 for M14 and 0.53 for MV3. (B) The column diagram of relative density of NEDD8 conjugation in cells

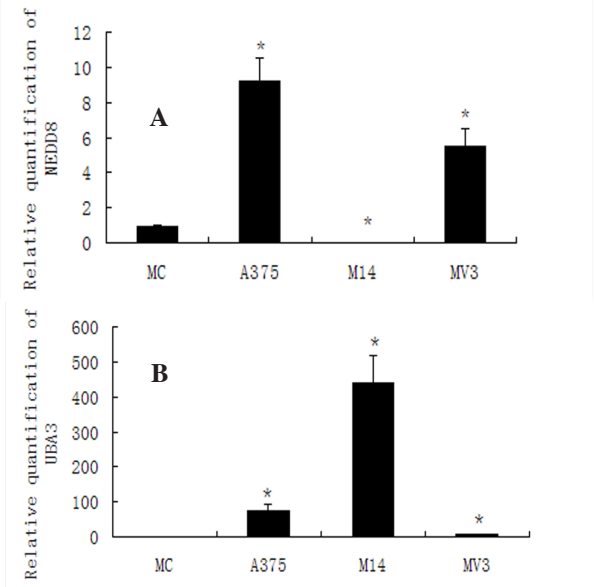

Figure2. The Relative Quantification of NEDD8 and UBA3 Gene in Melanoma Cell Lines and Melanocytes, with the β-actin Working as an Internal Reference. Statistical differences were analyzed with t-test. Significant differences were observed between every melanoma cell line and melanocytes. Every experiment was repeated three times. (Error bars = Standard Deviation, *P < 0.05 relative to the control of melanocytes)

lines and melanocytes

Gene expression of NEDD8 in three melanoma cell lines and melanocytes were test by real-time PCR . From the Figure 2A, we found that the expression of NEDD8 was higher in MV3 and A375 than that in melanocytes. However, in M14, in which the NEDD8 conjugation was the highest, the expression of NEDD8 was the lowest. Gene expression of UBA3 was higher in melanoma cell lines compared with it in melanocytes (Figure 2B). Contrary to NEDD8 gene, the UBA3 was the highest in M14. We chose M14 for further studies.

Picked out the stably transfected clone of shRNA-UBA3

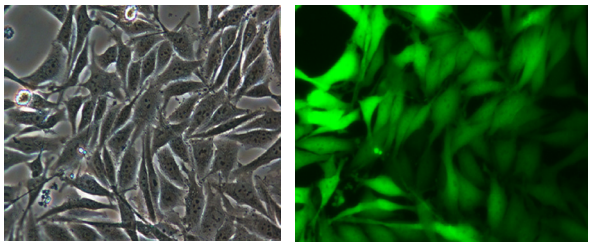

Figure 3. Stably Transfected Clones which Transfected with shRNA for UBA3. After transfected with shRNA-UBA3, selected with G418 and picked out the clones under fluorescence microscope, the cells expressing GFP was more than 98%. (A) The image observed under inverted microscope (10×40). (B) The image observed under fluorescence microscope (10×40)

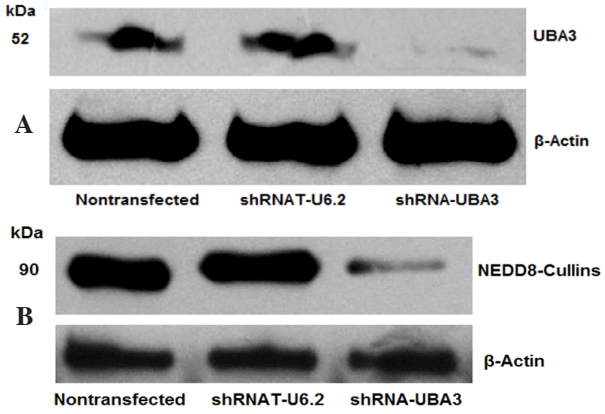

Figure 4. Decrease of NEDD8 Conjugation Following with the Transfection of shRNA-UBA3 in M14 Cell Line. (A) The relative quantification of UBA3. (B) The relative quantification of NEDD8 conjugation at 90 kDa

After transfected shRNA-UBA3 and shRNAT-U6.2 into M14, the transfection efficiency was about 30%. Picking out the clones which express the bright green fluorescent under fluorescence microscope, got the stably transfected clones. As shown in Figure 3, cells, picked out under fluorescence microscope, almost all expressed GFP that represented the success of transfection.

Interference the expression of UBA3 in M14 resulted in the depression of NEDD8 conjugation pathway

The interference effect against UBA3 was observed by Western blot (Figure 4). The optimum sequence against UBA3 was 5'-GGATCCCGTGCACGCTGGAACTTTATCTTC AAGAGAGATAAAGTTCCAGCGTGCATTT TTTCCA ACTCGAG (71 bp). As shown in Figure 4A, the level of UBA3 in M14/shRNA-UBA3 was lower than that in nontransfected M14 and M14/shRNAT-U6.2. As shown in Figure 4B, following inhibiting the expression of UBA3 effectively, the NEDD8 conjugation level in M14/shRNA-UBA3 was significant declined. The results above approved that after interfered the expression of UBA3 effectively, the NEDD8 conjugation decreased.

Depression of NEDD8 conjugation pathway inhibited the degradation of p53

P53 were degradated through UPS (Tsvetkov et al., 2010; Xirodimas and Scheffner, 2010; Germain, 2011). Interference the formation of NEDD8 conjugation could

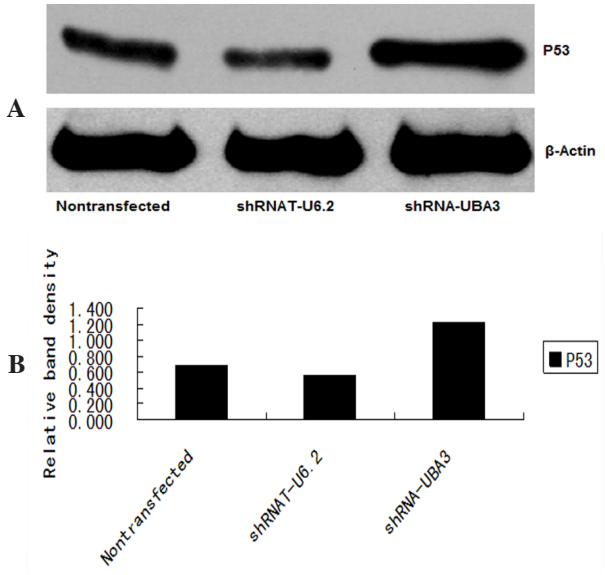

Figure 5. The Relative Band Density of p53 was Analyzed by Western Blot Assay, and the β-actin Level Worked as Loading Controls. (A) The relative density of p53. (B) The column diagram of relative density in cells

Table 1. The OD Value of the Transfected and Nontransfected M14 after Culture for 24, 48 and 72h.

Groups	24h	48h	72h
Nontransfected M14	0.5880±0.147	1.2468±0.199	1.9724±0.137
M14/shRNAT-U6.2	0.5498±0.016	1.2259±0.056	1.6789±0.120
M14/ shRNA-UBA3	0.3820±0.034*	0.8164±0.066**	1.3123±0.146**

Statistical differences were analyzed with t-test (M±SD, n=9, *P < 0.05 relative to M14/shRNAT-U6.2 group, **P < 0.05 relative to Nontransfected M14 group and M14/shRNAT-U6.2 group)

inhibit the ubiquitination and reduce the degradation of p53. After got the stably transfected clone, the level of p53 were observed by Western blot. The results showed that the density of p53 increased obviously in the group of M14/shRNA-UBA3 (Figure 5). The result manifested that interference of UBA3 could influence the degradation of p53 through depressing the positive effect of NEDD8 conjugation pathway on the ubiquitination of them. This also proved that interference of UBA3 could inhibit the pathway of NEDD8 conjugation successfully.

Inhibition of NEDD8 conjugation pathway could negatively regulate the proliferation of M14 in vitro

To observe the effect of NEDD8 conjugation pathway on the proliferation of melanoma cells, viability of cells was measured by CCK8 assay. The living cells could be stained by CCK8, however, the dead cells was nonstaining. The OD value, which represented the number of living cells, reflected the growth situation of cells. As shown in Table 1, the OD value of M14/shRNA-UBA3 was lower than that of nontransfected M14 and M14/shRNAT-U6.2 groups at 48 and 72h. However, at 24h, no difference was observed between nontransfected M14 and M14/shRNA-UBA3. Transfection of shRNA-UBA3 had negative effects on the growth of M14 accompanied with the inhibition of NEDD8 conjugation pathway.

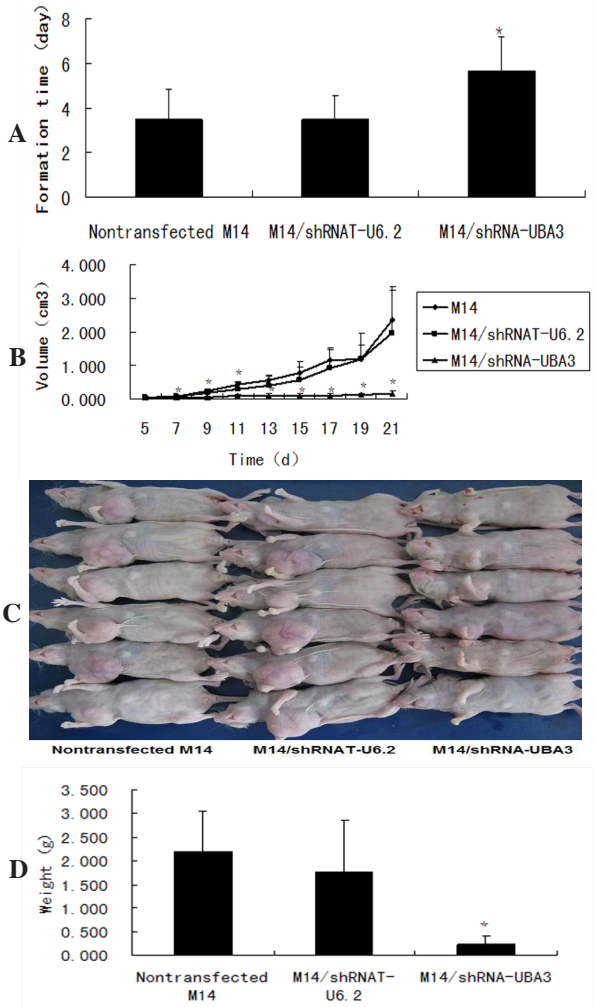

Figure 6. The Growth Speed of the Tumor Xenografts. (A) The formation times of the tumor xenografts. (B) Growth curves were determined by the tumor volume measured every other day. (C) The size of tumor xenografts when executed the nude mice. (D) The weight of tumor xenografts when executed the nude mice. (Error bars = Standard Deviation, n=6, *P < 0.05 relative to the two control groups)

Inhibition of NEDD8 conjugation pathway could negatively regulate the proliferation of M14 in vivo

The formation time of tumor xenografts for M14/shRNA-UBA3 group was 5.67±1.51 (M ± SD) days, which was later than that for the groups of nontransfected M14 and M14/shRNAT-U6.2 (Figure 6A). Seven days later, the tumor xenografts were touchable at the injection position of all nude mice. Measure the volume (0.5 × length × width^2) of tumor xenografts on alternate days. The results showed that the growth speed of subcutaneous tumors in the group of M14/shRNA-UBA3 was slower evidently than that in the groups of M14 and M14/shRNAT-U6.2 (Figure 6B, C). Execute the nude mice when the volumes of majority tumor xenografts were about 2 cm^3. Separated the tumor xenografts and weighed them.

The results showed that the weight of subcutaneous tumors of group M14/shRNA-UBA3 was lighter than that of group M14 and group M14/shRNAT-U6.2 (Figure 6D). Inhibition of NEDD8 conjunction pathway had significantly negative effects on M-14 proliferation ability.

Discussion

UPS could modulate the degradation of proteins including p53. The first commercial drug targeting UPS is proteasomes inhibitor namely velcade that has been verified anti-tumor activity in many malignant cells (O'Connor, 2005; Croghan et al., 2010). However, proteasomes inhibitor, which completely cuts off the UPS pathway irreversibly, makes masses of protein turn over disordered (Soucy et al., 2009). NEDD8 activating enzyme inhibitor and other reversible proteasome inhibitors were concerned to avoid these shortages (Demo et al., 2007; Soucy et al., 2009). MLN4924, one of NEDD8 activating enzyme inhibitor, verifies the feasibility of NEDD8 conjugation pathway as a new target against tumor (Soucy et al., 2009; Lin et al., 2010). NEDD8 conjugation pathway has effect on the proteins whose ubiquitination is dependent on SCF complex. Regulation of NEDD8 pathway is more special and may contribute to more safety profiles. For the reasons above, NEDD8 conjugation pathway captures our attention. In many carcinoma cell lines, levels of NEDD8 conjugation were up-regulated (Brownell et al., 2010). We speculate that up-regulated NEDD8 conjugation may be one reason for uncontrolled proliferation of malignant cells.

Previous research showed that a series of bands in a ladder-like pattern represented conjugation of proteins with NEDD8 multimers in Western blot analysis. Different cells or tissues had different ladder-like bands (Gong and Yeh, 1999). The band of 90 kDa, which was consistent with the molecular mass of NEDD8-cullins conjugation, was observed in all mammalian cell lines tested (Kamitani et al., 1997; Gong and Yeh, 1999; Huang et al., 2009). The band of 90 kDa was chosen to be observed in following test. For all cullin members, like CUL-1, CUL-2, CUL-3 and CUL-4A, have relatively similar molecular weights, the identity of 90 kDa NEDD8 conjugated band cannot be simply predicted in Western blot analysis. Nonetheless, most cullin family members are involved in cell cycle, which was closely related to the progression and development of tumor (Brownell et al., 2010).

We observed the NEDD8 conjugation level in three melanoma cell lines and melanocytes. The results showed that, compared with melanocytes, NEDD8 conjugation raised more remarkably in A375, M14, and MV3. The results above represented the relationship between conjugated NEDD8 and melanoma.

In cell lines or tissues, the band of 6 kDa, which represented free NEDD8, was invisible. To investigate the reason of free NEDD8 decline further, decline of the expression of NEDD8 gene or increase of NEDD8 conjugation, we studied the gene expression of NEDD8 in three melanoma cell lines. Interestingly, in M14 who with the highest NEDD8 conjugation, the expression of NEDD8 gene was down-regulated, even lower than that in melanocytes. This indicated that the expression of NEDD8 had an indirect relationship with NEDD8 conjugation, which was identical with the study of Kamitani (1997). Why the expression of NEDD8 gene decreased in M14? We presume that the remarkably boost of NEDD8 conjugation will give negative feedback to the expression of NEDD8 gene. In A375 and MV3, the expression of NEDD8 remained up-regulated, although the NEDD8 conjugation elevated. Maybe, the negative feedback would happen when the NEDD8 conjugation is high enough.

We observed the expression of UBA3 gene. UBA3 was increased sharply in M14 who with the highest NEDD8 conjugation. NEDD8 is activated by APPBP1/UBA3 dimer. The up-regulated UBA3 may be one reason of the enhanced NEDD8 conjugation pathway.

We investigated the role of NEDD8 conjugation in the proliferation of M14, which has the highest level of NEDD8 conjugation among the three cell lines. To interrupt the formation of NEDD8 conjugation, but did not affect the physiological function of free NEDD8 , UBA3, subunit of NEDD8-Activating enzyme, was chosen to be interfered. To verify the effect of inhibition of UBA3 on NEDD8 conjugation pathway, expression of p53 were determined after picking out the stable transfected clones of shRNA-UBA3. The results showed that p53 increased after transfection. The ubiquitination of p53 are regulated by the pathway of NEDD8 conjugation. Inhibition of NEDD8 conjugation pathway successfully could make the ubiquitination of p53 the degradation decline.

Malignant cells almost have the common character of uncontrolled proliferation (Glinsky et al., 1996). After the NEDD8 conjugation was interfered effectively, the proliferative capacity of M14 was declined. The test of subcutaneous tumor formation was carried out in nude mice. The formation time of tumor xenografts were later, and the growth speed of subcutaneous tumors was slower obviously, when the NEDD8 conjugation pathway was depressed. With the extension of time, the gap of growth speed between the test group and the control groups became more and more notable, which was consistent with the result of proliferative test in vitro. These results suggest that NEDD8 conjugation is required for cell proliferation. The pathway of NEDD8 conjugation has positive effect on the ubiquitination, which monitor the degradation of p53. The results showed that, when the pathway of NEDD8 conjugation was inhibited, the intracellular quantity of p53 were significantly increased which might have closely relationship with the restricted proliferation of melanoma.

Based on the results above, we conclude that high expression of NEDD8 conjugation could be involved in the malignant biologic behavior of melanoma. Interference of NEDD8 conjugation pathway by shRNA to UBA3, the subunit of NEDD8-Activating enzyme, could potentially depress the proliferative capacity of melanoma.

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