RESEARCH ARTICLE

Soluble Expression of Recombinant Human Smp30 for Detecting Serum Smp30 Antibody Levels in Hepatocellular Carcinoma Patients

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Abstract

Senescence marker protein 30 (SMP30), a hepatocellular carcinoma (HCC) associated antigen, was earlier shown by our research group to be highly expressed in HCC paracancerous tissues, but have low levels in HCC tissues. In order to detect anti-SMP30 antibody in serum of HCC patients, we established pET30a-SMP30 and pColdIII-SMP30 expression systems in *Escherichia coli*. However, the expression product was mainly in the form of inclusion bodies. In this research, we used several combinations of chaperones, four molecular chaperone plasmids with pET30a-SMP30 and five molecular chaperone plasmids with pColdIII-SMP30 to increase the amount of soluble protein. Results showed that co-expression of HIS-SMP30 with pTf16, combined with the addition of osmosis-regulator, and a two-step expression resulted in the highest enhancement of solubility. A total of 175 cases of HCC serum were studied by ELISA to detect anti- SMP30 antibody with recombinant SMP30 protein. Some 22 were positive and x^2 two-sided tests all showed $P>0.05$, although it remained unclear whether there was a relationship between positive cases and clinical diagnostic data.

Keywords: SMP30 - genetic engineering - soluble expression - molecular chaperone

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Introduction

It is well known that detection of cancer at early stage can prolong age of patients or decrease an incidence of cancer related death (Oyunsuren et al., 2006). Senescence marker protein-30 (SMP30), also known as regucalcin, has been reported in 1988 (Yamaguchi et al., 1988) as a calcium binding protein. Our previous research has shown that SMP30 is a HCC (Hepatocellular Carcinoma) associated antigen and its expression level in paracancerous tissues is higher than that in cancer tissues (Zhou et al., 2006). So we recombined a fusion protein (MBP-SMP30, 66kDa) with its 165 amino acids in C-terminal (18.5 kDa) as an antigen to detect anti-SMP30 antibody in serum of HCC patients. The positive rate is approximately 39% (Zhou et al., 2011). In this study, we have constructed and expressed a fusion protein with 6×His-tag (HIS-SMP30, 20 kDa) as an antigen for detecting anti-SMP30 antibody and other subsequent studies.

Materials and Methods

Materials

Recombinant plasmids of pMAL-c2-SMP30 and pET30a were self-owned by our team; plasmid pCold III, molecular chaperone plasmids (pG-KJE8, pGro7, pKJE7, pG-Tf2, pTf16), Sal I and Nde I were obtained from Takara Biotechnology (Dalian, China); E.coli .BL21 (DE3) and E.coli .BL21 (DE3) pLysS were bought from Tiangen (Beijing, China).

IPTG, PMSF, betaine, benzyl alcohol, and mouse anti-human SMP30 IgG were purchased from Sigma (St Louis, MO, United States); Ni-NTA affinity column was purchased from Solarbio (Beijing, China); ultrafiltration centrifuge tube was from Millipore Corporation (Bedford, MA, United States); anti-His-tag mouse IgG goat, anti-mouse IgG (H+L)-HRP were bought from Boster (Wuhan, China); ECL Plus Western Blotting Detection System was from Beyotime Institute of Biotechnology (Nantong, China).

Construction of expression plasmids

In this work, both genomic DNA isolation and plasmid isolation were performed according to laboratory manual (Sambrook et al., 2001), and all sequences engineered by PCR were verified subsequently by DNA sequencing. To amplify the target gene, recombinant plasmid of pMAL-c2-SMP30 was used as a template, Nde I restriction site (CATATG) and 6×histidine sequences (CACCATCATCATCATCAT) were added

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to forward primer, Sal I restriction site (GTCGAC) was added to reverse primer, the primer sequences were 5'-ATACATATGCACCATCATCATCATCATTACTCCC TCTTTCCTGATCACCAC-3' (forward) and 5'-AGTGT CGACTCATCCCGCATAGGAGTAGGGAG-3' (reverse), and yielded a 500bp product. The SMP30 cDNA containing the 6×His-tag was obtained by restricting digestion using Nde1 and Sal I endonucleases from PCR products and ligated into linearized vectors (pET30a and pCold III).

Construction of co-expression system

Each chaperone plasmid (pG-KJE8, pGro7, pKJE7, pTf16, and pG-Tf2, Takara) was transformed into BL21 (DE3) cells (Tiangen) and the transformed cells were selected with chloramphenicol. The E. coli competent cells transformed with each chaperone plasmid were prepared using standard methods and then transformed with recombinant pCold III/SMP30 plasmid followed by selection with chloramphenicol and ampicillin.The transformation was confirmed by PCR.

The BL21 (DE3) bacteria containing co-expression plasmids were induced with stepwise selection Method (Ran et al., 2010). In the first step, the expression of molecular chaperone proteins was induced with 1 mg/ml L-arabinose (aiming at the chaperones in pG-KJE8, pGro7, pKJE7, pTf16) or 5 ng/ml tetracycline (aiming at the chaperones in pG-Tf2) 30 min prior to adding IPTG according to the manufacturer's instructions. In the second step, when A600 = 0.5, the expression of targeting protein was induced by adding 0.6 mM IPTG at 15°C for 24h. The supernatant and sedimentation of bacteria were collected.

Adding adjuvant reagents

According to the result of stepwise selection Method, three genetically engineered bacteria containing co-expression plasmids (pKJE7/pColdIII-SMP30, pG-Tf2/pColdIII-SMP30 and pTf16/pColdIII-SMP30) were selected for the future study on using adjuvant reagents. In the first step to induce the expression of molecular chaperone proteins, 5 mM betaine was added to growth medium when A600 = 0.3. 10 min after adding betaine, 10 mM benzyl alcohol was added. In the second step, 0.6 mM IPTG was added to induce expression at 15°C for 22h with shaking at 225 r/min. Then, the cells were collected by centrifugation, resuspended with equivalent fresh LB medium without antibiotics or IPTG, and further cultured at 20°C, for 2h with shaking at 225 r/min. The cells were collected and lysed; their supernatant was collected to identify targeting protein by SDS-PAGE.

The identification of products by Western Blotting

The proteins in extracts were separated by SDS-PAGE and transferred to nitrocellulose paper; the membrane was then incubated in 5% skim milk powder for 1h. The membrane was washed with TBST and subsequently incubated for 1h with anti-His antibody and anti-human SMP30 antibody respectively. The washing steps were repeated, and the membrane was then incubated for 1h with horseradish peroxidase conjugated goat anti mouse IgG. The immunoreactive bands were visualized using the ECL plus Western Blotting Detection System. Images were obtained using ChemiDOC™ XRS Image Analyzer (Bio-Rad, Hercules, CA) and Quantity One software.

Detecting anti-SMP30 antibody with recombinant SMP30 protein

The bacteria were thawed on ice, re-suspended in 20 mM PBS (pH 7.4), and lysed by sonication. The lysate was centrifuged for 30 min at 12,000 rpm at 4°C, and the supernatant was purified by immobilized metal affinity chromatography (IMAC) on a Ni-NTA affinity chromatography column and concentrated with ultra filtration centrifuge tube, dissolved with 8M. The purified SMP30 protein was used in ELISA to detect anti-SMP30 antibody in HCC serum.

Results

The identification of expression plasmids

The recombinants pET30a-SMP30 and pCold III-SMP30 were successfully constructed and induced, and their expression was roughly shown in the SDS-PAGE. There was targeting protein in sedimentation but not in supernatant (Figure 1), Analysis by Quantity One displayed that both of them accounted for over 60% of total proteins and reached a purity of more than 95%.

The Co-expression of molecular chaperone plasmids with recombined plasmids

A co-expression system of molecular chaperone plasmids with recombined plasmids was successfully constructed. Our efforts to express SMP30 in E. coli using pET30a vector resulted in the formation of inclusion bodies (Figure 2A), and we were not able to generate active SMP30 after solubilization, purification, and refolding. Therefore, in order to obtain soluble SMP30, we explored a cold shock expression system using pCold III vector in conjunction with co-expression of molecular chaperones,

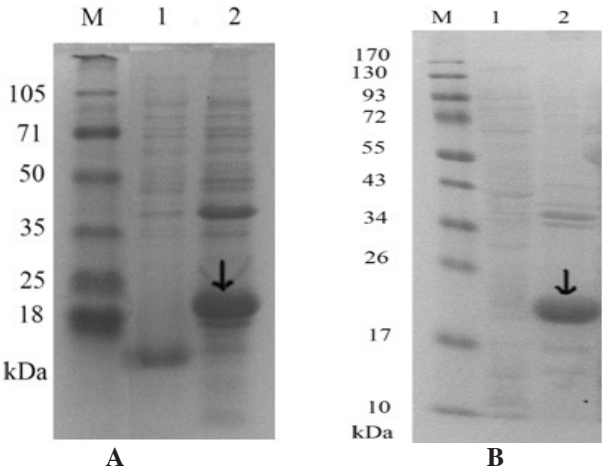

Figure 1. SDS-PAGE Analysis of the HIS-SMP30 Soluble Expression (stained with Coomassie blue). A: the soluble expression with pET30a-SMP30; lane 1: the soluble fragment; lane 2: insoluble fragment; B: the soluble expression with pCold III-SMP30; lane 1: the soluble fragment; lane 2: insoluble fragment. Marker lane sizes are in kDa, the arrows indicate HIS-SMP30

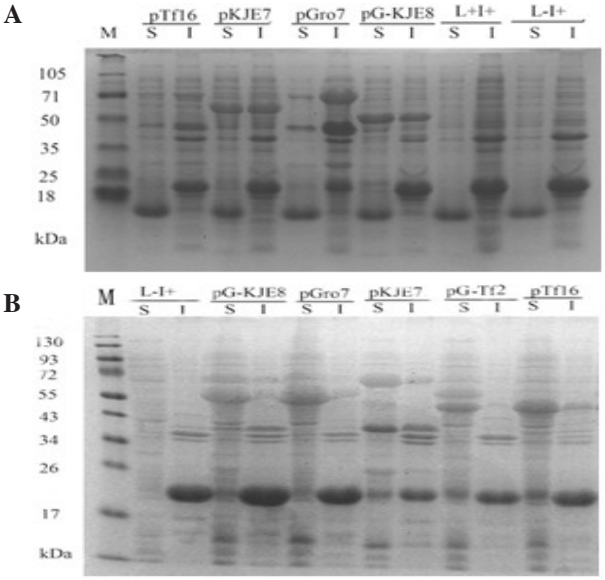

Figure 2. Effects of Chaperones Co-expression on the Solubility of HIS-SMP30 (SDS-PAGE gel with Coomassie blue stained). A: pET30a-SMP30 co-expression with different plasmids of chaperones; B: pCold III-SMP30 co-expression with different plasmids of chaperones. S: soluble fragment; I: insoluble fragment; L+I+: induction with L-Arabinose and IPTG; L-I+, induction with IPTG only. Marker lane sizes are in kDa

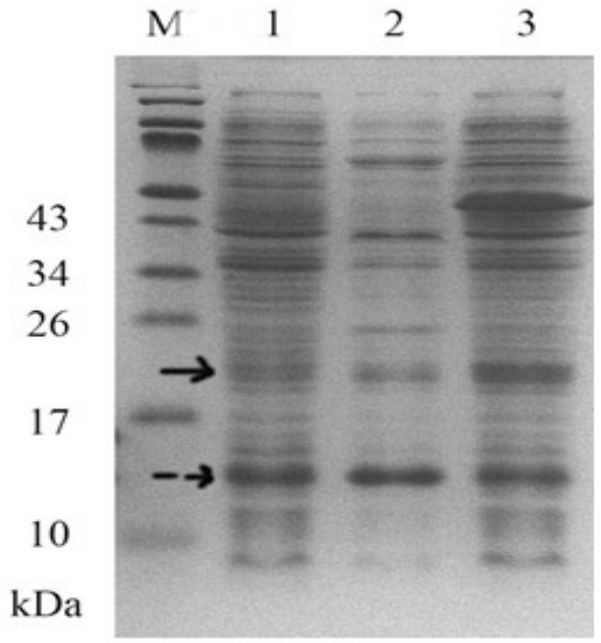

Figure 3. The Effect of Adjuvant on Improving the Solubility Expressed (SDS-PAGE gel stained with Coomassie blue). Lane 1-3: co-expression with pKJE7, pG-Tf2, pTf16 respectively. Marker lane sizes are in kDa. The solid arrow indicates HIS-SMP30 and the dotted arrow indicates degradation fragment

the solubility of SMP30 was improved in three (pTf16, pKJE7, and pG-Tf2) of five different combinations tested (Figure 2B). These three plasmids (pTf16, pKJE7, and pG-Tf2) of chaperone expression were selected for further studies. After adding betaine and benzyl alcohol (Figure 3), pTf16 co-expression system could more effectively promote the expression level of soluble SMP30 (24.7%) than the other two (4.9% and 11.5% respectively); its degradation fragment was also less (15.2%) than the other two (24.1% and 30.8% respectively).

Western blotting

Besides the soluble target protein in supernatant (Figure 4, lane 1, indicated by the solid arrows) and the protein purified from inclusion body (Figure 4, lane 2, the solid arrows) could combine with anti-His antibody and anti-SMP30 antibody, we found a degradation fragment (Figure 4, lane 1, shown by the dotted arrows) which could also combine with anti-His antibody and anti-SMP30 antibody. It has indicated that the degradation fragment was likely from N-terminal of HIS-SMP30.

The detection of anti-SMP30 antibody in HCC serum

22 of 175 cases HCC serum was positive, x^2 two-sided test all showed $P>0.05$, there was no statistical significance. It's not sure there is relationship between the positive cases and clinical diagnostic datas.

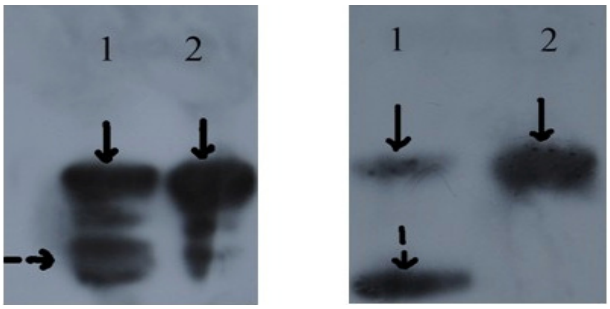

Figure 4. Western Blotting Analysis of the Targeting Protein in Supernatant and Inclusion Body. A: PVDF membrane combined with anti-HIS antibody; B: PVDF membrane combined with anti-SMP30 antibody. Lane 1-2: soluble fragment and HIS-SMP30 respectively. The solid arrows indicate HIS-SMP30, and the dotted arrows indicate degradation fragment

Discussion

The most commonly used expression system for recombinant expression of genes is Escherichia coli. However, recombinant proteins often aggregate in insoluble and inactive deposits called inclusion bodies because of their inability to attain proper tertiary structure due to anomalies in folding (Kiefhaber et al., 1991; Schrodel, 2005) Human SMP30 has a high probability (66%) of being insoluble when expressed in E. coli according to the predicted solubility calculated from the revised Wilkinson–Harrison solubility model (Wilkinson et al., 1991). Consistent with this prediction, our efforts to generate HIS-SMP30 using recombined plasmid expression system (pET30a-SMP30 and pCold III-SMP30) resulted in insoluble and inactive preparations.

Co-expression of chaperones with recombinant proteins has been traditionally used to increase the amount of soluble protein (Goloubinoff et al., 1989; Ventura et al., 2006). Because successful chaperone co-expression to improve solubility is a trial-and-error process that can not be predicted from properties of proteins (Baneyx

et al., 2004; Hoffmann et al., 2004), we explored nine combinations of chaperones four chaperones with pET30a-SMP30 and five chaperones with pColdIII-SMP30, and found that SMP30 was rescued from aggregation by co -expression of chaperones pKJE7, pG-Tf2 and pTf16, especially pTf16. This result was consistent with other studies (Nishihara et al., 2000; Moonsuk et al., 2010).

According to our research, SMP30 mainly existed in sedimentation (insoluble form) in pET30a-SMP30 co-expression system; but more SMP30 existed in supernatant (soluble form) in pColdIII-SMP30 co-expression system. As a heat-shock expression vector, pET30a has the absolute advantage in expressing velocity and quantity comparing with other expression vectors, at high temperature, the target proteins can express with high speed although they often form inclusion bodies. pCold III is a more ideal selection for soluble protein with biological activity, which is a cold-shock expression vector developed in 2004 by Qing et al. the use of pCold III enable us to express recombinant proteins more slowly at a lower temperature, which may lead to correct folding (Huang et al., 2012).

Betaine is an osmosis-regulator; the presence of physiological amounts of osmosis-regulator can increase osmolyte viscosity and reduce the thermodynamically less favorable accidental misfolding events, so significantly increase the stability of native thermolabile proteins (Diamant et al., 2001; Timasheff et al., 2002). In our experiments, pTf16 co-expression system added betaine and benzyl alcohol could promote the expression level of soluble SMP30 more effectively, which means adding adjuvant is an effective method for soluble expression. We also used a two-step growth procedure, wherein chaperones and target protein are accumulated in the first growth phase and target proteins then acquire native conformation in the second growth phase after the block of protein synthesis (de Marco et al., 2007). The results showed that the solubility of SMP30 was significantly enhanced during the second growth phase. In conclusion, the pTf16 co expression system we constructed could promote the expression level of soluble SMP30 more effectively with the help of adjuvant.

In this study, HIS-SMP30 was used as antigen to detect anti-smp30 antibody in HCC serum; in another study, MBP-SMP30 was used to detect anti-smp30 antibody in the same samples. We found their positive rates were similar (12.6% and 11.4%) in two studies, and the positive samples were also consistent. It means that the tags have little effect on the antigenicity of recombinant SMP30.

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References

Baneyx F, Mujacic M (2004). Recombinant protein folding and misfolding in Escherichia coli. *Nat Biotechnol*, **22**, 1399-408.

de Marco A, Deuerling E, Mogk A, Tomoyasu T, Bukau B (2007) . Chaperone-based procedure to increase yields of soluble recombinant proteins produced in E. coli. *BMC Biotechnol*, **7**, 32.

Diamant S, Eliahu N, Rosenthal D, Goloubinoff P (2001). Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. *J Biol Chem*, **276**, 39586-91.

Frank B, Jennifer M, Petersen R (2010). A cell-based screen for inhibitors of protein folding and degradation. *Cell Stress Chaperon*, **15**, 913-27.

Goloubinoff P, Gatenby AA, Lorimer GH (1989). GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in Escherichia coli. *Nature*, **337**, 44-7.

Hoffmann F, Rinas U (2004). Roles of heat-shock chaperones in the production of recombinant proteins in Escherichia coli. *Adv Biochem Eng Biotechnol*, **89**,143-61.

Huang L, Shakhnovich EI (2012). Is there an en route folding intermediate for Cold shock proteins? *Protein Sci*, **21**, 677-85.

Kiefhaber T, Rudolph R, Kohler HH, Buchner J (1991). Protein aggregation in vitro and in vivo: a quantitative model of the kinetic competition between folding and aggregation. *Biotechnology*, **9**, 825-9.

Moonsuk SC, Saxena AM, Chilukuri NW (2010). A strategy for the production of soluble human senescence marker protein-30 in Escherichia coli. *Biochem Bioph Res Co*, **393**, 509-13.

Nishihara K, Kanemori M, Yanagi H (2000). Overexpression of trigger factor prevents aggregation of recombinant proteins in Escherichia coli. *Appl Environ Microbiol*, **66**, 884-9.

Oyunsuren T, Sanduijav R, Davaadorj D, Nansalmaa D (2006). Hepatocellular carcinoma and its early detection by AFP testing in Mongolia. *Asian Pac J Cancer Prev*, **7**, 460-2.

Ran F, Gadura N, Michels CA (2010). Hsp90 co-chaperone Aha1 is a negative regulator of the saccharomyces MAL Activator and Acts early in the chaperone activation pathway. *J Biol Chem*, **285**, 13850-62.

Sambrook J, Russell DW (2001). Molecular cloning: a laboratory manual. *Cold Spring Harbor*, New York.

Schrodel A, de Marco A (2005). Characterization of the aggregates formed during recombinant protein expression in bacteria. *BMC Biochem*, **31**, 10.

Timasheff SN (2002). Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry*, **41**, 13473-82.

Ventura S, Villaverde A (2006). Protein quality in bacterial inclusion bodies. *Trends Biotechnol*, **24**, 179-85.

Wilkinson DL, Harrison RG (1991). Predicting the solubility of recombinant proteins in Escherichia coli. *Biotechnology*, **9**, 443-8.

Yamaguchi M, Mori S, Kato S (1988). Calcium-binding protein regucalcin is an activator of (Ca^{2+}-Mg^{2+})-adenosine triphosphatase in the plasma membranes of rat liver. *Chem Pharm Bull*, **36**, 3532-9.

Zhou SF, Mo FR, Bin YH, et al (2011). Serum immunoreactivity of SMP30 and its tissues expression in hepatocellular carcinoma. *Clin Biochem*, **44**, 331-6.

Zhou SF, Xie XX, Bin YH (2006). Identification of HCC-22-5 tumor-associated antigen and antibody response in patients. *Clinica Chimima Acta*, **366**, 274-80.