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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

add in the medium of RT112 cells and cultured at 37°C for 4 h in the cell incubator. The cells were then harvested and washed twice with PBS buffer. Then the cells were fixed by 4% paraformaldehyde and stained with 5 μg/ml DAPI and observed under a fluorescent microscope (Olympus, Japan).

2.8. R3P-mediated AIB1-siRNA delivery and pro-apoptosis effect in RT112 cells

70 pmol AIB1-siRNA (GGUGAAUCGAGACGGAAACTT) was mixed with 28 μg R3P or 2 μl Lipo, respectively. The PBS buffer was added into the mixture till the final volume is 50 μl. The mixture was incubated at 4°C for 60 min. PBS was used as a negative control. The mixture was added in the medium of RT112 cells and cultured at 37°C for 48 h or 72 h respectively in the cell incubator. At 48 h, the cells were harvested for Western blot analysis according to previous descriptions. At 72 h, the cells were stained for Annexin V staining according to the kit protocol (BD Biosciences, USA).

2.9. Statistical analysis

The Values were presented with means ± S.E.M, and data were analyzed by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered as different significant differences.

3. Results

3.1. Cloning of the fusion gene and inducible expression R3P

R3P was designed according to the schematic illustration of Fig. 1A. In this synthetic gene, ScFv that binds with FGFR3 was located in the N-terminal of R3P gene, and the truncated protamine that binds with siRNAs was fused with 3′ terminal of ScFv using a linker. The amino acid sequence of linker is GGGGSGGGGSGGGGS. The 6His-tag was put in the N-terminal of R3P gene. The fusion gene was amplified by PCR with specific primers containing restriction enzyme site, and its size is about 820 bp (Fig. 1B). The fusion gene was cloned into pET-20b expression plasmid, and the double digestion of recombinant plasmid was performed with *Nde*Iand *Xho* I (Fig. 1C). The results showed that the size of the digested fragment was consistent with that of PCR product. The recombinant fusion gene was further successfully confirmed by DNA sequencing.

The recombinant bacteria containing pET-20b-R3P were induced at 37°C by 0.5 mM IPTG at about 0.6 of OD_{600}. The cell pellet was collected and lysed by sonication. The total protein, supernatant and precipitation from bacteria extracts were isolated by centrifugation and analyzed by SDS-PAGE. The results showed that a size of 37 kDa recombinant protein was successfully induced (Fig. 1D), but more than 90% R3P protein exists in the inclusion body precipitation of bacteria extracts (Fig. 1E).

3.2. Denaturation, refolding, purification and identification of inclusion body protein

Inclusion body protein was washed, denatured and refolded according to the detailed descriptions in the Materials and methods section. The ratio of refolding protein attains 60%. Furthermore, the acquired R3P, which displayed better solubility and stability, was purified on Ni-NTA column chromatography. SDS-PAGE results displayed the purity of R3P exceeds 95% (Fig. 1F). Finally, the R3P yield reached 10 mg per liter of bacterial culture by inclusion body expression strategy and by Ni-NTA column chromatography.

To identify the authenticity of R3P, the immunological activity of R3P was assayed by Western blot with specific anti-protamine or anti-His antibody, respectively. The results showed that R3P has immunoblot reactivity with two kinds of antibodies, respectively (Fig. 2A). The

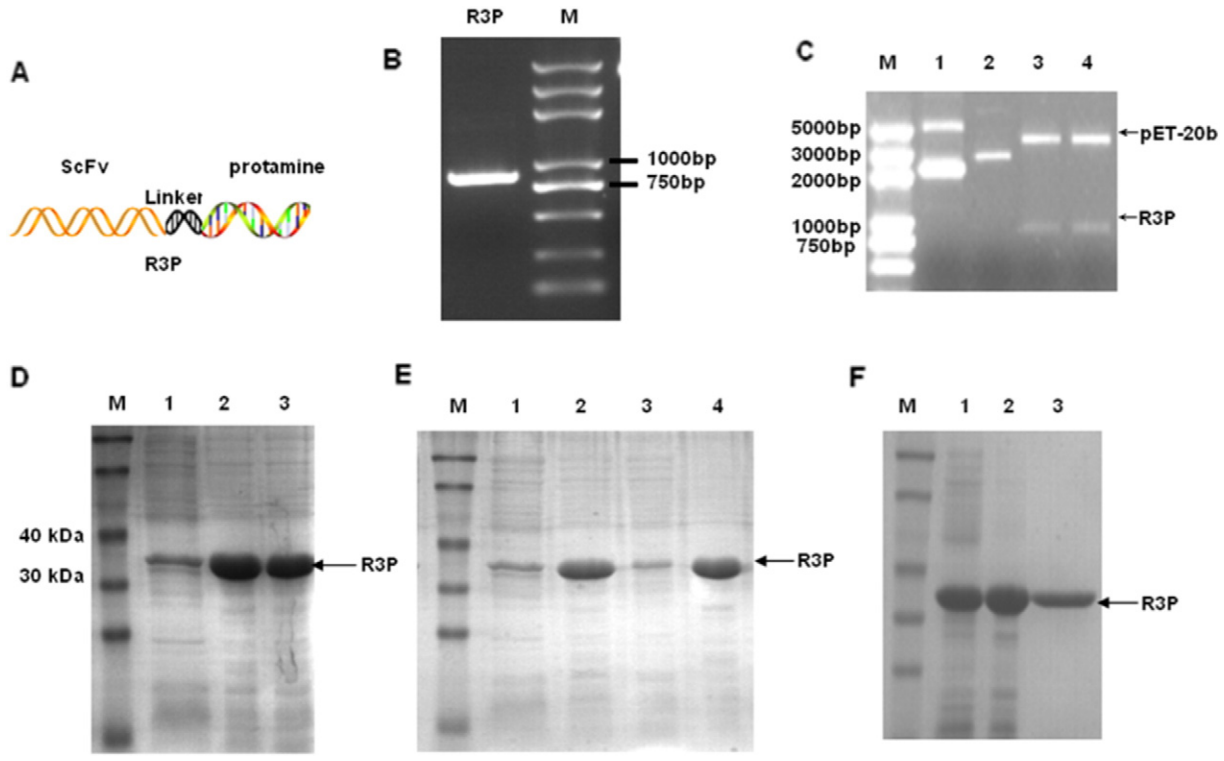

Fig. 1. Construction, expression and purification of R3P. (A) The schematic illustration of the composition of R3P. (B) The PCR product of R3P. (C) Identification of the recombinant plasmid by enzyme digestion. Lane 1, blank plasmid; Lane 2, the recombinant plasmid; Lane 3 and lane 4, the digested recombinant plasmid with *Nde* I and *Xho* I. (D) SDS-PAGE analysis of R3P. Lane 1, without IPTG; Lane 2 and lane 3, IPTG induction. (E) SDS-PAGE analysis of different expression levels of R3P in the supernatant (lane 1 and 3) and precipitation of bacterial R3P (lane 2 and lane 4). (F) SDS-PAGE analysis of R3P purification (lane 1 and lane 2, before purification; lane 3, after purification).

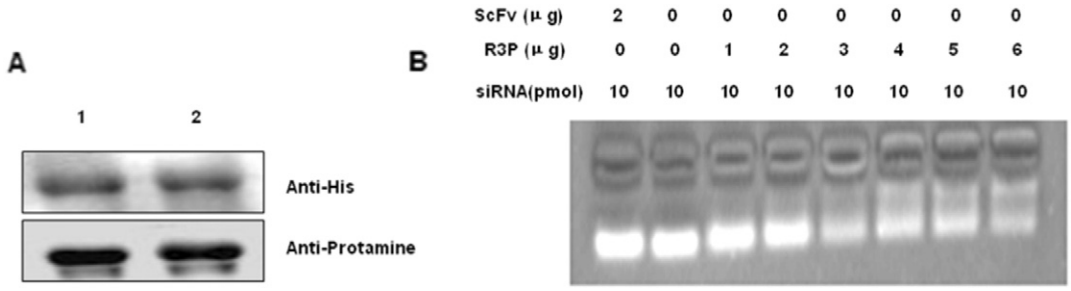

Fig. 2. Identification of R3P. (A) Western blot analysis of R3P with anti-His tag antibody and anti- protamine antibody. (B) Gel retardation assay of siRNA binding to R3P. Ten picomoles of noncoding siRNAs were mixed with increasing amounts of R3P in the PBS buffer and the mixture was then detected by electrophoresis on 2% agarose gels.

amino acids of R3P were analyzed by LC–MS/MS, and its sequence coverage rate attains 74% (Data not shown), which suggests that the recombinant protein is consistent with the expected result.

3.3. The ability of binding siRNAs of R3P

To detect the binding ability of R3P to siRNAs, agarose retarding experiment was performed. As shown in Fig. 2B, R3P protein was able to shift siRNAs in a concentration dependent manner. When the amount of R3P reaches between 3 μg and 4 μg, the siRNA binding ability tends to saturable condition. In other words, 1 μg R3P can efficiently bind to at least 2.5 pmol siRNAs. In contrast, 2.0 μg ScFv against FGFR3 displayed no siRNA binding ability compared with 2.0 μg R3P. These results also suggested that R3P can specifically bind to siRNAs *via* the role of protamine.

3.4. The ability to deliver siRNAs into FGFR3 positive cancer cells

To confirm whether R3P can efficiently deliver siRNAs into FGFR3-positive cells, a FGFR3 positive bladder cancer cell line, RT112, was chosen for delivery assay by immunofluorescence. In our previous study, we testified that ScFv from R3P can inhibit FGF9-induced FGFR3 phosphorylation in RT112 cells [16]. So in this study, ScFv that could not deliver siRNAs was used as a negative control. Fluorescent microscope analysis showed that the FAM-positive cells only exist in R3P/siRNA-treated RT112 cells (Fig. 3A), and no FAM fluorescent was found in control groups (ScFv/siRNAs). To further identify

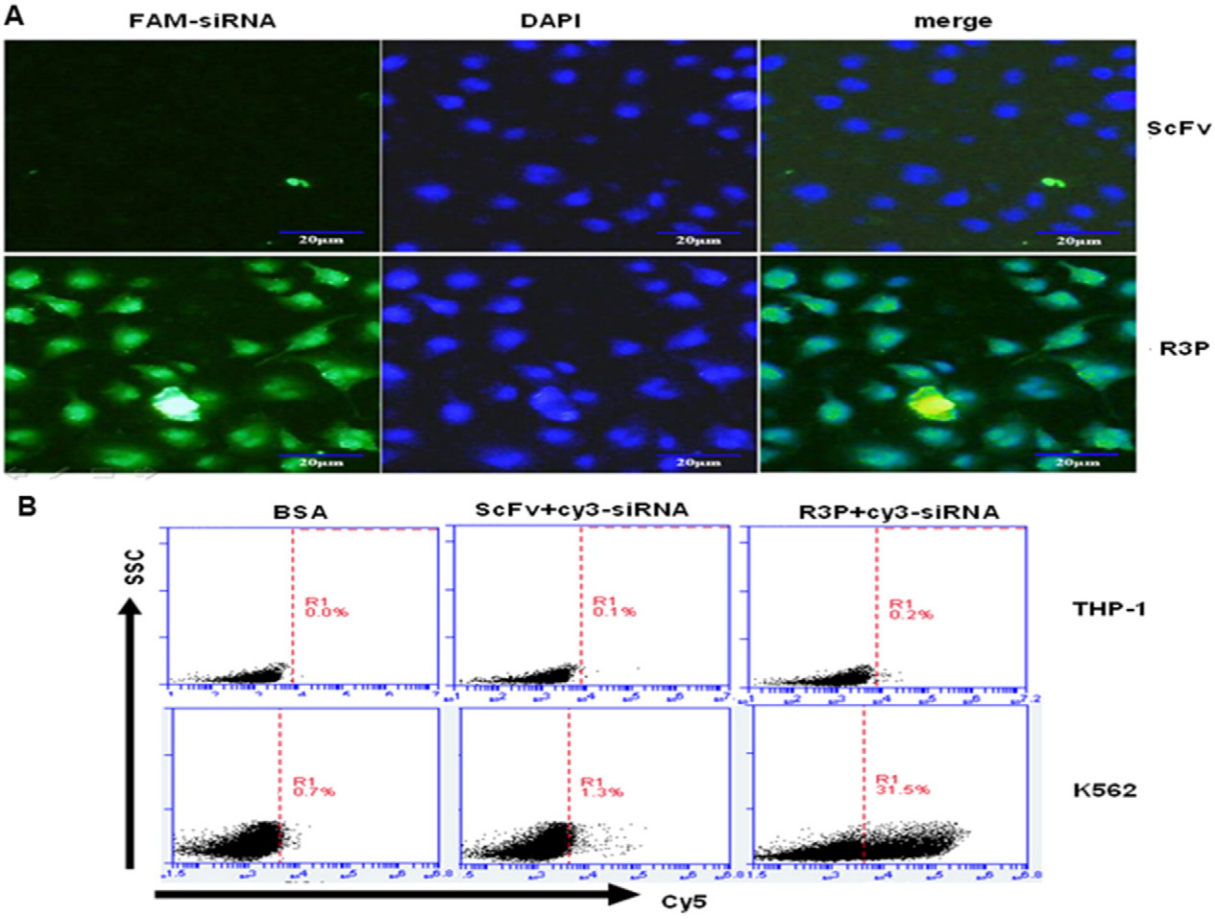

Fig. 3. Efficient siRNA transfer by R3P targeting FGFR3 positive cancer cells. (A) Fluorescent microscope analysis showed R3P, not ScFv, delivers siRNAs into RT112 cells (scale bar, 20 μm). (B) Flow cytometry results showed that R3P can efficiently bind and deliver siRNAs into FGFR3 positive K562 cells, not FGFR3 negative THP-1 cells.

the delivery ability of R3P, we chose FGFR3-positive leukemia cell line, K562 and FGFR3-negetive leukemia cell line, THP-1 as assay targets. Flow cytometry data showed more than 31% K562 cells are Cy3-positive, and no positive cells appears in treated THP-1 cells (Fig. 3B). These results displayed that R3P can specifically deliver siRNAs into FGFR3 positive K562 cells. These results also demonstrated that R3P, not ScFv, can efficiently deliver siRNAs into FGFR3 positive cells.

3.5. R3P delivers AIB1-siRNA to silence the AIB1 expression and induce RT112 cell apoptosis

To investigate the functionality of R3P-mediated siRNA delivery, we chose AIB1, a noted oncogene in bladder cancer [14,15] as a target. Indicated amounts of AIB1-siRNAs were combined with R3P or Lipo to treat RT112 cells. After 48 h, the AIB1 expression significantly downregulated in R3P/AIB1-siRNA group and Lipo/AIB1-siRNA group compared with control group (Fig. 4A). The better effects were observed in Lipo/AIB1-siRNA group compared with R3P/AIB1-siRNA group, suggesting Lipo has better effect in delivering siRNAs. Next, the apoptosis rate was analyzed by Annexin V staining at 72 h. The results showed that both R3P/AIB1-siRNA and Lipo/AIB1-siRNA significantly induced cell apoptosis (Fig. 4B, C), and Lipo/AIB1-siRNA also showed better pro-apoptosis effect. These results revealed that R3P not only binds and delivers siRNAs into FGFR3 positive cancer cells, but also delivered-siRNA could silence target gene expression by RNAi and then exert anti-tumor functions.

4. Discussion

In this study, we acquired a bifunctional recombinant R3P by inclusion body method in *E. coli*. Previously, we fused Sumo (Small ubiquitin-related modifier) in the N-terminal of R3P to acquire soluble recombinant protein. Sumo is an effective molecular companion for fusion protein to enhance soluble expression of proteins and decrease proteolytic degradation [18]. Our results showed that Sumo can promote soluble expression of R3P, but soluble R3P was easier to bind to the bacteria genomic DNA or RNA (Data not shown), which leads to the low binding ability to exogenous siRNAs and adverse biological safety. To resolve this problem, we removed Sumo fragment from the 5′-terminal of R3P gene and expressed R3P by inclusion body method. In order to avoid complicated steps and possible low activity, low amounts of protein were expressed by inclusion body method. Interestingly, our results showed that R3P produced by the inclusion body method did not bind to the bacteria genomic DNA or RNA by analysis of OD_{260} (Data not shown). Moreover, refolded R3P still had good binding activity and target ability. Unfortunately, the binding efficiency of R3P is poorer than our previous reported ScFv-9R [19]. We hypothesized that the recombinant ScFv-9R acquired by soluble expression might have higher right folding efficiency than that of

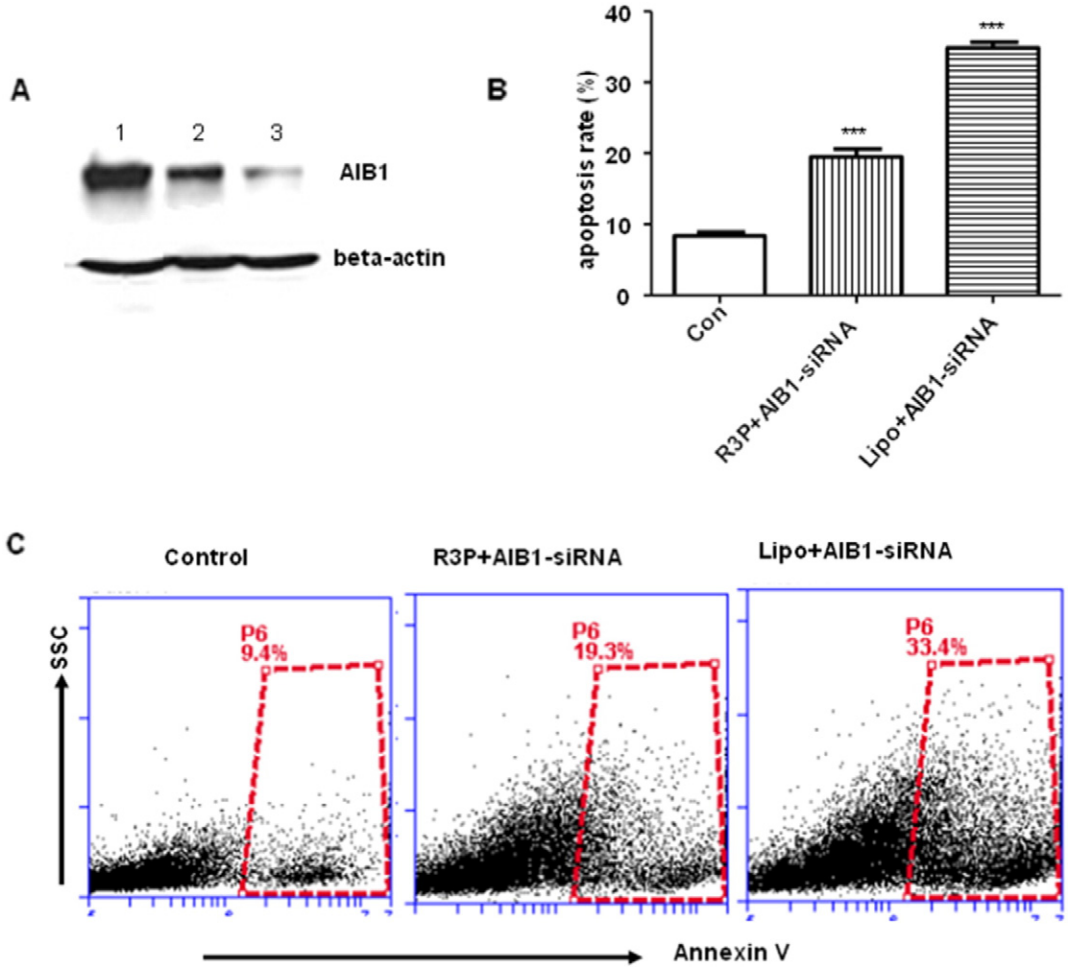

Fig. 4. AIB1-siRNA delivered by R3P induced RT112 cell apoptosis *in vitro*. (A) Western blot analysis of downregulated-AIB1 expression in R3P/AIB1-siRNA group (lane 2) and Lipo/AIB1-siRNA group (lane 3) compared with control group (lane 1). (B) Statistical analysis of C. Data were expressed as means ± S.E.M (n = 4). Significant differences compared with control were showed at $^{***}P < 0.001$. (C) Flow cytometry results showed that both R3P/AIB1-siRNA and Lipo/AIB1-siRNA significantly induced cell apoptosis compared with control group.

R3P acquired by inclusion body method. Therefore, we still need to further improve the methods of denaturation and refolding in the future. In a word, this study provides us a very good strategy that proteins which bind to nucleic acid in *E. coli* can be expressed by inclusion body method.

In our studies, FAM-siRNAs mixed with ScFv without protamine was not internalized. The FGFR3 negative THP1 cells transfected with ScFv/siRNAs or R3P/siRNAs also did not take up FAM-siRNAs as well. Only R3P can specifically deliver fluorescently labeled FAM-siRNAs into FGFR3 positive K562 cells and RT112 cells, as evaluated by fluorescence microscope and flow cytometry (Fig. 3). Next, we will visualize the R3P/siRNAs internalization into RT112 cells or K562 cells by confocal laser scanning microscopy. To determine the functionality of R3P-mediated siRNA delivery, AIB1, which promotes bladder cancer cell proliferation [15] was chosen as a therapy target. Our results showed that R3P/AIB1-siRNA induced cell apoptosis with the apoptosis rate of less than 20%. Presumably, there might be two major reasons for the low apoptosis rate. One possible reason is R3P/AIB1-siRNA only downregulates about 50% of AIB1 gene expression (Fig. 4A), the other half of AIB1 protein still plays a portion of functions for tumor promotion. The other possible reason is that tumor development usually involved numerous genes and multiple signaling pathways, so single gene interference is very difficult to completely inhibit tumor growth and is likely to induce cancer cell resistance to drugs. Based on this problem, a cocktail of siRNAs which can disturb several gene expressions would be a better choice. Yao's et al. [20] reports showed that a cocktail containing siRNAs of three genes is more effective than one siRNA alone in breast cancer suppression. In our next studies, siRNA cocktail containing several tumor promotion genes will be evaluated by R3P carrier *in vitro* and *in vivo*.

Additionally, how to enhance the binding capability of R3P and stability of R3P/siRNAs are also important issues to be resolved. Animal experiments need to be performed for detecting the inhibition functions of R3P/siRNAs for tumor growth *in vivo* in the future.

5. Conclusions

In summary, we successfully generated R3P by inclusion body expression method in *E. coli*, which showed bifunctional characteristics that not only binds to siRNAs but also delivers siRNAs into FGFR3-positive cancer cells. Our results also revealed that R3P, as a novel carrier, might have potential applications in antitumor studies and pharmaceutical development.

Conflict of interests

We declare that there is no conflict of interest regarding the publication of this work.

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