

Preparation of a monoclonal antibody against the carcinoembryonic antigen, glypican-3

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Abstract. The carcinoembryonic antigen, glypican-3 (GPC3), is a putative therapeutic target and diagnostic marker of hepatoma. In the present study, a monoclonal antibody (mAb) specifically against GPC3 was obtained via cloning the sequence of GPC3 via polymerase chain reaction and inserting it into a pET16b vector prior to transfection into *Escherichia coli* (*E. coli*) BL21. BALB/c mice were immunized with 20 µg purified antigen by intrasplenic embedding. Splenocytes and mouse myeloma cells SP2/0 were fused; then, the hybridoma cells were screened by an indirect ELISA. The properties of the mAb were examined by western blotting and immunofluorescence analysis against the purified protein. The results revealed that the prokaryotic expression vector of GPC3 had been successfully generated and GPC3 was stably expressed in *E. coli* BL21. A stable hybridoma cell line, 2F3, was generated in the present study, which produced mAbs against GPC3. The mAb 2F3 had a high antibody titer and the isotype was identified as IgG1/κ; 2F3 hybridomas had a median chromosome number of 98. Western blot and immunofluorescence analyses revealed that 2F3 specifically recognized recombinant and native GPC3. The 2F3 clone was proposed as a stable secretor of this mAb against GPC3. The results of present study indicated that the successful preparation of recombinant GPC3 protein and an anti-human GPC3 mouse mAb may provide a basis for developments in the diagnosis and treatment of liver cancer.

Introduction

Hepatoma, the most prevalent form of liver cancer, is the fifth most common malignant tumor, as well as the third leading cause of tumor-associated mortality worldwide (1); annually, ~500,000 new cases of hepatoma are diagnosed (2). China has the highest incidence of hepatoma and accounts for the majority (55%) of all newly diagnosed liver cancer cases globally (3,4). Novel diagnostic and therapeutic strategies are urgently required (5).

Glypican-3 (GPC3/OCI-5) is a 70 kDa protein comprising 580 amino acids. It is a heparan sulfate proteoglycan that is anchored on the cell surface via glycosylphosphatidylinositol (6). GPC3 promotes the growth of hepatoma cells by stimulating the canonical Wnt signaling pathway (7). The expression, properties and regulation of carcinoembryonic antigen GPC3 have been examined; the protein is overexpressed in hepatoma tissues and the degree of the overexpression correlates with the poor prognosis of patients (8-10). Therefore, in the future, GPC3 may not only serve as a potential diagnostic biomarker, but also as an immunotherapeutic target against hepatoma.

The expression of antibodies against GPC3 may serve as a potential therapeutic strategy against hepatoma. Therefore, a GPC3 prokaryotic expression vector was developed for the ectopic expression of GPC3 in *Escherichia coli* (*E. coli*) in the present study. Then, the purified GPC3 recombinant protein was used to immunize BALB/c mice via intrasplenic embedding, and subsequently a monoclonal antibody (mAb) was obtained and characterized.

Materials and methods

Experimental animals and cell lines. The present study was approved by the Institutional Animal Care and Use Committee of Tianjin First Central Hospital (Tianjin, China) and conformed to the Guide for the Use and Care of Animals published by the National Institutes of Health (NIH Publication number 85-23, revised 1996) (11). In total, eight female BALB/c mice (age, 8 weeks; weight, 18-20 g) were obtained from the Academy of Military Medical Science [Beijing, China; certificate no. SCXK (JUN) 2007-004]. Mice were housed at a temperature of 22-24°C, relative humidity

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of 45-60%, a 12-h light/dark cycle, with free access to food and water. Mouse myeloma cells (Sp2/0) and a human hepatoblastoma cell line (HepG2) were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Medical Sciences, Shanghai, China). Hepatoblastoma is a rare disease; however, it is the most common type of primary hepatic malignancy encountered in infants and young children (12). SP2/0 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). HepG2 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Clone of GPC3 gene. The template for GPC3 was created in accordance with the sequence from GenBank (accession no. NM_001164617; <https://www.ncbi.nlm.nih.gov/genbank/>); the primer sequences for amplification of the GPC3 gene were as follows: Forward, 5'-ACGGGATCCGCCAAGAACTACACCAATGC-3' with the *Bam*HI restriction site underlined; and reverse, 5'-ATTCTCGAGTTACTCCACCACACCTGCATACA-3' with the *Xho*I restriction site underlined (Sangon Biotech Co., Ltd., Shanghai, China). The sequence was inserted into pDNOR223-GPC3 (IMMUSOFT, Seattle, WA, USA). The primers were synthesized by Takara Bio, Inc. (Tokyo, Japan). The polymerase chain reaction (PCR) amplification conditions were the following: In total, 30 cycles of 98°C for 10 sec, followed by annealing at 55°C for 15 sec, extension at 72°C for 30 sec using an ABI 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR product was analyzed by electrophoresis on a 1.0% agarose gel. Following staining with ethidium bromide solution (10 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), gel images were obtained.

Expression and purification of fusion protein. PCR fragments were digested using *Bam*HI and *Xho*I restriction enzymes (10 U/μl; Takara Bio, Inc.) for 1 h at 37°C and then cloned into the *Bam*HI- and *Xho*I-digested pET16b vector encoding a His-tag (Novagen; Merck KGaA); successful cloning was confirmed by sequencing (Sangon Biotech Co., Ltd.). The recombinant plasmid was transfected into *E. coli* BL21 (DE3, Novagen; Merck KGaA) with the soluble fusion protein GPC3 expressed following induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Takara Bio, Inc.), and non-IPTG cells were used as control. The cultures were induced for 4 h at 37°C. Affinity chromatography was used to purify the expressed fusion protein via a His-tag. The target fusion protein was expressed in inclusion body; thus, the bacterial cells were lysed using 4 ml lysis buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl and 8 M urea. Lysed cells were subsequently mixed with 1 ml of a solution provided in the kit containing the Nickel chelating resin (GE Healthcare, Chicago, IL, USA). The binding buffer contained 50 mmol/l Na₂HPO₄, 300 mM NaCl, 6 M guanidine at pH 6.0-8.0 and was used as the mobile phase at a flow rate of ~1 ml/min at 4°C. The protein was eluted using the elution buffer containing 50 mmol/l Na₂HPO₄, 300 mM NaCl and 250 mM imidazole

at pH 8.0. The identity of the purified fusion protein was confirmed by SDS-PAGE and western blot analysis (13). The primary antibody used was rabbit anti-His-tag antibody (cat. no. ABIN398410; 1:5,000; antibodies-online GmbH, Aachen, Germany), and the secondary antibody was goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase antibody (cat. no. ABIN398323; 1:10,000; antibodies-online GmbH). The concentration of the purified protein was determined using a Bradford protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China).

Immunization of mice and preparation of hybridoma. Recombinant GPC3 protein (20 μg) was deposited onto nitrocellulose (NC) membranes (Sigma-Aldrich; Merck KGaA) in a drop-wise manner using a micro-syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). BALB/c mice were anesthetized with via isoflurane inhalation (1.0-2.0%, induction; 2.0-2.5% maintenance; Lunan Pharmaceutical Co., Ltd., Linyi, China) using a VMR Small Animal Anesthesia Machine System (Midmark Corporation, Orchard Park, NY, USA). An NC membrane was embedded into the spleen via a 1-cm surgical incision on the left side and the embedding procedure was repeated 2 weeks later. On day 21 following immunization, blood samples were obtained from the tail vein of mice. The serum was collected by centrifugation at 6,000 x g and 4°C for 20 min. An indirect ELISA was applied to assay serum antibody titers against GPC3 (13). The fusion protein (10 μg/ml) was used to coat microtiter plates, plates were incubated with dilutions of mice immunized serum. Subsequently, goat anti-mouse IgG-horseradish peroxidase (cat. no. sc-2005; 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added. The final injection was administered intraperitoneally with 100 μg of fusion protein antigen purified following chromatography and after 3 days, mice were sacrificed prior to harvesting of the spleens. Splenocytes from the immunized mice were collected and subsequently fused with pre-prepared SP2/0 cells with the standard methodology (14). In brief, the cells were washed twice with RPMI-1640 medium and pre-warmed 50% polyethylene glycol 4000 (Sigma-Aldrich; Merck KGaA), and then added to the cell pellet slowly with continuous agitation. Hypoxanthine-aminopterin-thymidine selection media (Sigma-Aldrich; Merck KGaA) was used to select for fused cells under 37°C in a humidified environment with 5% CO₂. The non-fused dead cells were removed by replacing the medium. The fused cells were seeded with RPMI-1640 medium into 96-well plates; the hybridoma culture supernatants were then initially screened by an indirect ELISA (15). Positive hybridomas, which secreted antibodies against the recombinant GPC3 protein, were cloned at least four times by a limiting dilution assay in Hypoxanthine-thymidine selection media at 37°C in a humidified environment with 5% CO₂. Single positive hybridoma clones were expanded 4 days at 37°C in 75-cm² culture flasks and cryopreserved in liquid nitrogen [Air Products and Chemicals (Tianjin) Co., Ltd., Tianjin, China].

Production of mAbs. Selected positive clones of 5x10⁶ hybridoma cells were injected into the peritoneum of three sterile female BALB/c mice (age, 8 weeks; weight, 18-20 g) (The Academy of Military Medical Science) primed with

pristane (cat. no. P2870; Sigma-Aldrich; Merck KGaA). Mice were housed at a temperature of 22–24°C, relative humidity of 45–60%, with a 12-h light/dark cycle. After 10 days, ascitic fluid (3–5 ml) was first drained from the peritoneum for when the body weight of mice increased to 20–30%; ascites collection was conducted for a total of three times. Multiple harvests were conducted two days apart; the second and third harvests were performed two days apart. Providing mice manifested signs of pain or were due for final ascites harvest, mice were euthanized by cervical dislocation prior to ascites collection. Subsequently, mAbs against GPC3 were purified using G-Sepharose 4B (Sigma-Aldrich; Merck KGaA), the affinity purification column (size, 5x0.7 cm) was filled with a sepharose cross-linked with protein G. In total, 10 ml ascitic fluid sample was clarified using centrifugation at a speed of 10,000 x g for 10 min at 4°C prior to column-based purification. The supernatant at pH 7.0 was loaded through the protein G-agarose column at a flow rate of 1 ml/min at 4°C following by washing the column using 5 volumes of washing buffer containing 20 mmol/l Na₂HPO₄, 150 mmol/l NaCl and 2 mmol/l EDTA at pH 7.0 at the same flow rate and temperature. The antibodies were eluted using the elution buffer containing 100 mM glycine at pH 2.7. The immunoglobulin-containing fraction was identified by measuring its absorbance at 280 nm.

mAb analysis. The mAb isotypes were determined using a mouse mAb isotyping kit (cat. no. 501240; Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocols.

Chromosome counting of the hybridomas. The chromosome number of the hybridomas was determined in accordance with the International System for Chromosome Nomenclature 2013 (16). Cell division was inhibited in metaphase by adding 200 µg/ml colcemid (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 18 h. The chromosome number was then assessed using an Ni-U fluorescence microscope (Nikon Corporation, Tokyo, Japan; magnification, 100x).

Western blot analysis. For western blotting, HepG2 cells were collected and lysed in radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.). In total, 50 µg protein was loaded in each lane of the separation gel following protein concentration determination (Bradford's assay). Proteins were separated by 12% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Merck KGaA) with Mini PROTEAN (Bio-Rad Laboratories, Inc., Hercules, CA). Following three washes with Tris-buffered saline with 0.1% Tween 20, the membrane was incubated with purified mAb (1:1,000) at 37°C for 2 h. Followed by washing as aforementioned, then goat anti-mouse IgG-horseradish peroxidase (cat no. sc-2005; 1:5,000; Santa Cruz Biotechnology, Inc.) was used as a secondary antibody and incubated at room temperature for 1 h. The membranes were washed again and blots were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.). All experiments were repeated three times.

Immunofluorescence. HepG2 cells were plated onto a glass slide and cultured overnight at 4°C; following washing with PBS, cells were fixed with ice-cold 4% paraformaldehyde for

1 h and were blocked with bovine serum albumin (Beyotime Institute of Biotechnology) for 1 h at 37°C. Following three washes with PBS, cells were incubated with mAb in a humidified chamber overnight at 4°C followed by washing as aforementioned. A fluorescent-labeled goat anti-mouse IgG (cat. no. sc-2010; 1:5,000; Santa Cruz Biotechnology, Inc.) was applied as the secondary antibody and cells were incubated at room temperature for 1 h. Immunofluorescence was analyzed in 10 randomly selected fields of view using a Ni-U fluorescence microscope (magnification, x400).

Results

Construction of the prokaryotic expression vector. The PCR product (~510 bp) was analyzed by 1% agarose electrophoresis (Fig. 1). Subsequently, the recombinant plasmid was digested by incubation with two restriction endonucleases, *Bam*HI and *Xho*I. Gel electrophoresis revealed that the cleavage of *Bam*HI and *Xho*I had generated the target fragment, which was determined to have the expected size of 500 bp (Fig. 2). The fragment obtained from endonuclease cleavage was verified by sequencing (data not shown); the results were consistent with the sequence of GPC3 (Genbank, accession no. NM_001164617). These findings indicated the successful construction of the expression vector.

Expression, purification and identification of recombinant GPC3 protein. The plasmid pET16b-GPC3 was transfected into *E. coli* BL21 (DE3). Protein expression was induced by adding IPTG; a dense protein band with a molecular weight of ~19 kDa was detected in IPTG-induced cells (Fig. 3A). The location of the band corresponded to the theoretical molecular weight calculated for the recombinant GPC3 protein. Purification of the recombinant GPC3 protein by affinity chromatography via a His-tag was conducted with a purity of ≤95%. Following purification, there appeared to be only one clear protein band with a molecular weight of ~19 kDa (Fig. 3B). This result was further confirmed by western blotting (Fig. 3C).

Production of specific hybridomas. Following immunization, splenocytes were fused with Sp2/0 to establish hybridomas; these were cultured in 96-well culture microtitration plates. Hybridoma cells secreting antibodies were screened by an indirect ELISA and sub-cloned three times in order to guarantee the monoclonal behavior of the IgGs. Finally, we obtained three positive hybridoma cell clones against recombinant GPC3 following cell fusion and sub-cloning.

Identification of the mAbs against GPC3. A stable hybridoma clone designated as 2F3 and the corresponding antibody titers were determined to be 1:10⁵ in ascites. Furthermore, 2F3 was identified as being an IgG1/κ isotype. In addition, the median chromosome number of 2F3 was 98 (Fig. 4).

Western blot analysis. Western blot analysis demonstrated that mAb 2F3 yielded a positive signal with the total cell extracts of HepG2 cells at a molecular weight of ~70 kDa (Fig. 5).

Immunofluorescence analysis of the specific mAb against GPC3. HepG2 cells were observed via fluorescence

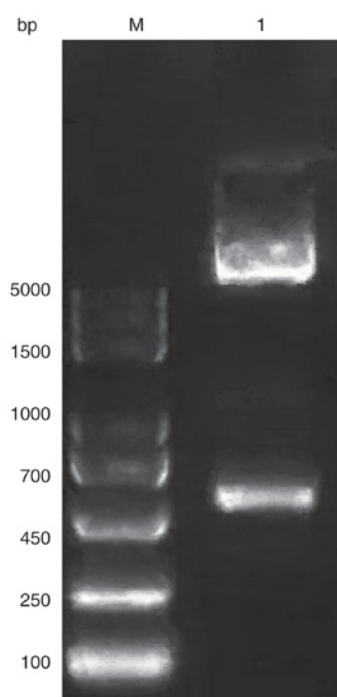


Figure 1. Agarose gel electrophoresis of PCR product. M, DNA marker; Lane 1, PCR product. PCR, polymerase chain reaction.

microscopy following staining with mAb 2F3 (Fig. 6). The results indicated that mAb 2F3 could react specifically with the native GPC3 protein in liver carcinoma cells.

Discussion

A confident diagnosis of cancer is a critical step prior to administering any treatment. The targeting of cancer cell-specific proteins could result in more effective cancer treatments with fewer side effects. Thus, developments in reliable tumor markers, and the detection of antibodies or technologies are urgently required. For example, Ramucirumab, a fully humanized monoclonal antibody, was demonstrated to exhibit clinical efficacy and a favorable safety profile in the treatment of a number of malignancies including advanced liver cancer (17). GPC3 protein is distributed in the cytoplasm and present in the membranes of hepatocytes in cancerous tissues (18,19); GPC3 has been considered as a promising biomarker for hepatoma-targeted therapeutics (20). In the present study, a fusion GPC3 protein was obtained from a prokaryotic expression system and an anti-human GPC3 mouse mAb was generated that specifically bound GPC3 protein in liver carcinoma cells.

In 1988, Filmus *et al* (21) isolated a developmentally-regulated cDNA clone, which was denoted as OCI-5, from a rat small intestine cell line. As the OCI-5 gene encodes a protein with extensive homology to the glypican family, the human gene was renamed as GPC3 (22). OCI-5 was determined to be located in the Xq26 region of the human chromosome, being bound onto the cell surface via a glycosylphosphatidylinositol anchor. There are six members of the glypican family (GPC1-6) (22). GPC3 is abundantly expressed in the placenta and numerous fetal tissues, including the liver, lung and kidney; however, GPC3 is significantly downregulated in the organs of adults (23). The GPC3 gene encodes a core protein of 580

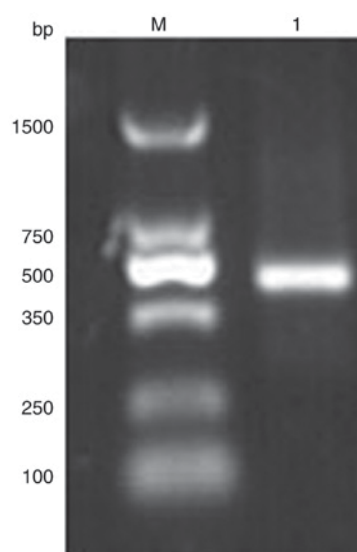


Figure 2. Gel electrophoresis of recombinant plasmid following *Bam*HI and *Xho*I double enzymatic digestion.

amino acids with a mass of 70 kDa. GPC3 can be released from the cell surface into the extracellular milieu (6). Accumulating evidence has indicated that GPC3 overexpression is closely associated with the malignant transformation of hepatocytes; GPC3 has been reported as a potential pathological diagnostic marker present in the serum of patients with hepatoma (24). In addition, GPC3 is known to be a carcinoembryonic antigen with an involvement in cell proliferation and metastasis by stimulating Wnt signaling. Significant GPC3 expression in hepatoma tissues is a putative prognostic factor predicting the poor outcome of patients with hepatoma (10). Thus, humanized anti-GPC3 mAb may possess notable cytotoxicity against GPC3-expressing human hepatoma cell lines *in vivo*; these effects may be mediated via antigen-dependent and/or complement-dependent cell cytotoxicity (25-27). Preliminary studies and clinical trials targeting GPC3 via humanized anti-GPC3 mAb in hepatoma have been conducted (28-31).

In addition, the recombinant proteins produced in prokaryotic expression systems are immunogens for the preparation of mAbs or polyclonal antibodies. The *E. coli* system offers numerous advantages for protein expression, including low cost, convenience and good fermentation potential (32). In the present study, the GPC3 recombinant protein was expressed in *E. coli* BL21 and the purified protein was used to immunize BALB/c mice in the generation of mAbs. The results of SDS-PAGE demonstrated that the human GPC3 recombinant protein was obtained with a high degree of purity.

Hybridoma technology for mAb production has become well-established during the past decades (33). Certain mAbs against GPC3 have been produced and are commercially available; however, the method and process of mAb preparation described in the present study varied from traditional methods. BALB/c mice were immunized with purified antigen via intrasplenic embedding. Furthermore, antibodies that recognize a conformational epitope could be easily obtained from mice immunized via intrasplenic embedding. Thus, we have successfully demonstrated a rapid, easy, feasible and reliable method to prepare mAbs by expressing recombinant protein following

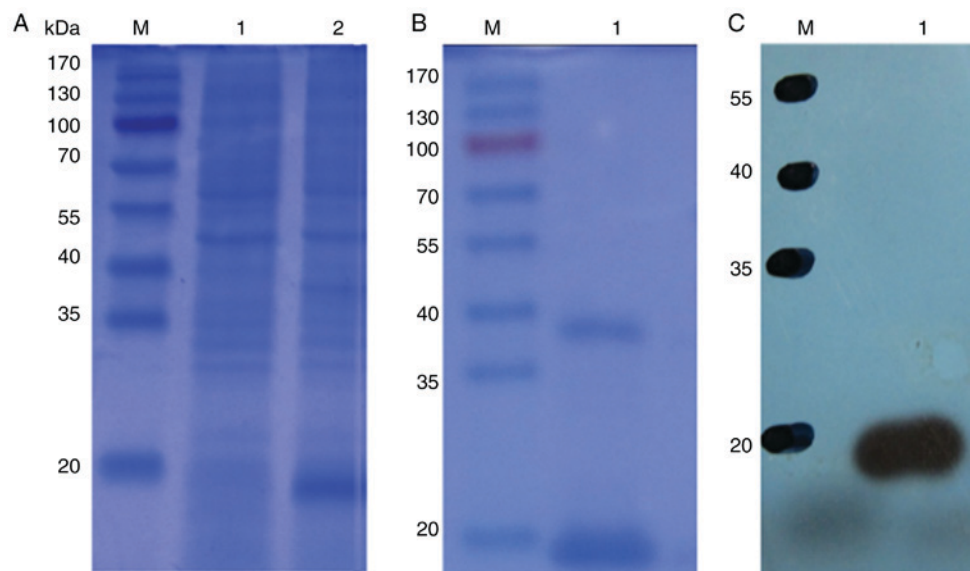


Figure 3. Expression, purification and identification of recombinant GPC3 protein. (A) Induction expression of recombinant GPC3 protein by SDS-PAGE analysis. Lane M, protein molecular weight marker; Lane 1, non-induction of GPC3; lane 2, induction of GPC3. (B) SDS-PAGE analysis of purification of recombinant GPC3 protein. M, standard protein molecular weight; lane 1, purified protein. (C) Confirmation of the recombinant protein by western blot analysis. The primary antibody was rabbit anti-His-tag monoclonal antibody. M, standard protein molecular weight; lane 1, purified protein. GPC3, glypican-3.

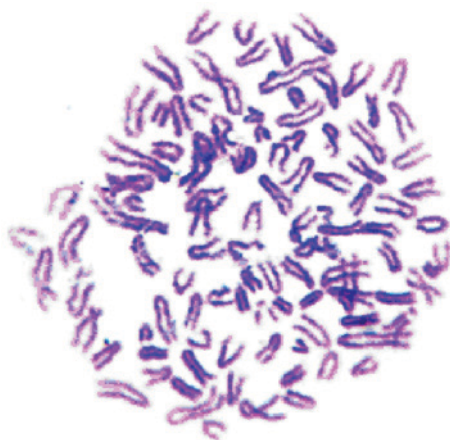


Figure 4. Chromosome map of mAb 2F3. The chromosome number of mAb 2F3 was counted following staining with colcemid; representative image (original magnification, x100). mAb, monoclonal antibody.

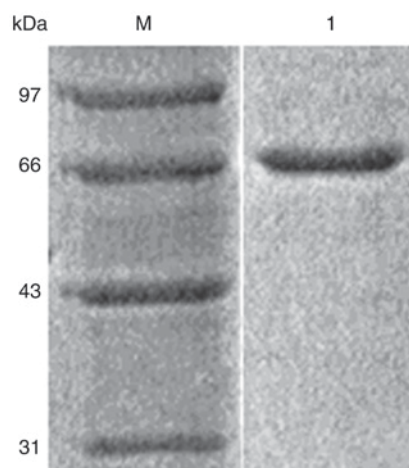


Figure 5. Western blot analysis with the purified monoclonal antibody 2F3. Lane M, protein molecular weight marker; lane 1, total extracts of HepG2 cells.

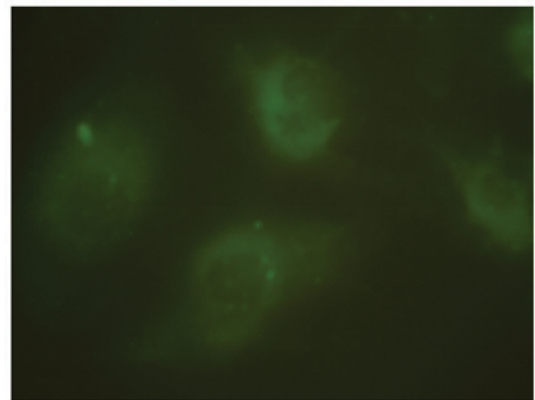


Figure 6. Immunofluorescence analysis of monoclonal antibody binding to native glypican-3 protein expressed in HepG2 cells (original magnification, x400).

optimization within *E. coli* and immunization via intrasplenic embedding. The results of the present study indicated that the specific mAb could be isolated by mouse hybridoma fusing and screening technology; the clone was denoted as 2F3 with the isotype of the generated mAb determined as IgG1/ κ . The mAb was present at a relatively high titer ($1:10^5$) in hybridoma supernatants and ascites as analyzed by indirect ELISA; its specificity was confirmed by western blot analysis, in agreement with previous reports that have demonstrated higher expression levels of GPC3 in HepG2 cells (34). Immunofluorescence experiments revealed strong signal intensity following interactions between 2F3 and GPC3 in HepG2 cells. Our results indicate that the bacterially expressed GPC3 protein is biologically active and that the 2F3 clone could specifically recognize the native GPC3 expressed in hepatoma cells.

A limitation of the present study is that the potential of the novel anti-GPC3 mAb in clinical settings was not investigated; thus, this antibody was not compared with other available antibodies. The present study aimed to generate the prokaryotic

expression of recombinant GPC3, and the preparation and characterization of its mAb. This novel mAb for GPC3 may provide a basis for therapeutic developments and applications in the future. The efficiency of the novel antibody in the early diagnosis of liver cancer requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW conceived and designed the present study. YZ, DQ, RL, YL, SS and YW collected, analyzed and interpreted the data. YL critically revised the manuscript for important intellectual content. YL and YW gave final approval for the version to be published. All authors read and approved the final manuscript, and agreed to be accountable for all aspects of the present study.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Tianjin First Central Hospital (Tianjin, China) and conformed to the Guide for the Use and Care of Animals published by the National Institutes of Health (NIH Publication number 85-23, revised 1996) (11).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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