

## Original Article

# Therapeutic effects of targeting RAS-ERK signaling in giant congenital melanocytic nevi

Qingxiong Yu\*, Min Wu\*, Lingling Sheng, Qingfeng Li, Feng Xie

*Department of Plastic and Reconstructive Surgery, Shanghai Ninth People's Hospital, Medical College of Shanghai Jiaotong University, Shanghai, P. R. China. \*Equal contributors.*

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**Abstract:** Most giant congenital melanocytic nevi (GCMN) exhibit an activating mutation in NRAS. Constitutive activation of the RAS-ERK signaling pathway induces proliferation in nevus cells and plays a pivotal role in melanoma development. In this study, we studied the efficacy of RAS-ERK pathway targeted therapy in GCMN. We isolated nevus cells from GCMN (GNCs) and compared the morphology of GNCs with normal melanocytes and the A375 melanoma cell line. Proliferation curves of GNCs and A375 cells were determined using Cell Counting Kit-8 assays. Cell cycle distribution was measured using flow cytometry. The RAS-ERK pathway inhibitors Vemurafenib and Trametinib, which are used in melanoma therapy, were applied. After inhibitor treatment, GNCs were analyzed for apoptosis and the protein expression of ERK, p-ERK, P38, p-P38 and P53. We found that compared with A375 cells, the cultured GNCs exhibited a higher G1 phase population and a lower proliferation rate. Both Vemurafenib and Trametinib treatment induced GNCs apoptosis in a dose-dependent manner, with Vemurafenib having a stronger effect. With inhibitor treatment, ERK activation was greatly suppressed, while the expression of p-P38 exhibited no obvious change. Vemurafenib treatment also increased the level of P53 protein in GNCs. These findings suggested that Vemurafenib treatment may be a potential therapeutic strategy for treatment of GCMN via targeting of the RAS-ERK pathway.

**Keywords:** Giant congenital melanocytic nevi, targeting therapy, Ras, extracellular regulated protein kinases

## Introduction

Giant congenital melanocytic nevi (GCMN) refer to benign nevi present at birth with diameters of greater than 20 cm projected in the adult [1, 2]. GCMN can cover any part of the body, and up to 80% of the body surface can be involved. Large areas of pigmented nevi are a heavy psychological burden on the patients and their parents. Previous studies have shown that the risk of malignant transformation of GCMN is much higher than in the normal population, approximately 5% to 8% over the lifetime of the patient [3-5].

The treatment of GCMN is still a challenge. Non-surgical treatments, such as laser therapy, chemical-peeling, and dermabrasion, only remove superficial pigment and nevus cells in the dermis. Recurrence of the pigment is common, and the remaining nevus cells deep in the lesion still have malignant transformation potential. Skin grafts and tissue expansion can

completely remove the lesion. However, even experienced surgeons become stranded when the nevus involves too large an area on the body.

Constitutive activation of the extracellular signal-regulated kinase (ERK) pathway has been found in the majority of melanocytic neoplasms [6, 7]. The underlying mechanisms for ERK activation differs between entities, but most of them are somatic mutation of RAS and BRAF. A previous study showed that 97.4% of GCMN samples harbored an NRAS<sup>Q61</sup> mutation [8, 9]. The abnormal RAS protein caused sustained activation of the RAS-ERK signaling pathway and proliferation of nevus cells. These findings prompted the possibility of small molecule inhibitor therapy for GCMN by targeting the RAS-ERK signaling pathway. Vemurafenib and Trametinib are inhibitors that target different components of the RAS-ERK pathway. They were approved by the FDA for metastatic melanoma and effectively prolonged the life span of

late stage melanoma patients [10, 11]. Therefore, we used these two drugs to study the therapeutic effect on nevus cells. Here, we treated isolated nevus cells from GCMN with Vemurafenib and Trametinib. Both Vemurafenib and Trametinib induced similar cell apoptosis in GNCs and A375 cells, and the expression of p-ERK in GNCs was markedly inhibited after drug application. In contrast, the expression of p-P38 was slightly improved. We demonstrated that RAS-ERK targeting therapy might serve as a potential drug for GCMN treatment.

## Materials and methods

### Cell culture

GNCs were isolated from human GCMN tissue. Lesion tissue was obtained at the time of surgical intervention, from patients admitted into the department of Plastic and Reconstructive Surgery of Shanghai Ninth People's Hospital between July 2015 and January 2016. The obtained specimens were washed with normal saline twice in sterile culture dishes. The subcutaneous fat was removed carefully with dissecting scissors, and the remaining skin tissue was cut into 2 mm strips and incubated for 24 h at 4°C in 0.25% Dispase II (Sigma-Aldrich). Then, the epidermis and dermis were carefully separated with tweezers, and the obtained dermis was then minced with scissors and incubated in 0.2% collagenase at 37°C for 1 h. After digestion, the dermis was filtered, and the disaggregated cells were centrifuged and resuspended in MeIM medium (ScienCell, America).

Melanocytes were isolated from normal prepuce tissue obtained from patients who underwent a circumcision. After rinsing in phosphate-buffered saline (PBS), the subcutaneous fat was removed from the skin pieces, and tissues were incubated in 0.25% Dispase II (Sigma-Aldrich) at 4°C overnight. The epidermis was then peeled from the dermis and transferred into 0.05% trypsin/0.02% EDTA (Life Technologies) for 3 min at 37°C. Enzymatic digestion was stopped by the addition of a trypsin inhibitor (Sigma-Aldrich). MeIM medium (ScienCell, America) was used to cultivate the corresponding cells.

A piece of dermis was used for isolation of fibroblast (FB) using a migration method as follows. The dermis was cut into 4×4 mm pieces,

attached to a culture dish, and covered with 10 ml of Dulbecco's modified of Eagle's medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (Sigma-Aldrich).

The A375 cells were purchased from Fudan IBS (Institutes of Biomedical Sciences) Cell Source Center and maintained in RPMI-1640 (Life Technologies) medium containing 10% fetal bovine serum and 1% penicillin/streptomycin solution.

### Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min and then blocked in 5% goat serum for 1 h at room temperature; then, the cells were immunostained with primary antibodies specific for Melan-A (Santa Cruz, USA), HMB-45 (Dako, USA), Ki-67 (Abcam, UK), S-100 (Abcam, UK) and SOX10 (Abcam, UK) overnight at 4°C. Cells were then washed in PBS and incubated with the appropriate secondary antibody (conjugated with Alexa Fluor 488 or Alexa Fluor 555) for 1 h at 4°C. The nuclei were stained with DAPI (Dako, USA). Cells stained with a secondary antibody without the primary antibody served as a negative control.

### Cell proliferation assays

A cell proliferation curve was obtained using Cell Counting Kit-8 (CCK-8, Dojindo, Japan) following the manufacturer's instructions as described previously. Briefly, nevus cells and A375 cells were seeded at  $2 \times 10^3$  cells/well in 96-well plates and cultured in 100  $\mu$ L of medium. After 6 h, 12 h, 24 h, 48 h and 72 h of incubation, 10  $\mu$ L of Cell Counting Kit-8 reagent was added to each well, and the plate was then incubated at 37°C for 2 h. The optical density (OD) values at 450 nm were measured using an MRX microplate reader (Dynex Technologies, New York, USA) according to the manufacturer's instructions. The assay was performed in triplicate.

### Cell cycle analysis

Nevus and A375 cells were seeded at  $1 \times 10^5$  cells per well in 6-well plates in triplicate. Following 72 h of culture, cells were trypsinized, centrifuged at 1000 rpm for 5 min and resuspended in cold PBS. The cells were pelleted by centrifugation and 1 mL of cold 70% ethanol

was added for fixation (overnight at 4°C). Then, the cells were centrifuged, the supernatant was discarded, and the cells were labeled with propidium iodide (50 µg/mL) in 0.2% Triton X-100 and RNase A (100 µg/mL) and incubated in the dark on ice for 30 min. The stained cells were then analyzed for DNA content with a flow cytometer (FACSCalibur, Becton-Dickinson, America).

## *Apoptosis detection*

A375 cells and nevus cells at passage 2 were seeded at  $2.5 \times 10^5$  cells per well in 6-well plates in triplicate, and fibroblast cells were seeded as a control. After 24 h of culturing, different concentrations of Vemurafenib and Trametinib were applied for treatment. The concentrations of Vemurafenib selected in this study were 50 nmol/L, 100 nmol/L, 500 nmol/L, 1000 nmol/L, 10 µmol/L, and 50 µmol/L; the concentrations of Trametinib were 100 nmol/L, 1000 nmol/L, 5000 nmol/L, 10 µmol/L, and 50 µmol/L. After 48 h of treatment, the cells were trypsinized for analysis. Cell apoptosis was measured with an Annexin V-FITC/PI detection kit (BD Bioscience, San Jose, CA, USA). Briefly, the cells were harvested, washed once with cold PBS, and resuspended in 500 ml of binding buffer, followed by staining with Annexin V-FITC and PI solution in the dark for 15 min at room temperature. The cells were centrifuged at 1000 rpm for 5 min, and the pellets were resuspended in 500 µL of binding buffer. After filtration, the percentage of apoptotic cells was determined using flow cytometry (BD FACSCalibur, San Jose, CA, USA). The experiment was performed in triplicate. Data are presented as the mean  $\pm$  SD.

## *Western blot analysis*

GNCs and A375 cells were seeded at an appropriate density (70%-75% confluent) in six-well plates 1 day before compound treatment. After Vemurafenib treatment at a concentration of 500 nmol/L and Trametinib treatment at a concentration of 1000 nmol/L for 2 days, the cells were harvested and lysed. Protein was extracted using RIPA lysis buffer with Complete Mini-Protease Inhibitor Cocktail (Roche, USA). After incubation on ice for 20 to 30 min, the lysates were centrifuged at 14,000 rpm for 15 min. The protein concentration of the lysates was determined. Equal amounts of total protein were separated on an SDS-PAGE gel, and the

fractionated proteins were then transferred from the gel onto a PVDF membrane (Millipore Corporation, Massachusetts). The membranes were blocked with 10% bovine serum albumin for 1 h at room temperature and then incubated with primary antibody overnight at 4°C. Antibodies to ERK, p-ERK, P38, p-P38, P53 and  $\beta$ -actin were used. Then, the membranes were incubated with appropriate peroxidase-conjugated secondary antibodies. The chemiluminescent signal was generated with Enhanced Chemiluminescence Plus Western Blotting Detection Reagents and detected with a Fujifilm LAS-3000 imager. Densitometric quantitation of specific bands was determined using Multi Gauge Software (Fujifilm).

## *Statistical methods*

The results are expressed as the mean  $\pm$  SD or the total number and percentage. ANOVA was used to compare groups, and Student's t-test for independent samples was used to compare 2 groups. The significance level was set as  $P < 0.05$ . Statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, IL, USA).

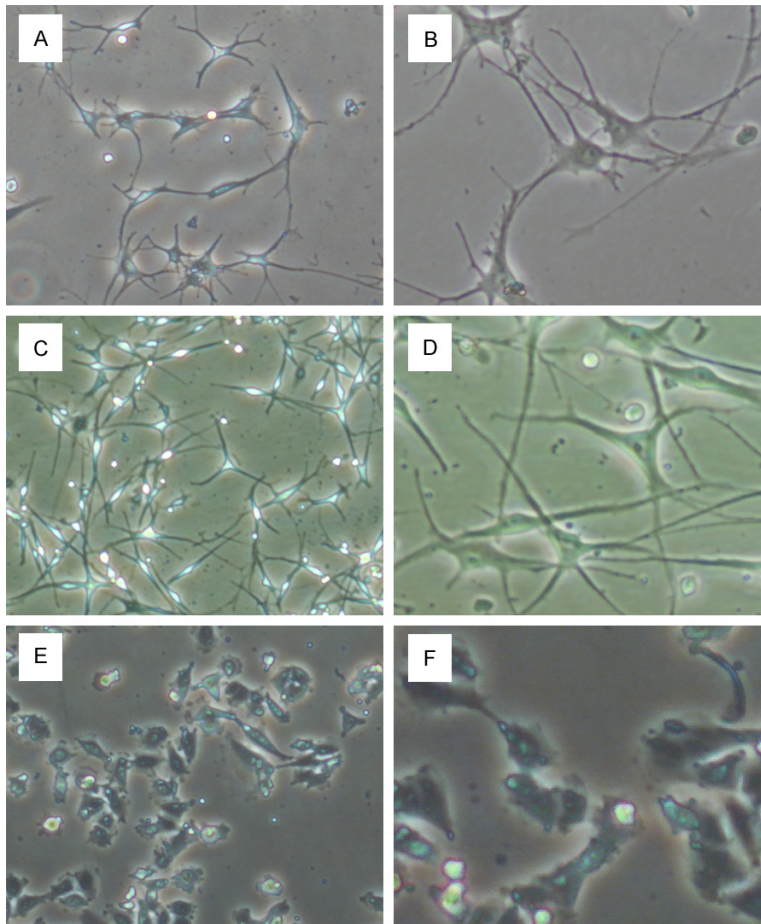
## **Results**

### *Morphology of GNCs is similar to that of melanocytes*

Primary GNCs isolated from GCMN lesions began to adhere 12 h after being inoculated into a petri dish. The separated cells were quite homogenous. They were short spindle or polygonal shaped with slim dendrites, with an average of two or three dendrites seen in every cell (**Figure 1C** and **1D**). No parallel or spiral growth characteristics could be seen. Compared with cultured normal human melanocytes, the morphology of GNCs was quite similar, except normal melanocytes possessed more dendrites and greater morphological diversity (**Figure 1A** and **1B**). A375 cells demonstrated aggressive growth with a polygonal shape, and no dendrites were observed (**Figure 1E** and **1F**). The morphology of GNCs was consistent and stable without a substantial number of hybrid cells after being transmitted to another generation.

### *GNCs were confirmed by immunofluorescence staining*

For confirmation of isolated GNCs, immunofluorescence staining was performed on cultured



**Figure 1.** Morphology of isolated GNCs is similar with normal melanocyte. A. Normal human melanocyte isolated from prepuce tissue, they are short spindle or polygonal shaped with slim dendrites (40×). B. Normal melanocyte (100×). C. Cultured GNCs from GCMN lesion demonstrated similar morphology with normal melanocyte (40×). D. Cultured GNCs (100×). E. A375 cell line demonstrated polygonal shape and no dendrite observed (40×). F. A375 cell line (100×).

GNCs. Melan-A (also designated MART-1) is a melanocyte differentiation antigen that is specific to melanocyte cell lines and retina. Nevus cells in the GCMN lesion demonstrated positive staining for Melan-A. HMB45 antibody labeled the cytoplasm of “immature” and “activated” melanocytes. In GCMN lesions, HMB45 staining was restricted to only the base layer and superficial layer. In cultured GNCs, immunofluorescence staining demonstrated that only a portion of the cells were positive for Melan-A and HMB45. This suggested that although the cultured GNCs had morphological uniformity, functional differences still existed. The GNCs were also positive for Ki-67, SOX10, and P16 staining. P16 is a tumor suppressor protein and plays an important role in cell cycle regulation

by decelerating cell progression from G1 phase to S phase. Both Ki-67 and P16 positive staining indicated that the GNCs were benign proliferating cells (**Figure 2**).

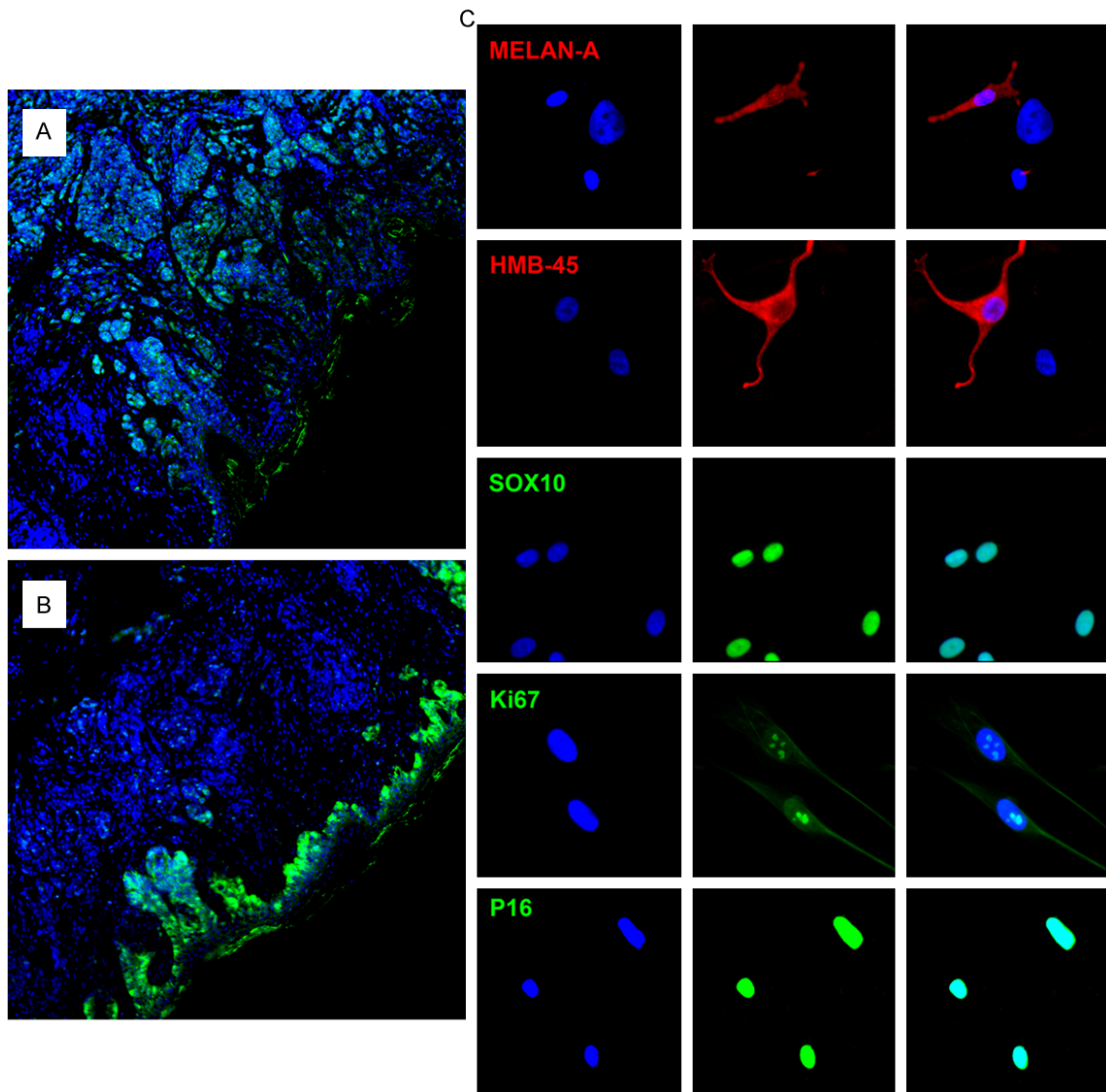
## *Cell cycle analysis of GNCs demonstrated a high G1/S ratio*

The cell cycle distribution of nevus cells and A375 cells was examined. We observed that the DNA content of GNCs in G1 phase was an average of  $54.13 \pm 0.64$  (arbitrary unit). The number of cells in G1 phase accounted for  $81.90\% \pm 1.61\%$  of the total, while the number of cells in S phase accounted for  $9.32\% \pm 1.12\%$  of the population (**Figure 3A**). The DNA content of A375 cells in G1 phase was an average of  $46.20 \pm 0.99$ . Cells in G1 phase accounted for  $41.03\% \pm 3.35\%$  of the total. The number of cells in S phase accounted for  $26.77\% \pm 4.62\%$  of the population (**Figure 3B**). The G1/S ratio of GNCs was 8.79, much higher than that of A375 cells (1.53). The percentage of GNCs in G1 was significantly higher than that of A375 cells ( $P < 0.05$ ,

see **Figure 3C**). These results demonstrated that the G1/S phase transition of GNCs was arrested compared with A375 cells; this cell cycle arrest may be regulated by P16 protein in GNCs, as mentioned previously.

## *Cellular proliferation rate of GNCs is lower than that of A375 cells*

In this study, we explored the proliferative properties of GNCs and A375 cells with CCK-8 assays. For GNCs, the OD values at 6 h, 12 h, 24 h, 48 h and 72 h were  $0.2757 \pm 0.0096$ ,  $0.2894 \pm 0.0185$ ,  $0.4342 \pm 0.0101$ ,  $0.5937 \pm 0.0106$ , and  $0.8506 \pm 0.0129$ , respectively. For A375 cells, the OD values at 6 h, 12 h, 24 h, 48 h and 72 h were  $0.4948 \pm 0.0273$ ,  $0.5303$



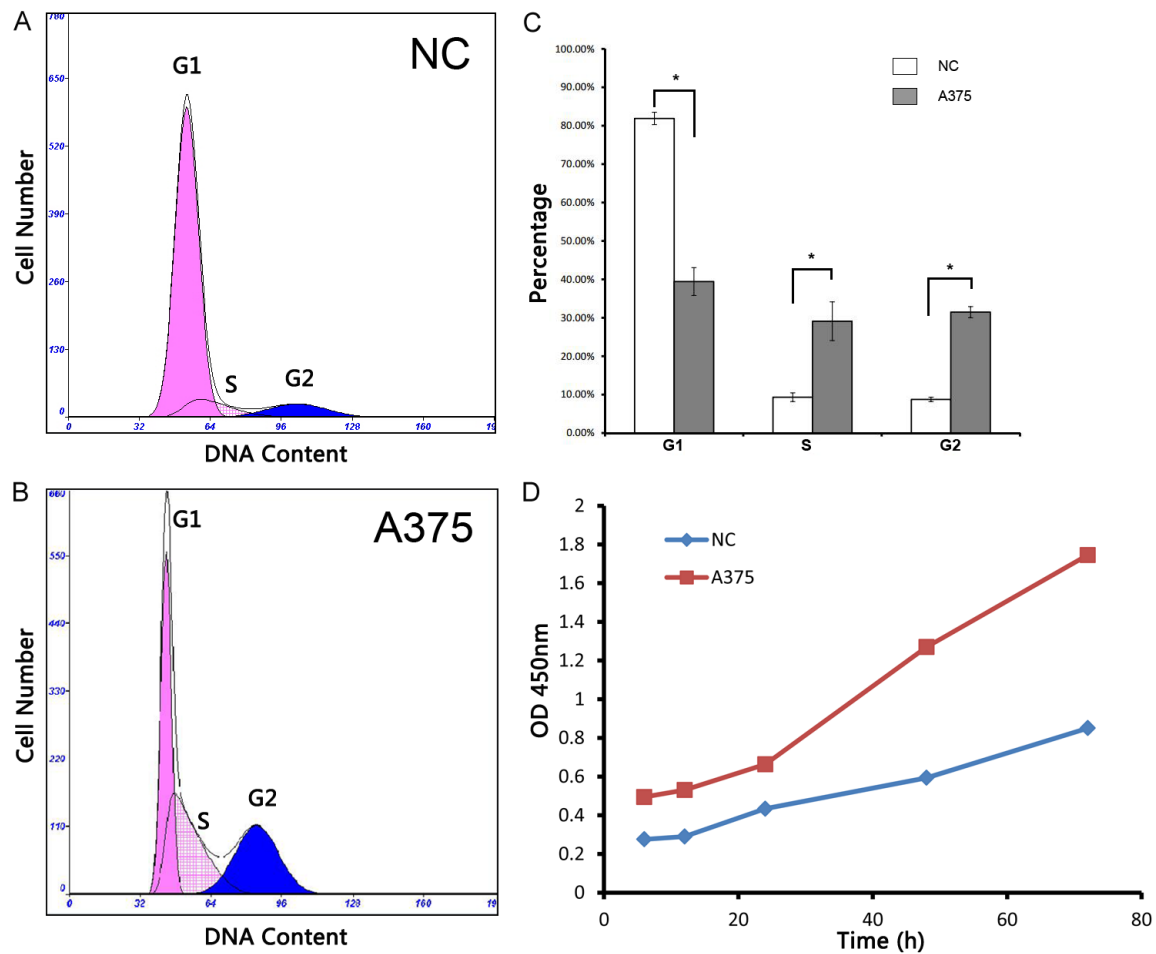
**Figure 2.** Isolated GNCs were confirmed with specific markers. A. GCMN lesion with Melan-A staining in dermal. B. HMB-45 staining was restricted to the epidermal and superficial dermal in GCMN lesion. C. Partial cultured GNCs demonstrated Melan-A and HMB-45 positive staining, all GNCs were positive staining in SOX10, Ki67 and P16.

$\pm 0.0234$ ,  $0.6633 \pm 0.0595$ ,  $1.2695 \pm 0.0598$  and  $1.7451 \pm 0.0835$ , respectively. According to the proliferation curve (**Figure 3D**), the proliferation rate of GNCs was lower than that of A375 cells. The results further demonstrated the cell cycle characteristics of GNCs.

#### *Vemurafenib and Trametinib promote apoptosis in GNCs and A375 cells*

By using the aforementioned cultured GNCs as an in vitro model, we evaluated the effects of Vemurafenib and Trametinib treatment in GCMN. The concentrations of Vemurafenib ap-

plied in this study were 50 nmol/L, 100 nmol/L, 500 nmol/L, 1000 nmol/L, 10  $\mu$ mol/L, 50  $\mu$ mol/L; and the concentrations of Trametinib were 100 nmol/L, 1000 nmol/L, 5000 nmol/L, 10  $\mu$ mol/L and 50  $\mu$ mol/L. Fibroblasts showed no significant apoptosis in the above concentration ranges of these two drugs (**Figure 4A**). The number of apoptotic cells in the GNC and A375 cell increased in a dose-dependent manner after drug application (**Figure 4D** and **4E**). Both Vemurafenib and Trametinib induced similar cell apoptosis in GNCs and A375 cells. Cell death was more significant in GNCs than in A375 cells. At the same drug concentrations,



**Figure 3.** GNCs demonstrate high G1/S ratio and low proliferation rate compared with A375 cells. A. Cell cycle distribution of GNCs. B. Cell cycle distribution of A375 cells. C. Cell cycle comparison between GNCs and A375 cells in each phase. D. Proliferation curve of GNCs and A375 cells. (\*:  $P < 0.05$ ).

Vemurafenib induced more cell apoptosis than Trametinib in both GNCs and A375 cells. These results demonstrated that Vemurafenib was more effective than Trametinib in the GNC model.

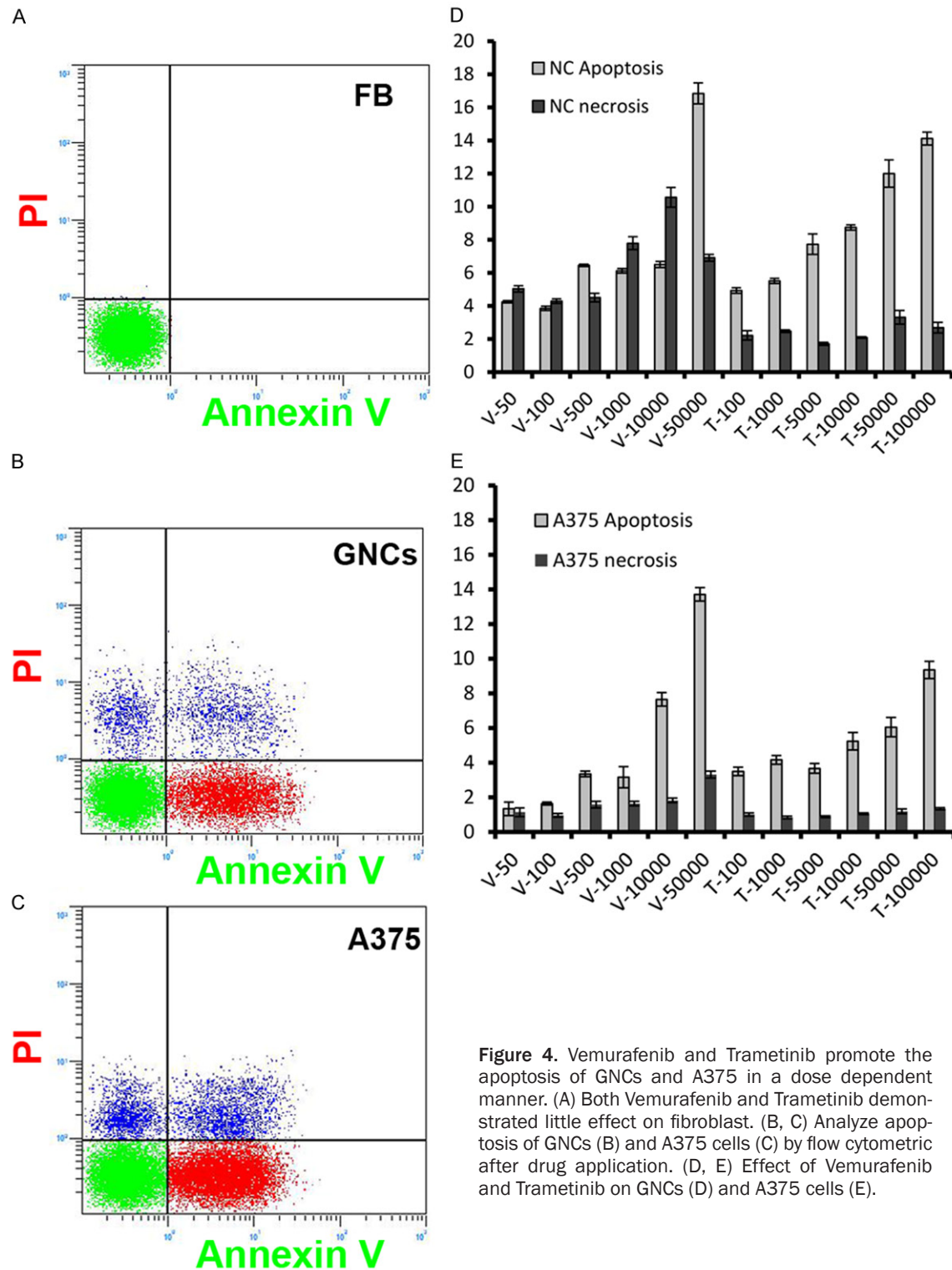
*Vemurafenib and Trametinib inhibit ERK but not P38 signaling and alter the expression of P53*

The RAS-ERK signaling pathway is constantly activated in GNCs and melanoma cells. To determine whether the applied drugs promote apoptosis of GNCs by inhibiting the ERK pathway, GNCs and A375 cells were treated with 500 nmol/L Vemurafenib and 1000 nmol/L Trametinib. Moreover, the phosphorylation of ERK and P38 were determined using western blotting. The results showed that both Vemurafenib and Trametinib clearly inhibited the

expression of p-ERK in GNCs (Figure 5A and 5C). In contrast, the expression of p-P38 was slightly improved. Vemurafenib significantly improved the expression of P53 in GNCs, while Trametinib had no influence on the expression of P53. In A375 cells, there was a base level of P53 expression (Figure 5B and 5D). Both Vemurafenib and Trametinib had little influence on the expression of P53 in A375 cells.

## Discussion

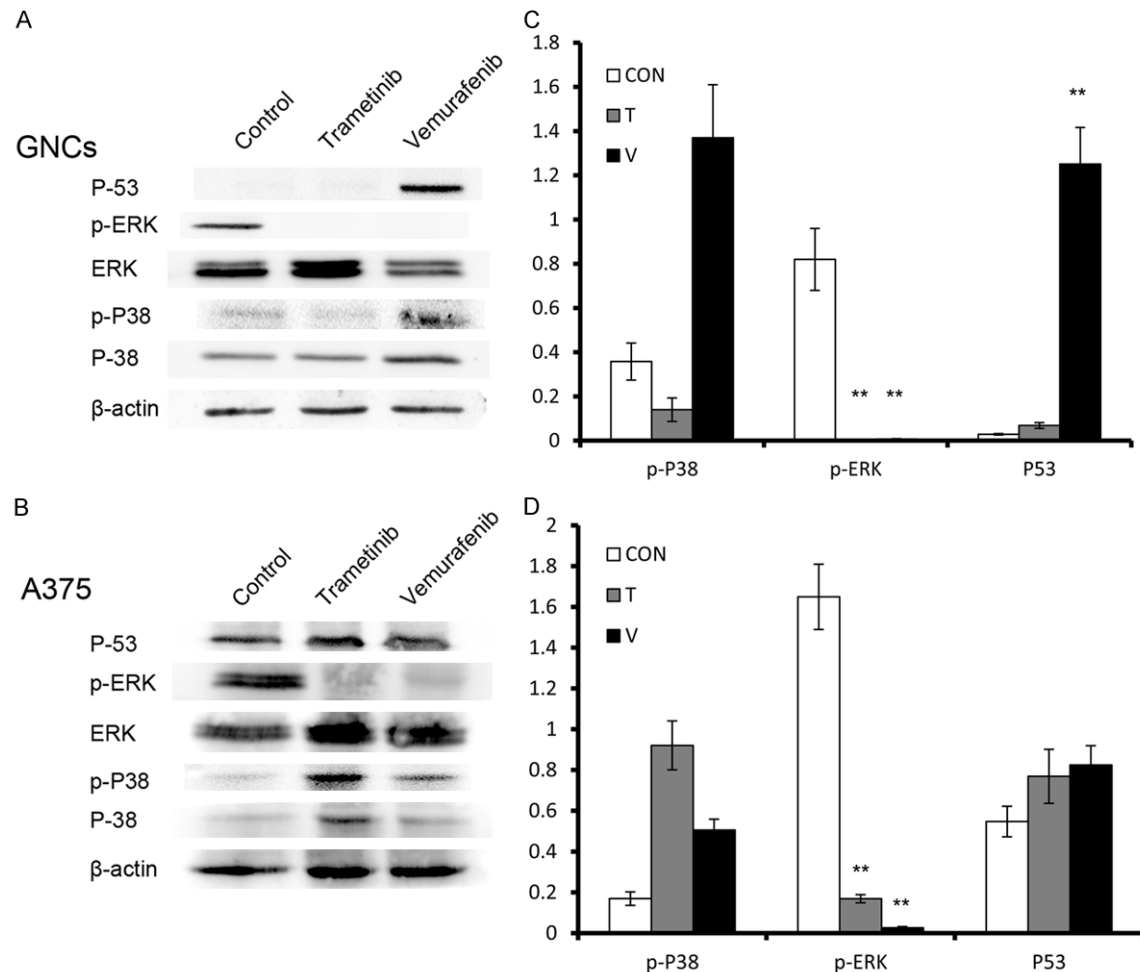
Currently, surgical resection and reconstruction remain the primary treatment for GCMN [12, 13]. However, with surgical treatment, the skin graft induces an unsatisfactory appearance and a donor site defect. Tissue expansion reconstruction is quite time and money consuming. For GCMN that involve large areas of skin, even surgical treatment cannot resurface



**Figure 4.** Vemurafenib and Trametinib promote the apoptosis of GNCs and A375 in a dose dependent manner. (A) Both Vemurafenib and Trametinib demonstrated little effect on fibroblast. (B, C) Analyze apoptosis of GNCs (B) and A375 cells (C) by flow cytometric after drug application. (D, E) Effect of Vemurafenib and Trametinib on GNCs (D) and A375 cells (E).

the lesion due to the limited availability of normal skin on the body. In recent years, targeted cell signaling therapy has been attracting increasing attention for the obvious therapeutic

effects and safety in tumor therapy. Drugs for targeted therapy have been developed for lung cancer, breast cancer and other types of tumors [14-16]. In this study, we show that tar-



**Figure 5.** Vemurafenib and Trametinib inhibit ERK signaling and alter the expression of P53 in GNCs. (A, B) Cultured GNCs (A) and A375 cells (B) were treated with Vemurafenib and Trametinib for 2 days, and WB analysis was done for ERK, P38 and P53. (C) Phosphorylation of ERK in GNCs was intensively inhibited after Vemurafenib and Trametinib treatment, Vemurafenib improve P53 level in in GNCs. (D) Phosphorylation of ERK in A375 cells was also inhibited after drug application but the P53 level was little influenced. (\*\*:  $P < 0.01$  vs control group).

getting the RAS-ERK pathway can effectively suppress the proliferation of GNCs and induce apoptosis. This may serve as a potential therapeutic strategy for GCMN treatment.

Due to activating mutations of the KRAS, NRAS and BRAF genes, the RAS-RAF-MEK-ERK signaling pathway is hyperactivated in a high percentage of tumors [17-19]. The use of compounds targeting components of ERK signaling, such as RAF or MEK inhibitors, has led to substantial improvement in clinical outcomes in metastatic melanoma and has shown promising clinical activity in additional tumor types [20-22]. GCMN is a benign congenital tumor. Research has demonstrated that 97.4% of GCMN lesions harbor N-RAS mutations. In addition,

further experiments have demonstrated that adult mice expressing NRAS<sup>Q61K</sup> in the melanocyte lineage accumulate more dermal and hair follicle melanocytes than normal mice [23]. Mutation of the N-RAS gene induces constant activation of the RAS-ERK pathway, which enhances the proliferation of melanoblasts and forms nevi. Based on these findings, we propose a strategy of targeting the RAS-ERK pathway as a therapeutic approach for GCMN. To our knowledge, this is the first report exploring targeted molecular therapy to treat GCMN.

The N-RAS gene is a highly evolutionarily conserved proto-oncogene. It is closely associated with cell proliferation, differentiation, apoptosis and migration. RAS protein is located inside the

cell membrane and is activated by binding of GTP. Activated RAS-GTP directly combines with its downstream protein Raf, relocating Raf inside the cell membrane and activating it. At the same time, phosphorylation of RAS switches GTP to GDP, which deactivates it. Oncogenic RAS mutations are single base substitutions that lead to the stabilization of GTP binding and constitutive activation of RAS and downstream signaling cascades. Activated Raf causes cascade amplification, triggering the mitogen-activated protein kinase (MAPK) pathway, ultimately sending signals into the nucleus, affecting cell proliferation and differentiation [24, 25]. Though RAS activation takes charge of the formation of GCMN, direct pharmacologic inhibition of RAS has been a major challenge. An alternative strategy is to target the downstream pathway of RAS.

Vemurafenib and Trametinib are both FDA approved targeting molecules used to treat metastatic melanoma [26, 27]. Vemurafenib is a BRAF inhibitor while Trametinib is an inhibitor of MEK1/2, with high specificity. Both drugs target different parts of the RAS-RAF-MEK-ERK pathway, blocking activation of the pathway. In this study, we observed that both Vemurafenib and Trametinib can inhibit proliferation and induce apoptosis of GNCs as well as A375 cells in a dose-dependent manner. The western blotting results demonstrated that both drugs markedly inhibit the phosphorylation of ERK. ERK is a downstream protein of the RAS-RAF-MEK-ERK signaling pathway. Our data show that targeting different components of this pathway eventually lead to similar inhibition of ERK. This presents many possibilities for developing targeting molecules. A375 cells demonstrate a basal level of P53 protein expression. P53 protein is a transcription factor that controls cell cycle initiation and promotes apoptosis. Both Trametinib and Vemurafenib slightly improve the expression of P53 in A375 cells. Interestingly, in GNCs, Trametinib has no influence on P53 expression, while Vemurafenib significantly improves P53 expression. The apoptosis analysis demonstrated that Vemurafenib has a stronger effect compared with the same concentration of Trametinib, and the upregulation of P53 induced by Vemurafenib may be one of the reasons. The precise mechanism still needs further research. The choice of targets for targeted therapy is quite important.

Targeting different components of the same pathway may result in similar but different results.

P38 is also a member of the mitogen-activated protein kinase family. Similar to ERK, it can cause a variety of biological responses after activation. It is responsive to stress stimuli such as cytokines, ultraviolet irradiation, and heat shock, and it is involved in cell differentiation, apoptosis and autophagy [28-30]. The results demonstrate that drug application had little influence on the phosphorylation of P38 protein. Thus, the drugs are specific to the RAS-ERK pathway. Since P38 is sensitive to stress stimuli, we speculate that the targeting drugs in our study have little impact on the physiological processes. For Vemurafenib, the treatment results in vitro are encouraging. We think that it may be due to Raf inhibition, which is upstream of the MAPK signaling pathway. This inhibition might have better results. Trametinib might have had little effect due to side signaling pathways.

This study explores the possibility of treating GCMN with small molecule targeted therapy. Our results demonstrate that targeting the RAS-ERK pathway can significantly reduce the proliferation of GNCs and promote apoptosis. Targeted therapy is quite specific and has little influence on normal tissue. We have only verified our conclusion in cultured GNCs, and therefore, the therapeutic effect needs to be further confirmed in an animal model. In addition, targeted drug treatment combined with immunotherapy may be a new approach to healing the disease in the future.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Qingfeng Li and Feng Xie, Department of Plastic and Reconstructive Surgery, Shanghai Ninth People's Hospital, Medical College of Shanghai Jiaotong University, Shanghai, China. Tel: (86) 021-23271699; Fax: (86) 021-

23271699; E-mail: dr.liqingfeng@yahoo.com (QFL);  
dr.xiefeng@yahoo.com (FX)

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