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IL-7 splicing variant IL-7δ5 induces human breast cancer cell proliferation via activation of PI3K/Akt pathway

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ABSTRACT

Various tumor cells express interleukin 7 (IL-7) and IL-7 variants. IL-7 has been confirmed to stimulate solid tumor cell proliferation. However, the effect of IL-7 variants on tumor cell proliferation remains unclear. In this study, we evaluated the role of IL-7δ5 (an IL-7 variant lacking exon 5) on proliferation and cell cycle progression of human MDA-MB-231 and MCF-7 breast cancer cells. The results showed that IL-7δ5 promoted cell proliferation and cell cycle progression from G1 phase to G2/M phase, associated with upregulation of cyclin D1 expression and the downregulation of $p27^{kip1}$ expression. Mechanistically, we found that IL-7δ5 induced the activation of Akt. Inhibition of PI3K/Akt pathway by LY294002 reversed the proliferation and cell cycle progression of MDA-MB-231 and MCF-7 cells induced by IL-7δ5. In conclusion, our findings demonstrate that IL-7δ5 variant induces human breast cancer cell proliferation and cell cycle progression via activation of PI3K/Akt pathway. Thus, IL-7δ5 may be a potential target for human breast cancer therapeutics intervention.

1. Introduction

IL-7 is a pleiotropic immune regulatory protein, predominantly produced by stromal cells and by cells at the inflammatory sites [1]. It is crucial for B- and T-cell development, as well as T-cell homeostasis, and mediates a plenitude of functions in health and disease [2]. Growing evidences show that IL-7 expression is closely correlated with tumor development and progression. IL-7 mRNA is detected in a variety of tumors, such as colorectal [3], renal [4], and central nervous system cancers [5]. IL-7 receptor mRNA is also expressed in many tumor cells including breast, lung, colon, renal and CNS cancer cells [5]. Following the binding of IL-7R to its ligand, a series of intracellular phosphorylation events occurred, such as the activation of the Janus kinases (JAK-1 and JAK-3), phosphoinositide 3 kinase (PI3K), and the signal transducers and activators of transcription 5 (STAT-5) [6]. IL-7 stimulates the proliferation of some types of cancers, such as lymphoma [7] and leukemia [8]. The enhancement effect of IL-7 on tumor cell proliferation has been shown to be derived from modulation of IL-7/IL-7R signaling downstream genes, such as $p27^{kip1}$ and cyclin D1 [9,10].

The activity of IL-7 in tumor cell proliferation has been proved using the 'canonical' form of IL-7, which spans six exons presented in 33 kb of the chromosomal band 8q [11]. On the basis of the central role of IL-7 in the development of the immune system and the enhanced risk of lymphoma formation, production of biologically active IL-7 protein should be tightly controlled. In general, alternative splicing may affect binding properties, cellular localization, stability and protein function [12]. In our previous study, we successfully cloned IL-7 and IL-7 variants produced by alternative mRNA exon splicing from several human cancer cell lines, and found that a differentially spliced IL-7 isoform lacking exon 5 (IL-7δ5) could phosphorylate STAT-5 in $CD4^+$ and $CD8^+$ T cells, promoting thymocyte maturation and T-cell survival [13]. However, whether IL-7δ5 has a role in tumor cell proliferation remains unclear. In this study, we sought to explore the impact of IL-7δ5 on breast tumor cell proliferation and its underlying mechanisms. Our results demonstrate that IL-7δ5 variant promotes human breast cancer cell proliferation and cell cycle progression via activation of PI3K/Akt pathway and suggest that IL-7δ5 may be a potential target for therapy against human breast cancers.

2. Materials and methods

2.1. Materials

LY294002 (PI3K inhibitor) was obtained from Merk. Cell culture reagents were obtained from Invitrogen. Akt, the total and phosphorylated protein antibodies, as well as cyclin D1, $p27^{kip1}$

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antibodies and horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody were purchased from Cell Signaling Technology (Boston, MA). All other reagents were from Sigma (St. Louis, MO) unless stated otherwise.

2.2. Cell lines and cell culture

The MDA-MB-231 and MCF-7 human breast cancer cell lines were originally purchased from the ATCC (Manassas, VA) and cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal calf serum), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY) in a humidified 5% CO_2/95% air atmosphere at 37 °C.

2.3. Recombinant IL-7δ5 protein expression and purification

The protein expression and purification were performed according to our previous study [13]. Briefly, IL-7δ5 cDNA was obtained using RT-PCR using primers which cover six exons of IL-7. The 5′ oligonucleotide primer included an *Bam*HI restriction site at the ATG start codon and the 3′ oligonucleotide harbored a *Xho*I restriction site. IL-7δ5 cDNA was subcloned into the pIZ/V5-His vector, and then transfected into HighFive insect cells (Invitrogen Carlsbad, CA USA). Proteins were purified by Ni–NTA affinity chromatography on a column according to its manufacturer's instructions.

2.4. MTT assay

Approximately 5×10^4 cells in 100 μL of serum-free DMEM were grown in 96-well plates and incubated overnight. Then cells were treated with increasing concentrations of IL-7δ5 with or without treatment with LY294002 (30 μM) for 72 h. After 72-h treatment, 20 μL of MTT (Sigma, St Louis, MO) labeling reagent (5 mg/mL) was added to the designated wells, and cells were incubated at 37 °C for another 4 h. The supernatant was removed, and then 150 μL dimethyl sulfoxide (DMSO) was added to the designated well. After the plate was incubated at 37 °C for 15 min, the absorbency was measured with a micro ELISA reader (Bio-Tek, Winooski, VT, USA) at a wavelength of 570 nm.

2.5. Cell cycle analysis by flow cytometry

After exposure to IL-7δ5 for 48 h, cells were fixed with 75% cold alcohol followed by incubation at 4 °C overnight. Then cells were washed with phosphate buffered saline (PBS), propidium iodide (PI) was added and cells were incubated at 4 °C for 30 min. After then, Cell cycle distribution was detected with an Epics-XL II flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA).

2.6. Western blotting

Cell lysates were separated by SDS/PAGE in 10% Tris–glycine gels and transferred to a NC membrane. For analysis of Akt and phosphor-Akt, blots were probed with their specific antibodies (diluted with 5% BSA to 1:1000). Nonphosphorylated total Akt bands were chosen as loading control for Akt activation. For analysis of cyclin D1 and $p27^{kip1}$, blots were probed with cyclin D1 and $p27^{kip1}$ specific antibodies (diluted with 5% BSA to 1:500), respectively. Membranes were probed with horseradish peroxidase (HRP)–labeled anti-rabbit secondary antibody (diluted with 5% BSA to 1:1200). Antibody binding was detected by enhanced chemiluminescence detection kit (ECL) (UK Amersham International plc).

2.7. Statistical analysis

Data were statistically analyzed using Unpaired Student's *t* test at a significance level *P* value of <0.05 and were presented as mean ± S.D., using Sigma Plot software (Jandel Scientific).

3. Results

3.1. rh-IL-7δ5 promotes MDA-MB-231 cell proliferation

To determine the effect of IL-7δ5 on tumor cell proliferation, MDA-MB-231 cells (5×10^4 cells) were suspended in 100 mL of DMEM and seeded in 96-well plates. Then these cells were incubated in the absence or presence of increasing concentrations of rh-IL-7δ5 for 72 h. Fig. 1 shows that rh-IL-7δ5 treatment stimulated MDA-MB-231 cell proliferation in a concentration-dependent manner. After 72-h of rh-IL-7δ5 (10 ng/ml) treatment, cell survival was increased by ~2 folds.

3.2. rh-IL-7δ5 promotes MDA-MB-231 cell cycle progression

The results from flow cytometric analysis indicated that after 48-h treatment, rh-IL-7δ5 could dese-dependently induce an obvious decrease in the percentage of cells in G_1 phase and an increase in the percentage of cells in G_2/M phase (Fig. 2A). Fig. 2B shows that after 48-h treatment, rh-IL-7δ5 significantly stimulated the expression of cyclin D1, whereas led to a substantial decrease in $p27^{kip1}$ expression. These results suggest that the enhancement effect of rh-IL-7δ5 on MDA-MB-231 cell proliferation may be derived from promoting cell cycle progression, which appears to be correlated with regulation of expression of some cell cycle-related proteins including cyclin D1 and $p27^{kip1}$.

3.3. The involvement of PI3K/Akt pathway in rh-IL-7δ5-promoted MDA-MB-231 cell cycle progression and proliferation

Many studies have clearly confirmed the involvement of PI3K/Akt pathway in cancer cell cycle progression and cell proliferation. Thus, we sought to explore the possible correlation between rh-IL-7δ5 and the PI3K/Akt pathway. As shown in Fig. 3A and B, treatment with the PI3K inhibitor LY294002 (30 μM) could block the enhancement effect of rh-IL-7δ5 on MDA-MB-231 cell proliferation and reversed its effect on cell cycle progression, respectively. Western blotting analysis showed that rh-IL-7δ5 treatment could stimulate PI3K/Akt signaling represented as increased levels of

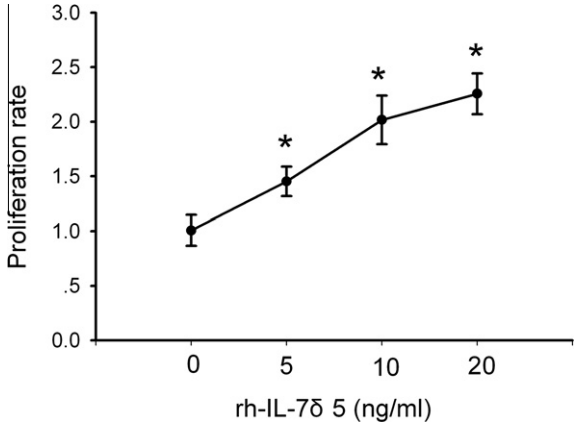

Fig. 1. The effect of rh-IL-7δ5 at indicated concentrations on MDA-MB-231 cell proliferation after 72-h treatment assayed by MTT. Bars are mean ± S.D. from four independent experiments. *Significantly different from control, $P < 0.05$.

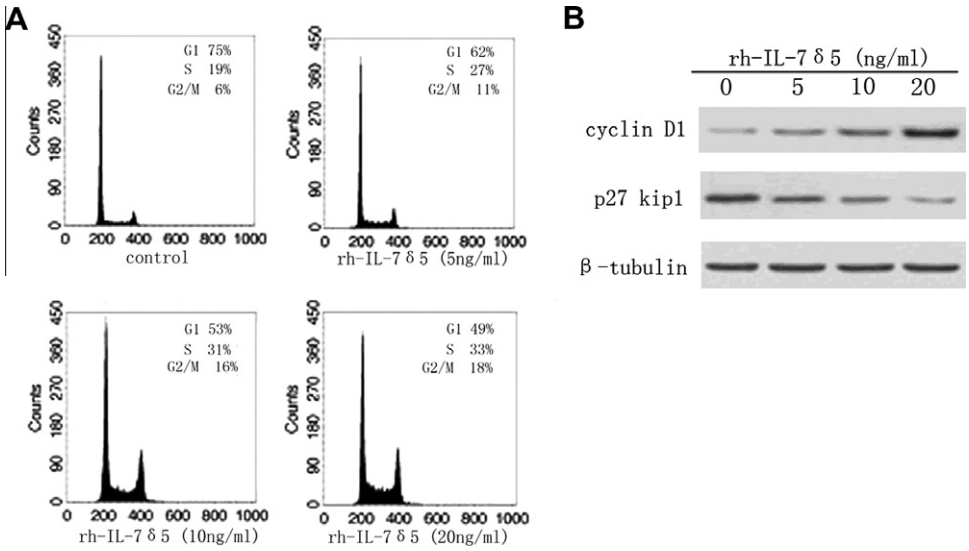

Fig. 2. The effect of rh-IL-7δ5 at indicated concentrations on cell cycle progression and the expression of cell cycle-related proteins in MDA-MB-231 cells. (A) The effect of rh-IL-7δ5 at indicated concentrations on cell cycle progression after 48-h treatment determined by flow cytometry assay. (B) The effect of rh-IL-7δ5 on the expression of cell cycle-related proteions in MDA-MB-231 cells after 48-h treatment determined by western blotting.

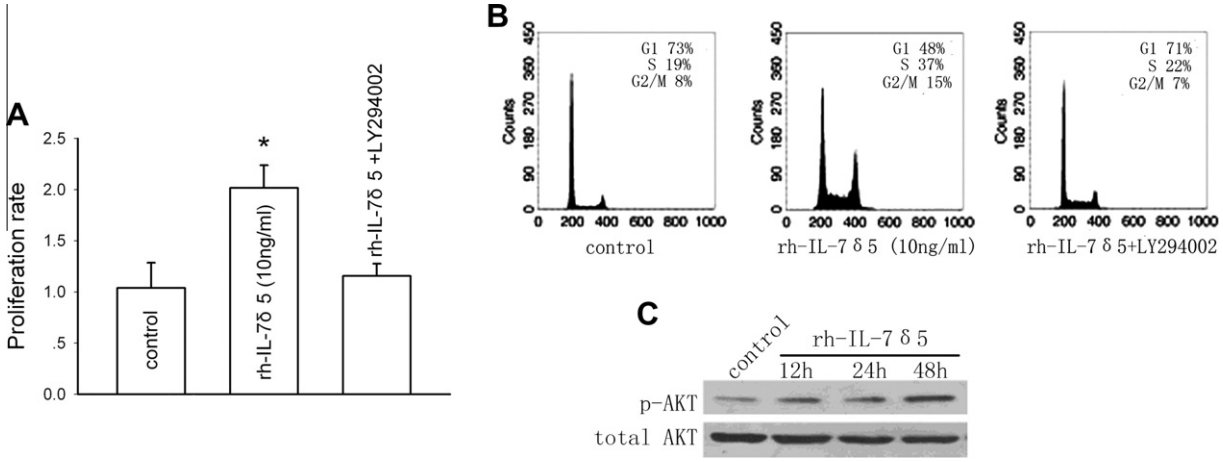

Fig. 3. The involvement of PI3K/Akt pathway in rh-IL-7δ5-enhanced cell cycle progression and cell proliferation of MDA-MB-231 cells. (A) The effect of inhibition of PI3K/Akt pathway by LY294002 (30 μM) on rh-IL-7δ5-enhanced cell proliferation. Bars are mean ± S.D. from five independent experiments. *Significantly different from control, $P < 0.05$. (B) The effect of LY294002 (30 μM) on rh-IL-7δ5-enhanced cell cycle progression. (C) The effect of rh-IL-7δ5 treatment at indicated times on the activity of PI3K/Akt pathway represented by the level of phosphorylated Akt.

phosphorylated Akt at the indicated times (Fig. 3C). Therefore, these results suggest that rh-IL-7δ5 can promote MDA-MB-231 cell cycle progression and cell proliferation, mainly through activation of PI3K-AKT pathway.

3.4. Role of rh-IL-7δ5 in enhanced cell proliferation in another breast cancer cell line MCF-7

To exclude the possibility that the observed effects are restricted to MDA-MB-231 cells, we further examined the effect of rh-IL-7δ5 on MCF-7 cell proliferation. Like in MDA-MB-231 cells, rh-IL-7δ5 treatment could significantly enhance MCF-7 proliferation after 72 h. When PI3K/Akt pathway was inhibited by LY294002 (30 μM), the enhancement effect of rh-IL-7δ5 was blocked (Fig. 4A). Furthermore, we also found that rh-IL-7δ5 treatment increased the activity of PI3K/Akt signaling in MCF-7 cells (Fig. 4B).

4. Discussion

This study investigates the role of IL-7 splicing variant IL-7δ5 in breast cancer cell proliferation. The results indicate that rh-IL-7δ5 can promote cell cycle progression and cell proliferation. We demonstrate that the enhancement effect of rh-IL-7δ5 on breast cancer cell cycle progression and cell proliferation is mainly derived from activation of PI3K/Akt pathway.

Alternative splicing is the process by which a single gene produces many different transcripts mediating a wide range of cellular events [14]. Up to date, several differentially spliced cytokine isoforms have been identified. For instance, IL-2δ2 and IL-2δ3 inhibit binding of the full-length IL-2 to the high affinity IL-2 receptor [15]. The alternatively spliced IL-4 isoform, IL-4δ2, a potent IL-4 inhibitor, is preferentially expressed in the thymic tissue and in the airway system upon mycobacterial infection [16]. In our previous study, we successfully cloned IL-7 and IL-7 variants produced by alternative mRNA exon splicing from several human cancer cell

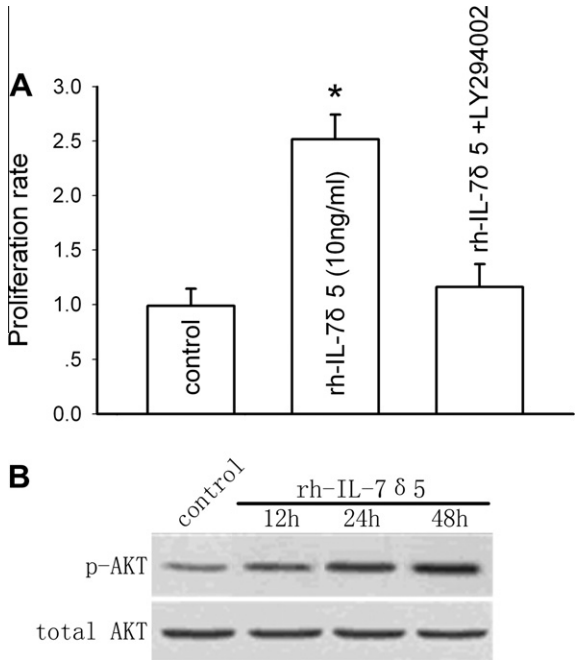

Fig. 4. The involvement of PI3K/Akt pathway in rh-IL-7δ5-enhanced cell proliferation of MCF-7 cells. (A) The effect of rh-IL-7δ5 on MCF-7 cell proliferation and inhibition of PI3K/Akt pathway by LY294002 (30 μM) on rh-IL-7δ5-enhanced cell proliferation. Bars are mean ± S.D. from five independent experiments. *Significantly different from control, $P < 0.05$. (B) The effect of rh-IL-7δ5 treatment at indicated times on the activity of PI3K/Akt pathway represented by the level of phosphorylated Akt.

lines, and found that IL-7δ5 could lead to enhanced T-cell survival [13].

Previous evidences had demonstrated the important role of IL-7 in the pathogenesis and progression of lymphomas [7,17]. In breast cancer cell lines, IL-7 could induce the growth of cells, while this effect involved PI3K and Jak3 [18]. IL-7 stimulated proliferation of lung cancer cells via up-regulates cyclin D1 [10]. However, the impact of IL-7δ5 on tumor cell proliferation remains unclear. In the present study, we found that IL-7δ5 promoted breast cancer cell proliferation and cell cycle progression of the cells probably via concomitant upregulation of cyclin D1 expression and down-regulation of $p27^{kip1}$ expression. cyclin D1 [19] and $p27^{kip1}$ [20] have well been defined as important regulators of cell cycle progression. Thus, our results suggest that IL-7δ5 stimulates breast can cell proliferation, probably through modulation of cyclin D1 and $p27^{kip1}$ expression.

The PI3K/Akt pathway is crucial in tumorigenesis because the p-Akt can regulate the cell proliferation, apoptosis, angiogenesis and cell cycle by activating the downstream cell receptors or effectors [21]. In this study, our results show that activation of PI3K/Akt pathway is sufficient to mediate IL-7δ5-enhanced breast cancer cell proliferation. IL-7 binds to a heterodimeric receptor consisting of an IL-7 specific chain (IL-7R) and the common gamma (γc) chain. IL-7δ5 appears to bind equally well to hIL-7R, but even better to the γc chain, compared with IL-7 [22]. The γc chain is associated with JAK3 whose activation results in phosphorylation of STAT5 [23]. IL-7/IL-7R signaling has been confirmed to play a pivotal role in growth of T cell acute lymphoblastic leukemia cells via activation of PI3K/Akt pathway [24]. Besides, IL-7 was shown to support T-cell survival via STAT5-mediated activation of Akt [25]. Therefore, combined with other reports, our results suggest that activation of PI3K/Akt pathway by IL-7δ5 may be derived from binding of IL-7δ5 to both IL-7R and γc chain, and is indispensible for IL-7δ5-mediated breast cancer cell proliferation.

Our work has some limitations. We cannot exclude the possibility that IL-7δ5 influences the expression of cell cycle-related proteins other than cyclin D1 and $p27^{kip1}$. Furthermore, it will be important to determine the enhancement effect of IL-7δ5 on breast cancer cell proliferation *in vivo*. Notwithstanding these limitations, the present study does demonstrate the role of IL-7δ5 in breast cancer cell proliferation *in vitro* and make clear the critical involvement of activation of PI3K/Akt pathway. Thus, intervention of IL-7δ5 may be a potential alternative against breast cancer cell proliferation.

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