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

Summary

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 recep-
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Receptors, 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 immunohistochemical 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-difluorophenacetyl-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% penicillin G-streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37°C in a humidified incubator with 5% CO₂ and passaged every 2-3 days after digestion with trypsin and ethylene-diaminetetraacetic 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 negative 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 AUA ATT-3' and antisense 5'-UUA UUA CAC UGC AGG UUG CTT-3'; siRNA-Negative control, sense 5'- UUC UCC GAA CGU GUC AGC 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% fetal bovine serum (HyClone, Logan, UT, USA). Cells at 50-50% confluence were transfected with the plasmid using LipofectamineTM 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Briefly, LipofectamineTM 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 LipofectamineTM. 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 lipofectamineTM 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 (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 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 GCC 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 intratumoral 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 1×10^5 B16F1 cells. When the tumors were palpable with a volume of around 100-150 mm³, the tumor-bearing mice were randomly divided into 5 groups (N=5). Mice receiving intratumoral injections of PBS, 50 µg siNotch1, 1×10⁴ 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×width²)/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, sc-579, Santa Cruz, USA) and β-actin antibody (1:100 dilution, ab8227, Abcam, UK) staining.
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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 significantly 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 intumoral injection on tumor tissues, the expression of Notch1 and its downstream hey1 protein in tumor tissues was deter-
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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.

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.

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
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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 $1.5 \times 10^{5} - 3.0 \times 10^{5}$ 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, partially 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).

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