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Over-expressed Fas improves the apoptosis of malignant T-cells in vitro and vivo

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Abstract Fas play a critical role in T-cell apoptosis by functioning as a major cell-surface death receptor. To explore a potential method that can improve the sensitivity to Fas-mediated apoptosis in malignant precursor T-cells. Fas gene was stable transfected into Jurkat cells to establish a new cell line named Jurkat-Fas with over-expressed Fas. RT-PCR, real-time RT-PCR, flow cytometry, and confocal microscopy assay were performed to detect the Fas level of mRNA and protein in the two cell lines. The sensitivities to Fas-mediated apoptosis of the two cell lines were evaluated by flow cytometry with Alexa Fluor 488 annexin V/PI staining in vitro. Tumor xenograft models were prepared with Jurkat and Jurkat-Fas cells for in vivo study. Fas mRNA and protein levels in Jurkat-Fas cell line were higher than that in Jurkat cell line. Compared to Jurkat cells, apoptosis rates of Jurkat-Fas cells were remarkably higher in vitro, and the tumor growth of Jurkat-Fas cells in nude mice was significantly inhibited in vivo. Stable over-expression of extrinsic Fas gene can significantly ameliorate the sensitivity to Fas-mediated apoptosis in human malignant T-cell, which indicates a novel strategy to improve therapeutic effects on precursor T-cell malignancy.

Keywords Fas · T-cell malignancy · Jurkat · Apoptosis

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Introduction

Precursor T-cell malignancy is a biologic unit in the World Health Organization classification referring to clinically and biologically heterogeneous group of neoplasm originating from T lymphocyte. Among the Precursor T-cell malignancies, T-cell lymphoblastic lymphoma (T-LBL) and T-cell acute lymphoblastic leukemia (T-ALL) are separated by a cut point of 25% bone marrow infiltration: the former is below 25% and the latter is above 25% [1].

Fas, a member of tumor necrosis factor receptor (TNFR) family, play a critical role in T-cell apoptosis by functioning as a major cell-surface death receptor. During inflammation reaction, activated and proliferative T-cells are cleared through Fas pathway which is termed as activation induced cell death (AICD) to avoid excessive accumulation of these T-cells [2]. Various defections in Fas including gene mutation, gene deletion, aberrant transcript, and deregulation of protein expression have been found in patients with T-cell malignancy [3–15]. Based on the fundamental role of Fas system in T-cell homeostasis and the frequent occurrence of Fas defections in patients with T-cell malignancy, Fas is identified as an attractive candidate for an important role in the pathogenesis of T-cell malignancy.

While the T-cells transfected with defective Fas gene present reduced sensitivity to Fas-mediated apoptosis, the effect of transfection with normal Fas gene in malignant T-cells is still unclear [8, 16]. To explore a potential method that can improve the sensitivity to Fas-mediated apoptosis in malignant precursor T-cell, this study focusing on the contribution of over-expressed Fas to the apoptosis in Jurkat cells were performed in both vitro and vivo.

Materials and methods

Malignant precursor T-cell line

The human malignant precursor T-cell line Jurkat was grown in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and a humidified atmosphere at 37°C and 5% CO_2.

Wild type Fas gene amplification

A peripheral blood sample of a healthy volunteer was obtained with institutional review board approval. The volunteer gave informed consent and the protocol adhered to the declaration of Helsinki principles. The mononuclear cells were separated using Ficoll reagent and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total cDNA was synthesized from the total RNA with superscript reverse transcriptase and random primers (Invitrogen, Carlsbad, CA, USA). Full-length Fas cDNA (1,167 bp) was amplified from the total cDNA by polymerase chain reaction (PCR) using high-fidelity *Taq* polymerase (Invitrogen, Carlsbad, CA, USA) with primers as described by Weller et al. [17]. The PCR products were gel-purified and directly sequenced from both sides on an ABI-PRISM3730 sequencing apparatus (Applied Biosystems, Carlsbad, CA, USA).

Eukaryotic vector construction

Fas cDNA was retrieved from agar gel and cloned into vector pMD-18T (Invitrogen, Carlsbad, CA, USA). The pMD-18T-Fas plasmid was transferred into competent cell DH5α to grow. The target gene of Fas cDNA was amplified using forward primer (5′-CCGGAATTCCACTTCGGAG GATTGCTCAAC-3′) and reverse primer (5′-CCGCTCG AGTATGTTGGCTCTTCAGCGCT-3′) with EcoRI and XhoI cutting sites (95°C for 25 s, 60°C for 25 s, 68°C for 75 s). After cut by EcoRI and XhoI (NEB), the target gene was cloned into eukaryotic expression vector pCDNA3.1(+) by ligase (NEB, Ipswich, MA, USA). The recombinant plasmid pCDNA3.1(+)-Fas was transferred into DH5α to grow and then extracted by QIAprep Spin Miniprep Kit (Qigan, Hilden, Germany). The recombinant plasmids were directly sequenced on an ABI-PRISM3730 sequencing apparatus (Applied Biosystems, Carlsbad, CA, USA).

Cell transfection

Jurkat cells were transfected with pCDNA3.1(+)-Fas using Attractene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were then grown in complete medium containing 500 mg/ml G418 (Sigma, St.Louis, MO, USA) for 7 days. The survive colonies were expanded continuously in 250 mg/ml G418 contained medium and were named as Jurkat-Fas cells.

Analysis of Fas mRNA level

RT-PCR

Total RNA (1 μg) of each cell line was isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify the full-length Fas cDNA using primers and method as described above with GAPDH as an internal control. The PCR products and DNA ladder molecular weight marker (TaKaRa BIO, Shiga, Japan) were loaded onto 1% agarose gel containing gel red in TAE. Images were acquired by imaging devices (Bio-Rad, Hercules, CA, USA).

Real-time RT-PCR

Total RNA and total cDNA of each cell line were synthesized as descried above. 40 cycles of real-time PCR were performed to amplify 110 bp of FAS cDNA using the

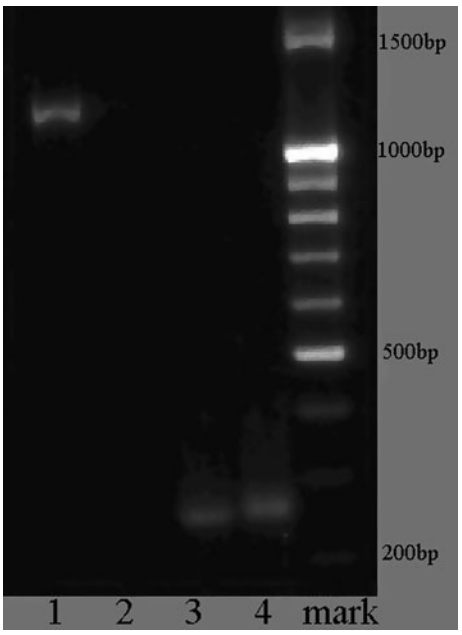

Fig. 1 Identification of Fas mRNA in the two cell lines by RT-PCR. Total RNA (1 μg) of each cell line was isolated and total cDNA was synthesized from the total RNA. RT-PCR was performed to amplify the full-length Fas cDNA with GAPDH as a control. *1* Fas in Jurkat-Fas cells, *2* Fas in Jurkat cells, *3* GAPDH in Jurkat-Fas cells, *4* GAPDH in Jurkat cells

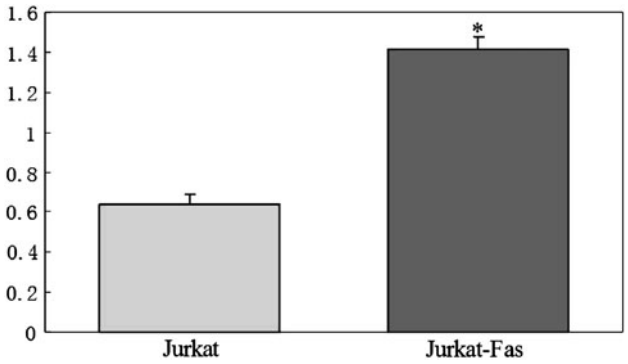

Fig. 2 Real-time RT-PCR analysis of Fas mRNA level in the two cell lines. Total RNA (1 μg) of each cell line was isolated and total cDNA was synthesized from the total RNA. 40 cycles of real-time PCR were performed to amplify 110 bp of FAS cDNA with GAPDH as an internal control. *Histograms* show relative expression level of FAS mRNA in the two cell lines. Data are represented as mean ± SEM ($n = 3$). *$P < 0.05$

primers and method described by Wu et al. [10] with GAPDH as an internal control. For each detection, the procedure was repeated three times.

Analysis of Fas protein level

Confocal microscopy assay

4×10^5 cells of each cell line were collected and washed twice with cold PBS. The cells were stained by PE anti-human Fas (eBioscience, San Diego, CA, USA) according to the manufacturer's instruction. After washed twice with cold PBS, the cells were scanned under confocal microscopy at a wavelength of 488 nm.

Flow cytometry

4×10^5 cells of each cell line were prepared and treated with PE anti-human Fas as described above. The fluorescence intensity was detected by flow cytometry with PE mouse IgG1 κ isotype control (eBioscience, San Diego, CA, USA) as an internal control. For each detection, the procedure was repeated three times.

Apoptosis assay

After treated with 25 ng/ml human soluble Fas Ligand (Enzo, Plymouth Meeting, PA, USA) for 0, 12, and 24 h, respectively, 4×10^5 cells of each cell line were collected and stained with Alexa Fluor 488 annexin V and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA). The apoptotic ratios were analyzed by flow cytometry. For each detection, the procedure was repeated three times.

Animal tumorigenesis

All animal procedures were approved by the Committee on Animal Experimentation of SOOCHOW University, and the procedures were complied with the NIH Guide for the Care and Use of Laboratory Animals. Eight SPF male BALB/c nude mice (4 weeks of age), obtained from the Center of Experimental Animals of SOOCHOW University, were separated into two groups averagely and randomly. After exposed to medial lethal dose of ray (4 Gy), the two groups of mice were subcutaneously inoculated with 5×10^7 Jurkat and Jurkat-Fas cells suspended in 100 μl PBS at the upper right armpit, respectively. All mice were injected with 0.5 ml 100 ng/ml human soluble Fas Ligand (Enzo, Plymouth Meeting, PA, USA) through caudal vein every 7 days from the injection of tumor cells. Tumor growth was monitored through calculating size in diameter (mm) using calipers every 4 days.

Statistical analysis

All data were expressed as Means ± SEM. Sample *t* test was conducted to compare the average of two numerical variables. All statistical analyses were performed using the

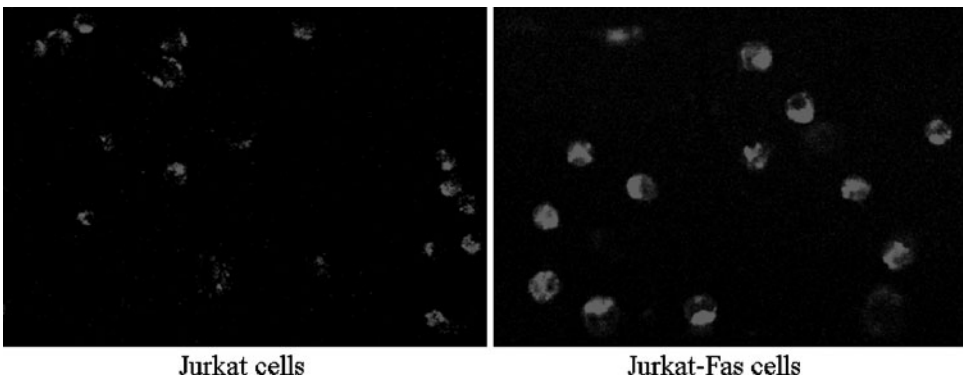

Fig. 3 Representative images of Fas protein. 4×10^5 cells of each cell line were scanned under confocal microscopy at a wavelength of 488 nm after treated with PE anti-human Fas

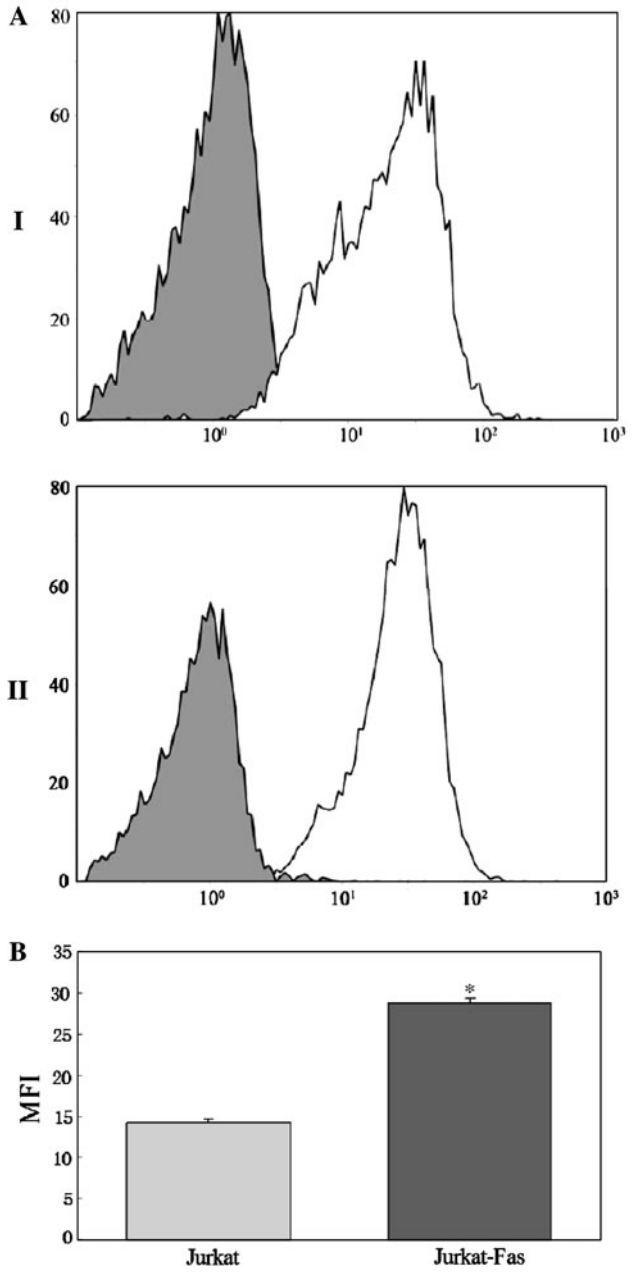

Fig. 4 Flow cytometry analysis of Fas protein level. 4×10^5 cells of each cell line were collected and treated with PE anti-human Fas. The fluorescence intensity was detected by flow cytometry with PE mouse IgG1 κ isotype as an internal control. **a** The isotype control is shown with the *curve filled in grey* and Fas is shown with *white filled curves*. *I* Jurkat cell, *II* Jurkat-Fas cell. **b** *Histograms* show relative mean florescence intensity (MFI) of PE signal in the two cell lines. Data are represented as mean ± SEM ($n = 3$). *$P < 0.05$

Statistical Analysis System (SAS V8.1 for Windows, SAS Institute Inc., NC, USA). Significant differences were accepted when $P < 0.05$.

Results

Successful construction of pCDNA3.1(+)-Fas

The sequencing results of PCR products and the recombinants plasmids pCDNA3.1(+)-Fas were consistent with GenBank accession no. NM_000043.3.

Expected over-expression of Fas by stable transfection

Over-expression of Fas mRNA

The gel electrophoresis image of Fas cDNA and GAPDH cDNA in Jurkat and Jurkat-Fas cell lines was shown in Fig. 1. The mean relative expression level of Fas mRNA was shown in Fig. 2. The mRNA level of Fas in Jurkat-Fas cells was significantly greater than that in Jurkat cells. ($P = 0.0007$).

Over-expression of Fas protein

The images of the Jurkat and Jurkat-Fas cells scanned by confocal microscopy displayed the Fas protein expressed on cell membrane surface (Fig. 3). The mean fluorescence intensity (MFI) calibrated by IgG1 κ isotype control standing for Fas protein expression level was shown in Fig. 4. The protein level of Fas in Jurkat-Fas cells were significantly greater than that in Jurkat cells. ($P < 0.0001$).

Increased sensitivity to Fas-mediated apoptosis via over-expression of Fas

After treated with 25 ng/ml FasL for 12 and 24 h, the apoptosis rates of Jurkat-Fas cells were about threefold and fourfold to Jurkat cell, respectively. The sensitivity to Fas-mediated apoptosis in Jurkat-Fas cells significantly increased compared to Jurkat cell. (12 h $P = 0.0012$; 24 h $P < 0.0001$) (Fig. 5).

Inhibition to tumorigenesis via over-expression of Fas in vivo

After 24 days from the injection of tumor cells, while all of the four mice injected with Jurkat cells began to erupt tumors and the tumors' sizes increased stably with time proceed, none of the other four mice injected with Jurkat-Fas cells developed any tumors during the 40 days of observation. (Fig. 6) Over-expression of Fas significantly inhibited the tumorigenesis in vivo. (d24 $P = 0.0048$; d28 $P = 0.0022$; d32 $P < 0.0001$; d36 $P = 0.0002$; d40 $P = 0.0004$) (Fig. 7).

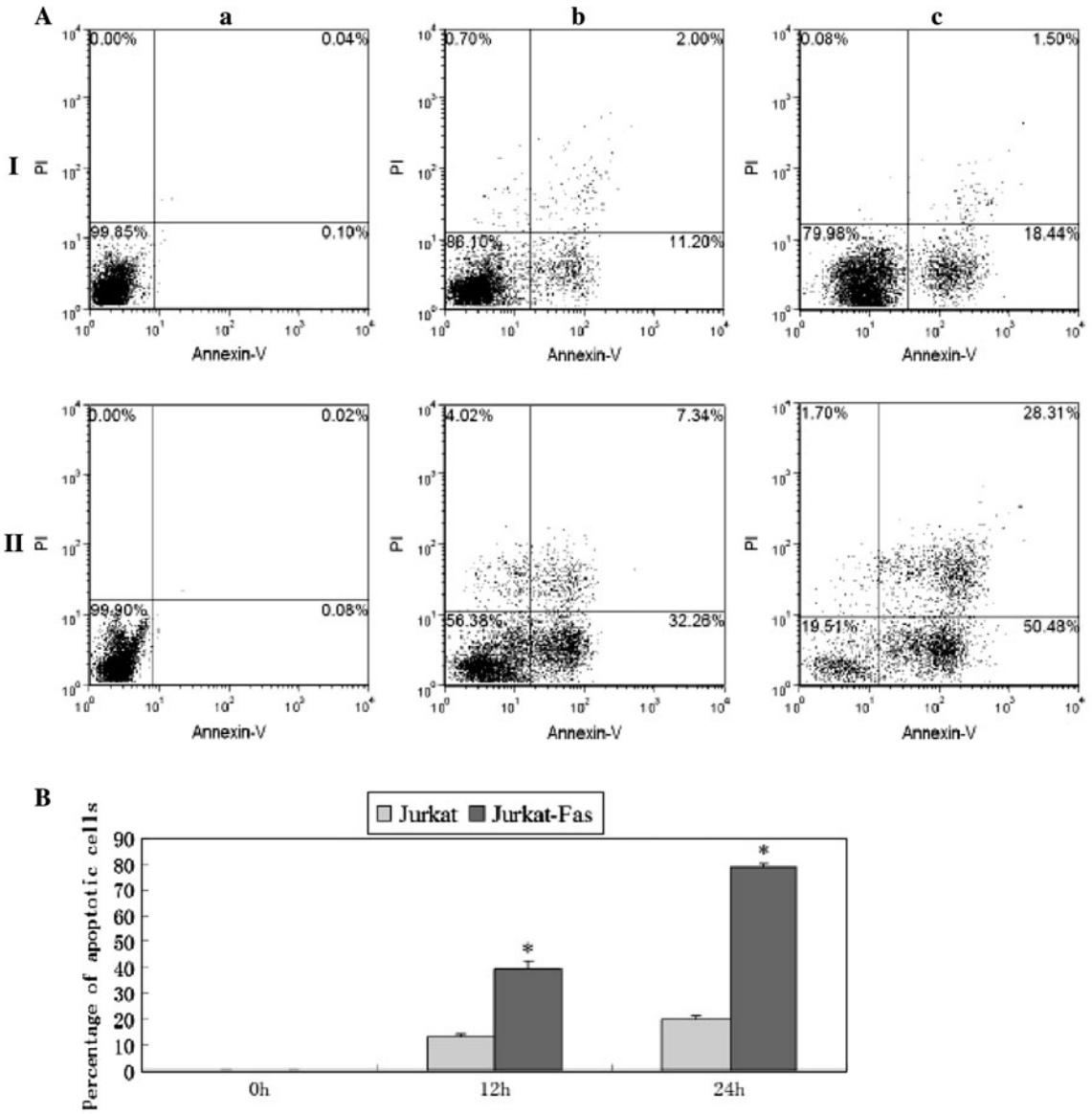

Fig. 5 Analysis of the sensitivity to Fas-mediated apoptosis. After treated with 25 ng/ml human soluble Fas ligand for 0, 12, and 24 h, respectively, 4×10^5 cells of each cell line were collected and stained with Alexa Fluor 488 annexin V and PI. The apoptotic ratios were analyzed by flow cytometry. **a** Representative record of annexin V/PI staining flow cytometry analysis of apoptotic cells. *I* Jurkat cell, *II* Jurkat-Fas cell; *a* 0 h; *b* 12 h; *c* 24 h. **b** *Histograms* show apoptosis rate in the two cell lines. Data are represented as mean ± SEM ($n = 3$). $^*P < 0.05$

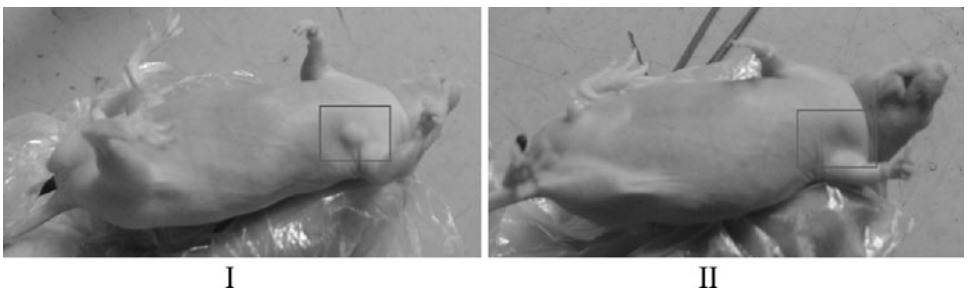

Fig. 6 Representative photographs of the tumorigenesis taken after the injection with tumor cells for 35 days. Injection sites and the tumors were marked with *rectangles*. *I* a nude mouse injected with Jurkat cells, *II* a nude mouse injected with Jurkat-Fas cells

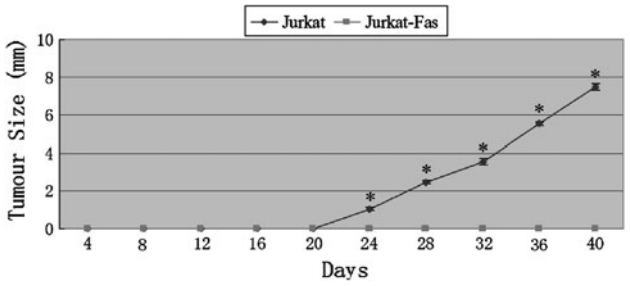

Fig. 7 The tumor growth tendencies in vivo. Eight SPF male BALB/c nude mice were separated into two groups averagely and randomly. After exposed in 4 Gy ray, the two groups of mice were subcutaneously inoculated with 5×10^7 Jurkat and Jurkat-Fas cells, respectively. All of them received 0.5 ml 100 ng/ml human soluble FasL through caudal vein every 7 days from injection of tumor cells. Tumor growth was monitored by calculating size in diameter (mm) every 4 days. *Polygrams* show the tumor growth in the two cell lines. Data are represented as Mean ± SEM ($n = 4$). *$P < 0.05$

Discussion

The incidence of T-LBL is about 30% of the non-Hodgkin's lymphoma (NHL) in children [1]. T-ALL constitutes about 25% of all adult ALL and the proportion in childhood ALL is 12–15% [1]. In T-LBL patients, the disease-free survival (DFS) rates of conventional chemotherapy achieved 23–56% [18–21]. The average survival rate of adult T-ALL was 40% with a very wide variation of 25–77% [1]. In general, the therapeutic outcomes of precursor T-cell malignancies remain poor especially of those progressive or relapse type [22]. So it is necessary to find a better therapy for patients with precursor T-cell malignancy. As an important development of medical science, gene therapy has become a hopeful method in curing tumors.

Since an imbalance between cell death and proliferation may result in tumor formation, enhancing apoptosis should be a way killing tumor cells directly and effectively [23]. Apoptosis may be more accurately referred to a mechanism of programmed cell death mediated principally by caspases now [24], which play important roles in eliminating the cells with unrepaired DNA damage, controlling cell number and proliferation as part of normal development, and removing self-reactive lymphocytes and lymphocytes with nonproductive rearrangements of the antigen receptor gene [25]. The two main pathways involved in apoptosis are the extrinsic pathway which is triggered through the Fas death receptor and the intrinsic pathway [26].

After triggered by its natural ligand (FasL), Fas recruits the relative proteins to form the death-inducing signaling complex (DISC). A cascade of caspase activation was initiated in turn subsequently which leads to apoptosis of the cell finally. Interestingly, while the Fas pathway was well recognized for the death related functions, maybe it was also involved in several inflammatory and proliferative signaling pathways [27]. In our study, over-expression of Fas resulted in significant increase of apoptosis but not proliferation in malignant T-cell line Jurkat, which was consist with the effects that were observed in other malignant T-cell lines, SeAx, SZ4, HH, and JFL, transfected with normal Fas gene [10]. In addition, over-expression of Fas in Jurkat-Fas cell line significantly resulted in the inhibition of tumorigenesis in vivo with the treatment of FasL in our study. To our knowledge, this is the first report in this filed. Both the vitro and vivo results supported that over-expression of Fas solidly enhanced malignant T Jurkat cells' sensitivity to Fas-mediated apoptosis. What is the mechanism determining the proceeding direction, apoptosis or proliferation, of the Fas pathway? This is a matter needing further study to resolve.

The comparison between Jurkat and Jurkat-Fas cell line demonstrated that the Fas protein level paralleled its mRNA level and the sensitivity to Fas-mediated apoptosis paralleled its protein level. Such a consistent relationship also was presented in other malignant T-cell lines [10, 28]. But the different result that the sensitivity to Fas-mediated apoptosis among malignant T-cell lines did not consistently parallel Fas protein levels also has been reported in one study [29]. As the relative published data are still minimal so far, whether over-expressed Fas always enhance the sensitivity to Fas-mediated apoptosis is still ambiguous.

In conclusion, our study demonstrated that stable transfection with Fas in Jurkat cell effectively improved its sensitivity to Fas-mediated apoptosis both in vitro and in vivo. Over-expression of Fas may be a potential method to promote the apoptosis of malignant T-cells with being triggered by FasL expressed on tumor infiltrating lymphocytes (TILs) and ameliorate the effectiveness of chemotherapeutic drugs working through Fas pathway.

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