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IL-2R α up-regulation is mediated by latent membrane protein 1 and promotes lymphomagenesis and chemotherapy resistance in natural killer/T-cell lymphoma

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Abstract

Background: Natural killer/T-cell lymphoma (NKTCL) is a highly aggressive non-Hodgkin lymphoma often resistant to chemotherapy. Serum level of soluble IL-2 receptor α (IL-2R α) is elevated in NKTCL patients and correlates significantly with treatment response and survival. In the current study we examined the potential role of IL-2R α by over-expressing IL-2R α in representative cell lines.

Methods: Levels of IL-2R α were evaluated in the human natural killer cell line NK-92 and the NKTCL cell line SNK-6. Lentiviral vectors were used to express latent membrane protein 1 (LMP1) in NK-92 cells, and IL-2R α in both NK-92 and SNK-6 cells. The biological effects of these genes on proliferation, apoptosis, cell cycle distribution, and chemosensitivity were analyzed.

Results: Expression of IL-2R α was significantly higher in SNK-6 cells than in NK-92 cells. Expressing LMP1 in NK-92 cells remarkably up-regulated IL-2R α levels, whereas selective inhibitors of the proteins in the MAPK/NF- κ B pathway significantly down-regulated IL-2R α . IL-2R α overexpression in SNK-6 cells promoted cell proliferation by altering cell cycle distribution, and induced resistance to gemcitabine, doxorubicin, and asparaginase. These effects were reversed by an anti-IL-2R α antibody.

Conclusions: Our results suggest that LMP1 activates the MAPK/NF- κ B pathway in NKTCL cells, up-regulating IL-2R α expression. IL-2R α overexpression promotes growth and chemoresistance in NKTCL, making this interleukin receptor a potential therapeutic target.

Keywords: Natural killer/T-cell lymphoma, Latent membrane protein 1, Epstein–Barr virus, Interleukin-2 receptor alpha

Background

Natural killer/T-cell lymphoma (NKTCL) is an aggressive non-Hodgkin lymphoma, with no standard treatment [1, 2]. Due to the over-expression of multi-drug resistance genes, NKTCL is typically resistant to

anthracycline-based chemotherapy (e.g., CHOP regimen) [3–5]. L-Asparaginase or pegaspargase have improved patient prognosis and thus increasingly used as first-line treatments in recent years [6–9]. Asparaginase-based regimens still fail in 20%–40% of the patients [6, 7, 9, 10], and few second-line treatments have been described for asparaginase-refractory cases [11].

Interleukin-2 (IL-2) is a pro-inflammatory cytokine that activates and maintains immune responses [12]. IL-2 exerts its biological effects by binding to its receptor IL-2R on the cell surface. IL-2R consists of three subunits. The α subunit, also known as CD25 or Tac antigen,

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does not participate on its own in IL-2-triggered signal transduction due to its very short intracellular domain. When the α subunit combines with IL-2R β and IL-2R γ , the affinity of IL-2R to IL-2 increases. Proteolytic cleavage of membrane-bound IL-2R α generates a soluble form of IL-2R α (sIL-2R α) [13]. In a variety of solid malignant tumors as well as hematologic malignancies, serum sIL-2R α is elevated and correlates with poor patient prognosis [14–18]. In a previous retrospective analysis from this research group, we reported elevated serum sIL-2R α in NK-TCL patients than in healthy individuals [19]. We also found significant correlation between higher sIL-2R α with poor chemotherapy response and patient prognosis [19].

In the present study, we examined the potential effects of IL-2R α over-expression on cell proliferation, cell cycle distribution and sensitivity to chemotherapeutic drugs in representative NK-TCL cell lines. Mechanistic investigation identified latent membrane protein 1 as a downstream effector.

Methods

Cell lines and culture

The human NK-TCL cell line SNK-6 and human natural killer cell line NK-92 (both were generously donated by professor Zhang Ming-zhi from the First Affiliated Hospital of Zhengzhou University) were incubated at 37 °C in an atmosphere of 5% CO₂. SNK-6 cells were cultured in RPMI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and recombinant human IL-2 (1000 U/ml; Sigma, USA). NK-92 cells were maintained in RPMI 1640 containing 10% FBS.

Construction of cell lines overexpressing LMP1 and IL-2R α

Lentivirus vectors expressing latent membrane protein-1 (LMP1) were constructed as described previously [20]. Briefly, full-length IL-2R α cDNA was inserted into the *NotI/BamHI* sites of the LV5 vector (GenePharma, China). Vector encoding IL-2R α and the packaging vectors pGag/Pol, pRev, and pVSV-G (GenePharma) were co-transfected into HEK293T cells using Lipofectamine 2000 (Beyotime, Shanghai, China). Supernatant containing recombinant lentivirus was collected 72 h after transfection. NK-92 and SNK-6 cells (1×10^4 cells/well) were separately infected for 24 h with the viruses in the presence of polybrene (5 μ g/ml). The medium was replaced with fresh medium, and cells were cultured another 48 h. Transfected cells were selected using puromycin (1.0 μ g/ml) for 7–10 days prior to expansion.

Western blot analysis

Western blot analysis was performed as described previously [20]. Briefly, cells were washed with ice-cold PBS and suspended in radioimmunoprecipitation assay (RIPA) lysis buffer (Biyuntian Biotech, Shanghai, China) containing 1% phenylmethylsulfonylfluoride. After centrifugation at 18,735 \times g for 10 min at 4 °C, the supernatant (10 μ g protein per lane) was fractionated using 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated at room temperature for 1 h with one of the following primary antibodies: IL-2R α , B-Raf, p-B-Raf, p38, p-p38, ERK, p-ERK (Abcam, Shanghai, China); LMP1, JNK, p-JNK (Santa Cruz, Shanghai, China); p65 and cyclins A1, A2, B1, and D (Boster); cyclin E (Proteintech, Wuhan, China); cyclin-dependent protein kinase (CDK) 1 (Abcam); and CDK2 and 4 (Boster). After extensive washing, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 20,000; Boster, Wuhan, China) at room temperature for 40 min. Signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia, Piscataway, NJ, USA). Results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sample protein concentration was determined using a BCA method.

Quantitative real-time PCR analysis

Quantitative RT-PCR was performed as described previously [20]. Briefly, total RNA was isolated using Trizol (Invitrogen, USA). Total RNA (1 μ g) was reverse-transcribed into cDNA using the Bestar™ qPCR RT Kit (DBI Bioscience, China). The qRT-PCR reaction was conducted in a total volume of 20 μ l containing 10 μ l DBI Bestar® SybrGreen qPCR Master Mix (DBI Bioscience), cDNA derived from 0.2 μ g of input RNA, 5 pM of each primer, and 7 μ l double-distilled H₂O. PCR reactions were carried out using a Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, USA) with the following steps: pre-denaturation at 95 °C for 2 min, followed by 40 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. Each reaction was performed three times. Fold differences in cDNA level relative to the GAPDH level were calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used: IL-2R α sense, 5'-AAATGACCCACGGGAAGAC-3'; IL-2R α antisense, 5'-TTGTGACGAGGCAGG AAGT-3'; LMP1 sense, 5'-CAACAACGGCAAGACTCC C-3'; LMP1 antisense, 5'-CCTCAAAGAAGCCACCCCT C-3'.

Measurement of sIL-2R α in culture supernatant

NK-92, SNK-6 and NK-92 transduced with lentivirus encoding LMP1, and NK-92 and SNK-6 transduced with lentivirus encoding IL-2R α or negative control lentivirus were centrifuged at $382\times g$ for 5 min. sIL-2R α concentration in the supernatant was measured using a sandwich enzyme-linked immunosorbent assay (Fine Biological Technology, Wuhan, China).

Cell proliferation and cytotoxicity assay

Cell proliferation was assessed using the Cell Counting kit-8 (CCK-8; Dojin, Tokyo, Japan). For cytotoxicity assay, SNK-6 cells were exposed to doxorubicin, gemcitabine, or asparaginase of varying concentrations for 24 or 48 h prior to CCK-8 assay. Optical density was measured at a wavelength of 450 nm using a Multiskan microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Relative fold drug resistance was calculated using IC₅₀ values.

Analysis of cell cycle distribution and apoptosis

Flow cytometry was used to determine cell cycle distribution and detect apoptosis. Upon 85% confluence, culture medium was removed and cells were suspended, centrifuged and fixed in precooled 70% ethanol for 1 h. The suspension was centrifuged again, the supernatant was removed, and the cells were washed with ice-cold PBS and stained with propidium iodide (PI; 50 μ g/ml, Sigma-Aldrich, St. Louis, MO, USA) in the presence of RNase A (100 μ g/ml; Fermentas[®], Shanghai, China). The suspension was passed through a 300-mesh filter, and DNA content of stained nuclei was analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA). Each experiment was performed in triplicate.

Apoptosis was analyzed using the Annexin V-APC/7-AAD Apoptosis Detection Kit (Lianke Bio, Hangzhou, China). The percentage of apoptotic cells was determined by flow cytometry on a BD FACS Calibur flow cytometer. All experiments were performed in triplicate.

Statistical analysis

Results are expressed as mean \pm SD. Statistical analysis was performed using SPSS 17.0 (IBM, Chicago, IL, USA). Inter-group differences were assessed for significance using Student's *t*-test. Differences were defined as statistically significant at $P < 0.05$ (2-sided).

Results

Expression of IL-2R α is higher in NKTCL cells than in natural killer cells

IL-2R α expression was significantly higher in SNK-6 cells than in NK-92 cells at both the mRNA (Fig. 1a) and protein levels (Fig. 1b). Similarly, the level of

sIL-2R α in culture supernatant was significantly higher in SNK-6 cells (Fig. 1c).

LMP1 acts via the MAPK/NF- κ B pathway to up-regulate IL-2R α in NK-92 cells

Lentivirus encoding LMP1 and negative control lentivirus infected cells efficiently at a multiplicity of infection of 300 (Fig. 1d, e). Western blot analysis revealed significantly higher LMP1 expression in NK-92 cells expressing lentivirus-encoded LMP1 at the mRNA (Fig. 1f) and protein levels (Fig. 1h) than in negative control lentivirus vector control. Similar results were observed with IL-2R α (Fig. 1g, h) as well as sIL-2R α in culture supernatant (Fig. 1i).

Up-regulation of these proteins correlated with increases in the levels of several proteins in the MAPK/NF- κ B pathway: p-Raf-B, p-p38, p-JNK, p-ERK, and p65 (Fig. 2a). When NK-92 cells expressing lentivirus-encoded LMP1 were treated with the following selective inhibitors of proteins in the MAPK/NF- κ B pathway, IL-2R α was down-regulated: SB590885, which inhibits B-Raf (Fig. 2b); PD98059, which inhibits ERK (Fig. 2c); SB203580, which inhibits p38 (Fig. 2d); SP600125, which inhibits JNK (Fig. 2e); and pyrrolidine dithiocarbamate (PDTTC), which inhibits NF- κ B (Fig. 2f). Similarly, inhibitors of the MAPK/NF- κ B pathway remarkably reduced expression of IL-2R α in SNK-6 cells constitutively expressing LMP1 (Fig. 3).

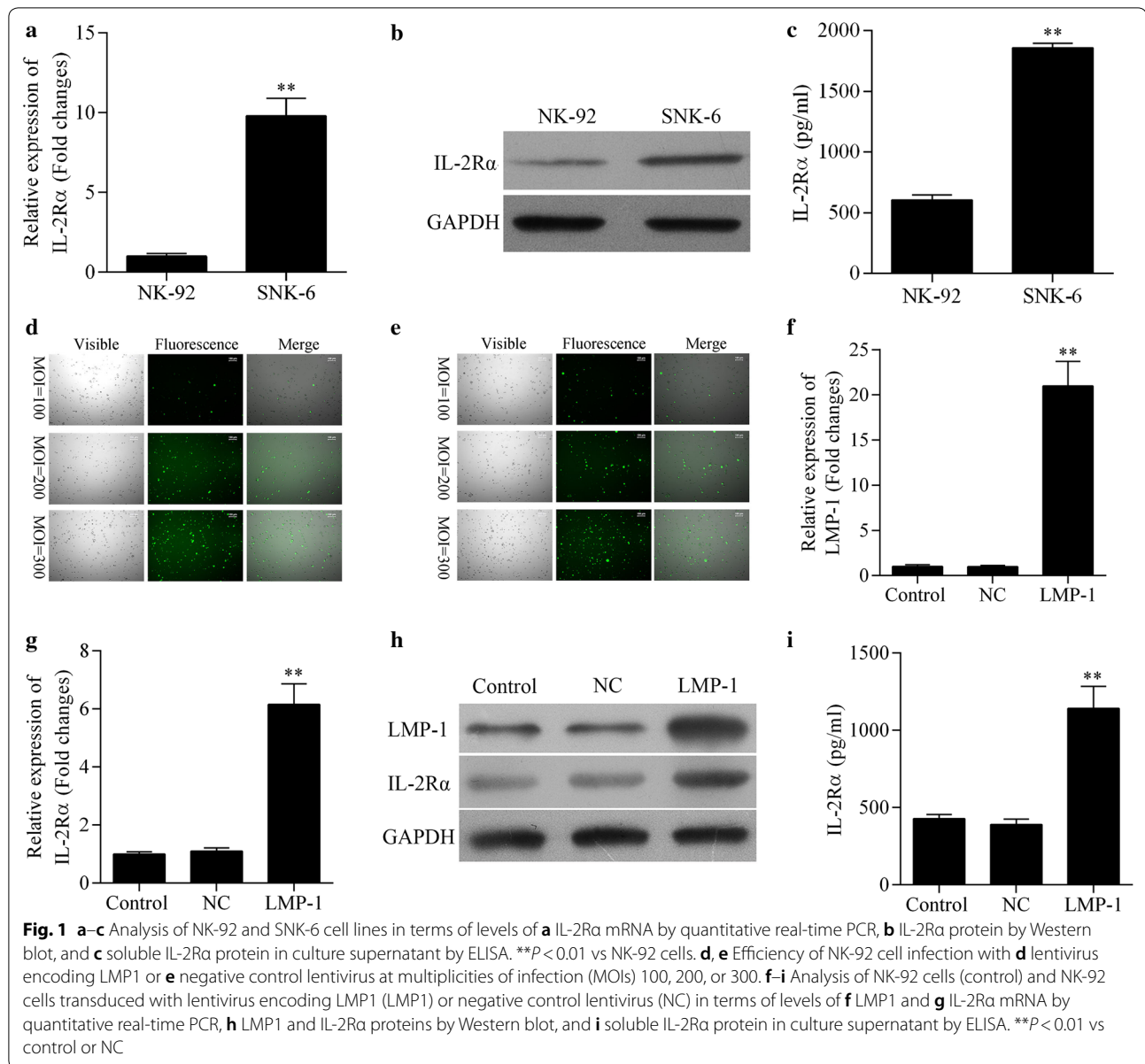
IL-2R α overexpression promotes proliferation of NK-92 and SNK-6 cells

The IL-2R α expression was significantly higher in cells expressing lentivirus-encoded IL-2R α at the mRNA (Fig. 4a) and protein levels (Fig. 4b) in comparison to the negative lentivirus control. Cells expressing lentivirus-encoded IL-2R α also secreted higher amount of sIL-2R α into the supernatant (Fig. 4c), and had higher rate of proliferation (Fig. 5a, b). IL-2R α overexpression did not significantly alter the percentage of apoptotic cells (Fig. 6).

IL-2R α overexpression decreased the percentage of cells in the G0/1 phase and increased the percentage in the S phase (Fig. 7). These changes were caused by increases in protein levels of cyclin A1, A2, B1, D, CDK1, and CDK4 (Additional file 1: Figure S1).

IL-2R α overexpression mediates chemoresistance in SNK-6 cells

IL-2R α overexpression in SNK-6 cells resulted in significantly higher IC₅₀ values against gemcitabine, doxorubicin, and L-Asp (Fig. 8). Addition of anti-IL-2R α antibody (0.2 μ g/ml; Beijing Shuanglu Pharmaceutical, Beijing, China) reduced all three IC₅₀ values.



Targeting of IL-2Rα to treat relapsed/refractory NKTCL

A 42-year-old man with stage IV NKTCL who relapsed after six cycles of GELOX (gemcitabine, oxaliplatin, and asparaginase) and who did not respond to two cycles of EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, and adriamycin) and two cycles of SMILE (dexamethasone, methotrexate, ifosfamide, asparaginase, and etoposide) was then given two cycles of pegaspargase and anti-IL-2Rα antibody basiliximab (2500 IU/m² pegaspargase given on day 1 + 20 mg basiliximab given on day 1 and 8, repeated every 3 weeks). This last treatment achieved partial remission and its toxicity was tolerable

(Additional file 2: Figure S2). Levels of Epstein–Barr virus DNA in plasma decreased from 4.2×10^3 copies/mL to 0.

Discussion

NKTCL is a rare, highly aggressive hematologic malignancy. The frequency with which the disease is resistant to conventional chemotherapy (e.g., CHOP and EPOCH) has made radiotherapy the primary therapy [5, 8, 21, 22]. Although novel regimens incorporating L-Asp or pegaspargase have significantly improved outcomes, 20%–40% of patients still experience treatment failure [6, 7, 9, 10]. Therefore, there is an urgent need to develop new

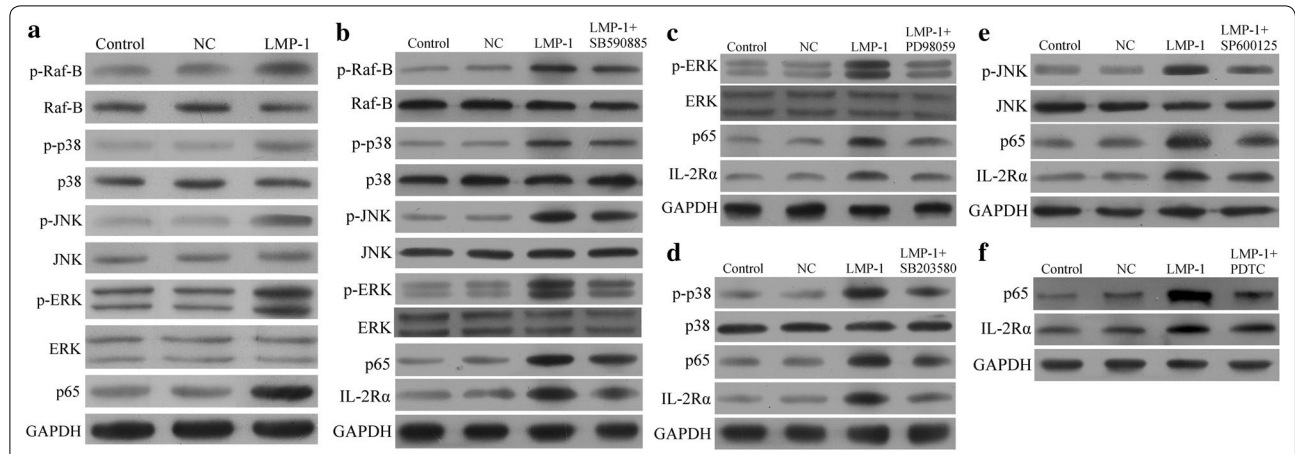


Fig. 2 LMP1 up-regulated IL-2Rα expression through the MAPK/NF-κB pathway in NK-92 cells. **a** NK-92 cells transduced with LMP1-encoding lentivirus led to higher levels of p-Raf-B, p-p38, p-JNK, p-ERK, and p65 than transduction with NC lentivirus. **b–f** NK-92 cells transduced with LMP1-encoding lentivirus were treated for 1 h with the following selective inhibitors of proteins in the MAPK/NF-κB pathway, leading to IL-2Rα down-regulation: **b** 0.1 μM SB590885 (B-Raf inhibitor), **c** 20 μM PD98059 (ERK inhibitor), **d** 10 μM SB203580 (p38 inhibitor), **e** 20 μM SP600125 (JNK inhibitor), and **f** 100 μM pyrrolidine dithiocarbamate (PDTIC; NF-κB inhibitor)

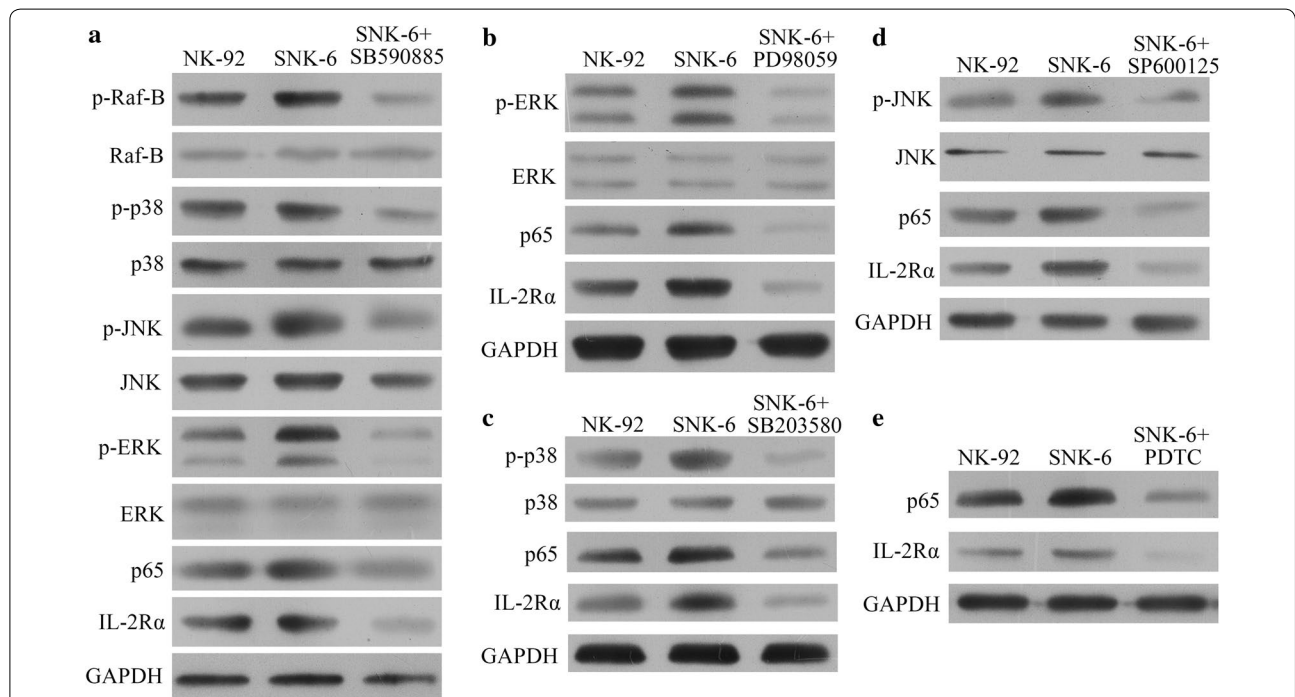
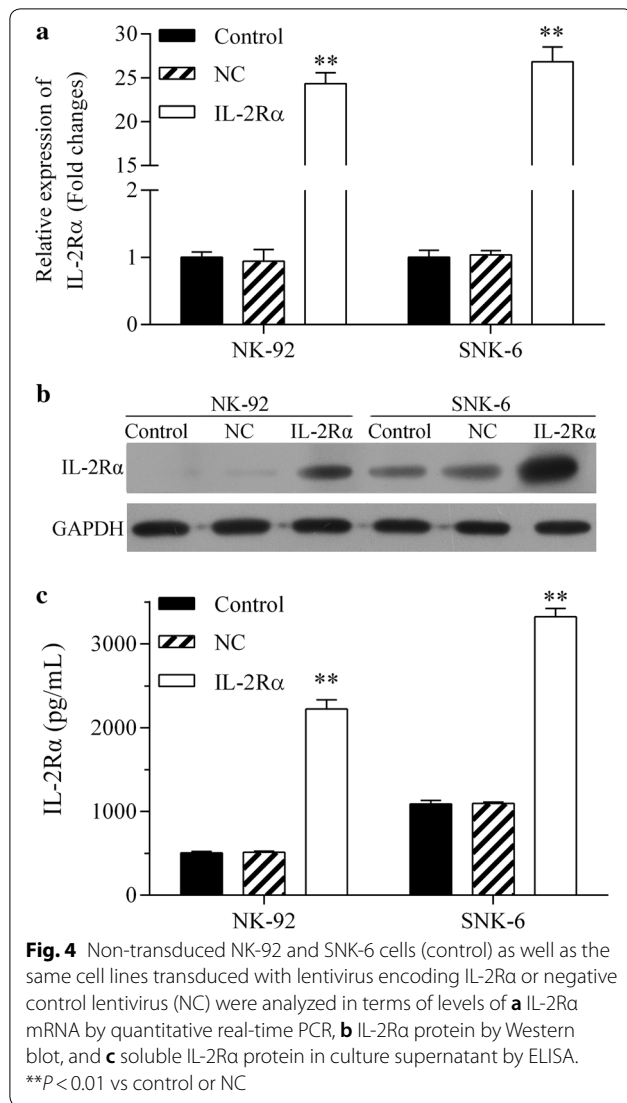


Fig. 3 Treating SNK-6 cells constitutively expressing LMP1 for 1 h with the following selective inhibitors of proteins in the MAPK/NF-κB pathway down-regulated IL-2Rα: **a** 0.1 μM SB590885 (B-Raf inhibitor), **b** 20 μM PD98059 (ERK inhibitor), **c** 10 μM SB203580 (p38 inhibitor), **d** 20 μM SP600125 (JNK inhibitor), or **e** 100 μM pyrrolidine dithiocarbamate (PDTIC; NF-κB inhibitor)

biological and genetic biomarkers to predict prognosis and guide therapy.

In previous clinical work, our group reported that sIL-2Rα levels in serum were significantly elevated in NKTCL, and that these levels correlated with

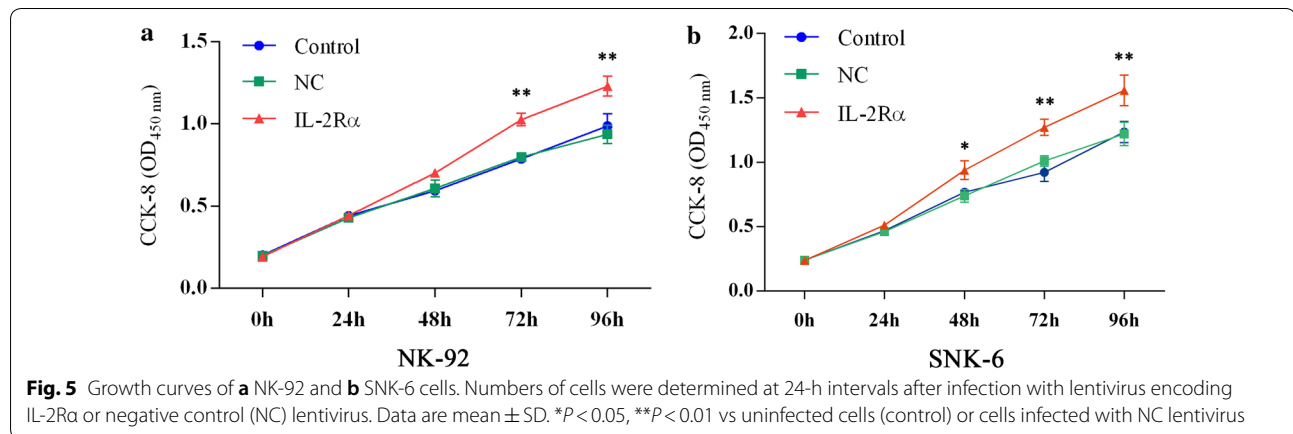
chemotherapy response and prognosis [19]. The present study extends and deepens that work by describing a pathway by which IL-2Rα overexpression promotes NKTCL tumor growth, and by showing the potential of anti-IL-2Rα antibody therapy. Here we describe several

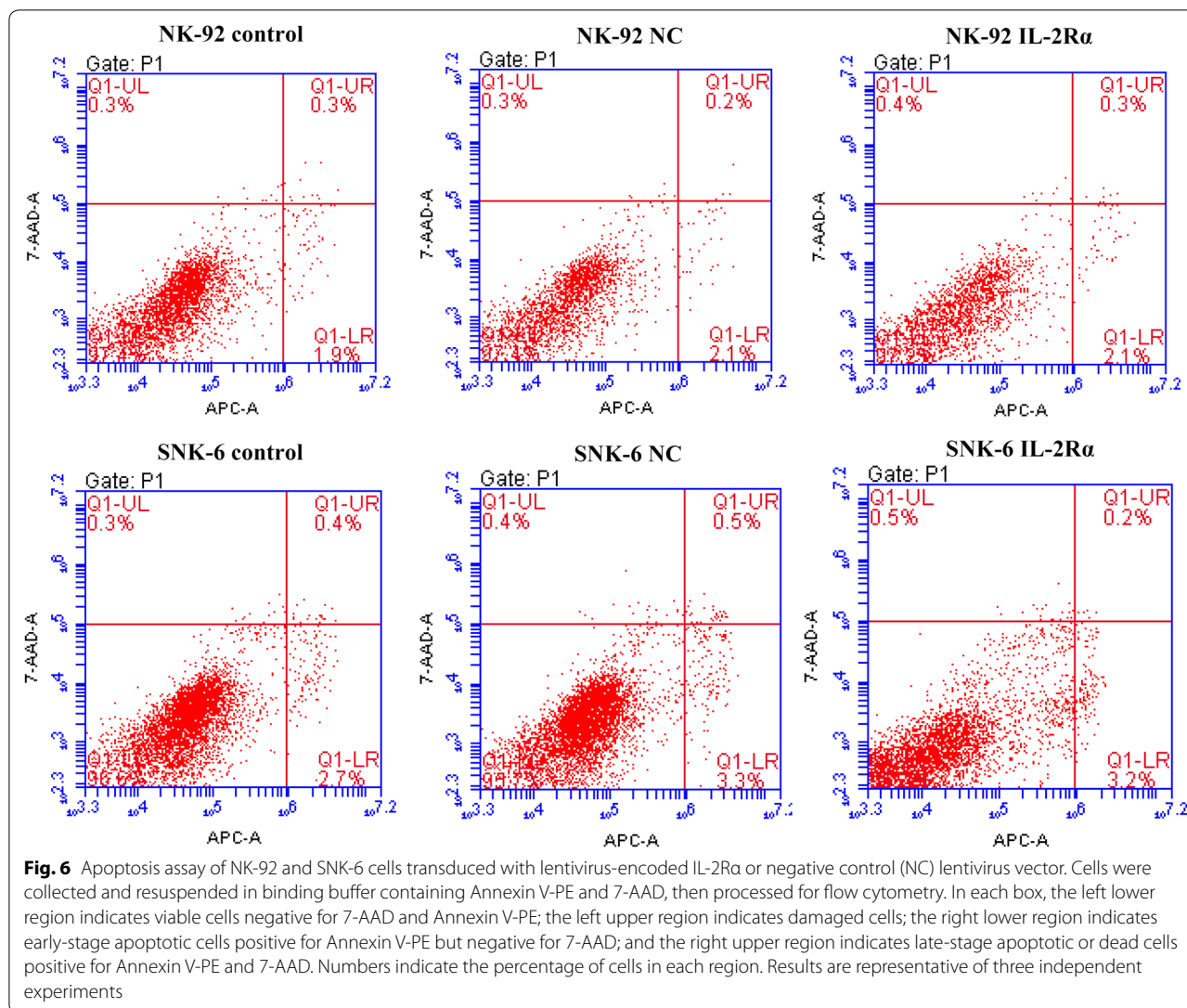


major findings: (1) LMP1 acts via the MAPK/NF- κ B pathway to up-regulate IL-2R α in NKTCL, (2) IL-2R α overexpression promotes NKTCL cell proliferation and cell cycle progression without affecting apoptosis, and (3) IL-2R α overexpression correlates positively with chemoresistance in NKTCL cells, and anti-IL-2R α antibody can restore chemosensitivity.

Elevation of various cytokines has been observed in both non-Hodgkin and Hodgkin lymphoma [23–25]. IL-2 acts as a potent immunomodulator and activates many immune cells, including antigen-specific T cells, B cells, and natural killer cells [12]. Elevated expression of the IL-2R α subunit of the IL-2 receptor has been reported in several types of malignancy, including NKTCL [26, 27]. The high affinity of IL-2R α for IL-2 led us to hypothesize that IL-2R α up-regulation may promote tumor cell proliferation and progression. Consistent with this idea, a preliminary study of NKTCL patients showed that in 53.8% of cases, IL-2R α was expressed on the tumor surface (data not shown). In the present study, IL-2R α expression was significantly stronger in NKTCL cells than in natural killer cells, and more sIL-2R α was present in supernatants of NKTCL cultures.

We provide here evidence that IL-2R α up-regulation in NKTCL may be mediated by the Epstein–Barr viral protein LMP1. The Epstein–Barr virus plays a pivotal role in NKTCL pathogenesis, and LMP1 regulates several cytokines and cytokine receptors in malignancies associated with the Epstein–Barr virus [28–30]. We found that LMP1 expression in NK-92 cells up-regulated IL-2R α and sIL-2R α levels, which was reversed by selective inhibitors of the MAPK/NF- κ B pathway. This is consistent with previous work showing that LMP-1 acts via the NF- κ B pathway to up-regulate IL-2R α in two other lymphomas associated with Epstein–Barr virus, Hodgkin’s lymphoma and Burkitt’s lymphoma [31].

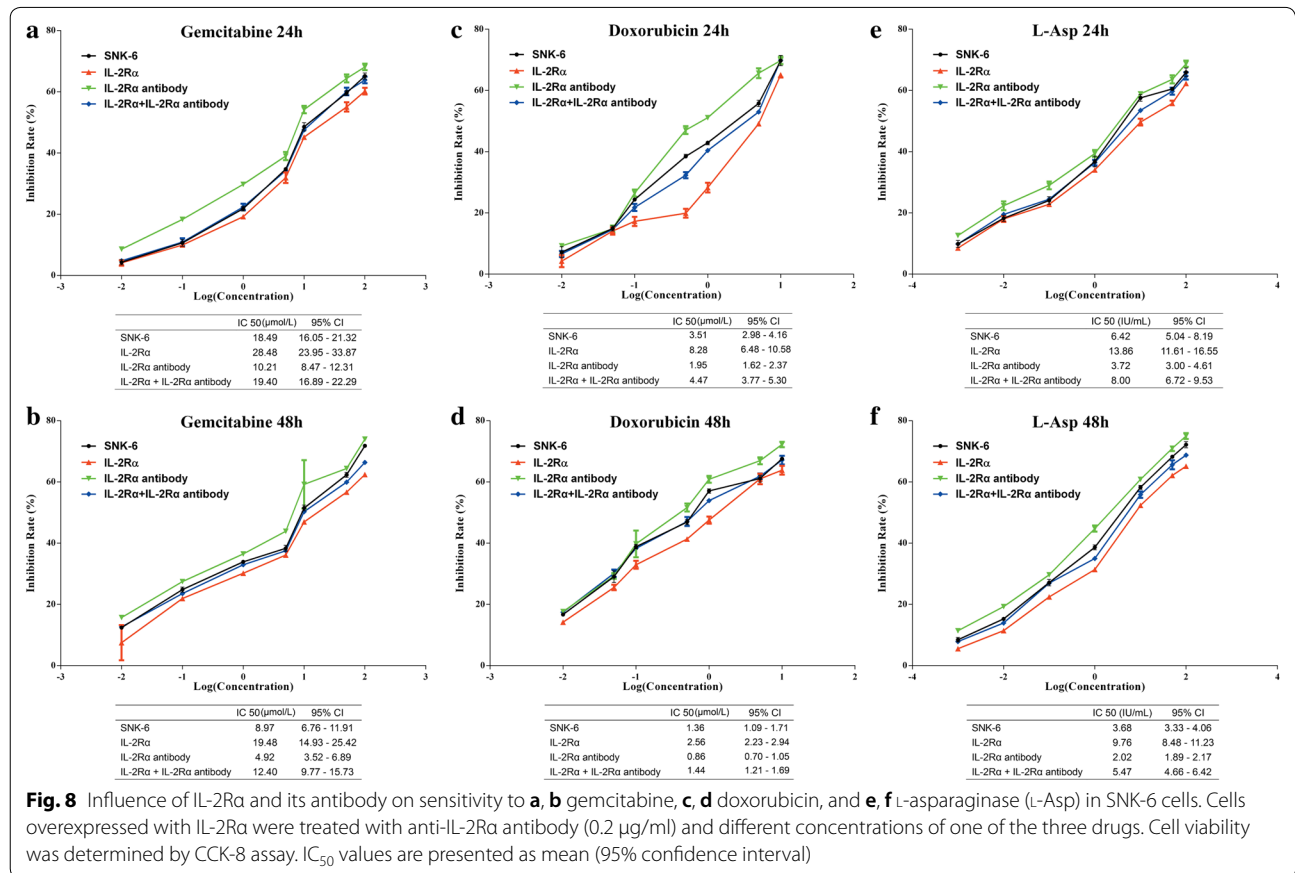
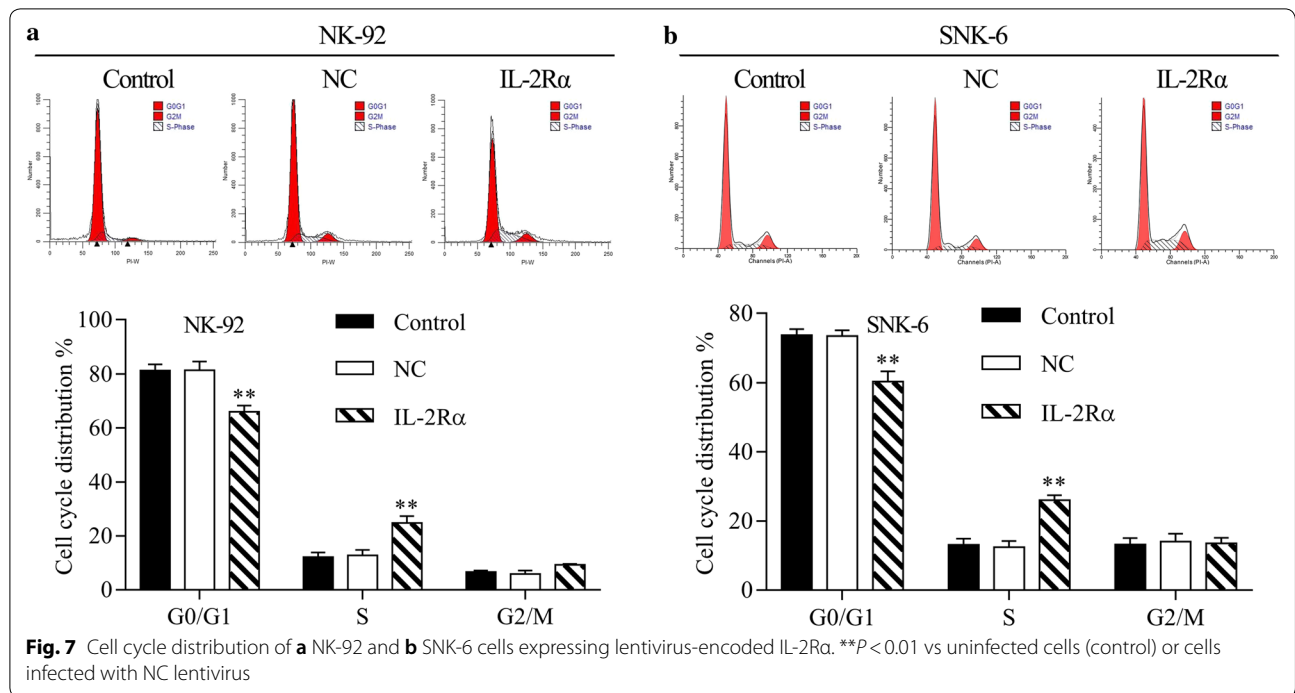




Elevated levels of sIL-2R predict inferior outcomes in several types of lymphoma, including B-cell and T-cell non-Hodgkin lymphomas [18, 32, 33]. In some hematological neoplasms, such as hairy-cell leukemia and adult T-cell leukemia, sIL-2R α is released by tumor cells constitutively expressing IL-2R α [34, 35]. Therefore, the serum level of sIL-2R α may reflect tumor burden and disease activity. The intracellular domain of IL-2R α is too short to elicit signal transduction on its own, but IL-2R α promotes IL-2 recycling back to the cell surface, creating an IL-2 reservoir at the surface, thereby potentiating its activity [36]. This activates oncogenic pathways such as JAK-STAT, MAPK, and PI3K, with mitogenic and anti-apoptotic effects [37, 38]. At the same time, sIL-2R α binds soluble sIL-2, and the complex activates tumor-friendly Treg cells rather than antitumor T cells [32]. In the present study, we report for the first time that IL-2R α

overexpression promotes NKTCL cell proliferation and cell cycle progression without affecting apoptosis. This is consistent with previous reports that IL-2 activates and promotes natural killer cell proliferation [39], and further work should clarify the mechanism(s) involved.

Resistance to chemotherapy is the major cause of treatment failure in NKTCL, especially in advanced disease [40]. While overexpression of multi-drug resistance genes may contribute to this chemoresistance [4], our previous work showed that elevated serum levels of sIL-2R α correlated with significantly inferior response rate to chemotherapy (mainly CHOP or EPOCH regimens) [19]. Consistent with this previous work, we report here that IL-2R α overexpression in NKTCL cells led to chemoresistance in vitro to gemcitabine, doxorubicin, and L-Asp. As further evidence for the involvement of IL-2R α , this chemoresistance was reversed by anti-IL-2R α antibody.



This suggests the potential of targeting IL-2R α for treating NKTCL. Indeed, we combined the anti-IL-2R α antibody basiliximab with pegaspargase to successfully treat a patient with relapsed/refractory NKTCL. Both basiliximab and the humanized monoclonal anti-IL-2R α antibody daclizumab have been used to treat autoimmune diseases such as multiple sclerosis as well as prevent transplant rejection. We plan to conduct a prospective phase 2 clinical trial to evaluate the efficacy of combination of basiliximab and pegaspargase in patients with relapsed/refractory NKTCL [41].

Our results should be interpreted with caution given several limitations. We did not investigate which IL-2-associated signaling pathways may help mediate the tumor-promoting effects of IL-2R α in NKTCL, nor did we examine the immunomodulatory effects of IL-2R α in NKTCL. The role of serum sIL-2R α in lymphomagenesis needs to be further investigated. Thorough study of these questions will improve our understanding of the role of IL-2R α in the pathogenesis and progression of NKTCL.

Conclusions

In NKTCL, LMP-1 acts via the NF- κ B pathway to up-regulate IL-2R α , and promotes tumor growth. Further studies are warranted to understand how IL-2R α exerts its oncogenic effects and to explore IL-2R α as a therapeutic target.

Additional files

Additional file 1: Figure S1. Levels of several cell cycle proteins were increased in SNK-6 cells when IL-2R α was overexpressed, as detected by Western blot. Control samples came from uninfected cells, while negative control samples came from cells infected with NC lentivirus.

Additional file 2: Figure S2. Positron emission tomography-computed tomography of a 42-year-old man with refractory NKTCL before and after treatment with anti-IL-2R α antibody (basiliximab) and pegaspargase. Before treatment, diffuse infiltration of lymphoma with high metabolic activity was observed (*right panel*). After two cycles of treatment, partial remission was observed (*left panel*).

Abbreviations

NKTCL: natural killer/T-cell lymphoma; IL-2R α : IL-2 receptor α ; L-Asp: L-asparaginase; IL-2: interleukin-2; LMP1: latent membrane protein-1.

Authors' contributions

LW and QQC designed this study. LW, XWB, and YJZ performed the experiments, analyzed the data and wrote the manuscript. LW, YZH, and QYL collected data. ZJX provided patients and data. All authors read and approved the final manuscript.

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Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data that support the findings of this study have been deposited in the Research Data Depository (<http://www.researchdata.org.cn/>) with Approval Number RDDDB2018000417. The data are available from the author upon reasonable request and with permission from this Depository.

Consent for publication

The patient in the current study provided written informed consent for collection and publication of his medical information.

Ethics approval and consent to participate

This study protocol was approved by the Ethics Committee of Zhujiang Hospital of Southern Medical University, and complied with relevant national regulations.

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