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ORIGINAL PAPER

Expression of IL-13Rα2 in liver cancer cells and its effect on targeted therapy of liver cancer

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Abstract
Purpose Liver cancer is the third leading cause of cancer-related deaths globally. The number of liver cancers diagnosed in the world is increasing at an alarming rate. It is of great significance to find the new targets of the tumor cells and specific medicine. This research investigated the expression of interleukin-13 receptor α2 (IL-13Rα2) in different liver cancer cell lines and liver cancer tissues, and assessed the cytotoxin DT389-hIL13-13E13K (IL-13 and diphtheria toxin fusion protein) targeted killing effect on liver cancer cells. Based on study above, we further analyzed the function of IL-13Rα2 on the targeted liver cancer therapy. The results will provide a novel strategy and an alternative way for liver cancer therapy.
Methods The expression of IL-13Rα2 in different liver cancer cell lines and tissues were analyzed by RT-PCR and immunohistochemistry. Cytotoxicity assay of DT389-hIL13-13E13K was performed in eight different concentrations in liver cancer cell lines in vitro. At the same time, siRNA-mediated knockdown was introduced to assess the role of IL-13Rα2 in liver cancer therapy.
Results Two out of four tested liver cancer cell lines and 27 out of 33 (81.82%) liver tissues expressed the IL-13Rα2. The fusion protein DT_{389}-hIL13-13E13K showed a moderate cytotoxicity to the cancer cell line BEL-7402 in vitro, which 50% inhibition (IC_{50}) concentration occurred at 1.4×10^{-5} M. Besides, the sensitivity to fusion protein DT_{389}-hIL13-13E13K was decreased in siRNA-transfected liver cells compared with control ones. These results suggest that IL-13Rα2 chain is a specific target for IL-13-directed fusion protein.
Conclusions We reported the expression of IL-13Rα2 in liver cancer cell lines and tissues as well as investigated the cytotoxin (DT389-hIL13-13E13K) targeted killing efficiency of liver cancer cells and potential role of IL-13Rα2 in the cancer treatment.

Keywords Liver cancer · IL-13Rα2 · Targeted therapy · siRNA

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Introduction

Liver cancer is one kind of high-grade malignant tumor. Current estimates indicate that liver cancer is the sixth most common cancer worldwide and the third leading cause of cancer-related deaths (Forner et al. 2006). There are as many as 598,000 cases of death out of 626,000 new cancer cases every year because of very poor prognosis. In these new cases, 55% occur in China alone, which marks an urgent situation for both the prognosis and the treatment of

liver cancer (Parkin et al. 2002). Curative resection is still the main treatment of liver cancer patients. However, the success of this approach is limited by the tumor size and the function left as well as the metabolism of the liver. Moreover, the resection has a high relapse rate and is also unsuitable to the late stage patients (Greten et al. 2005). In this case, finding the new treatment targets of the tumor cells and specific medicine are of great significance.

Interleukin-13 (IL-13) is a Th2 cell-derived pleiotropic immune regulatory cytokine (Fluckiger et al. 1994), which has been shown to share many effects of IL-4 including growth-promoting expression of CD23 and enhancement of B-Cell DNA synthesis. IL-13 receptor complex exists at least in two different types, IL-13Rα1 and IL-13Rα2, in which IL-13Rα2 chain is a major binding component of the IL-13R complex and has a high affinity with IL-13. IL-13α2 is overexpressed in many different types of cancer cells including gliomas, human pediatric brain tumors, AIDS-Kaposi's sarcoma, Ovarian cancer, oral squamous cell carcinoma, and other human head and neck cancer, but not expressed at a very low level in corresponding normal human cells (Joshi et al. 2000; Husain and Puri 2000; Kawakami et al. 2003, 2004a, b; Kioi et al. 2006, 2009). Some other researches demonstrated that the combination approach of injecting IL-13Rα2 plasmid in established tumors followed by IL-13 cytotoxin administration showed profound antitumor activity against human breast tumors in xenografted immunodeficient mice (Kawakami et al. 2004a, b). These results suggest that the targeting of IL-13Rα2 may represent a valuable approach for the therapy of some kinds of tumors (Li et al. 2002). Previous studies of our lab had also proved that recombinant IL-13 cytotoxin (DT389-hIL13-13E13K), which is composed of IL-13 and a mutated short form of Diphtheria toxin (DT) had potent antitumor activity both in vitro and in vivo (Du et al. 2008). However, there is no such research published about IL-13α2 expression and associated therapy targeting IL-13α2 in liver cancer. In this case, we analyzed the IL-13α2 expression level in both liver cancer cells and tissues, and assessed the cytotoxin (DT389-hIL13-13E13K) targeted killing effect on liver cancer cells at various concentrations. Besides, we investigated the sensitivity of IL-13 cytotoxin in liver cancer cells after IL-13Rα2 siRNA transfection. Our research will potentially provide a novel theory as well as treatment to the therapy of liver cancer.

Materials and methods

Cell lines

Human embryonic kidney 293 cell line (HEK293), human glioma U251 cell line (U251), and liver cancer cell lines BEL-7402, SMMC-7721, HuH7, HepG2 were cultured in high glucose DMEM (Sigma) containing 10% FBS (Gibco), 1 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin. All the cells were incubated at 5% CO_2 at 37°C in cell incubator.

RT-PCR of liver cancer cells

To detect the mRNA expression in cell lines, total RNA was isolated from U251, HEK293 and four liver cancer cell lines BEL-7402, SMMC-7721, HuH7, and HepG2 using TRIZOL reagent (Invitrogen). RT-PCR was performed for 35 cycles for IL-13Rα2 and 28 cycles for β-actin. PCR mixture was first incubated for 5 min at 94°C, and then denatured for 50 s at 94°C, annealed for 60 s at 62°C for IL-13Rα2 and at 64°C for β-actin, elongated for 60 s at 72°C. The specific forward and reverse primers of human IL-13Rα2 are 5′-GTGAAACATGGAAGACCATC-3′ and 5′-GTGAAATAACTGGATCTGATAGGC-3′. The forward and reverse primers of human β-actin are 5′-GTGGGGCG CCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGC ACGATTTC-3′. (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.). The amplification products were resolved in 1.5% agarose gel and photographed.

Immunofluorescence assay

For further analysis of the IL-13Rα2 expression in liver cancer, liver cancer cell lines and liver cancer tissue chip (Chaoying Biotechnology Co., Ltd. and Imagenex Corporation) were used for the immunofluorescence assay. Experiment was done according to the manufacturer's instructions. In brief, tissue chip were deparaffinized by xylene treatment and washed with an alcohol gradient (from 100 to 50%). Cell line slides and tissue chips were washed with PBS and blocked with goat serum. After that, they were incubated with the specified primary monoclonal antibody to IL-13Rα2 (BD) for 1 hour at room temperature followed by washing with PBS and subsequently incubated for 1 h with secondary antibody (goat anti-mouse IgG FITC, Jackson). After three washes with PBS and dehydration, the slides and chips were viewed in a Nikon fluorescence microscope using appropriate filters.

siRNA to IL-13Rα2 and analysis by real-time PCR

The sequence of sense siRNA to IL-13Rα2 was G CCT ATC AGA TCC AGT TAT. Plasmid was extracted in accordance with the manufacturer's instructions (Tiangen Biochemistry Technology Co., Ltd.) and transfected into liver cell line BEL-7402. The transfected cells were

selected by G418, the concentration of which is 400 μg/ml. Expression levels of IL-13Rα2 in BEL-7402 and RNA-interfered BEL-7402 were assessed by real-time PCR (Applied Biosystem) whose condition is the same as PCR mentioned in the RT-PCR except elongated at 60°C with 40 cycles. β-actin was used as the house keeping control. By analysing Ct value and standardizing with β-actin, IL-13Rα2 expression level of BEL-7402 and RNA-interfered BEL-7402 was compared.

Protein synthesis inhibition assay

IL-13 and diphtheria toxin fusion protein (DT_{389}-hIL13-13E13K) were provided by our lab (Du et al. 2008). The cytotoxic activity was tested using the manufacturer's instructions of Cell Counting Kit-8 (CCK8, Dojindo Molecular Technologies, Inc.) which can directly show all the living cells. Liver cancer cell lines SMMC-7721 and BEL-7402 were used as test cell lines; cell lines HEK293, and U251 were used as the negative and positive control, respectively. Typically, cells were cultured in DMEM medium for 24 h at 37°C. Then cytotoxicity assay was performed with or without seven different concentrations of DT389-hIL13-13E13K (10^{-6}, 5×10^{-7}, 2.5×10^{-7}, 10^{-7}, 5×10^{-8}, 10^{-8}, 10^{-9} M) in different tumor cell lines in vitro. The solution buffer was used as the blank control. Each concentration was repeated in three wells. The DT_{389}-hIL13-13E13K inhibition rate was identified after 72 h with 10 μl CCK-8 in each of 96 wells. After incubating for an additional 2 h, cells were harvested and $OD_{450\,nm}$ was measured.

The cytotoxic activity of the siRNA infected cell was tested with the similar method as above except for adding 10 μl CCK-8 after 48 h of incubation. Data were collected by the average of triplicates. The $OD_{450\,nm}$ of the blank control cells were regarded as 100% proliferation and by this the cell proliferation rate can be obtained. The concentration of DT389-hIL13-13E13K at which 50% inhibition effect occurred was analyzed after all the procedures.

Result

IL-13Rα2 expression analysis by RT-PCR and immunofluorescence assay

To determine the expression of IL-13Rα2 in liver cancer cell lines, four liver cancer cell lines BEL-7402, SMMC-7721, HuH7, HepG2 and U251, and HEK293 were cultured, respectively, and RT-PCR analysis was performed. Among these six cell lines, U251, BEL-7402, and SMMC-7721 expressed IL-13Rα2; HuH7, HepG2, and HEK293 did not express IL-13Rα2. Moreover, BEL-7402 cell line had more IL-13Rα2 than SMMC-7721 cell line, and it was suggested that expression level of IL-13Rα2 was different in different cell lines (Fig. 1A). The fraction of the amplification is 453 bp. The housekeeping gene β-actin which is 392 bp was positive in all the cell lines. To further confirm IL-13Rα2 expression, two liver cell lines BEL-7402 and SMMC-7721 were tested by Immunofluorescence which approved that IL-13Rα2 receptor was expressed in these cell lines (Fig. 1B).

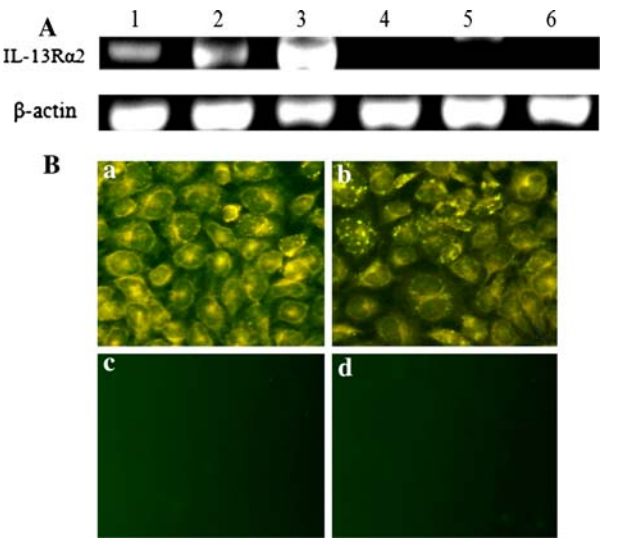

Fig. 1 IL-13Rα2 chain expression in different liver cancer cell lines. **A** RT–PCR analysis of liver cancer cells, *lane 1* was U251, which was positive control; *Lane 2–lane 5* were SMMC-7721, BEL-7402, HuH7 and HepG2 respectively, SMMC-7721 and BEL-7402 expressed IL-13Rα2; *Lane 6* was the HEK293 cell line, which was used as the negative control. **B**: **a** was the result of the immunofluorescence which approved the expression of IL-13Rα2 chain on liver cell line BEL-7402, **b** was result of another liver cell line SMMC-7721, **c** and **d** were negative stain of HEK293 and HuH7 cell lines (200×)

IL-13Rα2 expression analysis by liver cancer tissue chips

To further investigate the expression of IL-13Rα2 in liver cancer tissue, 33 cases of liver cancer tissue in the chip were analyzed by immunofluorescence. Twenty-seven cases out of 33 were positive, which was as many as 81.82%. Thirteen cases of normal liver tissue in the chip were also assessed and only two of them (15.38%) were positive for IL-13Rα2 which suggested that liver cancer tissues highly expressed IL-13Rα2 (Fig. 2). The cells in cancer area always had high-density nuclear staining which were of different shape and varied dramatically. We randomly measured the diameters of 30 cells and their nuclears in five visual fields by SimplePCI5.0 and counted the karyoplasmic ratio. The mean of karyoplasmic ratio is 1.351 and obviously over 1.

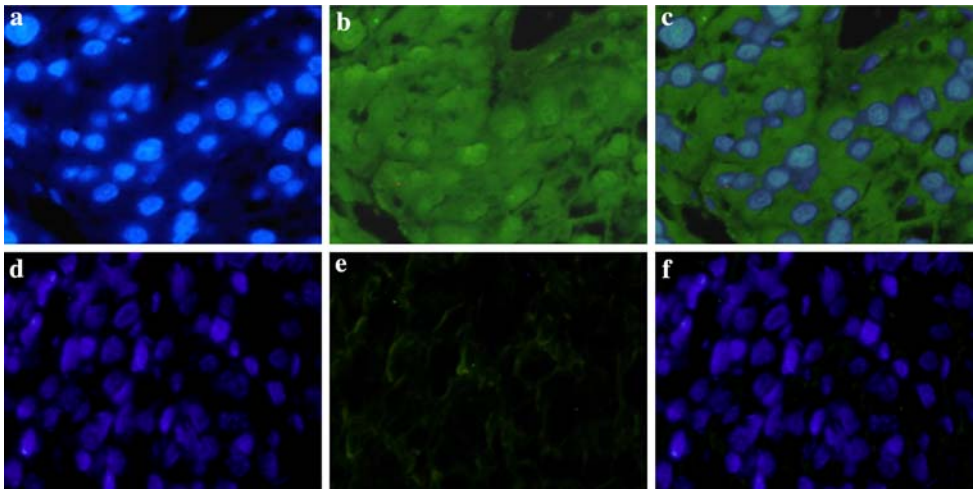

Fig. 2 The expression of IL-13Rα2 in liver cancer tissue. This figure randomly showed one cancer tissue that expressed IL-13Rα2 chain (*upper row*) and one normal liver tissue that did not express IL-13Rα2 chain (*lower row*). **a** and **d** stand for the nuclear staining by DAPI, **b** and **e** were stained by the specific mice anti-human IL-13Rα2 monoclonal antibody (secondary antibody is goat anti-mice IgG FITC), **c** was the merge of image **a** and **b**, **f** was the merge of image **d** and **e** (200×)

Cytotoxicity assay

The cell death rate by CCK8 kit was measured to indicate the cytotoxicity of the fusion protein DT389-hIL13-13E13K to U251 cell lines, liver cancer cell lines BEL-7402, SMMC-7721, and HEK293 cell line which do not express IL-13Rα2. Results showed that there was clear killing effect at the concentration of 10^{-8} mol/l to U251 cell line which acted as the positive control. There was also a moderate cytotoxicity to BEL-7402 cell line (Fig. 3). Dose-dependent killing effects of U251 and BEL-7402 cell line were seen with the IC_{50} 1.78×10^{-7} and 1.4×10^{-5} M, respectively, at 72 h. However, HEK293 and SMMC-7721 were not killed obviously by the fusion protein DT389-hIL13-13E13K (Fig 3B). Moreover, the fusion protein DT389-hIL13-13E13K also showed quite weak cytotoxicity to SMMC-7721 as well as no toxicity against HEK293 even at high concentration 10^{-6} M.

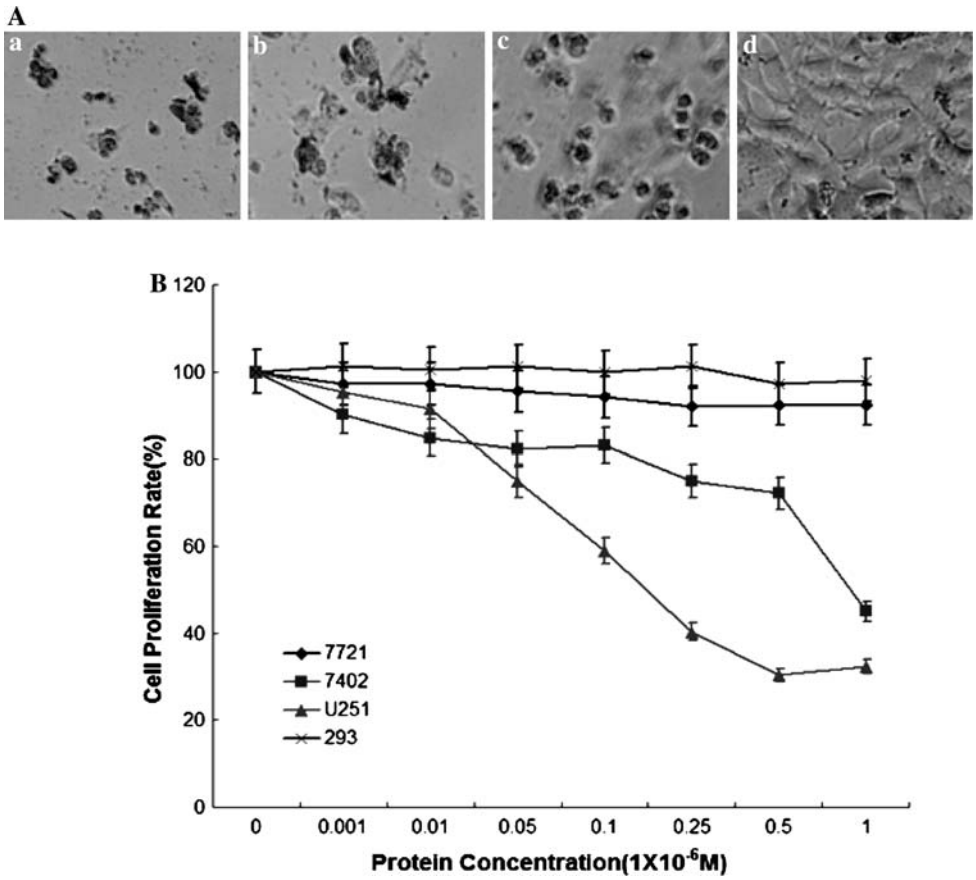

Fig. 3 The images of different cells and cell proliferation assay result at 72 h after adding DT_{389}-hIL13-13E13K: the conditions of the cells with 10 μl different concentration of protein DT_{389}-hIL13-13E13K in every 100 μl culture medium was identified after 72 h. Cell proliferation rate can be obtained by comparing $OD_{450\,nm}$ value of test cells with that of control cells. **A**: **a** was the human glioma U251 cells under the protein concentration of 10^{-6}M which was the positive control group. **b** and **c** were the liver cancer cells BEL-7402 and SMMC-7721 respectively under the same concentration, while d was the negative control group HEK293 (100×). **B** Cell proliferation assay result at 72 h after using different concentration of DT_{389}-hIL13-13E13K. In this figure, an obvious killing effect was showed on U251 cells; there was medium cytotoxicity for cancer cell line BEL-7402; the liver cancer cell line SMMC-7721 and HEK293 were not sensitive to this protein even at the highest concentration

Real-time PCR result of IL-13Rα2 expression in siRNA effected BEL-7402 cells

According to the outcome of the cytotoxicity assay, we examined siRNA sequence to target the IL-13Rα2 expression. BEL-7402 cell line was selected to be transfected with siRNA. Figure 4 shows IL-13Rα2 siRNA transfected BEL-7402 cells resulted in a 63% knocked down reduction when compared to untransfected cells. IL-13Rα2 gene expression was assessed by Real-time PCR analyses on cDNA level. This level could show the real expression result, and we standardized IL-13Rα2 result by intra-standard β-actin.

Effect of DT389-hIL13-13E13K on BEL-7402 cells transfected with IL-13Rα2 siRNA in vitro

We also analyzed the sensitivity of BEL-7402 cells transfected with IL-13Rα2 siRNA to the cytotoxic activity of DT389-hIL13-13E13K. Based on our previous results (Du et al. 2008), the experiment was done on four various concentrations 5×10^{-6}, 10^{-6}, 10^{-7}, and 10^{-8} M. Interleukin-13 (IL-13) was added in as a factor with the hope of understanding the effect of this cytokine to the cytotoxin. Results indicated that the cytotoxic effect of DT389-hIL13-13E13K on IL-13Rα2 siRNA-transfected BEL-7402 cells decreased comparing with the effect on control original BEL-7402 cells. The cytotoxicity of DT389-hIL13-13E13K after siRNA effect was modestly abrogated with the IC_{50} from 2.87×10^{-6} (original cells) to 4.07×10^{-5} M (siRNA-transfected cells) (Fig. 5). However, the result is not statistically significant. The HEK293 cells which express no IL-13Rα2 chain was not affected by the DT389-hIL13-13E13K -induced cytotoxicity at the concentration of 10^{-6} M or lower level. However, 10 ng/ml IL-13 had little effect on the fusion protein DT389-hIL13-13E13K cytotoxicity, which was out of our expectations.

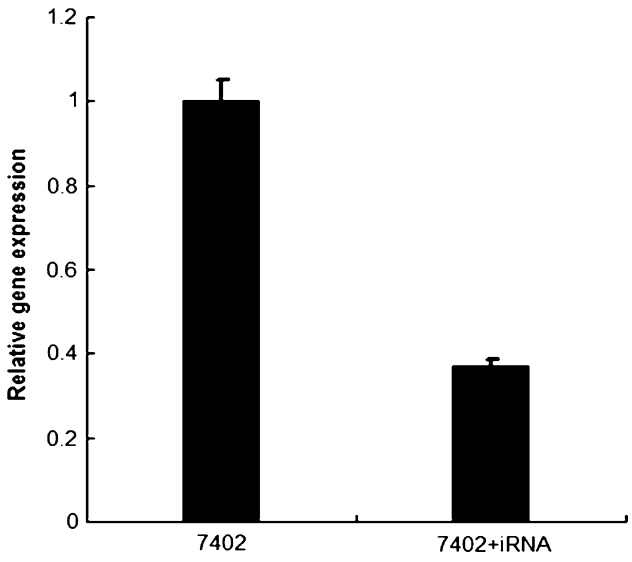

Fig. 4 Different gene expression by real-time PCR analysis: the gene expression was visible decreased after being interfered by plasmid which is only 37% of the original expression. It means that the expression of the receptor was interfered by the plasmid to a certain extent

Discussion

Liver cancer is a quite common disease all over the world, especially in China and other Asian countries (Jiang et al. 2006). Previous report had indicated that IL-13Rα2 was expressed in various tissues including liver (Donaldson et al. 1998). Similarly, there is another recent research demonstrating that IL-13Rα2 was overexpressed in activated hepatic stellate cells (Shimamura et al. 2008). In this study, it was proved that two out of four liver cancer cell lines (BEL-7402, SMMC-7721) expressed the IL-13Rα2 chain. Further studies by tissue chip testified that IL-13Rα2 was expressed in liver tissue at extremely high level of 81.82%.

Nowadays, combination therapy is developing rapidly. Meanwhile, much more attention was paid on the ones which include IL-13Rα2. Fusion protein IL-13-PE38QQR which targets IL-13Rα2 has been investigated in several Phase I/II clinical trials with very good results (Kunwar et al. 2006; Vogelbaum et al. 2007). Some other investigators transfected IL-13Rα2 chain into human breast and pancreatic cancer cell lines that do not naturally express this chain. The result showed that IL-13-PE38QQR could effectively inhibit breast and pancreatic cancer derived after transfection under the assistance of IL-13 and IL-8. This combination approach showed profound antitumor activity against human breast tumors in xenografted immunodeficient mice (Kawakami et al. 2001). Besides, no remarkable toxicity changes of serum chemistry, hematology, and organ histology were detected from mouse by the combination therapy. Taken together, this combination therapy may be applied safely and be a new treatment for breast cancer (Kawakami et al. 2004a, b).

Previous report described a 3–10 times higher affinity of IL13E13K-PE38 than wtIL13-PE38 (Kioi et al. 2004). Other studies demonstrated that DT_{390}IL13 fusion protein had a very valuable function to cure IL-13Rα2 overexpressed cancer as well. Joshi et al. (2000)). Based on studies above, we designed our fusion protein and chose the test concentration. The research of DT389-hIL13-13E13K cytotoxicity assay showed that DT389-hIL13-13E13K had different toxicity effect on different tumor cell lines. The positive control U251 cell line which highly expressed the IL-13Rα2 had a great sensitivity to the fusion protein DT389-hIL13-13E13K. Cell death and proliferate inhibition could be seen at 24 h (data not shown), while IC_{50} is 1.78×10^{-7}M at 72 h. Meanwhile, the growth was

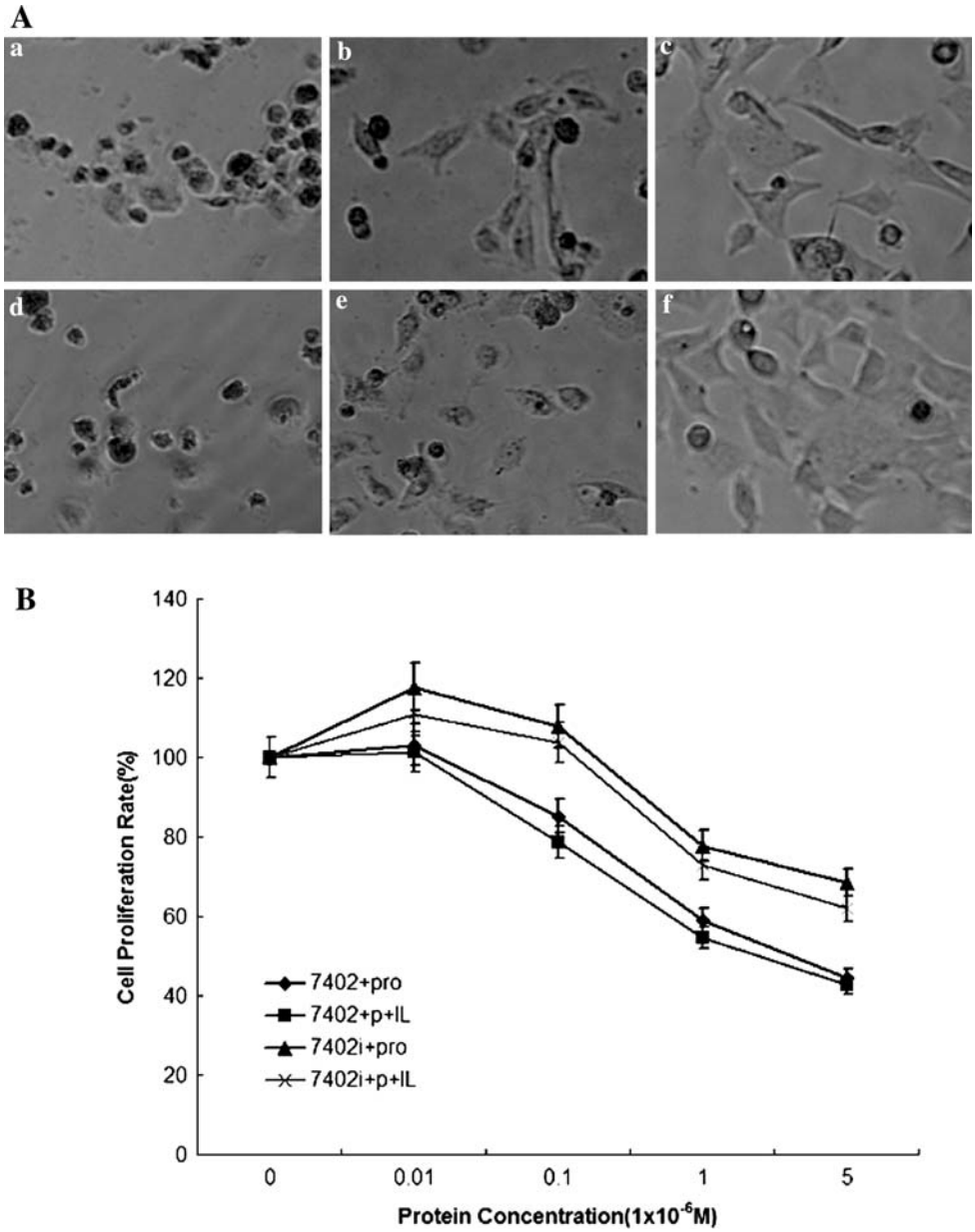

Fig. 5 The photos of different cells and cell proliferation assay after 48 h using of DT389-hIL13-13E13K, **A**: **a**, **b** and **c** were the liver cancer cells BEL-7402, siRNA infected BEL-7402 and the negative control human embryo kidney cell line HEK293 under the concentration of 5×10^{-6} M respectively; while **d**, **e** and **f** were the liver cancer cells BEL-7402, siRNA infected BEL-7402 and the negative control human embryo kidney cell line HEK293 under the concentration of 10^{-6} M respectively (100×). **B** Cell proliferation assay using different concentration of DT389-hIL13-13E13K. There was a sensitive decline in all the concentration of the transfected BEL-7402 cells

inhibited in liver cancer cell lines at 24 h and cell death phenomenon was seen after 48 h; IC_{50} is 1.41×10^{-5}M on BEL-7402 cells at 72 h. The result suggested that the cytotoxicity of DT389-hIL13-13E13K obviously appeared and reached the maximum efficiency at 48 h. HEK293 cell line that naturally does not express IL-13Rα2 was chosen as a negative control. The results showed that the DT389-hIL13-13E13K did not cause damage to HEK293 even at the highest level after 72 h, which suggested that the DT389-hIL13-13E13K did not cause toxicity for normal cells. Other research also mentioned that protein DT_{390}IL13 which is quite similar to DT389-hIL13-13E13K did not cause renal histopathological change even at 30 times of the efficacy dose (Joshi et al. 2000). These results confirmed that DT389-hIL13-13E13K may have targeted antitumor activity by IL-13Rα2 mediation. At the same time, it is speculated that the difference of DT389-hIL13-13E13K killing efficacy in BEL-7402 and SMMC-7721 was due to the different expression level of IL-13Rα2.

To further investigate the role of IL-13Rα2 chain in liver cancer, we introduced the siRNA method into our research. Similar researches were done in other cancers earlier. Koji Kawakami et al. transfected siRNA of IL-13Rα2 chain into glioblastoma multiforme (GBM) cells and assessed the binding intensity and sensitivity of IL-13 cytotoxin to interfered-GBM cell. Their results showed that both binding intensity and cytotoxicity of cytotoxin IL13-PE38QQR to interfered-GBM cells decreased. In following study, they transferred IL-13Rα2 gene into GBM cells which are native IL-13R-expressing cells, and then used these over-expressed

GBM cells to make tumor model. The results found that overexpressed GBM cell tumors were more sensitive to cytotoxin IL13-PE38QQR, and that tumor sizes were much smaller in IL-13Rα2-transfected tumors than the control group. These data indicate that IL-13Rα2 chain in GBM cells is essential for IL-13 cytotoxin-induced cytotoxicity (Kawakami et al. 2005).

We designed and transfected siRNA into the liver cell line which expressed IL-13Rα2 chain. Up to 63% of IL-13Rα2 chain was interfered on the mRNA level by measuring of real-time PCR. Besides this, these cells decreased the cytotoxic activity of IL-13 cytotoxin DT389-hIL13-13E13K at 48 h at diverse protein concentrations. These results further suggested that the presence of this chain on tumor cells may play a crucial biologic role in tumor cell killing. However, the interference effects of this siRNA is not so obvious because there were some difference in the cytotoxic study but not significant. In vivo researches need to be done after getting a better result. The information about the function both of fusion protein DT389-hIL13-13E13K and IL-13Rα2 chain provided here is of very significant for the improvement of later receptor researches as well as broadened the kinds of tumors that can be treated by this IL-13 cytotoxin therapy or other combination treatment.

In summary, we found the specific expression of IL-13Rα2 chain in some of liver cancer cell lines and tissues which had not been discovered earlier. In addition, we proved the killing effect of fusion protein DT389-hIL13-13E13K in some liver cancer cells in which IL-13Rα2 was expressed. These results combined with our previous ones (Du et al. 2008) lead to the hypothesis that this cytotoxin will have comprehensive effects on high IL-13Rα2 expressed tumors and that IL-13Rα2 chain plays a pivotal role in IL-13 cytotoxin-mediated killing for liver cancer cells. Our research will provide a new treatment for specifically targeted killing of majority of interleukin-13 (IL-13) receptor-expressing liver cancer, and hopefully, in the end lead to improved quality of life and survival of patients.

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