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Research article

Expression, purification and biological effect of a novel single chain Fv antibody and protamine fusion protein for the targeted delivery of siRNAs to FGFR3 positive cancer cells

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ABSTRACT

Background: Gain-of-function of fibroblast growth factor receptor 3 (FGFR3) is involved in the pathogenesis of many tumors. More and more studies have focused on the potential usage of therapeutic single-chain Fv (ScFv) antibodies against FGFR3. RNA interference (RNAi) has been considered as a promising therapeutic method against cancer. A tool which can deliver small interference RNAs (siRNAs) into FGFR3 positive cancer cells is very promising for anti-tumor therapy.
Results: In this study, a novel fusion protein R3P, which consists of FGFR3-ScFv and protamine, was generated in *Escherichia coli* by inclusion body expression strategy and Ni-NTA chromatography. Its yield reached 10 mg per liter of bacterial culture and its purity was shown to be higher than 95%. 1 μg of R3P could efficiently bind to about 2.5 pmol siRNAs and deliver siRNAs into FGFR3 positive RT112 and K562 cells. Annexin V staining results showed that R3P can deliver the amplified breast cancer 1 (AIB1) siRNAs to induce RT112 cell apoptosis.
Conclusion: These results indicated that R3P was a promising carrier tool to deliver siRNAs into FGFR3 positive cancer cells and to exert anti-tumor effect.

1. Introduction

RNA interference (RNAi) has been considered as a promising therapeutic method against cancer [1,2]. However, how to deliver small interference RNAs (siRNAs) to the targeting sites has become a main obstacle for application due to no effective carrier tool [1,3]. To develop a carrier which not only binds to siRNAs but also targets and delivers siRNAs into specific cancer cells is very important for the application of RNAi drug. In the past ten years, a new type of fusion protein containing single chain Fv (ScFv) and basic polypeptide (*e.g.*, protamine, 9-arginine) was created by combination of antibody technique and RNAi theory. The ScFv which contains a variable heavy chain (V_H) and a variable light chain (V_L) has the high binding affinity with the antigen of cell surface and excellent internalization properties [4], and the basic polypeptide can bind to nucleic acid. Therefore, this fusion protein containing ScFv and basic polypeptide can be used as a novel carrier to deliver siRNAs into ScFv-targeting cancer cells. Up to now, many fusion proteins have been studied to target different types of cancer cells. For example, Su [5] expressed the fusion protein containing anti-prostate specific membrane antigen (anti-PSMA) ScFv and truncated protamine in *Escherichia coli*, and demonstrated that this fusion protein can specifically deliver siNotch1 into PSMA-positive prostate cancer cells and inhibit cell proliferation and promote apoptosis both *in vitro* and *in vivo*. Zhang et al. [6] reported a fusion protein composed of anti-EGFR ScFv and truncated protamine can efficiently inhibit EGFR positive cervical carcinoma cell proliferation by RNAi-mediated knockdown of hWAPL gene.

Fibroblast growth factor receptor 3 (FGFR3), one member of fibroblast growth factor receptor family, is a receptor tyrosine kinase involved in the tumorigenesis of many malignancies, such as bladder cancer [7], multiple myeloma (MM) [8] and hepatocellular carcinoma [9]. Overexpression of FGFR3 correlates with shorter overall survival

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of t(4;14)-positive multiple myeloma, which occurs in about 15%–20% of multiple myeloma patients [10]. FGFR3 frequently mutates in approximately 70% of papillary and 16%–20% of muscle-invasive bladder carcinomas [11]. Overexpression or activating mutations in FGFR3 promotes tumor growth, metastasis and resistance to drugs by activating MEK–ERK, PI3K-AKT and JAK/STAT signals [7,10,11]. As early as 2005, ScFv against FGFR3 was first reported to inhibit proliferation of bladder cancer cell line RT112 *in vitro* [12,13]. Immunotoxin (anti-FGFR3 ScFv-fused toxin) exhibited significantly anti-tumor activity in RT112 tumor xenografts by inducing cell apoptosis [13].

In this study, we designed and expressed a novel recombinant fusion protein R3P, which was composed of an anti-FGFR3 ScFv and truncated protamine. The amplified breast cancer 1 (AIB1) protein, which plays essential roles for bladder cancer promotion [14,15] was chosen to detect the functions of R3P. Our results provide evidences that R3P can effectively deliver AIB1-siRNA into FGFR3-positive bladder cancer cells and exert anti-tumor function.

2. Materials and methods

2.1. Materials

Prime STAR®GXL DNA Polymerase was purchased from TaKaRa (Japan). Restriction enzymes *Nde*I and *Xho*I were provided by NEB (New England, USA). Ni-NTA agarose was purchased from GE healthcare (Sweden). Anti-His tag antibody was provided from Proteintech (USA). Anti-protamine antibody was obtained from Abcam (USA). HRP-labeled secondary antibody was purchased from Cell Signaling (USA). Lipofectamin™ 2000 (Lipo), *E. coli* DH5α and *E. coli* BL21 (DE3) strains were obtained from Invitrogen (USA). BCA protein assay kit and ECL kit was purchased from Thermo (USA). AIB1-siRNA and negative control siRNAs were synthesized by GenePharma (China). Annexin V staining kit was purchased from BD Biosciences (USA).

2.2. Design of fusion gene and construction of recombinant plasmid

The FGFR3-ScFv sequence comes from our prior report [16] and the sequence of truncated protamine comes from Li et al. report [17]. The R3P fusion gene, containing FGFR3-ScFv and protamine was synthesized by ZoonBio Biotechnology Co.(China). The primers used for sub-clone were designed as follows: P1 (5′ GGAATTCCATATGCATC ATCATCATCATCACCAGG TGCAGCTGCAGCAG AG 3′) was designed according to the 5′ terminal sequence of ScFv fragment; P2 (5′ CCGCTCGAGTTAGCTGCGGCGGCGGCGGC 3′) contained 3′ terminal sequence of protamine. Furthermore, the R3P containing 6His-tag and restriction enzyme sites was generated by PCR, using P1 and P2 as the forward and reverse primers, using synthetic R3P as the template. PCR parameters consisted of 5 min of Prime STAR®GXL DNA Polymerase activation at 98°C, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, extension at 68°C for 60 s, and then a final single extension at 68°C for 5 min. The fusion gene was digested with *Nde*I and *Xho*I, and inserted into the pET-20b expression vector. Finally, the identified recombinant plasmid was confirmed by DNA sequencing (Sangon, Shanghai).

2.3. Inducible expression of recombinant R3P and refolding of inclusion bodies

A single colony from *E. coli* BL21 (DE3) harboring pET-R3P was grown overnight in the LB medium (1% peptone, 0.5% yeast extract, and 1% sodium chloride, pH 7.0), and incubated in a shaker at 37°C. A 3 ml aliquot of resulting culture was inoculated into 300 ml fresh LB medium and incubated at 37°C and 200 rpm until OD_{600} was 0.6 to 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM final concentration for 4 h induction at 37°C. The cell pellets were collected by centrifugation and lysed by sonication. The supernatants were harvested by centrifugation at 12,000 × g for 30 min at 4°C, and the remaining pellets (insoluble fraction) containing inclusion bodies were resuspended into an equal volume of lysis buffer. Both soluble and insoluble fractions were analyzed by 12% SDS-PAGE.

The precipitation of sonicated recombinant bacteria was resuspended with washing buffer A (20 mM Tris/HCl,4 mM NaCl, 2 mM urea,2% Triton X-100, pH = 8.0), vortexed for 20 min at 4°C and centrifugated 12,000 × *g* for 15 min, then the supernatant was removed and the precipitation was washed one time with 50 mM Tris/HCl (pH = 8.0). Furthermore, the inclusion bodies were dissolved with renaturing buffer (50 mM Tris/HCl, 50 mM NaCl, 10 mM β-mercaptoethanol, 8 M urea, pH = 8.0). After denatured at 4°C for 4 h, the sample was centrifuged at 12,000 × g for 30 min and the supernatant was collected and dialyzed stepwise with refolding buffer (20 mM Tris/HCl, 500 mM NaCl, 0.3 mM GSSH, 1.5 mM GSH, pH = 8.0) containing 6, 4, 2, 1, 0 M urea for 12 h, respectively.

2.4. Purification and identification of R3P

The refolded R3P protein was purified by Ni-NTA chromatography. The Ni-NTA resin was washed with wash buffer I (20 mM Tris–HCl, pH 8.0) until OD_{280} of effluent reached base line. 6His-tagged R3P was harvested from the column with elution buffer (20 mM Tris–HCl containing 200 mM imidazole, pH 8.0). The purity of R3P was assessed by SDS-PAGE and its concentration was determined with BCA protein assay kit according to the kit protocol.

The immunogenic activity of purified R3P was assayed by Western blot. Total bacteria protein was boiled in an equal volume of sample loading buffer. Protein samples were electrophoresed on 12% of SDS-PAGE, and then electrophoretically transferred onto PVDF membrane. The nonspecific binding of transferred membrane was blocked with 5% non-fat milk powder at 4°C overnight. The membrane was incubated with a polyclonal anti-His tag antibody (1:1000) or a polyclonal anti-protamine antibody (1:1000), then washed and incubated with a 1:1000 dilution of secondary HRP-conjugated antibody. Immunoreactive bands were visualized using an ECL kit.

The specific band of R3P from the SDS-PAGE gel was pooled and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). This technical service was supported by Beijing Protein Institute Co. Ltd.

2.5. Gel retardation assay

10 pmol noncoding siRNAs (UUCUCCGAACGUGUCACGUTT) were mixed with increasing amounts of R3P in the PBS buffer, and incubated at 4°C for 60 min. 2 μg ScFv against FGFR3 was applied to as a negative control. The mixture was then performed electrophoresis on 2% (*w*/*v*) agarose gels and detected by EB staining.

2.6. Flow cytometry

70 pmol Cy3 labeled-siRNA was mixed with 28 μg of R3P in the PBS buffer at 4°C for 1 h, then added in the 2×10^5 K562 cells or THP-1 cells at 4°C for 1 h, and further culture in 37°C of incubator for 1 h. The cells were washed twice with PBS buffer, and suspended in 100 μl PBS buffer. Bovine serum albumin (BSA) and ScFv against FGFR3 were used as controls compared with R3P. Cy3 fluorescence level was assayed by BD C6 flow cytometry.

2.7. Immunofluorescence

70 pmol noncoding siRNAs labeled with FAM were mixed with 28 μg of R3P in the PBS buffer totally 50 μl, and incubated at 4°C for 60 min. ScFv against FGFR3 was used as a negative control. The mixture was