Biological characterisation and application of human MTH1 and monoclonal antibody preparation

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Received July 20, 2018; Accepted December 7, 2018

DOI: 10.3892/or.2018.6942

Abstract. Human MutT homolog 1 (MTH1) hydrolyses oxidised nucleotide triphosphates, thereby preventing them from being incorporated into DNA; MTH1 has been found to be elevated in many types of cancers, including lung, stomach cancer, melanoma and breast cancer. Thus, tumour-targeted hMTH1 may be valuable for developing novel anticancer therapies. In the present study, we prepared human MTH1 protein and its monoclonal antibody (mAb). The *hMTH1* gene was cloned into the prokaryotic expression vector pET28a and optimally expressed in the *E. coli* Transetta (DE3) strain. Using an Ni-NTA column and a G-50 gel filtration column, 20.1 mg of active hMTH1 was obtained from 1,000 ml of bacterial culture, and the purity was over 98%, as detected by high-performance liquid chromatography (HPLC). The half maximal inhibitory concentration (IC_{50}) of TH287 (hMTH1 inhibitor) was determined to be 3.53±0.47 nM using the recombinant hMTH1 protein (rhMTH1). The enzyme activity assay showed the Michaelis constant (K_m) and the catalytic constant (k_{cat}) of the protein were 106.13±48.83 μM and 3.64±0.58 sec^{-1}, respectively. The anti-hMTH1 mAb was obtained via the hybridoma technique and validated by western blot analysis. In addition, an immunofluorescence assay (IFA) and ELISA determined that the mAb could efficiently bind to natural hMTH1 expressed on the human breast cancer cell line MCF-7. Taken together, the results showed the rhMTH1 is an active protein and has practical applications for inhibitor selection, and our prepared hMTH1 mAb will provide a valuable tool for the further characterisation of hMTH1 and antitumour medicinal development in future.

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Key words: MTH1, optimised expression, enzyme activity assay, hybridoma, monoclonal antibody

Introduction

Reactive oxygen species (ROS) are generated by tightly regulated enzymes during normal cellular metabolic processes, and are known to convert deoxyribonucleoside triphosphates (dNTPs) into oxidised DNA bases in the dNTP pool when they are overproduced (1-4). The damaged nucleotides are integrated into the DNA double chains during the process of replication, resulting in DNA structural damage that may ultimately lead to apoptosis (5-7). MutT homolog 1 (MTH1) can hydrolyse the oxidised forms of dGTP (8-10), thus, preventing 8-oxo-dGTP from being incorporated into DNA and leading to G to T transversions (11). The production and elimination of ROS is tightly regulated in normal cells during cellular metabolism, thus the role of MTH1 is non-essential for normal cell survival (12,13). Unlike normal cells, tumours produce high levels of oxidants due to chronically hyperactivated mitogenic and pro-survival signalling, as well as metabolic alterations, and have a greater reliance on MTH1 function. Thus, hMTH1 may play a crucial role in the survival of many cancers, such as melanoma and breast cancer (3,14), and the expression level of hMTH1 is positively correlated with the malignant degree of lung cancer and gastric carcinoma (15,16). Data were published in 2014 lending further support to the hypothesis that MTH1 has a role in the prevention of DNA damage and cancer cell survival. It was shown that MTH1 inhibitors, such as TH287, TH588 and (S)-crizotinib, were sufficient to produce DNA breaks and induce tumour suppressor responses (13,17). Researchers have expressed recombinant human MTH1 protein in *Escherichia coli* for crystallisation (18-20) and inhibition experiments (13,17,21). However, there have been few reports on the optimised expression of hMTH1, as well as its purity and enzymatic activity, which are key factors for inhibitor screening and the functional analysis of hMTH1

protein. In addition, to generate an anti-hMTH1 specific mAb, highly bioactive hMTH1 is required.

Since Köhler and Milstein (22) proposed the method of hybridoma technology and prepared mouse monoclonal antibodies, many antibodies specific to various antigens have been obtained (23). They have applications in the diagnosis and therapy of cancer and infectious diseases. At present, the available therapeutic anticancer mAbs recognise extracellular or cell surface proteins. However, most tumour targets are nuclear or cytoplasmic tumour-associated proteins. Therefore, generating therapeutic mAbs that recognise intracellular tumour antigens will facilitate possible cancer target selection and enhance therapeutic potency. Various efficient and safe methods of delivering an antibody into a living cell have been introduced, such as cell-penetrating peptides (CPPs), micelles, liposomes and even pH-sensitive antibodies (24-27).

In our experiment, we optimised the gene sequence for optimal expression in *E. coli*, and the optimal induction conditions of OD_{600}, inducer concentration, temperature and time were obtained. The rhMTH1 was verified to have high purity by high-performance liquid chromatography (HPLC) analysis. A series of assays were used to determine the enzymatic activity of MTH1. We injected soluble hMTH1 protein into mice as an antigen and produced an anti-hMTH1 mAb. We showed that the anti-hMTH1 mAb had a high binding affinity for hMTH1 by western blotting, ELISA and an immunofluorescence assay. In summary, the bioactive rhMTH1 was suitable for the selection of hMTH1 inhibitors and the MTH1 mAb may be a novel tool for the detection of hMTH1 in tumour cells, with potential for further functional investigation and clinical applications.

Materials and methods

Strains, plasmids and culture medium. The *E. coli* Transetta (DE3) chemically competent cells were purchased from TransGen Biotechnology Co., Ltd. (Beijing, China). *Nco*I, *Eco*RI and T4 DNA ligases were purchased from New England Biolabs, Ltd. (Beijing, China). Isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin, malachite green and ammonium molybdate were obtain from Sangon Biotechnology Co., Ltd. (Shanghai, China). The dGTP and inorganic pyrophosphatase were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-His mouse monoclonal antibody (cat. no. D191001) and goat anti-mouse IgG conjugated to horseradish peroxidase (cat. no. D110087) were purchased from Sangon Biotechnology Co., Ltd. Six-week-old female BALB/c mice were obtained from the College of Animal Science and Technology Yangzhou University (Yangzhou, China). Freund's adjuvant, bovine serum albumin (BSA), polyethylene glycol (PEG4000, 50% w/v), hypoxanthine/aminopterin/thymidine (HAT) and hypoxanthine/thymidine (HT) were all from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen (Thermo Fisher Scientific, Inc.). Mouse monoclonal antibody subtype identification kit was purchased by ProteinTech Group Inc. (Wuhan, China). The anti-mouse IgG (cat. no. A7028), FITC-conjugated goat-anti-mouse IgG (cat. no. A0568) and DAPI were from Beyotime Institute of Biotechnology (Shanghai, China). The MCF-7 cell line and SP2/0 myeloma cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Construction of recombinant hMTH1 expression plasmids. The hMTH1 sequence (GenBank accession number D38594.2) was optimised by Nanjing GenScript Co., Ltd., (Nanjing, China) to adapt the *E. coli* expression system, and the GenBank accession number of the optimised sequence was MH193376. The hMTH1 gene was amplified by PCR using forward (5'-CCATGGGCATGGGTGCA-3') and reverse primers (5'-GAATTCGACCGTATCAACTTCG-3'), cleaved with *Nco*I/*Eco*RI restriction enzymes, then ligated with pET-280a plasmids to generate a series of recombinant expression vectors. The constructed vectors were transformed into *E. coli* Transetta (DE3) chemically competent cells, and the negative control was pET28a in this experiment. The positive clones were confirmed by colony PCR using the T7 forward and the reverse primers.

Optimised expression and purification of hMTH1 protein. For protein expression, pET-28a-MTH1/Transetta (DE3) single bacterial colonies were cultured in LB medium supplemented with 100 μg/ml kanamycin at 37˚C in a shaking incubator at 200 rpm until the OD_{600} of the culture medium reached 0.5. IPTG (0.4 mM) was then added to the culture medium and incubated at 25˚C and 160 rpm for 12 h. The culture was centrifuged at 5,938 x g for 15 min, and the pellet of induced recombinant strains was resuspended in cell lysis buffer (50 mM Tris, 500 mM NaCl, 5 mM DTT, 0.1‰ PMSF; pH 7.5) and then placed in an ice bath for ultrasonic lysis (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) for 30 min (250 Watts, 5 sec, 5 sec). The strain cell lysate was centrifuged at 1,3361 x g for 20 min to separate the precipitate and soluble parts. Supernatant and pellet were subjected to 12% SDS-PAGE electrophoresis to assess the expression levels and solubility of the recombinant protein.

To improve the soluble expression of rhMTH1 protein, the induction conditions of the initial strain OD_{600} value (0.5/0.8), induction temperature (25/20/16˚C), IPTG concentration (0.1/0.2/0.4 mM) and induction time (12/16/20 h) were optimised. The supernatant and precipitate of rhMTH1 (15 μl/lane) at different conditions were detected by 12% SDS-PAGE and Tanon Image software 1.0 (Tanon Science and Technology, Co., Ltd., Shanghai, China) was used for semi-quantitative analysis of the percentage fraction of soluble rhMTH1 by densitometry. In this experiment, the percentage of soluble rhMTH1 in the total protein was calculated.

hMTH1 was purified using an Ni-NTA column (GE Healthcare, Chicago, IL, USA) and eluted by different concentrations of imidazole (50, 100 and 250 mM, respectively). The target sample was identified by 12% SDS-PAGE and concentrated to 1 ml by ultrafiltration. SDS-certified samples were further purified by Sephadex G-50 Gel filtration chromatography (GE Healthcare). The LC system connected to the Ni-NTA and gel filtration columns was provided by Nanjing University Puyang Institute of Scientific Instruments (Nanjing, China). After adding the sample to a G-50 column, it was eluted by Tris-HCl elution buffer (25 mM Tris, 75 mM NaCl and 10% glycerine) at a flow rate of 0.07 ml/min, and the collected solution was detected by 12% SDS-PAGE.

Western blot analysis. For western blotting, rhMTH1 protein was separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes using a wet transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 300 mA for 30 min. The membrane was blocked with 5% non-fat dried milk diluted in TBST at 37˚C for 2 h and incubated with anti-His antibody (dilution 1:5,000) in TBST plus 3% non-fat milk at 4˚C overnight, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (dilution 1:5,000) for 2 h at 37˚C. Visualisation via enhanced chemiluminescence was performed in a gel imager (Tanon Science and Technology, Co., Ltd.).

To analyse the specificity of the hMTH1 mAb by western blotting as described above, the prepared anti-hMTH1 mAb was used as the primary antibody.

Purity and yield determination of recombinant hMTH1. The purity of the sample was determined via high-performance liquid chromatography (HPLC; Shimadzu Corp., Tokyo, Japan). Tris-HCl buffer in the sample was replaced with 0.05 mol/l phosphate buffer (25 mM $NaHPO_4$, 25 mM NaH_2PO_4, 300 mM NaCl; pH 6.7) for HPLC analysis by concentration and dilution. A total of 20 μl sample (20.1 mg/ml) was loaded on the TSK-GW-4000 column (Tosoh Corp., Tokyo, Japan) and eluted with 0.05 mol/l phosphate buffer at a speed of 0.8 ml/min.

The concentration of total soluble protein was determined by bicinchoninic acid (BCA) assay and the purity of the soluble rhMTH1 in the total soluble protein was assessed by HPLC or SDS-PAGE.

Enzymatic activity determination. The enzymatic assay was based on the hydrolysis reaction of dGTP catalysed by recombinant hMTH1 protein to form dGMP and pyrophosphate. The inorganic phosphates of the enzyme coupling reaction product can be quantitatively determined after an excess of pyrophosphatase is added to the assay. The absorbance of inorganic phosphate was measured with malachite green (82.2 μM malachite green, 0.364 mM ammonium molybdate, 17% concentrated sulfuric acid, 0.17% Tween-20) at OD_{630} nm (28).

The half-maximal effective concentration (EC_{50}) of hMTH1 was determined by malachite green measurement. Human MTH1 was diluted in assay buffer (100 mM Tris-acetate, 40 mM sodium chloride, 10 mM magnesium acetate, 1 mM DTT and 0.005% Tween-20; pH 7.5) to generate eight different concentrations (0, 0.006, 0.015, 0.045, 0.075, 0.12, 0.18 and 0.24 nM). In the assay, rhMTH1 (0-0.24 nM) and assay buffer were added to 96-well plates and incubated with shaking for 15 min at 25˚C. dGTP and inorganic pyrophosphatase were added to a final concentration of 0.1 mM and 0.2 U/ml, respectively, and incubated with shaking for 45 min at 25˚C. The malachite green assay reagent was added, followed by incubation with shaking for 45 min at 25˚C. The absorbance of the assay plate was read at 630 nm using a full-wavelength microplate reader (Thermo Fisher Scientific, Inc.). The EC_{50} value was determined by fitting a dose-response stimulation curve to the data points using non-linear regression analysis in GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), where y represents the absorbance at 630 nm and x is log(MTH1). Moreover, the concentration of MTH1 at which it reached its maximum enzymatic activity was used for the determination of the TH287 IC_{50} value.

TH287 IC_{50} value determination by recombinant MTH1. The method of determining the IC_{50} value of TH287 was similar to that of the enzymatic activity assay described above. TH287 (provided by Professor Lai Yisheng, China Pharmaceutical University, Nanjing, China), known to be an hMTH1 inhibitor, was diluted in assay buffer to generate eight different concentrations from 100 to 0.4 nM. Briefly, the assay was developed with a final hMTH1 concentration of 0.18 nM, according to the calculation mentioned above. A dilution series of TH287 was included on the assay plate as well as a negative control (lacking enzyme) and a positive control (lacking inhibitor). The IC_{50} value was determined by fitting a dose-response inhibition curve to the data points using non-linear regression analysis in GraphPad Prism 5 software (GraphPad Software, Inc.), where y is the rate of inhibition and x is log(TH287).

Determination of K_m and k_{cat} for hMTH1. To draw the standard curve of inorganic phosphate, inorganic phosphate was diluted in the assay buffer to generate a series of concentrations (0-24 μM) and incubated with malachite green in the plate at 25˚C for 45 min. The standard curve was made and analysed using GraphPad Prism 5 software (GraphPad Software, Inc.) to assess the linear relationship of the absorbance at 630 nm with different concentrations (0, 3, 6, 9, 12, 15, 18 and 24 μM) of inorganic phosphate. hMTH1 activity was assayed in a reaction mixture containing assay buffer and various amounts of dGTP (0, 40, 80, 120 and 160 μM). The mixtures were incubated at 25˚C for 3 min with 1.5 nM hMTH1 protein. Malachite green assay reagent (50 μl) was added, followed by incubation with shaking for 45 min at 25˚C. The hydrolysed products of nucleoside triphosphates were quantified using the inorganic phosphate standard curve, and the Lineweaver-Burk curve was plotted using GraphPad Prism 5.0 (GraphPad Software, Inc.). The Michaelis-Menten parameters (K_m and V_{max}), catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were calculated using the following equations:

$$1/V = K_m/V_{max}[S] + 1/V_{max} \text{ and } k_{cat} = V_{max}/[S]_{hMTH1}$$

Immunisation of mice and ELISA determination. A total of 50 μg of the purified hMTH1 was emulsified with an equal volume of Freund's complete adjuvant, and then injected into the back and limbs of three six-week-old female BALB/c mice, while the negative control was an unimmunised and healthy BALB/c mouse. For the following two booster immunisations, 50 μg hMTH1 in Freund's incomplete adjuvant was injected into each mouse at three-week intervals. Three days before cell fusion, the mouse with the highest antibody titre was injected intravenously with 50 μg hMTH1 without Freund's adjuvant. Procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH Pub. no. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of the China Pharmaceutical University (Nanjing, China).

The serum titres of immunised mice were monitored by indirect ELISA as follows: rhMTH1 (1 μg/ml) in 0.1 M

carbonate coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$; pH 9.6) was used to coat plates, which were incubated at 37˚C for 2 h and blocked with 3% BSA at 37˚C for 2 h. Mouse serum was used as the primary antibody and was incubated for 2 h at 37˚C. The negative control was incubated with serum from non-immunised mice. Subsequently, the plate was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000) at 37˚C for 1 h. Finally, TMB was added to develop the colour, and the result was measured using a full-wavelength microplate reader at 450 nm after the reaction had been stopped by 2 M H_2SO_4.

Production and purification of the monoclonal antibody against hMTH1. In this experiment, nine six-week-old female BALB/c mice were used for immunisation and antibody production. They were ~18 g and lived in a room with constant temperature and luminosity. In addition, we provided food and water on time every day. Spleen cells were collected from the immunised mice and suspended in DMEM, and then mixed with SP2/0 myeloma cells at a ratio of 10:1 in the presence of PEG 1450. The feeder layer cells from BALB/c mice were plated into 96-well plates to create an environment conducive to the growth of fused cells. The selection of fused cells was performed with HAT medium and positive hybridoma clones were selected by indirect ELISA. A cell line with high affinity was obtained after being subcloned three times via the limiting dilution method. 0.5 ml sterile paraffin oil was injected intraperitoneally into five adult female BALB/c mice before the hybridoma injection. After 1 week, $1x10^6$ hybridoma cells were injected into each mouse. The anti-hMTH1 was purified from mouse ascites by protein A Sepharose chromatography (GE Healthcare) according to the manufacturer's protocol. The purified MTH1 mAb was analysed by SDS-PAGE and western blotting.

Isotype analysis. The subtype of the hMTH1 mAb was detected with a mouse monoclonal antibody (mAb) isotyping reagent (IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgM, κ, λ) (ProteinTech, Inc., Chicago, IL, USA). The heavy and light chain types of the hMTH1 mAb were determined by the absorbance at OD_{450} nm.

hMTH1 monoclonal antibody affinity constant determination. Indirect ELISA was performed to determine the affinity constant (K_{aff}) of the hMTH1 mAb. The rhMTH1 was diluted to two concentrations [(Ag')=1 μg/ml and (Ag)=2 μg/ml] with 0.1 M carbonate coating buffer (pH 9.6), and the plate was coated. The primary antibody was serially diluted to 10^5, 10^4, 10^3, 10^2, 10, 1, 0.1 and 0.01 ng/ml and added to the 96-well plate, while the negative control was incubated with 1% BSA. The subsequent procedures were the same as for the indirect ELISA described above. The EC_{50} values [$(Ab')_t$ and $(Ab)_t$] were determined by fitting a sigmoidal dose-response curve to the data points in GraphPad Prism 5 software, where y represents the absorbance at 450 nm and y is the concentration of the hMTH1 antibody. The affinity constant was finally determined using the following equation: $K_{aff} = (n-1)/2\ [n(Ab')_t - (Ab)_t]$, where $n = (Ag)/(Ag')$.

Specificity of the purified monoclonal antibody. The specificity and affinity of this mAb against hMTH1 was determined by cell ELISA, which was similar to the indirect ELISA. First, $1x10^4$ MCF-7 cells were seeded in 96-well plates and cultured at 37˚C with 5% CO_2 until they reached 90% cell density. Subsequently, the cells were fixed with 4% paraformaldehyde for 20 min. Triton X-100 (0.5%) was used to disrupt the cell membrane at room temperature for 20 min. The rest of the steps were the same as for the ELISA. The prepared anti-hMTH1 was used as the primary antibody and the negative control was 1% BSA. The absorbance of the plate was measured at a wavelength of 450 nm.

Immunofluorescence assay. To analyse the specificity of the MTH1 mAb to MTH1 expressed on cells, a tumour cell line, MCF-7, that overexpresses MTH1 was grown on 6-well plates at 37˚C with 5% CO_2 until 50% cell density was achieved. The plate was washed with PBS between each step. The plate was fixed in 4% paraformaldehyde for 20 min at 25˚C, blocked with 10% FBS and 0.5% Triton X-100 for 2 h at 25˚C, and incubated with prepared anti-hMTH1 mAb (100 μg/ml) or anti-mouse IgG (100 μg/ml) at 4˚C overnight. The negative control was incubated in PBS with 10% FBS. Thereafter, the cells were incubated with FITC-conjugated goat anti-mouse IgG (dilution 1:500) for 1 h at 25˚C and counterstained with DAPI (dilution 1:10) for 10 min at 25˚C. The cells were then observed under a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Results

Human MTH1 gene optimisation and expression plasmid construction. To improve the expression level, the MTH1 DNA sequence was analysed and the codon was optimised according to the ideal codon for *E. coli* (GenBank accession no. MH193376) (Fig. 1). The hMTH1 gene was inserted into the C-terminal His-tag on the backbone of pET-28a, as shown in Fig. 2A. The 691 bp gene band was obtained by colony PCR using the T7 forward and reverse primers, which demonstrated that the hMTH1 gene was subcloned into the pET-28a plasmid (Fig. 2B).

Expression optimisation of hMTH1. The plasmid pET28a-hMTH1 was transformed into competent *E. coli* Transetta (DE3) cells for protein expression. After inducing with 0.4 mM IPTG for 12 h at 25˚C and 160 rpm, the recombinant protein of 21 kDa in size was successfully expressed, with a 9.37% percentage of soluble MTH1 in the total protein (Fig. 2C). As previously reported, the amounts of various recombinant products in the soluble fraction may be improved effectively through optimisation of the induction conditions (29). Therefore, four parameters, including the OD_{600} value, IPTG concentration, induction temperature and induction time were investigated in a univariate analysis. The percentage fraction of hMTH1 was determined through densitometric semi-quantitative analysis using TanonImage software (version 1.0). The results showed that the optimal conditions were OD_{600} = 0.5 with induction at 16˚C for 20 h with 0.4 mM IPTG, and the percentage of soluble hMTH1 in the total protein expressed in the *E. coli* increased to 13.86% (Fig. 3A and B).

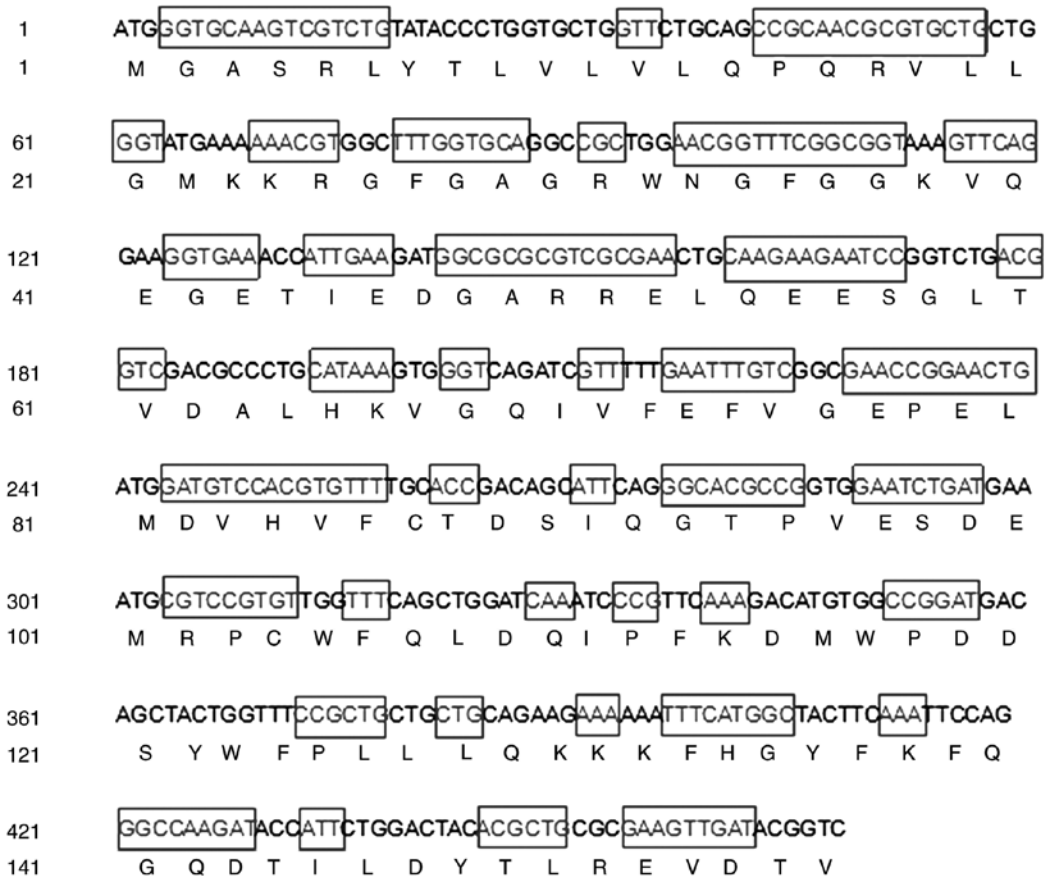

Figure 1. The amino acid sequence and gene sequence of hMTH1 (GenBank accession no. D38594.2). The codons of the hMTH1 nucleotides in the frame were optimised (GenBank accession no. MH193376). hMTH1, human MutT homolog 1.

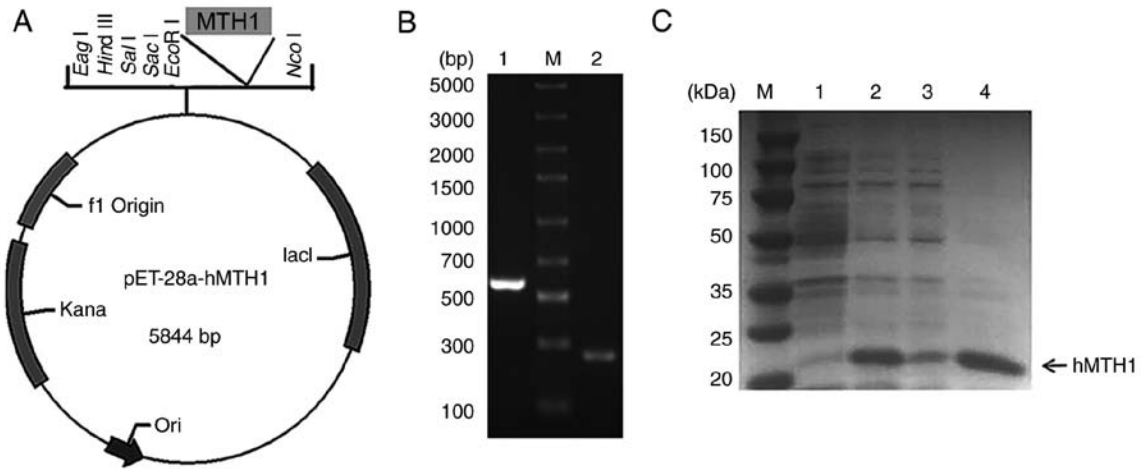

Figure 2. (A) Construction of the pET-28a-hMTH1 expression vector. The hMTH1 gene was inserted between *Nco*I and *Eco*RI sites, and thus was fused at the C-terminal His-tag. (B) Results of colony PCR. Lane M, DNA marker; lane 1, PCR verification of pET-28a-hMTH1; lane 2, PCR verification of pET-28a vector control. (C) 12% SDS-PAGE electrophoresis analysis of expression. Lane M, protein marker; lane 1, total protein of recombinant *E. coli* cells without adding IPTG; lane 2, total protein of recombinant *E. coli* cells with 0.4 mM IPTG; lane 3, supernatant of sonicated cells; lane 4, precipitate of sonicated cells. hMTH1, human MutT homolog 1; IPTG, isopropyl-β-D-thiogalactopyranoside.

Purification and determination of hMTH1. The human MTH1 protein we designed contained His-tags, thus an Ni-NTA column was used for purification and the eluents were detected by 12% SDS-PAGE (Fig. 4A). A total of 100 and 250 mM imidazole eluting solution was treated with Sephadex G-50 (GE Healthcare) for further purification after ultra-filtering to 1 ml. There were no other bands apart from the target protein in the 12% SDS-PAGE (Fig. 4B). In the western blot experiment, anti-His antibody was used to target proteins with His-tags, and the black band on the PVDF membrane proved that the purified protein we obtained was hMTH1 (Fig. 4A and B). In addition, HPLC was used to assess

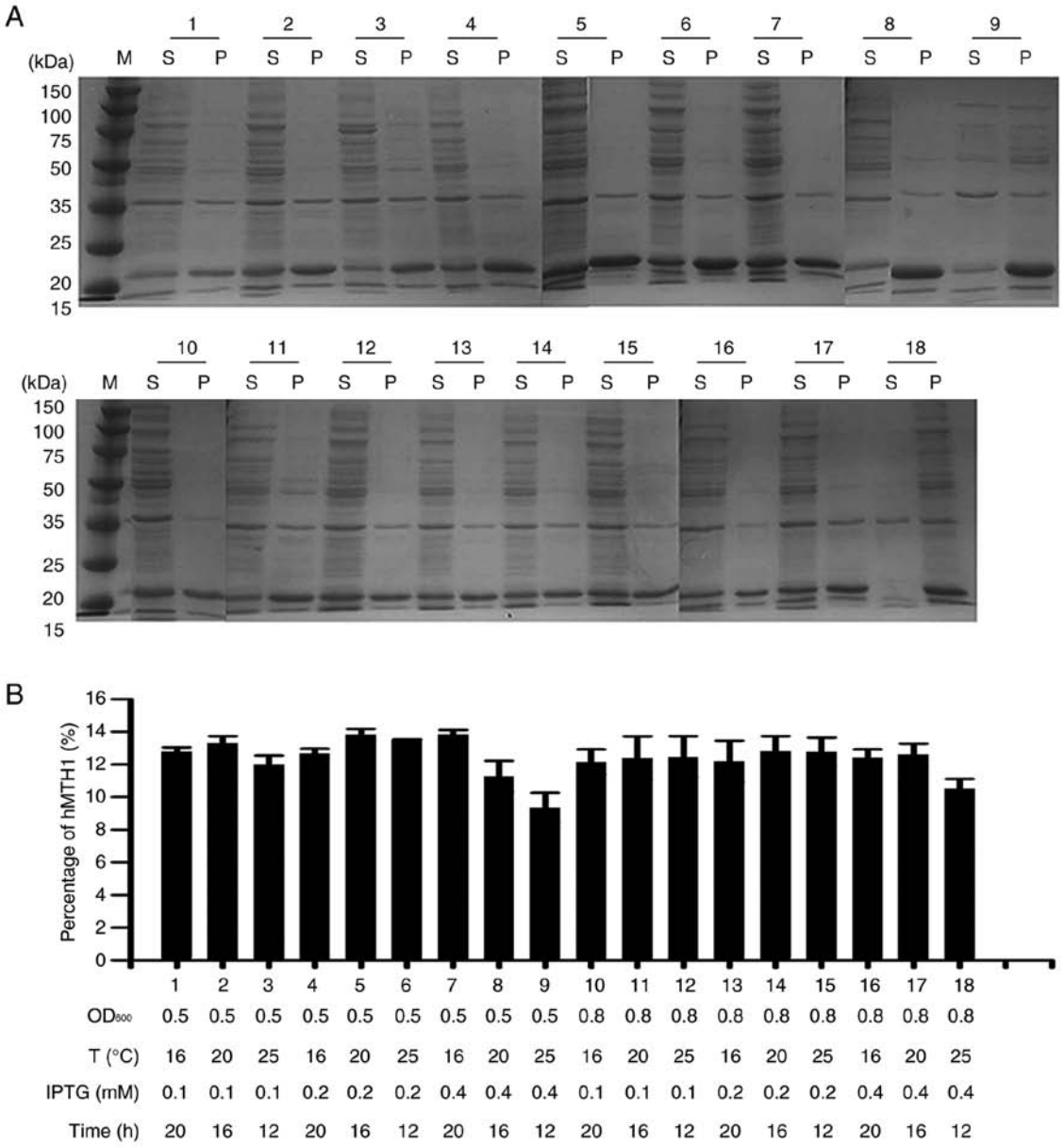

Figure 3. Optimisation of MTH1 expression by univariate analysis. (A) SDS-PAGE analyses of MTH1 expression with different induction conditions. Lane M, protein molecular weight marker; lane S, soluble supernatant after cell disruption; lane P, insoluble precipitate. (B) The percentage of soluble hMTH1 in the total protein was calculated by densitometric semi-quantitative analysis of the SDS-PAGE. Data shown are the average from three independent experiments. hMTH1, human MutT homolog 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

the sample purity, with the results revealing that the retention time of the protein was 14.556 min and the purity was >98% (Fig. 4C).

After two critical steps of purification, including purification by Ni column and Sephadex G-50, 20.1 mg hMTH1 was obtained with >98% purity from 1 l medium and the total yield was calculated to be 12.2% (Table I).

Recombinant hMTH1 enzyme activity and TH287 IC_{50} value determination. The enzymatic assay was based on the hydrolysis reaction of dGTP catalysed by the recombinant hMTH1 protein to form dGMP and pyrophosphate. As shown in Fig. 5A, the concentration required for the 50% maximal effect (EC_{50}) of rhMTH1 was 0.08543 nM. TH287 IC_{50} was determined using 0.18 nM rhMTH1, which reached its maximum enzymatic activity, and this concentration was consistent with the reference value (13). The IC_{50} curve of TH287 represented a clear S-type trend with no apparent data offset (Fig. 5B). The IC_{50} of TH287 was 3.53±0.47 nM, similar to the reference value (13). It was confirmed that rhMTH1 protein with high activity was obtained and could accurately verify the IC_{50} value of TH287 in an hMTH1 inhibitor screening model, as well as having the potential to be used to screen other MTH1 inhibitors.

Kinetic parameters of hMTH1 for dGTP. According to the linear equation of the standard curve, y = 0.0465x + 0.0082, with a correlation coefficient (R^2) of 0.9947 (Fig. 5C), the inorganic phosphate (Pi) which was the hydrolysed product of nucleoside triphosphates was able to be quantified. In the enzymatic reaction, the reaction mixtures, containing various concentrations of substrates (0-160 μM for dGTP),

Table I. Purity and yield of hMTH1.

Purification step	Total soluble protein (mg)[a]	Soluble hMTH1 (mg)[a]	Purity of hMTH1 (%)[b]	Yield (%)
Cell lysate	410.0	164.0	40.0	100.0
Supernatant	162.0	81.0	50.0	49.4
Ni column	57	42.8	75.0	26.1
G-50	20.1	20.1	>98%	12.2

[a]The protein concentration after each step was determined by bicinchoninic acid (BCA); [b]The purity of protein was evaluated by SDS-PAGE or HPLC. hMTH1, human MutT Hhomolog 1; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

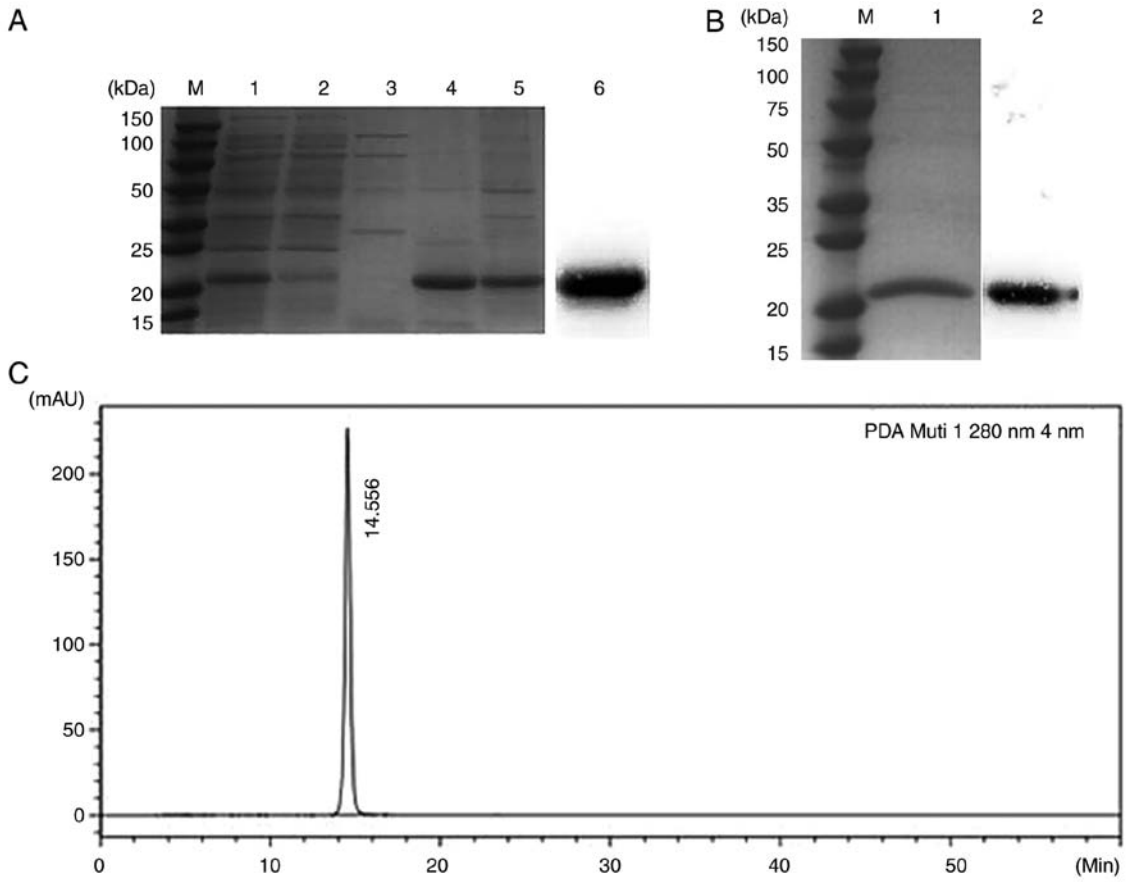

Figure 4. Purity analysis. (A) Results of 12% SDS-PAGE after purification by Ni-NTA affinity chromatography. Lane M, protein maker; lane 1, supernatant of sonicated cells; lane 2, flow-through collected from the Ni-NTA column; lane 3, 50 mM imidazole eluent; lane 4, 100 mM imidazole eluent; lane 5, 250 mM imidazole eluent; lane 6, western blot analysis for recombinant hMTH1. The primary antibody used in the western blot analysis was the anti-His monoclonal antibody. (B) Results of 12% SDS-PAGE after purification by G-50 Gel filtration chromatography. Lane M, protein marker; lane 1, the eluent collected by G-50 Gel filtration chromatography; lane 2, the recombinant protein hMTH1 that was analysed by western blot analysis. The primary antibody was the anti-His monoclonal antibody. (C) HPLC analysis of rhMTH1. Only one peak was detected. hMTH1, human MutT homolog 1; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; rhMTH1, recombinant hMTH1 protein.

were incubated with 1.5 nM rhMTH1 for 48 min at 25˚C. According to the 1/(dGTP)-1/V curve that was plotted using GraphPad Prism 5.0, kinetic analysis of the dGTP substrate with rhMTH1 revealed a K_m of 103.63±34.64 μM and a kcat of 3.64±0.58 sec^{-1} as well as a catalytic efficiency (k_{cat}/K_m) of 0.037±0.0064 $sec^{-1}\cdot\mu M^{-1}$ (Fig. 5D).

Production, purification and identification of the hMTH1 monoclonal antibody. Indirect ELISA was used to screen the highest titre among the mouse antiserum samples. The serum of three immunised mice was harvested and diluted into a series of concentrations (1:10,000; 1:20,000; 1:40,000; 1:80,000; 1:160,000; 1:320,000; 1:640,000; and 1:1,280,000) after three immunisations. The titre results in Fig. 6A show that the antiserum titre of mouse three, was the highest, therefore spleen cells from this mouse were used to fuse with SP2/0 myeloma cells to generate antibody hybridoma cells. After subcloning three times, a positive cell line, named 4A4, with

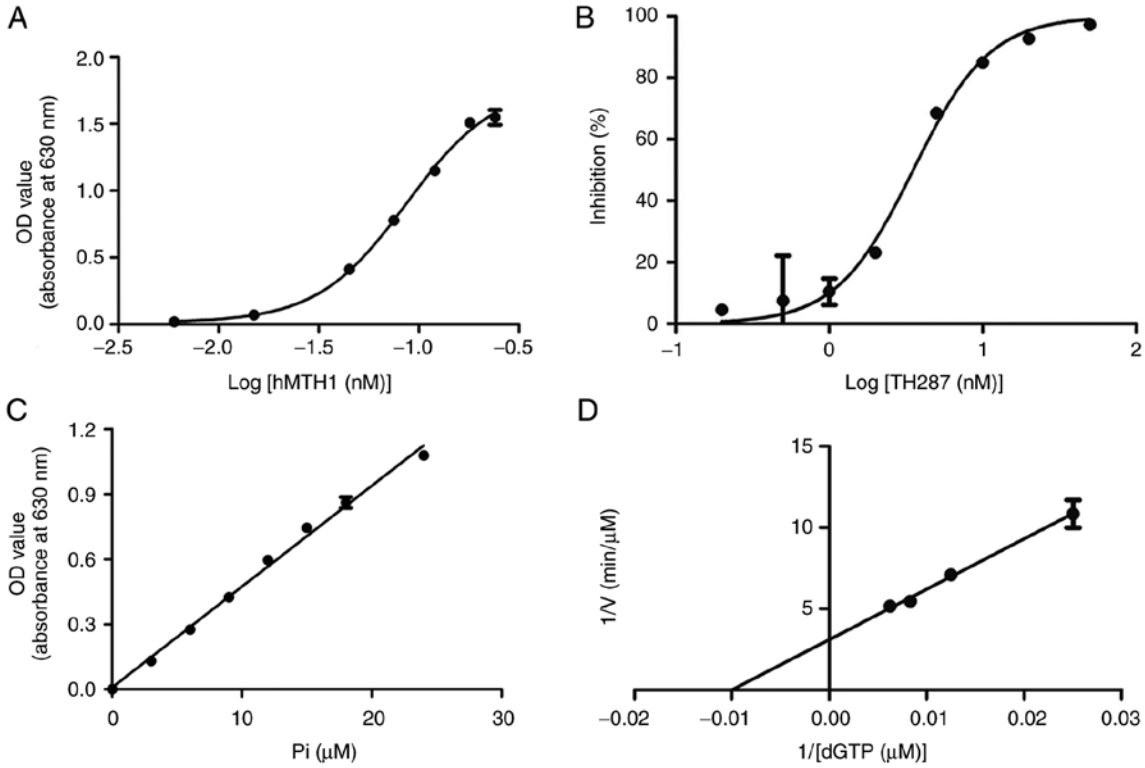

Figure 5. MTH1 enzyme activity assay. (A) EC_{50} value of rhMTH1 as determined by malachite green measurement. The EC_{50} of MTH1 was 0.08543 nM and the x-axis represents the concentration of log(MTH1), while the y-axis represents the absorbance at 630 nm. (B) IC_{50} value of TH287 as determined by malachite green measurement using 0.18 nM rhMTH1. The IC_{50} value of TH287 was 3.53±0.47 nM and the x-axis represents the concentration of log(TH287), while the y-axis represents the percentage of inhibition. (C) Phosphate standard curve. The equation was $y=0.0465x+0.0082$ and was used to calculate the consumption of dGTP. (D) Lineweaver-Burk plot for hMTH1. The purified rhMTH1 has a K_m of 103.63±34.64 μM and V_{max} of 0.33±0.053 μM/min. Data are shown as the average ± SD from three independent experiments. Pi, inorganic phosphate; hMTH1, human MutT homolog 1; rhMTH1, recombinant hMTH1 protein.

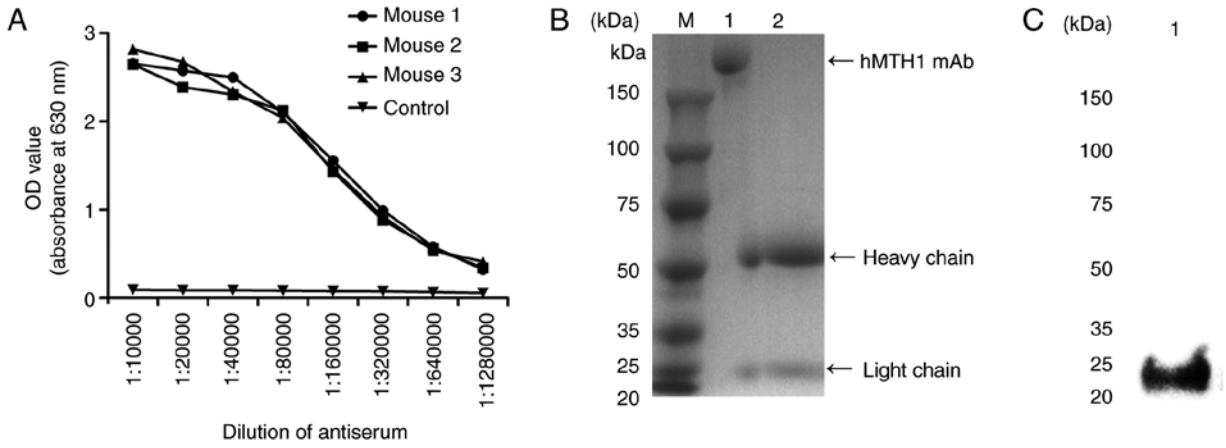

Figure 6. Characteristic determination of MTH1 monoclonal antibody (mAb). (A) The titre of the mouse antiserum, detected by indirect ELISA. Mouse 1, 2 and 3 were immunised with rhMTH1, and the negative control was an unimmunised healthy BALB/c mouse. (B) hMTH1 mAb was detected by SDS-PAGE. Lane M, the protein marker; lane 1, the sample mixed with non-reduced loading buffer. Lane 2, the monoclonal antibody treated with reduced loading buffer. (C The specificity of hMTH1 mAb was identified by western blot analysis. Lane 1, the recombinant protein hMTH1 was analysed by western blot analysis. The primary antibody was the prepared anti-hMTH1 mAb. hMTH1, human MutT homolog 1; rhMTH1, recombinant hMTH1 protein.

a relatively high binding affinity to hMTH1, was successfully obtained. After that, $1x10^6$ hybridoma cells were injected into BALB/c mice intraperitoneally to induce the formation of ascites containing mAb directly against hMTH1 protein. Within 7 to 10 days, ascites were collected and purified via protein A column (GE Healthcare). The hMTH1 mAb was identified by SDS-PAGE. The integrated MTH1 mAb was ~170 kDa with one clear heavy chain at 55 kDa and a light chain at 23 kDa, with a purity >90% (Fig. 6B). The specificity against hMTH1 was identified by western blot analysis. The results demonstrated that the hMTH1 mAb was able to specifically combine with the recombinant hMTH1 (Fig. 6C).

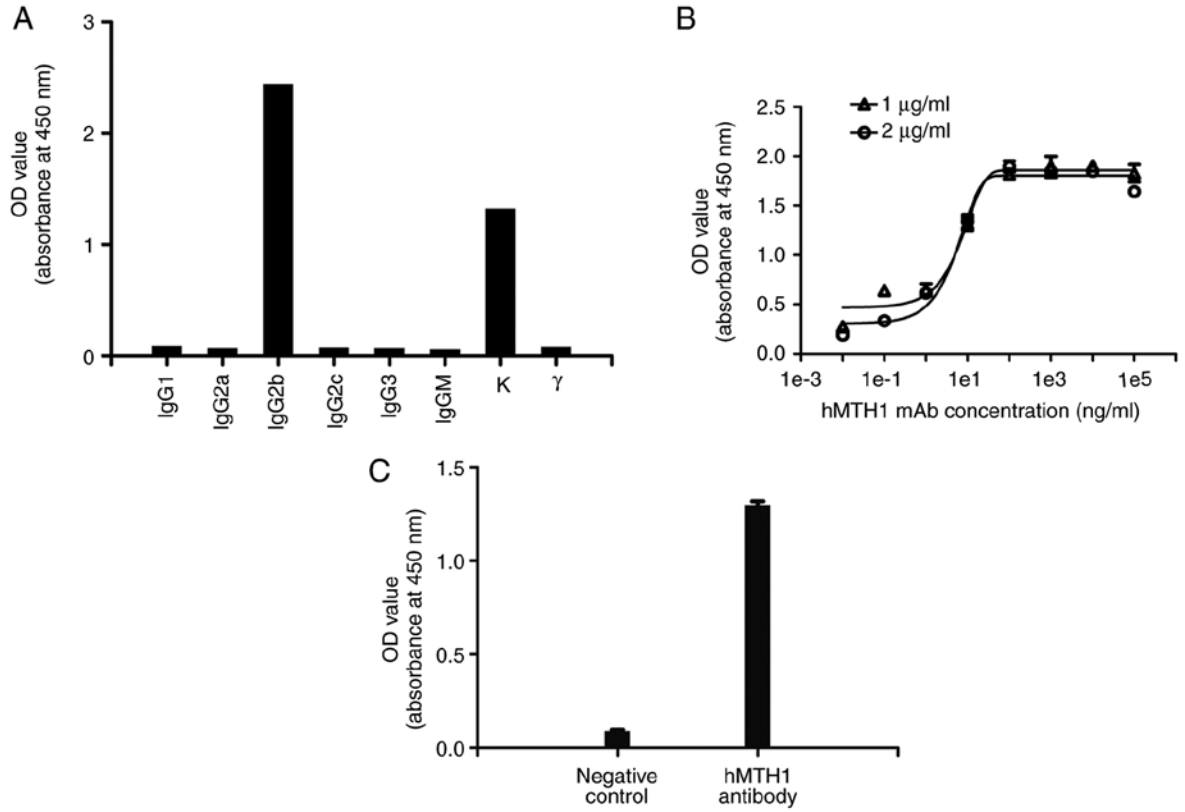

Figure 7. The isotype, affinity and specificity of the hMTH1 monoclonal antibody (mAb) were tested by ELISA. (A) The results of the MTH1 mAb isotyping. The type of heavy chain was IgG2b and the type of light chain was κ. (B) The results of the hMTH1 monoclonal antibody affinity constant determination. Two concentrations (1 and 2 μg/ml) of hMTH1 were utilised in indirect ELISA to calculate the affinity constant, which was 8.73×10^{-9} M. (C) The results of the cell ELISA. The MCF-7 cells were used to confirm the specificity of the hMTH1 antibody against natural hMTH1. The negative control was 1% BSA. hMTH1, human MutT homolog 1.

Specificity and affinity of the purified monoclonal antibody. The isotype of the hMTH1 mAb was detected using a subtyping kit. The results demonstrated that the type of heavy chain was IgG2b and the type of light chain was kappa (κ), as shown in Fig. 7A. Sigmoid curves were plotted using the OD values against the antibody concentrations (1e-2, 1e-1, 1e1, 1e2, 1e3, 1e4, 1e5 ng/ml) in two different antigen concentrations, as shown in Fig. 7B. The EC_{50} of the antibody at concentrations of 1 and 2 μg/ml of rhMTH1 in this experiment were 8.739 and 4.377 ng/ml, respectively. Therefore, the affinity constant of the anti-hMTH1 antibody was 8.73×10^{-9} M.

Cell ELISA was used to further validate the specificity of the purified mAb against hMTH1 in human breast cancer MCF-7 cells. hMTH1 mAb purified from ascites had a specific binding capacity to the natural hMTH1 expressed on MCF-7 cells (Fig. 7C).

Immunofluorescence assay. It has been reported that MCF-7 cells express a high level of MTH1, as previously measured using mass spectrometry (30) and novel probes (31). Therefore, we performed an immunofluorescence experiment to further confirm the binding to natural hMTH1, as shown in Fig. 8. hMTH1 mAb purified from ascites served as the primary antibody to incubate with the MCF-7 cells. After incubation with FITC-conjugated goat-anti-mouse IgG and counterstaining with DAPI, the hMTH1 mAb-treated cells displayed obvious green fluorescence compared with the negative control and mouse IgG treatment. The results also showed that green fluorescence was mainly accumulated in the cytosol of the MCF-7 cells, with a minor proportion at the mitochondria and nuclei, which corresponds with the distribution of hMTH1. This indicates that the purified MTH1 mAb was able to specifically combine with hMTH1 expressed on the MCF-7 cells.

Discussion

MutT homolog 1 (MTH1) protein is a homologous enzyme of MutT and is widely distributed in mitochondria and the nucleus (32). The human *MTH1* gene is located in the second sub-band of the second band of the short arm of the seventh chromosome, with a total length of ~12 kb and a total of six exons. It is an essential sanitiser of the free nucleotide pool that prevents lethal DNA damage in cancer cells, which has been known to maintain the growth of tumours and promotes their development.

In the present study, we developed a brief protocol to produce hMTH1 in a cost- and time-effective manner through an optimised *E. coli* expression system and generated an antibody against hMTH1. The present study showed that the percentage of soluble hMTH1 in the total protein was 9.37% without any optimisation. After optimisation, the percentage improved to 13.86%. We were thus able to acquire 20.1 mg hMTH1 protein from 1,000 ml medium with over 98% purity, and the total yield was 12.2%. In the analysis of enzyme

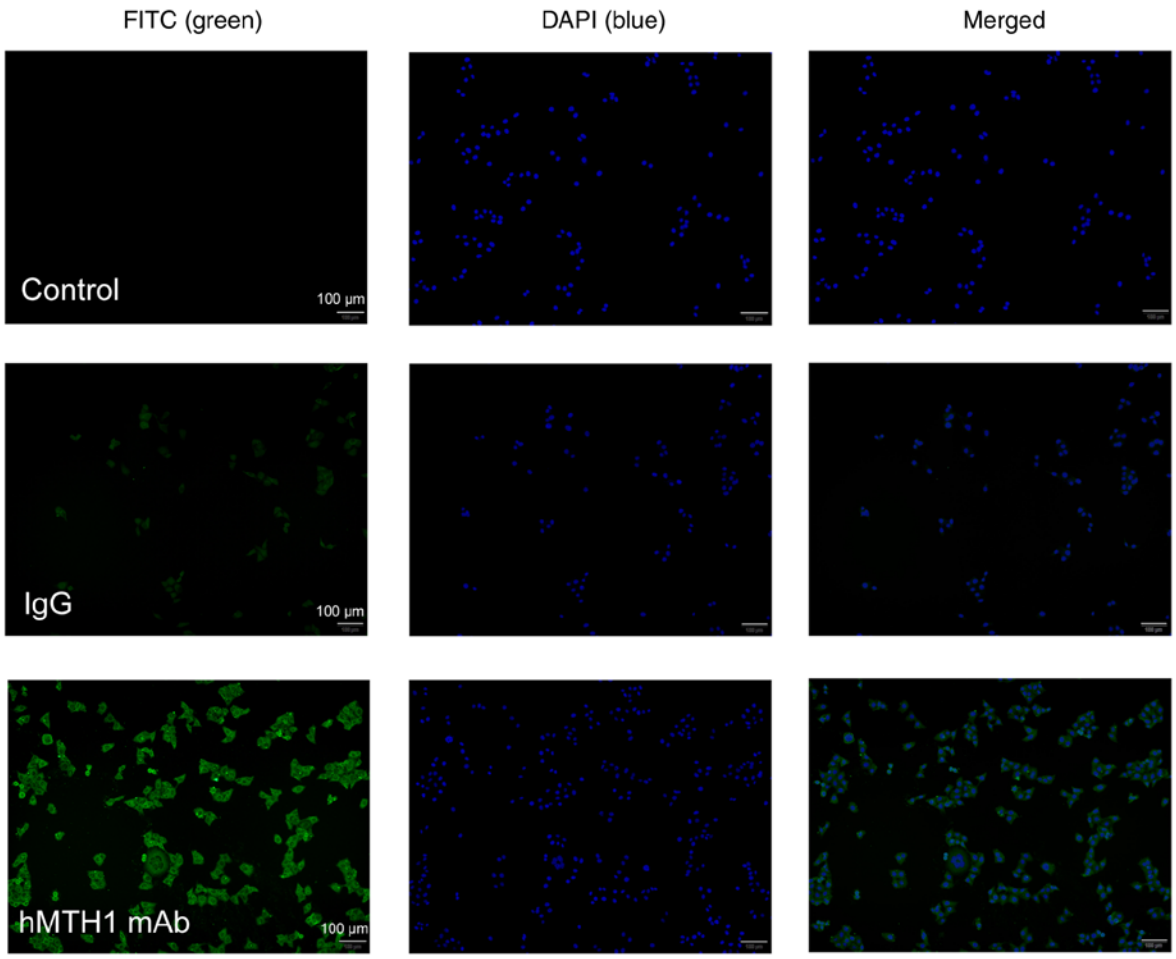

Figure 8. Immunofluorescence of cultured MCF-7 cells with the MTH1 monoclonal antibody (mAb). Green fluorescent labelling of protein stained with anti-hMTH1 mAb, blue nuclear staining with DAPI, and merged images of FITC and DAPI are presented. Calibration bar, 100 μm for all images. hMTH1, human MutT homolog 1.

activity determination, the EC_{50} of rhMTH1 was 0.08543 nM and the IC_{50} of TH287 was 3.53±0.47 nM, which were similar to the reference values (13). It was demonstrated that we obtained rhMTH1 protein with high purity and activity, which accurately verified the IC_{50} of TH287 in the hMTH1 inhibitor screening model and may be used to screen other MTH1 inhibitors. Fujikawa *et al* reported that the purified hMTH1 has a K_m of 258 μM and k_{cat} of 15.7 sec^{-1} (8,33) after purification by DEAE Sephacel column, HiTrap Butyl Sepharose 4FF column and HiPrep 26/60 Sephacryl S-100 HR gel filtration chromatography (34). In the present study, the Michaelis constant (K_m) and the catalytic constant (k_{cat}) were 103.63±34.64 μM and 3.64±0.58 sec^{-1}, respectively. From this perspective, our protein appears to have greater enzymatic activity.

To the best of our knowledge, the success rate of hybridoma cell construction may be affected by many factors, such as the growth state of the myeloma cells, the concentration of PEG, the ratio of spleen cells to myeloma cells, and the number of feeder layer cells (35). In the present study, 7% PEG was used for cell fusion at 1 min of incubation. Additionally, $1x10^5$ feeder layer cells were plated into each well of the 96-well plates to provide growth factors for the cells to survive and proliferate *in vitro*. Western blotting and ELISA analysis showed that this mAb had a high affinity for hMTH1. In addition, the results of the immunofluorescence assay determined that the hMTH1 mAb purified from ascites had specific binding capacity to the natural hMTH1 expressed on the human breast cell line MCF-7. Though hMTH1 is a cytoplasmic protein, various methods have been reported to transduce antibodies into cells (24-27). MTH1 is a non-essential enzyme in normal cells that is overexpressed in tumours, so its antibodies could play a role in targeted therapy for the broad-spectrum treatment of cancers.

To summarise, we reported an expression and purification method for human recombinant hMTH1 protein, by which milligrams of high purity (>98%) protein with bioactivity could be produced in 5 working days. The ability to produce milligram quantities of bioactive hMTH1 with simple steps will certainly facilitate *in vitro* or *in vivo* studies. This expression and purification strategy should also have reference value for the production of hMTH1 on a large scale. Using the rhMTH1, the anti-hMTH1 mAb was obtained via a hybridoma technique, and it showed high affinity and binding ability. Considering that this mAb has good selectivity to its corresponding antigen, it is expected to have potential for the functional and mechanistic study of hMTH1 in tumours, and for antitumour drug development in the future.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Fundamental Research Funds for the National Natural Science Foundation of China (grant no. 81603017), the Natural Science Foundation of Jiangsu Province (grant no. BK20160765) and the Innovation Ability Construction Plan of Hainan Province (grant nos. SQ2 017JSKF0027 and SQ2018JSKF0001).

Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

XJ and YLi conceived and designed the study; YLa provided and characterised the hMTH1 inhibitor; CC and XL wrote the manuscript, optimised the hMTH1 expression, prepared the hMTH1 antibody and performed the statistical analyses; MG, RD and NK expressed and purified hMTH1; LW prepared the hMTH1 inhibitor. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH Pub. no. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of the China Pharmaceutical University (Nanjing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J: Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39: 44-84, 2007.
2. Luo M, He H, Kelley MR and Georgiadis MM: Redox regulation of DNA repair: Implications for human health and cancer therapeutic development. Antioxid Redox Signal 12: 1247-1269, 2010.
3. Rai P: Human Mut T Homolog 1 (MTH1): A roadblock for the tumor-suppressive effects of oncogenic RAS-induced ROS. Small GTPases 3: 120-125, 2012.
4. Puigvert JC, Sanjiv K and Helleday T: Targeting DNA repair, DNA metabolism and replication stress as anti-cancer strategies. FEBS J 283: 232-245, 2016.
5. Nakabeppu Y: Cellular levels of 8-oxoguanine in either DNA or the nucleotide pool play pivotal roles in carcinogenesis and survival of cancer cells. Int J Mol Sci 15: 12543-12557, 2014.
6. Gao T, Gu S, Liu F, Li L, Wang Z, Yang J and Li G: Investigation of MTH1 activity via mismatch-based DNA chain elongation. Anal Chim Acta 905: 66-71, 2016.
7. Nakabeppu Y, Ohta E and Abolhassani N: MTH1 as a nucleotide pool sanitizing enzyme: Friend or foe. Free Radic Biol Med 107: 151-158, 2017.
8. Fujikawa K, Kamiya H, Yakushiji H, Nakabeppu Y and Kasai H: Human MTH1 protein hydrolyzes the oxidized ribonucleotide, 2-hydroxy-ATP. Nucleic Acids Res 29: 449-454, 2001.
9. Bialkowski K, Szpila A and Kasprzak KS: Up-regulation of 8-oxo-dGTPase activity of MTH1 protein in the brain, testes and kidneys of mice exposed to ^{137}Cs γ radiation. Radiat Res 172: 187-197, 2009.
10. Waz S, Nakamura T, Hirata K, Koga-Ogawa Y, Chirifu M, Arimori T, Tamada T, Ikemizu S, Nakabeppu Y and Yamagata Y: Structural and kinetic studies of the human nudix hydrolase MTH1 reveal the mechanism for its broad substrate specificity. J Biol Chem 292: 2785-2794, 2017.
11. Maki H and Sekiguchi M: MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature 355: 273-275, 1992.
12. Tsuzuki T, Egashira A and Kura S: Analysis of *MTH1* gene function in mice with targeted mutagenesis. Mutat Res 477: 71-78, 2001.
13. Gad H, Koolmeister T, Jemth AS, Eshtad S, Jacques SA, Ström CE, Svensson LM, Schultz N, Lundbäck T, Einarsdottir BO, *et al*: MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool. Nature 508: 215-221, 2014.
14. Wang JY, Liu GZ, Wilmott JS, La T, Feng YC, Yari H, Yan XG, Thorne RF, Scolyer RA, Zhang XD and Jin L: Skp2-mediated stabilization of MTH1 promotes survival of melanoma cells upon oxidative stress. Cancer Res 77: 6226-6239, 2017.
15. Kennedy CH, Cueto R, Belinsky SA, Lechner JF and Pryor WA: Overexpression of *hMTH1* mRNA: A molecular marker of oxidative stress in lung cancer cells. FEBS Lett 429: 17-20, 1998.
16. Borrego S, Vazquez A, Dasí F, Cerdá C, Iradi A, Tormos C, Sánchez JM, Bagán L, Boix J, Zaragoza C, *et al*: Oxidative stress and DNA damage in human gastric carcinoma: 8-Oxo-7'8-dihydro-2'-deoxyguanosine (8-oxo-dG) as a possible tumor marker. Int J Mol Sci 14: 3467-3486, 2013.
17. Huber KV, Salah E, Radic B, Gridling M, Elkins JM, Stukalov A, Jemth AS, Göktürk C, Sanjiv K, Strömberg K, *et al*: Stereospecific targeting of MTH1 by (S)-crizotinib as an anticancer strategy. Nature 508: 222-227, 2014.
18. Svensson LM, Jemth AS, Desroses M, Loseva O, Helleday T, Högbom M and Stenmark P: Crystal structure of human MTH1 and the 8-oxo-dGMP product complex. FEBS Lett 585: 2617-2621, 2011.
19. Koga Y, Inazato M, Nakamura T, Hashikawa C, Chirifu M, Michi A, Yamashita T, Toma S, Kuniyasu A, Ikemizu S, *et al*: Crystallization and preliminary X-ray analysis of human MTH1 with a homogeneous N-terminus. Acta Crystallogr Sect F Struct Biol Cryst Commun 69: 45-48, 2013.
20. Carter M, Jemth AS, Hagenkort A, Page BD, Gustafsson R, Griese JJ, Gad H, Valerie NC, Desroses M, Boström J, *et al*: Crystal structure, biochemical and cellular activities demonstrate separate functions of MTH1 and MTH2. Nat Commun 6: 7871, 2015.
21. Ellermann M, Eheim A, Rahm F, Viklund J, Guenther J, Andersson M, Ericsson U, Forsblom R, Ginman T, Lindström J, *et al*: Novel class of potent and cellularly active inhibitors devalidates MTH1 as broad-Spectrum cancer target. ACS Chem Biol 12: 1986-1992, 2017.
22. Köhler G and Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497, 1975.
23. Fan X, He C, Jing W, Zhou X, Chen R, Cao L, Zhu M, Jia R, Wang H and Guo Y: Intracellular osteopontin inhibits toll-like receptor signaling and impedes liver carcinogenesis. Cancer Res 75: 86-97, 2015.
24. Cornelissen B, Hu M, McLarty K, Costantini D and Reilly RM: Cellular penetration and nuclear importation properties of ^{111}In-labeled and ^{123}I-labeled HIV-1 tat peptide immunoconjugates in BT-474 human breast cancer cells. Nucl Med Biol 34: 37-46, 2007.
25. Lee Y, Ishii T, Kim HJ, Nishiyama N, Hayakawa Y, Itaka K and Kataoka K: Efficient delivery of bioactive antibodies into the cytoplasm of living cells by charge-conversional polyion complex micelles. Angew Chem Int Ed Engl 49: 2552-2555, 2010.

26. Yamada Y, Perez SM, Tabata M, Abe J, Yasuzaki Y and Harashima H: Efficient and high-speed transduction of an antibody into living cells using a multifunctional nanocarrier system to control intracellular trafficking. J Pharm Sci 104: 2845-2854, 2015.
27. Shin SM, Choi DK, Jung K, Bae J, Kim JS, Park SW, Song KH and Kim YS: Antibody targeting intracellular oncogenic Ras mutants exerts anti-tumour effects after systemic administration. Nat Commun 8: 15090, 2017.
28. Baykov AA, Evtushenko OA and Avaeva SM: A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. Anal Biochem 171: 266-270, 1988.
29. Malik A, Alsenaidy AM, Elrobh M, Khan W, Alanazi MS and Bazzi MD: Optimization of expression and purification of HSPA6 protein from *Camelus dromedarius* in *E. coli*. Saudi J Biol Sci 23: 410-419, 2016.
30. Coskun E, Jaruga P, Jemth AS, Loseva O, Scanlan LD, Tona A, Lowenthal MS, Helleday T and Dizdaroglu M: Addiction to MTH1 protein results in intense expression in human breast cancer tissue as measured by liquid chromatography-isotope-dilution tandem mass spectrometry. DNA Repair 33: 101-110, 2015.
31. Ji D, Beharry AA, Ford JM and Kool ET: A Chimeric ATP-Linked nucleotide enables luminescence signaling of damage surveillance by MTH1, a cancer target. J Am Chem Soc 138: 9005-9008, 2016.
32. Nakabeppu Y: Regulation of intracellular localization of human MTH1, OGG1, and MYH proteins for repair of oxidative DNA damage. Prog Nucleic Acid Res Mol Biol 68: 75-94, 2001.
33. Fujikawa K, Kamiya H, Yakushiji H, Fujii Y, Nakabeppu Y and Kasai H: The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein. J Biol Chem 274: 18201-18205, 1999.
34. Yakushiji H, Maraboeuf F, Takahashi M, Deng ZS, Kawabata S, Nakabeppu Y and Sekiguchi M: Biochemical and physicochemical characterization of normal and variant forms of human MTH1 protein with antimutagenic activity. Mutat Res 384: 181-194, 1997.
35. Ling S, Xiao S, Xie C, Wang R, Zeng L, Wang K, Zhang D, Li X and Wang S: Preparation of monoclonal antibody for brevetoxin 1 and development of Ic-ELISA and colloidal gold strip to detect brevetoxin 1. Toxins 10: E75, 2018.