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ORIGINAL ARTICLE

Small interfering RNA (siRNA)-mediated knockdown of Notch1 suppresses tumor growth and enhances the effect of IL-2 immunotherapy in malignant melanoma

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Summary

Purpose: *Malignant melanoma (MM) is a highly aggressive neoplasm that is resistant to conventional therapies. In this study, we aimed to investigate the effects of antitumor and immune enhancement of Notch1 knockdown on MM.*

Methods: *Mouse melanoma cells B16F1 were transfected with a small interfering RNA (siRNA) targeting Notch1 (siNotch1).*

Results: *The expression of Notch1 and its downstream hey1 was significantly decreased, resulting in reduced cell proliferation in vitro. Furthermore, intratumoral injection of siNotch1 successfully inhibited the expression of Notch1 and hey1, which suppressed tumor growth, and increased the number of tumor-infiltrating CD8⁺ T lymphocytes and IFN-γ secretion in vivo, especially when combined with IL-2 immunotherapy.*

Conclusion: *These results suggested that siRNA-mediated Notch1 knockdown might be an effective method for the inhibition of tumor growth both in vivo and in vitro, and might potentially enhance the effect of IL-2 immunotherapy in MM. Notch1 knockdown concurrently administered with IL-2 might be a novel therapeutic approach for the treatment of MM.*

Key words: *immunotherapy, malignant melanoma, Notch1, proliferation, small interfering RNA*

Introduction

Notch signaling plays an important regulatory role in cell differentiation, proliferation and apoptosis [1]. The mammalian Notch signaling pathway is composed of 4 receptors (Notch1 to Notch4) and 5 ligands (Delta-like-1,-3,-4, and Jagged-1,-2) in mammals [2]. Notch receptor releases its extracellular and transmembrane domain (NICD) after being cleaved by TNF-α-converting enzyme (TACE) and γ-secretase complex upon activation of Notch signaling by receptor-ligand binding, it translocates into the nucleus, where it interacts with CSL protein (CBF-1/suppressor of hairless/Lag-1) and mastermind-like (MAML1) protein, leading to the transcription of Notch target genes [3,4].

Recent studies have shown that Notch plays a dual role as either an oncogene in breast cancer, medulloblastoma, colorectal cancer, pancreatic cancer, melanoma and leukemia, or as a tumor suppressor in squamous cell carcinoma, prostatic carcinoma, hepatocellular carcinoma, and small cell lung cancer [5]. Among the 4 re-

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ceptors, the regulatory role of Notch, especially Notch1 overexpression, in tumor growth has become a research hotspot recently. Purow et al. have demonstrated that Notch1 downregulation by RNA interference inhibits the proliferation of glioma in animal models [6]. Similar results have also been reported in clear renal cell carcinoma [7], prostate cancer cells [8], pancreatic cancer cells [9], and ovarian cancer [10] growth and T-cell lymphoblastic leukemia cells [11]. The overactivation of Notch1 has been confirmed by immunohistochemiscal analysis [12,13]. It has been suggested that Notch signaling is oncogenic, and is one of the early events in the progression of melanocytic tumors [12].

Since Notch has been identified as an important receptor that promotes the development of a variety of cancers, recent studies have focused on the search of therapeutic approaches targeting the Notch pathway, such as dissociation of receptor/ligand binding, interaction with NICD to prevent its translocation into the nucleus, treatment with Notch antisense and monoclonal antibodies, etc. Our previous study has shown that interference of both Notch coactivation factor MAML1 blocks the activation of Notch pathway in both human and mouse melanoma cells [14], suggesting a potential new treatment strategy. In the past, researchers successfully inhibited tumor growth and induced cell apoptosis in Kaposi's sarcoma [15] and melanoma [13] by blocking the cleavage and activation of all Notch receptors with γ-secretase inhibitor N-[N-(3,5-diflu-orophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester. However, the application of this therapeutic drug was limited due to its non-specific effects [16].

MM is one of the most highly aggressive tumors, which is resistant to conventional chemotherapy and radiotherapy, and thereby has a fatal outcome in most cases. Currently, there is an urgent need for a more effective treatment for MM. IL-2 was the first immunotherapeutic agent for melanoma that was approved by the US Food and Drug Administration (FDA). Generally, high-dose IL-2 is required to achieve effective antitumor effects. Nevertheless, the tolerance due to the toxicity and hemodynamic instability associated with high-dose IL-2 has been a very serious problem during therapy [17].

In this study, we investigated the inhibitory effects of siRNA-mediated Notch1 knockdown on the growth of melanoma *in vitro* and *in vivo*. We first examined the expression of Notch1 gene in mouse melanoma cells B16F1 transfected with siRNA targeting Notch1 by RT-PCR and Western blot, and evaluated the effects of siRNA-mediated knockdown of Notch1 on the proliferation of B16F1s *in vitro* by MTT assay and cell clone assay. We further assessed the effects of antitumor and immune enhancement of siNotch1 intratumoral injection in tumor-bearing mice. The current study may provide insights on the development of novel molecular therapeutic approach for the treatment of MM.

Methods

Cell culture

Murine malignant melanoma cell line B16F1 of low metastatic potential was kindly provided by Dr Yuru Meng at the University of Chicago. B16F1 cells were cultured as adherent cells in Dulbecco's modified Eagle medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA) and 1% penicilin G-streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and passaged every 2-3 days after digestion with trypsin and ethylene-nediaminetetraacetic acid/EDTA (Invitrogen, Carlsbad, CA, USA) when they reached a confluence of 70–80%.

siRNA preparation and transfection

The siRNA oligo targeting Notch1 (siNotch1) and negtive control (siNC) were purchased from Genepharma (Shanghai, China). The two complementary oligonucleotides of the siRNAs were as follows: siRNA-Notch1-mus-4650, sense 5'-GCA ACC UGC AGU GUA AUA ATT-3' and antisense 5'-UUA UUA CAC UGC AGG UUG CTT-3'; siRNA-Negative control, sense 5'- UUC UCC GAA CGU GUC ACG UTT-3' and antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'. B16F1 cells were seeded in a 6-well plate and placed in antibiotics-deprived DMEM supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA). Cells at 30-50% confluence were transfected with the plasmid using LipofectamineTM 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, Lipofectamine™ 2000 and plasmid were diluted respectively by 100 μl of opti-MEM. Cells were divided into 3 groups and incubated in 7.5 μl of the Lipofectamine™. Three groups were established in the experiment: A: siNotch1 group transfected with 75 pmol siNotch1; B: siNC group transfected with 75 pmol siNC; and C: mock group (mock transfection with lipofectamine™ 2000). After 4 hrs of incubation at 37°C, the medium was replaced with normal culture medium and cells were incubated for an additional 8 hrs. Cells were then observed under a fluorescence microscope (Olympus 1×71, Tokyo, Japan) for transfection efficiency assay.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

At 24 hrs after transfection, total RNA was extracted using RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions. RNA concentration was determined at optical density (OD) 260 and its quality was measured at OD260/OD280 (Nanodrop opND-1000, USA). Total RNA (1μg) was reverse-transcribed in 20μl of reaction mixture using a PrimeScript® RT reagent kit. RT-PCR was performed using a SYBR® Premix Ex Taq™ (Takara, Dalian, China). Briefly, the reaction mixture containing 2 μl of cDNA, 0.4 μl of forward primer (10μM), 0.4 μl of reverse primer (10μM), 10 μl of SYBR Premix Ex Taq™ (2×), and 7.2 μl of RNase-free water was prepared. The PCR was performed using a LightCycler480 instrument (Roche Diagnostics, USA). The two-step cycling program was as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and extension at 60°C for 20 s. The melting curve program was performed at the end of each reaction. The relative expression of mRNA was determined by the comparative Ct Method (ΔΔCT Method). The sequences of primers were as follows: Notch1 (forward primer: 5'-TCA ATG TTC GAG GAC CAG ATG-3' and reverse primer: 5'-TCA CTG TTG CCT GTC TCA AG-3'); β-actin (forward primer: 5'-GAC CCA GAT CAT GTT TGA GAC C-3' and reverse primer: 5'-ACG ACC AGA GGC ATA CAG G-3').

Western blot analysis

At 72 hrs after transfection, cells were washed 3 times with PBS, lysed with RIPA buffer containing 1mM PMSF on ice for 20 min, and centrifuged at 12,000 g at 4 °C for 10 min. Total protein concentration was determined by Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma, USA). The cytosolic fraction was collected and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% running gel. The proteins were transferred to a polyvinylidene fluoride membrane (PVDF, Invitrogen, Carlsbad, CA, USA). The membrane was incubated sequentially in tris-tween buffered saline (TTBS) with 5% bovine serum albumin at room temperature for 1 h, with first antibody at 4°C overnight, and with secondary antibody at room temperature for 2 hrs. The membrane was then washed thoroughly with TTBS. Protein loading was assessed by anti-β-actin (ab6276, Abcam,UK) staining. The intensity of the immunoreactive band was quantified with ECL-detecting reagents (Sigma,USA) by densitometry and normalized against β-actin.

Cell cloning assay

Cells were trypsinized, centrifuged, and resuspended in a 6-well plate at a density of 100 cells per well. After 14 days of culture at 37°C, adherent cells were washed twice with PBS, and fixed with 4% paraformaldehyde at room temperature for 15 min. Colonies were visualized using a crystal violet solution.

MTT assay

Cells transfected with siNotch1 and siNC, and mock cells (800 cells per well) were plated into 96-well plates. B16F1 cells without any treatment were used as controls. Cell proliferation was assessed at different time points (0, 24, 48, and 72 hrs) using MTT assay kit (Sigma, USA) according to the manufacturer's instructions. Briefly, MTT was added to the culture medium for a final concentration of 1 mg/ml. Cells were incubated at 37°C for an additional 4 hrs. The cell lysates were dissolved with DMSO (150 μl/well) at room temperature for 10 min. The absorbance of each well was detected under a wavelength of 490 nm (ELX800NB, USA). The test was repeated independently three times.

Construction of animal model and introtumoral injection

Female C57BL/6 mice, 6-8 weeks old and weighing approximately 18-22 g, were purchased from the Laboratory Animal Center at the Southern Medical University (Guangzhou, China). This study was in strict accordance with the institutional guidelines for animal care and treatment. To construct the animal model, 25 mice were injected subcutaneously with 1×10^6 B16F1 cells. When the tumors were palpable with a volume of around 100-150 mm^3, the tumor-bearing mice were randomly divided into 5 groups (N=5): Mice receiving intratumoral injections of PBS, 50 μg siNotch1, 1×10^4 IU IL-2 (Peprotech, USA), or both. All treatments were performed twice a week (on days 0, 3, 7, 10, 14, 17, 21, 24 after the first injection), resulting in a total of 8 injections for each mouse. Xenografted tumors were measured twice a week. Tumor volume was calculated using the following formula: $(length\times width^2)/2$. On day 24, all mice were sacrificed. The tumor masses were removed within 5 min, fixed with 4% polyoxymethylene for 36 hrs, dehydrated sequentially in an ascending series of ethanol solutions, cleared with toluene, and embedded in paraffin wax.

Hematoxylin & eosin and immunohistochemical staining

Paraffin-embedded specimens were cut into 4 μm sections, and baked at 60°C overnight, deparaffinized with xylene and rehydrated, and washed with flowing water. Sections were then placed in Harris acidified hematoxylin for 2 min, and rinsed in water. Slides were placed in eosin solution for 30 sec and sequentially rinsed with ethanol,xylene. Coverslips were applied. Sections were baked overnight at 60°C, deparaffinized with xylene, and rehydrated. Sections were then immersed in mineral chameleon for 5 min and oxalic acid for 2 min to remove melanin. Sections were further submerged into citrate, autoclaved for antigen retrieval, and treated with 3% hydrogen peroxide. Sections were incubated with Notch1 antibody (1:50 dilution, sc-6014, Santa Cruz, USA) , Ki-67 antibody (1:100

dilution, 12202, Cell Signaling Technology, Boston, USA), PCNA antibody (1:150 dilution, sc-7907, Santa Cruz, USA) overnight at 4°C. Sections in the negative control group were incubated with PBS. Sections were incubated in the Polymer Helper for 20 min and then in horseradish peroxidase (HRP) labeled secondary antibody anti-goat, anti-rabbit and anti-rat IgG kit (PV-9003/PV-6001, ZSGB-BIO, China) for 20 min. DAB (Notch1, CD8 and Ki-67) or AEC (PCNA) were used as final chromogens and hematoxylin as the nuclear counterstain. Sections were observed under a 400× microscope (Olympus BX51, Tokyo, Japan) and 100 tumor cells were counted in 5 randomly selected fields. The expression of Notch1, Ki-67 and PCNA was assessed using the labeling index that was calculated as the percentage ratio of the number of positive staining cells to the total number of tumor cells [18].

Flow cytometric analysis

At each indicated time point, the tumor tissues were obtained from the mice, minced into small fragments, and mechanically dispersed in 3-5 ml of cold RPMI medium. The tumor cells were digested in 1 mg/mL collagenase IV (Sigma, USA), 0.1 mg/mL DNase (Sigma, USA) in RPMI 1640 at 37 °C for 1 h. The single-cell suspensions were adjusted to a concentration of 1×10^5 cells in 100 µl of PBS, stained with 1 µg of the following fluorochrome labeled antibodies, CD3, CD8 or matched isotype control antibodies (Miltenyi Biotech, Germany) on ice for 30 min, and then washed once with PBS. The cells were acquired on a FACSCablibur flow cytometer (BD Biosciences, New Jersey, USA), and 10,000-20,000 gated events were collected. The collected data were analyzed using the forward scatter (FSC) and side scatter (SSC) bivariate plot by FlowJo software 7.6. A region was drawn around the cluster of lymphocytes, and the fluorescence histogram was gated on them. This gate, once established, was then applied to the analysis of all subsequent samples.

IFN-γ secretion assay

Freshly excised tumor tissues were minced and homogenized in a tissue homogenate machine for 1 min. The supernatants were collected after centrifugation, and subjected to ELISA using mouse ELISA kits (Ebioscience, USA) according to the manufacture's protocol for the detection of IFN-γ secretion.

Statistics

All data were presented as means ± SD and analyzed using SPSS13.0. Results of RT-PCR, Western blot analysis, cell cloning assay and immunohistochemical analysis were compared by one-way ANOVA. Results of MTT assay and animal tumor volume were analyzed by factorial ANOVA. P values less than 0.05 were considered statistically significant.

Results

siNotch1 successfully downregulated the expression of Notch1 and its downstream hey1

At 24 hrs after transfection, the expression of Notch1 mRNA was measured by RT-PCR. It was shown that Notch1 mRNA expression in siNotch1 group was significant lower compared with siNC (negative control) and mock (blank control) group ($p<0.01$). At 72 hrs after transfection, the expression of Notch1 and its downstream hey1 protein in siNotch1 group was also significantly lower than that in both siNC and mock groups ($p< 0.01$, Figure 1).

Inhibitory effects of siNotch1 on cell growth in vitro

The effects of siNotch1 on cell proliferation were investigated by MTT assay and colony formation assay. As shown in Figure 2C, the OD490 values in the siNotch1 group were significantly lower compared with siNC and mock groups on days 2, 3, and 4 ($p<0.01$, Figure 2C). Moreover, the number of cells colonies in siNotch1 group was significantly lower than that in siNC and mock groups ($p<0.01$, Figures 2A & B), suggesting that Notch1 knockdown inhibited the growth of melanoma cells.

In vivo antitumor effect of the combined treatment with siNotch1 and IL-2

A tumor-bearing mouse model was successfully constructed by implantation of B16F1 cells into C57BL/6 mice (N=5, 5 groups) (Figure 3). These mice were then given an intratumoral injection of siNotch1, IL-2 or both to evaluate the antitumor effect of siNotch1 or in combination with IL-2. Treatment with siNC did not result in any suppressive effect on tumor growth, whereas treatment with either siNotch1 or IL-2 moderately inhibited tumor growth. Furthermore, the tumor growth was remarkably suppressed in those mice concurrently treated with IL-2 and siNotch1 (Figure 3B). Subsequent histological analysis on the tumor specimens demonstrated that the tumor diameter in the combined treatment group was significantly reduced compared with siNC and mock groups ($p<0.05$). The tumor growth was measured and growth curves were made accordingly.

To further assess the inhibitory effect of siNotch1 intatumoral injection on tumor tissues, the expression of Notch1 and its downstream hey1 protein in tumor tissues was deter-

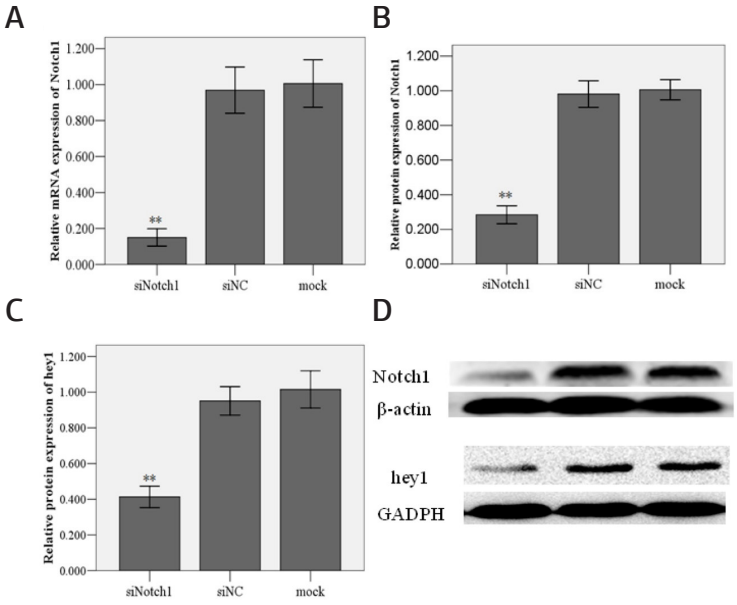

Figure 1. siNotch1 transfection successfully downregulated the expression of Notch1 and its downstream hey1. **A:** Relative expression of Notch1 mRNA (p<0.01). Notch1 mRNA expression is siNotch1 group was significantly lower than in siNC and mock group (p<0.01). **B:** Relative expression of Notch1 protein (p<0.01). **C:** Relative expression of hey1 protein (p<0.01). **D:** Western blot analysis showing the expression of Notch1 and hey1. The expression of Notch1 and its downstream hey1 protein in siNotch1 group was significantly lower than that in both siNC and mock groups. **p<0.01, compared with the control group.

mined by immunohistochemistry and Western blot analysis. The relative expression level of Notch1 protein in the siNotch1 group was markedly reduced compared with the control groups (p<0.05, Figure 3E). The expression of hey1 protein in the siNotch1 group was also significantly lower compared with the control groups (p<0.05, Figure 3D). These results indicated that the tumor-suppressive effect was primarily attributed to the intratumoral injection of siNotch1.

Downregulation of Ki-67 and PCNA expression by siNotch1 intratumoral injection

The expression of Ki-67 and PCNA in tumor tissues was measured by immunohistochemical staining to evaluate the tumor proliferation indexes *in vivo*. As shown in Figure 4, the expression of Ki-67 and PCNA protein in siNotch1 group was significantly lower compared with siNC, PBS, and IL-2 groups. However, it was down-regulated after *in situ* injection of siNotch1 (p<0.05, Figure 4), suggesting that the growth and proliferation of tumors in tumor-bearing mice were inhibited after siNotch1 treatment.

The immune enhancing effects of siRNA-mediated Notch1 knockdown in melanoma

To evaluate the immune enhancing effects of repeated intratumoral injections of siNotch1 and IL-2, tumor samples were subjected to hematoxylin & eosin staining and CD8 flow cytometric analysis. As shown in Figure 5A, the number of lymphocytes infiltrated in the tumor in siNotch1 and IL-2 groups was significantly higher compared with siNC and PBS groups.

We further assessed the immune enhancing effects of siNotch1 by detecting the presence of $CD8^+$ cytotoxic T cells in single-cell suspensions on day 24 using flow cytometry. As shown in Figure 5B, the number of $CD8^+$ T lymphocytes infiltrated in the tumor tissue and tumor intercellular substance in siNotch1, IL-2 and the combined treatment groups was significantly higher compared with siNC and PBS groups (p<0.05, Figure 5C). Further, tumor homogenate supernatants were collected for ELISA to determine the level of IFN-γ secretion *in vivo*. As shown in Figure 5D, IFN-γ secretion in siNotch1 and IL-2 groups was significantly increased compared with siNC and PBS groups (p<0.05, Figure 5D). Moreover, the IFN-γ secretion in the combined treatment

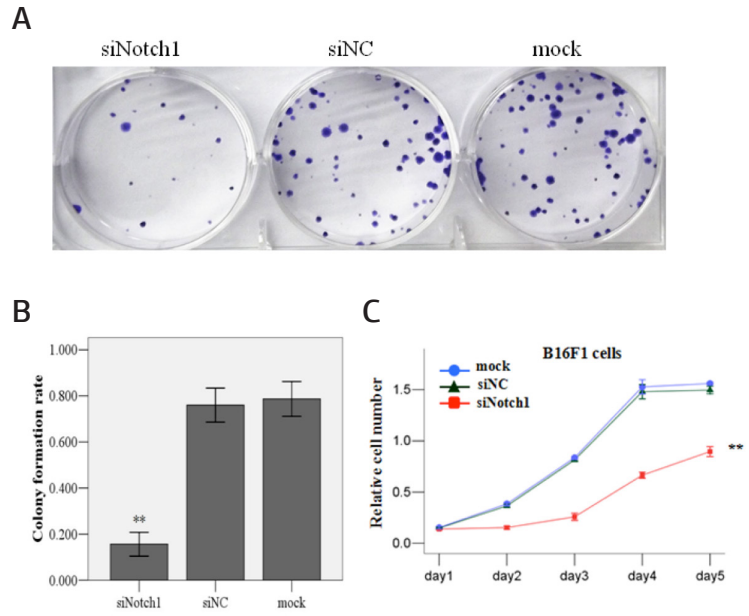

Figure 2. siNotch1 in the growth of melanoma cells *in vitro*. **A:** Colony formation assay. Cell colonies were visualized in a crystal violet solution. **B:** Quantification analysis of colony assay. The number of cell colonies in siNotch1 group was significantly lower than in siNC and mock groups ($p<0.01$). **C:** Growth curves of three groups in MTT assay. The OD490 values in the siNotch1 group were significantly lower compared with siNC and mock groups on days 2,3,4 and 5. ** $p<0.01$, compared with the control group.

group was significantly higher than in siNotch1 and IL-2 groups ($p<0.05$, Figure 5D). These results revealed that Notch1 knockdown promoted strong immune responses against tumors and enhanced the effects of IL-2 immunotherapy *in vivo*.

Discussion

MM is one of the most common fatal malignancies of young adults. Although the mortality rate of MM has remained stable, its incidence is increasing. In 1998, IL-2 immunotherapy was approved by FDA which has led to continuous complete remission over a long-time follow-up (>5 years) [19]. As antitumor agent, IL-2 increases the number of antigen-specific cytotoxic T lymphocytes and NK cells, and activates these cells to attack cancer cells. The tumor-inhibitory effects of local IL-2 administration have been confirmed in the Grimaldi et al. study [20]. Rosenberg et al. successfully inhibited the growth of melanoma tumor with $1.5x10^5$- $3.0x10^5$ U/mouse IL-2 in an animal model [21]. Nevertheless, the application of IL-2 immunotherapy has been restricted due to its associated complications including capillary leak syndrome, cardiac tachyarrhythmias, and seizures. Currently, there is an urgent need for a novel or combined therapy for the treatment of MM.

It has been well known that Notch pathway was crucial for the regulation of cell fate. Hurlbut et al. have found that Notch interacts with a variety of pathways such as receptor tyrosine kinases (RTKs), Hedgehog (HH), Wnt/Wingless (Wnt), Janus kinase/signal transducers and activators of transcription (Jak/STAT), transforming growth factor-β/decapentaplegic (TGF-β), platelet-derived growth factor

(PDGF/PDGFR), vascular endothelial growth factor (VEGF), phosphatidylinositol3-kinase(PI3K/Akt), Ras, mammalian target of rapamycin (mTOR), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and hypoxia-inducible factor (HIF). Among these, Jak/STAT, RTK and Wnt have been shown to regulate cell proliferation, patially through communicating with Notch [22]. Therefore, we speculate that the blockage of Notch signaling might reduce cell growth through changing these pathways.

The proliferative activity is considered as an important trait of malignant tumors. Identification of the overproliferating fraction within the tumor cell population has been proved to be useful in the diagnosis and prognosis of various tumors. Ki-67 expression has been commonly used for the diagnosis of several tumors, such as breast cancer [23], meningioma [24], prostate cancer [25]

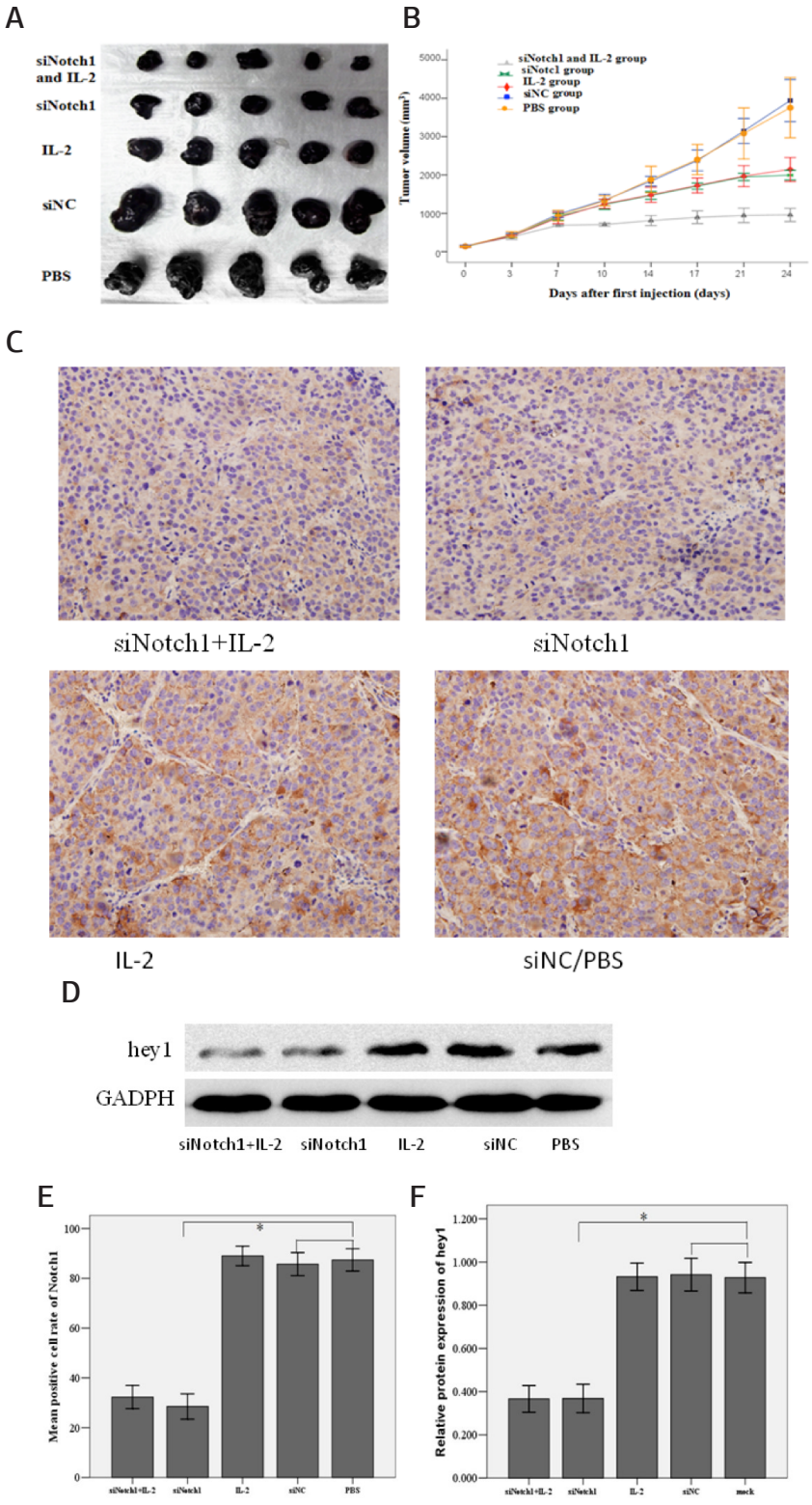

Figure 3. Antitumor effects of the combined treatment with IL-2 and siNotch1 in B16F1 tumor-bearing mice. **A:** Pictures of tumor specimens in each group on day 24 after the first injection ($p<0.01$, compared with the control group). **B:** Tumor growth curve after intratumoral injection of siNotch1 and IL-2. Treatment with siNC did not result in any suppressive effect on tumor growth, whereas treatment with either siNotch1 or IL-2 moderately inhibited tumor growth. The tumor growth was remarkably suppressed in those mice concurrently treated with IL-2 and siNotch1. **C:** DAB staining showing the expression level of Notch1 protein in different groups. (×400). **D:** Western blot analysis of hey1 expression in tumor tissue. **E:** Quantification analysis of Notch1 positive rate after intratumoral injection. The relative expression level of Notch1 protein in the siNotch1 group was markedly reduced compared with the control groups ($p<0.05$). **F:** Relative expression level of hey1 protein in different groups after intratumoral injection. The expression of hey1 protein in the siNotch1 group was significantly lower compared with control groups ($p<0.05$). *$p<0.05$, compared with the control group.

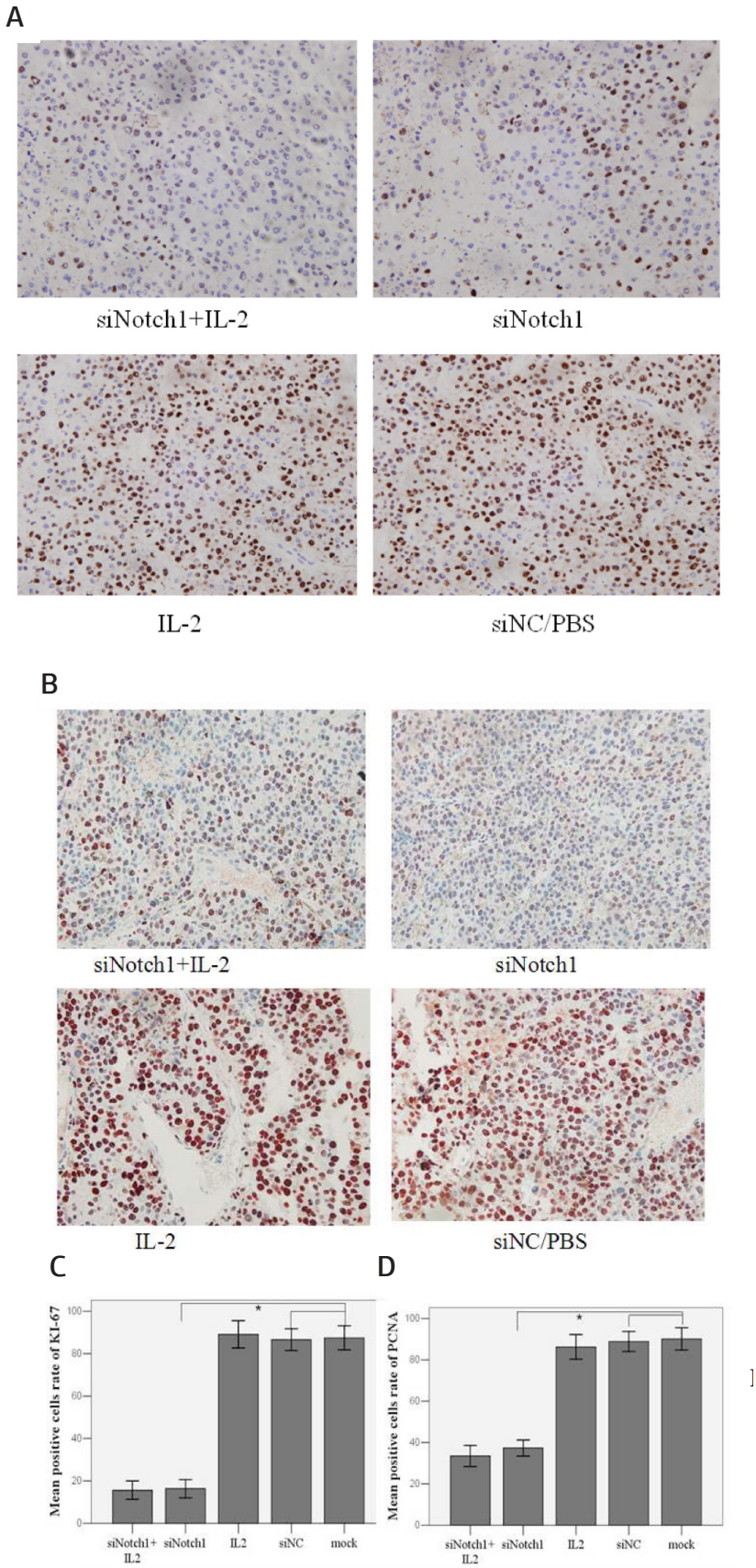

Figure 4. Down-regulation of Ki-67 and PCNA expression after siNotch1 intratumoral injection. **A:** DAB staining showing reduced Ki-67 expression after intratumoral injection (×400). **B:** AEC staining showing reduced PCNA expression after intratumoral injection(×400). **C:** Quantification analysis of Ki-67 positive cell rate after intratumoral injection. The expression of Ki-67 in siNotch1 group was significantly lower compared with siNC, PBS and IL-2 groups ($p<0.05$). **D:** Quantification analysis of PCNA positive cell rate after intratumoral injection. The expression of PCNA in siNotch1 group was significantly lower compared with siNC, PBS and IL-2 groups ($p<0.05$). *$p<0.05$, compared with the control group.

and melanoma [26]. It is expressed in G1, S, and G2 phases during the cell cycle of proliferating cells. Proliferating cell nuclear antigen (PCNA) is another nuclear protein that increases in the G1 phase, peaks in the G1/S interphase, and decreases in the G2 phase [27]. Expression of PCNA has been used as a functional tool for the evaluation of cellular proliferation indexes in a variety of malignancies [28-31]. In this study, the expression of Ki-67 and PCNA were measured to detect the proliferation of melanoma tumors.

Our results showed that the proliferation of melanoma cells in vitro and tumor growth in a mouse model was dramatically reduced after down-regulation of Notch1 with siRNA. Moreover, $CD8^+$ T lymphocytes infiltration and IFN-γ secretion were observed in melanoma tissue after subcutaneous injection of siNotch1. The expression of Ki-67 and PCNA in the siNotch1 group was lower compared with negative groups, suggesting that the siRNA-mediated Notch1 knockdown resulted in reduced cell proliferation and immune enhancement. It is worth noting that the strongest antitumor effects was observed in the combined treatment group with siNotch1 and IL-2.

Although the molecular mechanism of siNotch1-mediated proliferation reduction remains unclear, it might be associated with the downregulation of Akt and NF-κB signaling. It has been shown that siNotch1 disrupts the function of activated Akt signaling and induces cell apoptosis in T-ALL [32], nasopharyngeal carcinoma [33], and melanoma [34]. Hyperactivated PI3K/Akt pathway has also been observed in human melanoma, which promotes cell proliferation and cell cycle [35]. NF-κB is one of the major mediators of Notch1-induced transformation, and inhibition of the pathway can efficiently suppress tumor growth both *in vitro* and *in vivo* [36]. In addition, immunosuppressive cytokines, such as TGF-β, VEGF and PGE2, are produced during the progression of melanoma to create an immunosuppressive microenvironment that can facilitate tumor growth and block antitumor immune responses. Notch1 expression is positively correlated with the expression of PGE2, VEGF and TGF-β in tumor cells [37-39]. Therefore, Notch1 knockdown may lead to the downregulation of melanoma-derived immunosuppressive cytokines *in vivo* and the enhancement of immune responses against tumors.

In summary, siRNA-mediated Notch1 knockdown effectively inhibited tumor growth both *in vivo* and *in vitro*, promoted a favorable immune microenvironment, and enhanced the effect of IL-2 immunotherapy in MM. The combined treatment with siNotch1 and IL-2 might be a potential therapy for the treatment of melanoma. However, clinical trials should be performed to further verify its efficacy.

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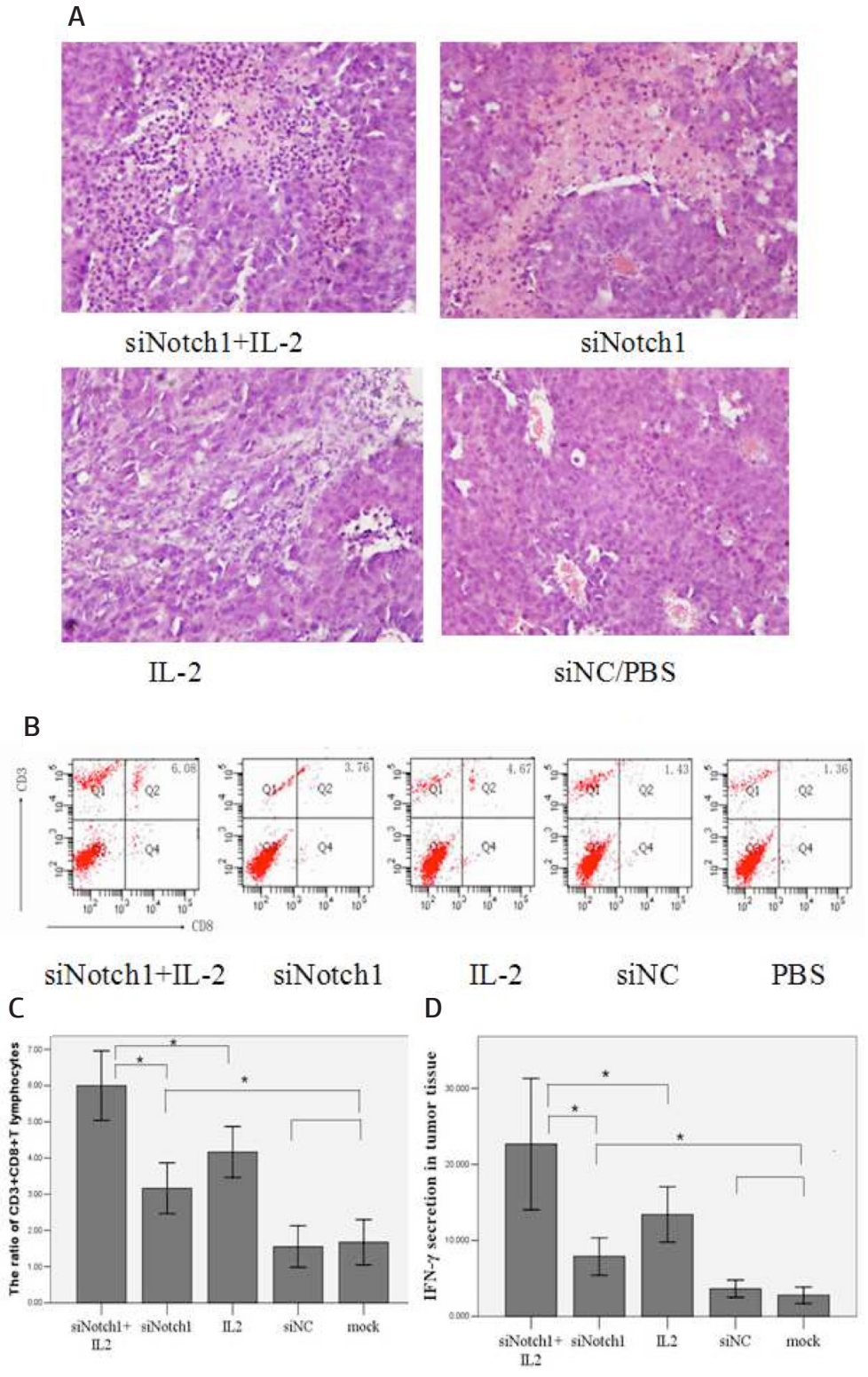

Figure 5. A: Hematoxylin & eosin staining (×400) of each group. A large number of lymphocytes were detected in the combined siNotch1 and IL-2 treatment group, whereas a moderate number of lymphocytes were observed in siNotch1 and IL-2 groups. **B:** Flow cytometry analysis of CD3+ CD8+ cytotoxic T lymphocytes. **C:** Quantitative analysis of the ratio of CD3+ CD8+ cytotoxic T lymphocytes. The number of CD3+CD8+ T lymphocytes infiltrating the tumor tissue in siNotch1, IL-2 and the combined treatment groups was significantly higher compared with siNC and PBS groups ($p<0.05$). **D:** The level of IFN-γ in IL-2 and siNotch1 groups were significantly higher compared with siNC and PBS groups ($p<0.05$). The level of IFN-γ secretion in the combined siNotch1 and IL-2 group was significantly higher than that in both IL-2 and siNotch1 groups ($p<0.05$).

References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284:770-776.
2. Lai EC. Notch signaling: control of cell communication and cell fate. Development 2004;131:965-973.
3. Penton AL, Leonard LD, Spinner NB. Notch signaling in human development and disease. Semin Cell Dev Biol 2012;23:450-457.
4. Wu L, Aster JC, Blacklow SC, Lake R, Artavanis-Tsakonas S, Griffin JD. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. Nat Genet 2000;26:484-489.
5. Koch U, Radtke F. Notch and cancer: a double-edged sword. Cell Mol Life Sci 2007;64:2746-2762.
6. Purow BW, Haque RM, Noel MW et al. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. Cancer Res 2005;65:2353-2363.
7. Sjolund J, Johansson M, Manna S et al. Suppression of renal cell carcinoma growth by inhibition of Notch signaling in vitro and in vivo. J Clin Invest 2008;118:217-228.
8. Ye QF, Zhang YC, Peng XQ, Long Z, Ming YZ, He LY. Silencing Notch-1 induces apoptosis and increases the chemosensitivity of prostate cancer cells to docetaxel through Bcl-2 and Bax. Oncol Lett 2012;3:879-884.
9. Wang Z, Zhang Y, Li Y, Banerjee S, Liao J, Sarkar FH. Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells. Mol Cancer Ther 2006;5:483-493.
10. Rose SL, Kunnimalaiyaan M, Drenzek J, Seiler N. Notch 1 signaling is active in ovarian cancer. Gynecol Oncol 2010;117:130-133.
11. Guo D, Ye J, Dai J et al. Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. Leuk Res 2009;33:678-685.
12. Massi D, Tarantini F, Franchi A et al. Evidence for differential expression of Notch receptors and their ligands in melanocytic nevi and cutaneous malignant melanoma. Mod Pathol 2006;19:246-254.
13. Balint K, Xiao M, Pinnix CC et al. Activation of Notch1 signaling is required for beta-catenin-mediated human primary melanoma progression. J Clin Invest 2005;115:3166-3176.
14. Kang S, Xie J, Miao J, Li R, Liao W, Luo R. A knockdown of Maml1 that results in melanoma cell senescence promotes an innate and adaptive immune response. Cancer Immunol Immunother 2013;62:183-190.
15. Curry CL, Reed LL, Broude E, Golde TE, Miele L, Foreman KE. Notch inhibition in Kaposi's sarcoma tumor cells leads to mitotic catastrophe through nuclear factor-kappaB signaling. Mol Cancer Ther 2007;6:1983-1992.
16. Panelos J, Massi D. Emerging role of Notch signaling in epidermal differentiation and skin cancer. Cancer Biol Ther 2009;8:1986-1993.
17. Balint K, Xiao M, Pinnix CC et al. Activation of Notch1 signaling is required for beta-catenin-mediated human primary melanoma progression. J Clin Invest 2005;115:3166-3176.
18. Bologna-Molina R, Mosqueda-Taylor A, Molina-Frechero N, Mori-Estevez AD, Sanchez-Acuna G. Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors. Med Oral Patol Oral Cir Bucal 2013;18:e174-9.
19. Antony GK, Dudek AZ. Interleukin 2 in cancer therapy. Curr Med Chem 2010;17:3297-3302.
20. Grimaldi AM, Cassidy PB, Leachmann S, Ascierto PA. Novel approaches in melanoma prevention and therapy. Cancer Treat Res 2014;159:443-455.
21. Rosenberg SA, Mule JJ, Spiess PJ, Reichert CM, Schwarz SL. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. J Exp Med 1985;161:1169-1188.
22. Hurlbut GD, Kankel MW, Lake RJ, Artavanis-Tsakonas S. Crossing paths with Notch in the hyper-network. Curr Opin Cell Biol 2007;19:166-175.
23. Railo M, Nordling S, von Boguslawsky K, Leivonen M, Kyllonen L, von Smitten K. Prognostic value of Ki-67 immunolabelling in primary operable breast cancer. Br J Cancer 1993;68:579-583.
24. Perry A, Stafford SL, Scheithauer BW, Suman VJ, Lohse CM. The prognostic significance of MIB-1, p53, and DNA flow cytometry in completely resected primary meningiomas. Cancer 1998;82:2262-2269.
25. Mucci NR, Rubin MA, Strawderman MS, Montie JE, Smith DC, Pienta KJ. Expression of nuclear antigen Ki-67 in prostate cancer needle biopsy and radical prostatectomy specimens. J Natl Cancer Inst 2000;92:1941-1942.
26. Tu TJ, Ma MW, Monni S et al. A high proliferative index of recurrent melanoma is associated with worse survival. Oncology-Basel 2011;80:181-187.
27. Tsurimoto T. PCNA, a multifunctional ring on DNA. Biochim Biophys Acta 1998;1443:23-39.
28. Barboza CA, Pereira PL, Freitas RA, Costa AL, Souza LB. Proliferating cell nuclear antigen (PCNA) and p53 protein expression in ameloblastoma and adenomatoid odontogenic tumor. Braz Dent J 2005;16:56-61.
29. Mesquita RA, Orsini SC, Sousa M, de Araujo NS. Proliferative activity in peripheral ossifying fibroma and ossifying fibroma. J Oral Pathol Med 1998;27:64-67.
30. Zhang W, Meng R, Fu C, Yu D. [Gene expression significance of beta-catenin, p53 and PCNA in PJS polyposis]. Zhonghua Wai Ke Za Zhi 2002;40:104-106.
31. Lim DY, Tyner AL, Park JB, Lee JY, Choi YH, Park JH. Inhibition of colon cancer cell proliferation by the dietary compound conjugated linoleic acid is mediated by the CDK inhibitor p21CIP1/WAF1. J Cell Physiol 2005;205:107-113.
32. Gutierrez A, Look AT. NOTCH and PI3K-AKT path-

ways intertwined. Cancer Cell 2007;12:411-413.

33. Chen SM, Liu JP, Zhou JX et al. Suppression of the notch signaling pathway by gamma-secretase inhibitor GSI inhibits human nasopharyngeal carcinoma cell proliferation. Cancer Lett 2011;306:76-84.
34. Bedogni B, Warneke JA, Nickoloff BJ, Giaccia AJ, Powell MB. Notch1 is an effector of Akt and hypoxia in melanoma development. J Clin Invest 2008;118:3660-3670.
35. Stahl JM, Sharma A, Cheung M et al. Deregulated Akt3 activity promotes development of malignant melanoma. Cancer Res 2004;64:7002-1010.
36. Vilimas T, Mascarenhas J, Palomero T et al. Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. Nat Med 2007;13:70-77.
37. Thurston G, Kitajewski J. VEGF and Delta-Notch: interacting signalling pathways in tumour angiogenesis. Br J Cancer 2008;99:1204-1209.
38. Sjolund J, Bostrom AK, Lindgren D et al. The notch and TGF-beta signaling pathways contribute to the aggressiveness of clear cell renal cell carcinoma. PLoS One 2011;6(8):e23057.
39. Zhou L, Wang DS, Li QJ, Sun W, Zhang Y, Dou KF. The down-regulation of Notch1 inhibits the invasion and migration of hepatocellular carcinoma cells by inactivating the cyclooxygenase-2/Snail/E-cadherin pathway in vitro. Dig Dis Sci 2013;58:1016-1025.