RESEARCH ARTICLE

A Novel Approach to Cloning and Expression of Human Thymidylate Synthase

Ying-Tao Lv, Pei-Juan Du, Qiao-Yan Wang, Yuan Tan, Zong-Bin Sun, Zhong-Liang Su, Cong-Min Kang*

Abstract

Thymidylate synthase (TS) catalyzes the transfer of a methyl group from methylenetetrahydrofolate to dUMP to form dTMP. It is a primary target in the chemotherapy of colorectal cancers and some other neoplasms. In order to obtain pure protein for analysis of structure and biological function, an expression vector TS-pET28b (+) was constructed by inserting wild-type human thymidylate synthase (hTS) cDNA into pET28b (+). Then an expression strain was selected after transformation of the recombined plasmid into Rosetta (DE3). Fusion protein with His-tag was efficiently expressed in the form of inclusion bodies after IPTG induction and the content was approximately 40.0% of total bacteria proteins after optimizing expression conditions. When inclusion bodies were washed, dissolved and purified by Ni-NTA under denatured conditions, the purity was up to 90%. On SDS-PAGE and West-blotting, the protein band was found to match well with the predicted relative molecular mass-36kDa. Bioactivity was 0.1 U/mg. The results indicated that high-level expression of wild-type hTS cDNA can be achieved in prokaryotes with our novel method, facilitating research into related chemotherapy.

Keywords: Wild-type human thymidylate synthase cDNA - prokaryotic expression - rare codon

Asian Pac J Cancer Prev, 14 (12), 7523-7527

Introduction

Thymidylate synthase (TS, E 2.1.1.45), consisting of two identical subunits with molecular weight of 36.0 kDa, catalyzes the reductive methylation of dUMP to produce dTMP (Carrers et al., 1995). This process is the only de novo source of dTMP, which is subsequently metabolised to thymidine triphosphate (dTTP), exclusively for incorporation into DNA during synthesis and repair (Ackland et al., 2006). In the rapid proliferative cells, it can result in apoptosis to decrease the level of TS. This phenomenon is exploited in therapeutic protocols utilizing TS inhibitors, such as raltitrexed, pemetrexed, or prodrugs such as 5-fluorouracil and 5-fluorodeoxyuridine that are metabolized to TS inhibitors. Two antifolates, Raltitrexed and Pemetrexed, are licensed anticancer drugs. Previous small-sized studies showed lower thymidylate synthase (TS) expression in adenocarcinoma of the lung, which may explain higher antitumor activity of TS-inhibiting agents such as pemetrexed. Other antifolates, Raltitrexed and Pemetrexed, are licensed anticancer drugs. Previous small-sized studies showed lower thymidylate synthase (TS) expression in adenocarcinoma of the lung, which may explain higher antitumor activity of TS-inhibiting agents such as pemetrexed.

To exploit new TS inhibitors, it’s important to study the structure and properties of TS and also the crystal structures of hTS. It is reported that a new crystal form of human TS (hTS) which allows binding studies by soaking crystals in artificial mother liquors containing ligands that bind in the active site. Using this approach, crystal structures of hTS complexes with FdUMP and dUMP were obtained, indicating that this form should facilitate in high-throughput analysis of hTS complexes with drug candidates. Crystal soaking experiments using oxidized glutathione revealed that hTS binds this ligand (Jarmula 2010).

Steadman et al. (1998) utilized site-directed mutagenesis to create 12 mutant proteins with substitutions at residue 216. The result proved that a serine residue corresponding to Ser216 in human TS is an essential site to binding dUMP. Phan et al. (2001) found that TS functions as an RNA binding protein that regulates the expression of its own mRNA translation, the emergence of resistance to the treatment is often related to the increased level of TS in cancer cells, which have been linked to the elimination of TS binding to its own mRNA upon drug binding. During the early stage, TS was obtained from human cells (Lockshin et al., 1979; Dolnick et al., 1977). With the development of gene recombination and prokaryotic expression system, it is feasible to expression human TS in E. coli. Davisson et al. (1989) isolated a full-length cDNA...
DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from TaKaRa Biotechnology (Dalian, China). All other chemicals were from Sigma.

**Bacterial strains, plasmids and culture medium**

Escherichia coli DH5α was utilized for subcloning propagation of plasmids, BL21 (DE3) (Meck, China) and Rosetta (DE3) (Meck, China) were respectively used for subclone vector and expression vector. pcDNA3.1zeo-TS was gifted by Dr. Maria Zajac-Kaye (Basic Research Laboratory and Molecular Therapeutic Program, National Institute of Health). The strains were maintained on LB agar plates and grown at 37 °C unless otherwise.

**DNA manipulations**

All cloning techniques including PCR, restriction digestion, ligation, E. coli transformation, DNA preparation were described previously (Sambrook et al., 1989).

**Construction of subclone vector for amplification of human TS cDNA**

According to the sequence of TS gene (GenBank ID: NM_001071), the primers were designed as the following, upstream primer: 5'-ATCAT ATGTA CGCGG CCATG CCTGT G-3' (NdeI) and downstream primer: 5'-GCGCTC GAGAA CCCTA AACAG CCATT TCCAT TTG-3' (XhoI). The PCR product was purified by DNA gel extraction kit (Sangon, Shanghai). Specific fragments about 1 kb were connected to pMD18-T vector, then transformed into competence cells of E. coli DH5α. By blue-white spot screening, identification of plasmid PCR (Figure 1), the positive strain was sequenced in the DNA sequencing department of Biosune systems Biology, Shanghai. The subclone vector was named as TS-pMD18-T (Figure 1A).

**Construction of E. coli expression vector for synthesis of human TS**

The full length of human TS cDNA in TS-pMD18-T was isolated by a double digestion of NdeI, XhoI and cloned into the multiple cloning site (MCS) downstream of the expression vector pET-28b (+) that was previously digested with the same two enzymes. The recombinant plasmid was transformed into E. coli DH5α, identified by PCR, double enzymes digestion (Figure 3) and named TS-pET-28b (+) (Figure 1B).

**Expression and expression form analysis of recombinant human TS**

E. coli BL21 (DE3) and Rosetta (DE3) with TS-pET-28b (+) was activated in LB broth, containing 50μg/ml kanamycin, and 34μg/ml chloromycetin at 37°C with 200 rpm shaking. 500 μl of activated culture was transferred into 10ml of fresh LB broth with the mentioned antibiotics under the same condition for about 1h. When OD600 reached 0.5-0.7, IPTG (final concentration = 1.5 mM) was added to induce the synthesis of human TS. After 8h of induction at 37°C, E. coli was harvested by 15min of centrifugation at 8000 rpm and resuspended in 3 mL of buffer A (50 mM phosphate buffer, pH 8.0). The samples were taken out and analyzed by 15% SDS-PAGE (Figure 4A), The rest were sonicated (50 cycles with 10s on/15s off) in ice using a sonicator JY92-11 system (Scientz Biotechnology Co, Ltd, Ningbo) and then centrifuged at 13000rpm for 15min. The supernatant and resuspended precipitation were analyzed by 15% SDS-PAGE (Figure 4a) and West-blotting.

**Purification and refolding of recombinant human TS with 6xHis-tag**

Because there is 6xHis oligonucleotide sequence between the promoter and MCS, human TS had a 6xHis-tag in the N-terminal. A nickel affinity column was utilized for purification of the recombinant human TS.

About 200 ml of culture broth was centrifuged at 8000rpm for 15 min , and cells were resuspended in Buffer B (8M urine, 100 mM NaH₂PO₄, 100 mM Tris–HCl, pH 8.0). The cells were lysed by ultrasonication (20 times, 5S×5S) for further purification and then filtered through 0.45 μm microporous membrane. The filtrate was chromatographed on Ni-NTA Superflow Cartridge (1ml, QIAGEN in China), which was preequilibrated with buffer B. After it was washed with the same buffer, the recombinant human TS was respectively eluted with 10 volumes of buffer B containing 200mM imidazole (Figure 5). The elution buffer was assayed protein concentration by Bradford method and dialysed with 20 mM Tris–HCl, 2 mM EDTA, 0.1% PEG, 0-6 M urine, 0.1 mM GSSG, 0.2 mM GSH, pH7.4, 0.025 mg/ml Protein under 4°C.

**Bioactivity assay of recombinant human TS**

Bioactivity assay was carried out on the basis of Williams’s method (Steadman et al., 1998). Enzyme
activity was measured at 37°C. The purified enzyme was incubated in Morrison buffer (Ellis and Morrison 1982) containing 1mM dUMP and 150 μM (6R)-CH2H4folate. The absorbance change at 340nm due to the conversion of CH2H4folate to H2folate (ε340=6.4 mM⁻¹cm⁻¹ (Wahba et al., 1961) was monitored for 1-10min. One unit of enzyme activity is defined as the amount of enzyme required to synthesize 1μmol of dTMP per minute under these conditions.

Results

The subclone and expression vector construction of human TS cDNA

After constructed the subclone vector TS-pMD18-T, we carried on preliminary identification using PCR. The product was analyzed by 1% agarose gel electrophoresis, and the result showed that there was a band about 1kb (Figure 1), proved human TS cDNA had insert into pMD18-T. We sent DH5α with TS-pMD18-T to sequence analysis; The result showed that there was no nucleotide mutation.

Since TS-pET-28b (+) contain NdeI, XhoI site. We digested it with NdeI, XhoI (Figure 3a), and identified with PCR (Figure 3b). The result of restriction enzyme digesting showed that there were two DNA bands about 1kb, 5kb, which were similar as human TS cDNA and pET-28b (+). Expression vector construction of human TS cDNA was constructed.

Expression of human TS in E. coli

TS-pET-28b (+)/BL21 (DE3) and TS-pET-28b (+)/Rosetta (DE3) were respectively induced under 37°C. The OD600 of the cultures grown under IPTG induction for 16h were all about 1.5, which was about two fold less than the values shown by the non-induced TS-pET-28b (+)/Rosetta(DE3), or the pET-28b (+)/BL21 (DE3) pET-28b (+)/Rosetta (DE3) (both induced and non-induced) grown for the same duration, respectively. The lower absorption from the induced TS-pET-28b (+)/BL21 (DE3) and a TS-pET-28b (+)/Rosetta (DE3) cultures suggested that the cultures were expressing recombinant human TS, and the expression of recombinant proteins may retard the cell growth and result in lower absorption.

When total cells of TS-pET-28b (+)/BL21 (DE3) and TS-pET-28b (+)/Rosetta (DE3) after inducing were analyzed by SDS-PAGE (Figure 4a) and Western-blotting
(Figure 4b), there was a significantly protein band on the TS-pET-28b (+)/Rosetta (DE3) track at 36.0kDa which matched well with human TS relative molecular mass (Carrers et al., 1995), however, the TS-pET-28b (+)/BL21 (DE3) was not significantly. The reason is that human TS gene has many E. coli rare codons, and E. coli rare codons have significant effect on the expression of human TS (Hori et al., 1990; Joan et al., 1997). Rosetta (DE3) has T7-promoter and have added the rare codon tRNA. In order to lighten the effect of rare codon to the expression of human TS, we selected Rosetta (DE3) as the expression host. By location analysis (Figure 4b), human TS expressing in Rosetta (DE3) was mainly on inclusion body.

Purification and refolding of recombinant human TS with 6×His-tag

Human TS was found to be expressed in Rosetta (DE3) as inclusion bodies from the SDS-PAGE analysis (Figure 4b). Attempts to solubilize human TS from these pellets with Buffer B failed, unless cells were lysed by ultrasonication. Human TS became soluble under these conditions and could be subjected to further purification using Ni-NTA cartridge. The use of successive stepwise elution on the gel allowed the best elution (Buffer B containing 200mM imidazole) and finally purification of protein to 89% (Figure 5).

Bioactivity assay of recombinant human TS

Under room temperature conditions, determination of enzyme activity employed ultraviolet spectrophotometry. Experimental results see Table 1. The linear equations obtained is \( Y=0.0145X+0.054 \) (R=99.98%), including the reaction time X and the amount of the resultant dTMP Y . Then the specific activity of human TS after renaturation calculated was \( SP=0.0186/0.15=0.124 \) U/mg (protein concentration was 0.15 mg/ml). The result is similar with the standard specific activity of human TS (Steadman 1998).

Discussion

In the early stage of this experiment, we transformed TS-pET-28b (+) into the general expression host BL21 (DE3), the result (Figure 4a) showed that the yield of human TS was low. We thought that the conditions (temperature, inducing time, and IPTG concentration) may be not the optimal, and carried out the orthogonal experiments to optimizing the expression conditions. But the expression level was never improved. We thought the reason may be that codon preference in E. coli and eukaryotic organisms is different, and exists many rare codon of E. coli in human TS cDNA. Grosjean (Zhang et al., 1989) found that there are eight types rare codons (AGA, AGC, AUA, CCG, CCT, CTC, CGA, et al), and lack of corresponding tRNA in E.cloi. Kane (Fu et al., 2007) proved the codons (CUA, CGA, CCC, GGA) had
negative effect on the expression of eukaryotic gene in E. coli. When the rare codons occurs continuously, the expression will be inhibited, sometimes even be terminated. By site-directed mutagenesis converting the rare codon to high-frequency codon, it can help to lighten the effect of rare codon on the expression level in E. coli (Joan et al., 1997; Liu et al., 2007). The other way to improve the yield is to add the rare codon tRNA to the expression host (Fu et al., 2007; Liu et al., 2007).

TS gene is eukaryote gene (Davisson et al., 1989). By analysis to wild-type human TS cDNA, there are many rare codon of E. coli (Table 2). Rosetta (DE3) contains six rare codon tRNA (AUA, AGG, AGA, CUA, CCC and GGA), which is expressed by a plasmid compatible with pET vectors. Afterwards, we transformed TS-pET-28b (+) into Rosetta (DE3). By SDS-PAGE analysis (Figure 4a), it’s very obvious that the expression level of human TS in Rosetta (DE3) is more than the BL21 (DE3). By orthogonal experiments to optimizing the expression conditions, the yield is up to 40% of the E. coli protein.

In summary, rare condon has important influence on the expression of human TS in E. coli. By adding the rare codon tRNA, the yield is improved. Human TS is high expressed by a novel method.

Acknowledgements

This research is supported by the National Natural Science Foundation of China (grant numbers 21072111 and 21272131).

References