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Soluble Expression of IFNα2-Tα1 Fusion Protein in *Escherichia coli* by N-terminal SUMO Fusion and its Anti-Proliferative Activity

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ABSTRACT

Interferon alpha 2 (IFNα-2) is a type of cytokine with both antiviral and anticancer activities. Binding of IFNα-2 to its receptor on cell surface leads to activation of interferon stimulated genes which mediate its anti-proliferative and anti-angiogenic properties. Similarly, Thymosin alpha 1 (Tα-1) helps to fight against different infections such as cancer and hepatitis with its immune modulating properties as well as through its direct action on target cells. The recombinant expression of some proteins in *E. coli* produces inclusion bodies which are misfolded proteins. The objective of this study was soluble expression of IFNα2-Tα1 fusion protein in *E. coli* using pET-SUMO vector and determination of its biological activity. SUMO-IFNα2-Tα1 was successfully expressed in soluble form with IPTG induction to final concentration 0.5 mM at 37°C for 4 h and purified using affinity chromatography. SUMO tag was removed from SUMO-IFNα2-Tα1 by SUMO protease and recombinant IFNα2-Tα1 was collected in flow through by affinity chromatography. The MW (~23 kDa) of IFNα2-Tα1 was determined by 12 % SDS-PAGE and its integrity was confirmed by Immuno blot analysis using anti-interferon α-2 and anti-thymosin α-1 antibodies. Purified IFNα2-Tα1 demonstrated anti-proliferation activity as assessed by MTT assay. This study also showed that N-terminal fusion of SUMO with IFNα2-Tα1 is effective for its soluble expression and to make its purification process more convenient.

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Authors' Contribution
MSA and IG performed the experiments. ZA did statitical analysis and prepared the manuscript. MAA designed the experiments and supervised the research.

Key words
Soluble expression, SUMO fusion, Immobilized metal affinity chromatography, MTT assay, IPTG.

INTRODUCTION

The production of recombinant biopharmaceuticals is a major concern of biotechnology industry for the treatment of different diseases. Cytokines are used to inhibit tumor growth and elimination of HBV infection through immune activation or by inducing different signaling pathways (Yuchen and Ulrike, 2017). Interferon alpha (IFNα) is a widely used cytokine for treatment of hepatitis and cancer. IFNα-2 is a form of IFNα which is approved by FDA for treatment of different types of cancers either alone or in combination with other therapeutic drugs (Ningrum, 2014). It is being used for hepatitis treatment in combination with ribavirin, lamivudine or adevofir and also used for treatment of cancer in combination with cytarabinvinblastine, 5-fluorouracil, tamoxifen, or interleukin-2 for treatment of cancer (Wang *et al*., 2002; Leader *et al*., 2008). IFNα-2 shows anti-proliferative activity by directly inhibiting the cancerous cells growth by apoptosis, cell cycle inhibition or differentiation. It also acts indirectly by activating immune cells such as natural killer cells and induction of other cytokines for inhibition of cancer growth (Sarkar *et al*., 2003).

Tα1 is small peptide of 28 amino acids which stimulate immune response and causes differentiation of T cells (Rustgi, 2005). Tα1 enhance response of immune system cells such as natural killer cell-mediated cytotoxicity, differentiation of $CD4^+$ and $CD8^+$ T cells, dendritic cells maturation through toll like receptors activation, stimulation of Th1 type immune response and anti-tumor cytotoxic T cells (Xiaoning *et al*., 2015). It is either used alone or in combination for therapy of various disorders and it is found very effective in reducing the tumor growth (Qin *et al*., 2009). The use of Tα1 in combination with IFNα or interleukin 2 give elevated biological effect by restoring cytotoxic action in conditions where immune response is suppressed by tumors or anti-cancer drugs and also demonstrate tumor regression in different in different types of mice models (Garaci *et al*., 2012).

Cloning and expression of therapeutically important proteins is preferred in *E. coli* due to its simple genetics and rapid growth rate. The expression of heterologous recombinant proteins in the form of inclusion bodies require several steps to get soluble active protein before

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purification (Wang *et al*., 2006; Mohammed *et al*., 2012). Multiple approaches have been used for soluble expression of eukaryokic proteins in *E. coli* (Cabrita *et al*., 2006; Burgess-Brown *et al*., 2008; Gustafsson *et al*., 2004; Rabhi-Essafi *et al*., 2007). Production of target protein as a fusion protein increases the efficiency of its soluble expression in *E. coli.* glutathione S transferase (Rabhi-Essafi *et al*., 2007), Nus A (De Marco *et al*., 2004), Maltose binding protein (Kapust and Waugh, 1999) and small ubiquitin like modifying protein (SUMO) (Butt *et al*., 2005; Zhu *et al*., 2013) are widely used fusion tags for soluble expression and simplify purification process of target proteins (Zhu *et al*., 2013). SUMO shields the target protein by using its chaperoning properties and enhance its solubility (Satakarni and Curtis, 2011).

In this study, IFNα2-Tα1 was expressed in soluble and biologically active form in *E. coli* by using pET-SUMO vector. The IFNα2-Tα1 fusion gene was expressed with N-terminal SUMO fusion under T7 promoter of pET-SUMO vector, purified by immobilized metal ion chromatography and its anti-proliferation activity was determined by MTT assay in comparison with commercial IFNα2b.

MATERIALS AND METHODS

Construction of pETSUMO-IFNα2-Tα1 recombinant vector

IFNα2-Tα1 gene was amplified by polymerase chain reaction with forward primer (IT-SUMO-F) (5'-TGTGATCTGCCTCAAACC-3') and reverse primer (IT-SUMO-R) (5'-T TAGTTCTCGGCCTCCTAC-3') in a reaction mixture with pTZ-IFNα2-Tα1 vector (constructed in previous study) as template, dNTPs (2.5 mM), $MgCl_2$ (2 mM), 1X PCR buffer (1 X) and Taq polymerase (5 units). The temperature profile was set as initial denaturation (1X) at 94°C for 5 min followed to 35 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 1 min and final extension (1X) at 72°C for 20 min. Amplicon (IFNα2-Tα1 gene) was analyzed by 1% agarose gel electrophoresis and gel purified with geneJET gel extraction kit (Thermo Scientific). IFNα2-Tα1 gene was directly inserted in to pET SUMO vector (Invitrogen) following the instructions of the manufacturer (Champion™ pET SUMO Protein Expression System, Invitrogen) using T4 DNA ligase.

Expression analysis

Recombinant pET SUMO-IFNα2-Tα1 vector was transformed in to competent cells of *E. coli* BL21 (DE3) and expressed under T7 promoter of pET SUMO expression vector (Invitrogen). 10 ml LB medium (1 % tryptone, 1 % yeast extract, 0.5 % NaCl, pH 7.2) containing kanamycin (40 µg/ml) was inoculated with a colony of recombinant clones and incubated at 37°C, 180 rpm until OD 0.7 at 600 nm is reached. Culture with OD_{600} 0.7 was cetrifuged at 8000 rpm for 10 min at 4°C and pellet was resuspended in 5 ml fresh LB medium containing kanamycin (40 µg/ml). After that, 50 ml terrific broth (TB) medium supplemented with glucose (1%), kanamycin (40 µg/ml) was inoculated with 2 ml of above culture and incubated at 37°C, 180 rpm until OD 0.5 at 600 nm is reached. Un-induced sample (5 ml) was collected to use as control for expression analysis and pelleted at 8000 rpm for 10 min at 4°C. The remaining culture was induced with IPTG to 0.5 mM and placed in incubator for 4 h at 37°C, 180 rpm. Induced cell culture was centrifuged at 8000 rpm for 15 min at 4°C and both pellet and supernatant were stored at -20°C for analysis.

Bacterial cell lysis and SDS-PAGE analysis

The expression of recombinant IFNα2-Tα1 induced by IPTG was checked on 12% SDS-PAGE in both soluble and insoluble cytoplasmic fractions. Induced and un-induced cell pellets were resuspended in lysis buffer (50 mM Tris-Cl pH 6.1, 5 mM EDTA, 1mM PMSF, 0.5% Triton X-100) and lyzed by sonication on ice for 30 min with intervals of 30 seconds. The cell extract were centrifuged at 15,000 rpm for 30 min at 4°C and supernatants were collected (soluble fraction) in a tube. Cell pellets (insoluble fraction) were resuspended in lysis buffer and 12% SDS-PAGE (Laemmli, 1970) was used to check presence of target protein both soluble and insoluble fractions. The protein concentration was estimated using a Bradford assay (Bradford, 1976).

Purification and SUMO protease cleavage

The column was packed with Ni^{2+} sepharose resin and washed with wash buffer (10 column volume). After equilibration of column with 10 column volume of equilibration buffer (50 mM Tris-Cl pH 7.4, 5 mM EDTA, 10 mM imidazole, 150 mM NaCl), soluble cytoplasmic fraction containing SUMO-IFNα2-Tα1 with His_6 tag at its N terminal end was filtered through 0.22 µm syringe filter and loaded on to column. The column was washed with 10 column volume of binding buffer to wash unbound proteins and bound SUMO-IFNα2-Tα1 was eluted with 3 column volume of elution buffer (50 mM Tris-Cl pH 7.4, 5 mM EDTA, 150 mM NaCl, 500 mM imidazole). Fractions were collected, dialyzed with 1X PBS and analyzed by absorbance at 280 nm. Presence of SUMO-IFNα2-Tα1 was further analysed by 12% SDS-PAGE (Laemmli, 1970). Purified SUMO-IFNα2-Tα1 was concentrated by freeze dryer and concentration was adjusted to 1 mg/ml. The SUMO protease digestion reaction was prepared

in 1X SUMO protease buffer with SUMO-IFNα2-Tα1 (0.2 mg/ml) and SUMO protease (20 Units). Digestion reaction was incubated at room temperature for 5 h and analyzed by 12 % SDS-PAGE gel. Digestion reaction was dialyzed with 1X PBS and IFNα2-Tα1 was purified by Ni-sepharose column. IFNα2-Tα1 was collected in flow through and concentrated by freeze dryer. Purified IFNα2-Tα1 was analyzed by 12 % SDS-PAGE and total protein concentration was determined by Bradford assay (Bradford, 1976).

Immuno blot analysis

Purified IFNα2-Tα1 was resolved on 12 % SDS PAGE and transferred to nitrocellulose membrane with transblot semi-dry apparatus (Biorad) at 18 volts for 2 h using buffer (50 mM Tris-Cl buffer pH 8.3, 192 mM glycine, 20 % (v/v) methanol, 0.1 % SDS). Non-specific sites on membrane were blocked by incubating membrane in 5 % (w/v) skimmed milk in 1X TBS-T buffer (920 mM Tris pH 7.6, 13 mM NaCl, 0.1 % (v/v) Tween 20) for 1 h at 4°C. The membrane was divided into two parts and one part of membrane was incubated with mouse anti-interferon α-2 antibody (1:3000 dilutions) and other part was incubated with mouse anti-thymosin α-1 antibody separately for 1 h at room temperature. Each blot was washed for three times with 1X TBS-T buffer (10 min/wash) and membranes were incubated in goat anti-mouse IgG-alkaline phosphatase conjugated (1:5000 dilution in 1X TBS-T) at room temperature for 2 h. After washing, the color reaction was developed by incubating the blots in BCIP/NBT substrate solution in carbonate buffer (pH 9.8). The reaction was stopped by washing membranes in water and strips were air dried and analysed.

MTT assay

Anti-proliferative activity of IFNα2-Tα1 using its different concentrations was compared with commercial IFN-α2b using MTT assay on HepG2 cell line. Briefly, MTT stock solution (5 mg/ml) was prepared and filtered by 0.22 µm syringe filter. The HepG2 cells were seeded in triplicate at 10,000/cm^2 density in a 48-well cell culture plates, respectively for sample and reference protein. Culture medium was replaced with different concentrations (1-10 ng) of both proteins in fresh culture medium after 24 h of seeding. After 24 h of proteins treatment, 10 µl MTT stock solutions was added to each well of 48-well plate and the cells were incubated at 37°C for 4 h in 5% CO_2. After 4 h, the medium was removed and cells were washed with 1X PBS buffer. MTT solubilizing agent (200 µl/well) was added to the cells and plates were incubated for 10 min at room temperature. Purple color at varying intensity was observed as MTT crystals solubilized. The contents (150 µl/well) were transferred to 96-well micro titer plate and absorbance was taken at 560 nm. The graph was plotted for percentage cytotoxicity against each concentration of both proteins.

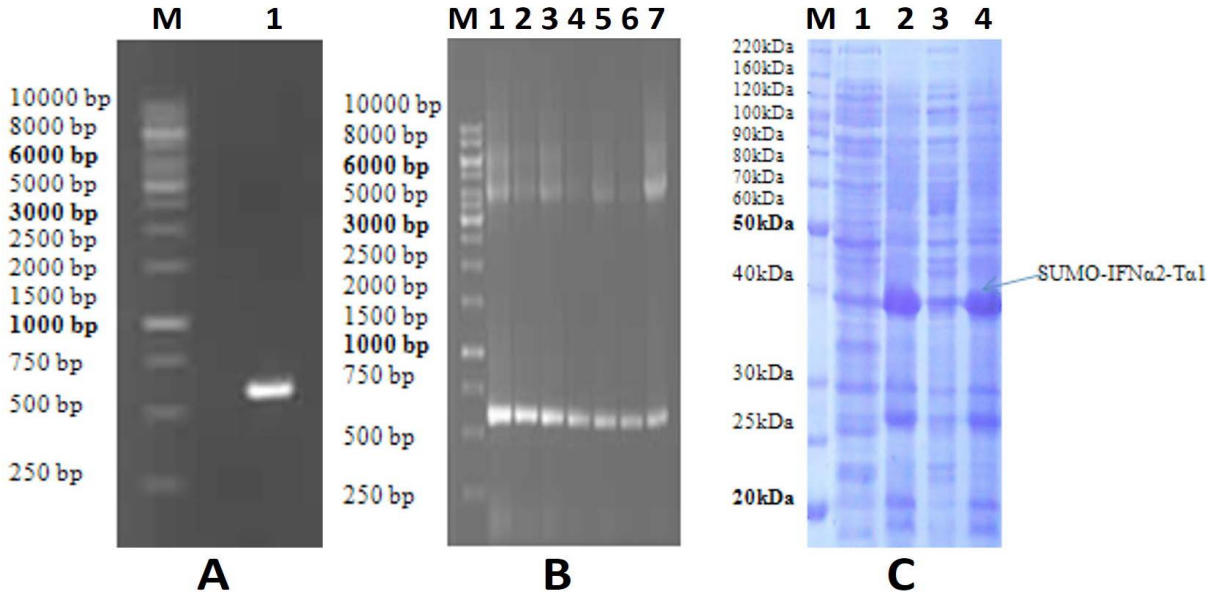

Fig. 1. **A,** Analysis of IFNα2-Tα1gene by 1% agarose gel electrophoresis (Lane M, DNA ladder; Lane 1, IFNα2-Tα1gene (591 bp)). **B,** Analysis of recombinant *E. coli* BL21 (DE3) clones by colony PCR(Lane M, DNA ladder; Lane 1-7, recombinant clones). **C,** Analysis of SUMO- IFNα2-Tα1 fusion protein by 12 % SDS-PAGE (Lane M, protein ladder; Lane 1, un-induced cell fraction; Lane 2, total cell lysate; Lane 3, insoluble cell pellet; Lane 4, soluble cell fraction).

RESULTS

Construction of pETSUMO-IFNα2-Tα1 vector and its expression analysis

IFNα2-Tα1 gene was (Fig. 1A) cloned in to pET SUMO expression vector Invitrogen). The pET SUMO expression vector uses SUMO to increase soluble expression of native protein and also simplify its purification process. IFNα2-Tα1gene was cloned in pET SUMO expression vector under T7 promoter with N-terminal SUMO fusion partner and His_6 tag for affinity purification. *E. coli* cells were transformed with recombinant pET SUMO-IFNα2-Tα1 vector and colony PCR was used to screen recombinant transformed clones (Fig. 1B). Expression of the SUMO-IFNα2-Tα1 was optimized with respect to IPTG concentration, induction time and temperature. 12 % SDS–PAGE analysis showed successful expression of SUMO-IFNα2-Tα1 in soluble fraction after induction with 0.5 mM IPTG for 4 h at 37°C (Fig. 1C). Band of ~ 38 kDa was observed on gel following staining with coomassie brilliant blue. It was also observed that ~ 80 % of SUMO-IFNα2-Tα1 was expressed in soluble form (Fig. 1C, lane 4) and ~ 20 % fusion protein was observed in insoluble pellet (Fig. 1C, lane 3).

Purification and immuno blot analysis

SUMO-IFNα2-Tα1 fusion protein with N-terminal His_6 tag was purified by affinity chromatography on Ni^+-sepharose column. SUMO-IFNα2-Tα1 was eluted from column and analyzed by 12 % SDS-PAGE which appeared with molecular weight of ~38 kDa (Fig. 2A). Purified SUMO-IFNα2-Tα1 was subjected to cleavage reaction by SUMO protease for removal of SUMO tag and analyzed by 12 % SDS-PAGE. The presence of two bands of IFNα2-Tα1 and SUMO on 12 % SDS-PAGE gel confirmed the successful cleavage of SUMO tag (Fig. 2B). IFNα2-Tα1 was purified from cleavage reaction by Ni^+ sepharose column and IFNα2-Tα1 was collected in the flow through. After removal of SUMO tag, purified IFNα2-Tα1 was resolved by 12 % SDS-PAGE which showed single band of ~23 kDa (Fig. 2C). The identity of purified IFNα2-Tα1 was confirmed by immuno blotting using mouse anti-interferon α-2 and mouse anti-thymosin α-1 antibodies respectively as shown in Figure 2D and E.

IFNα2-Tα1 fusion protein demonstrates anti-proliferative activity

Anti-proliferative activity of recombinant IFNα2-Tα1 was evaluated using MTT colorimetric assay on HepG2 cell line in comparison with commercial IFN-α2b. Recombinant IFNα2-Tα1 and commercial IFN-α2b showed a significant anti-proliferative activity after 24 h of treatment on HepG2 cells. Interestingly, IFNα2-Tα1 showed more anti tumor cell proliferation as compared to commercial IFN-α2b. The viability of HepG2 cells decreased up to 65 % at 10 ng/ml concentration of IFNα2-Tα1as compared to 52 % decrease with commercial IFN-α2b at same concentration (Fig. 3).

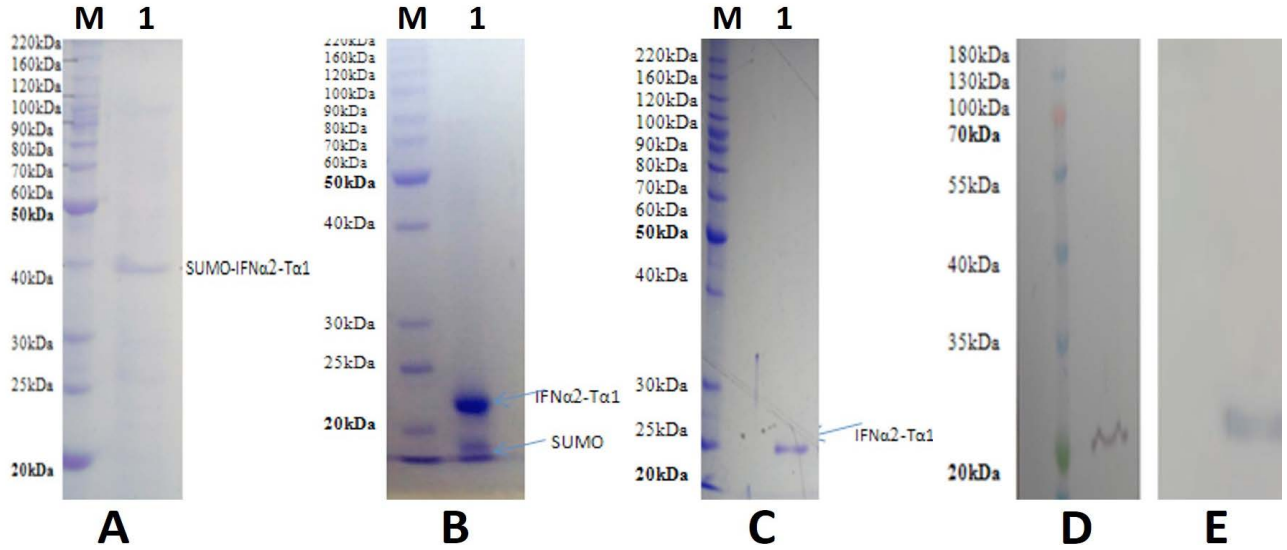

Fig. 2. Purification and immuno blot analysis. **A,** Purification of recombinant SUMO-IFNα2-Tα1 fusion protein by Ni^+ affinity chromatography (Lane M, Protein marker; Lane 1, Purified SUMO-IFNα2-Tα1). **B,** Analysis of SUMO-IFNα2-Tα1 cleavage reaction with SUMO protease by 12 % SDS-PAGE (Lane M, Protein marker; Lane 1, FNα2-Tα1 and SUMO bands after digestion). **C,** Purification of FNα2-Tα1 by Ni^+ affinity chromatography from cleavage reaction (Lane M, Protein marker; Lane 1, purified FNα2-Tα1). **D,** Immuno blot analysis with anti-interferon α2 antibody. **E,** Immuno blot analysis with anti-thymosin α-1 antibody.

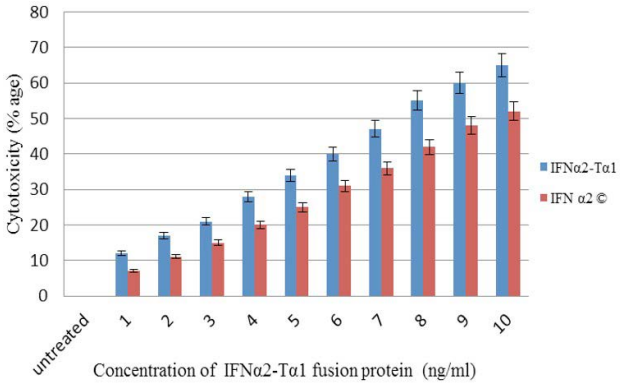

Fig. 3. Anti-proliferative activity of IFNα2-Tα1 fusion protein in comparison with commercial IFNα2 on HepG2 cell line using MTT assay.

DISCUSSION

E. coli provides less expensive and simple approach for expression of hetrologous recombinant proteins (Studier, 2005). However, majority of cytokines express in the form of incorrectly refolded aggregates called inclusion bodies in *E. coli* (Rosano and Ceccarelli, 2014). Therefore, it is a need to establish approaches for soluble expression of cytokines in *E. coli* expression system (Mohammed *et al*., 2012; Baneyx, 1999). The use of highly soluble partner in fusion with target protein is an effective strategy for soluble expression of protein of interest (Jana and Deb, 2005). In this study, we described a strategy to express IFNα2-Tα1 fusion protein in soluble and biologically active form in *E. coli* expression system using pET SUMO vector (invitrogen). The fusion of SUMO with target protein facilitates folding of target protein through its chaperon-like effect (Malakhov *et al*., 2004; Butt *et al*., 2005) and also helps it to fold in correct conformations for soluble expression (Kong and Guo, 2011).

IFNs have wide range of therapeutic applications including proliferative disorders (Wang *et al*., 2002). Recombinant human IFN-a2b is widely used in monotherapy or in combined therapy with other drugs, such as cytarabin, vinblastine, 5-fluorouracil, tamoxifen, or interleukin-2 on cancer treatment, and combined with the nucleoside analogs lamivudine, adevofir, entecavir, telbivudine, or ribavirin for treatment of different diseases (Ningrum, 2014; Foster, 2010; Lin and Young, 2014; Wang *et al*., 2002). Tα1 is a polypeptide of 28 amino acids which modulate immune response and decreases tumor cell growth (Jian-Hua *et al*., 2012; Belinda *et al*., 2016). Tα1 had been expressed in fusion with proteins like thymopentin (Juan *et al*., 2008) and human serum albumin (Jian-Hua *et al*., 2010). The anti-proliferative activity of Tα1 had been observed in lung adenocarcinoma and HepG2 hepatoma cells of humans (Qin *et al*., 2009). Our study showed that fusion of Tα1 with IFNα2 is more effective in inhibiting proliferation of tumor cells. Although we need more in vivo trials to accurately explain the mechanism of action of IFNα2-Tα1 fusion protein.

Briefly, we cloned IFNα2-Tα1 gene in pET SUMO expression vector (Invitrogen) downstream of SUMO gene by following instructions given in manual (Champion™ pET SUMO Protein Expression System, Invitrogen) with His_6-tag at N-terminal end of SUMO tag for purification by immobilized metal ions chromatography. Recombinant pET SUMO- IFNα2-Tα1was transformed in *E. coli* BL21 (DE3) and induced with IPTG to final concentration 0.5 mM in TBG medium. 12 % SDS–PAGE analysis confirmed that about 80% SUMO-IFNα2-Tα1 is expressed in soluble form in soluble cytoplasmic fraction soluble with molecular weight ~38 kDa (Fig. 1C). Similarly in other studies, SUMO is fused with Fibroblast growth factor 21 (Huiyan *et al*., 2014), bovine ß-lactoglobulin (Ponniah *et al*., 2010), interferon consensus (IFN-con) (Karolina *et al*., 2014), APRIL (Jie *et al*., 2014) for their soluble expression in *E.coli.* SUMO-IFNα2-Tα1 fusion protein was purified from soluble cell fraction by Ni^+ affinity chromatography and subjected to cleavage reaction by SUMO protease for removal of SUMO. The ability of SUMO protease to cleave partner proteins with high reliability and efficiency prove SUMO fusion system the best choice for soluble expression of target proteins (Malakhov *et al*., 2004). Electrophoretic analysis of purified SUMO-IFNα2-Tα1 (Fig. 2A) and cleavage reaction confirmed successful purification and cleavage of SUMO tag (Fig. 2B). After removal of SUMO tag, the IFNα2-Tα1 was analyzed on 12 % SDS-PAGE and showed single band of ~23 kDa (Fig. 2C). The identity of purified IFNα2-Tα1 was confirmed by immuno blot analysis with mouse anti-interferon α-2 and mouse anti-thymosin α-1 antibodies respectively (Fig. 2D, E). Anti-proliferative activity of IFNα2-Tα1 demonstrated that it is more active than single commercial IFN-α2b in inhibiting tumor cell proliferation. The viability of HepG2 cells decreased up to 65 % at 10 ng/ml concentration of IFNα2-Tα1as compared to 52 % decrease with commercial IFN-α2b (Fig. 3).

CONCLUSION

It is concluded that fusion of SUMO at N-terminal end of IFNα2-Tα1 is effective for its soluble expression to make purification process simple. This is a first report on soluble expression of IFNα2-Tα1 in *E. coli* by using pET SUMO expression vector showing anti-cancer activity.

Statement of conflict of interest

Authors have declared no conflict of interest.

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