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A novel antibody targeting CD24 and hepatocellular carcinoma *in vivo* by near-infrared fluorescence imaging

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ABSTRACT

Liver cancer is one of the most common malignant cancers worldwide. The poor response of liver cancer to chemotherapy has whipped up the interest in targeted therapy with monoclonal antibodies because of its potential efficiency. One promising target is cluster of differentiation 24 (CD24), which is known to beover-expressed on hepatocellular carcinoma (HCC), providing prospect for HCC targeted diagnosis and therapy. In this study we developed a novel CD24 targeted monoclonal antibody G7mAb based on hybridoma technology and then generated a single-chain antibodyfragment (scFv) G7S. Firstly, ELISA, western blot, and flow cytometry assays demonstrated specific binding of CD24 by G7mAb and G7S. Further, G7mAb was demonstrated to have similar binding capacity as ML5 (a commercial Anti-CD24 Mouse Antibody) inimmunohistochemical assay. Further more, a near-infrared fluorescent dye multiplex probe amplification (MPA) was conjugated to G7mAb and G7S to form G7mAb-MPA and G7S-MPA. The near-infrared fluorescence imaging revealed that G7mAb and G7S aggregate in CD24 + Huh7 hepatocellular carcinoma xenograft tissuevia specific binding to CD24 *in vivo*. In conclussion, G7mAb and G7S were tumor targeted therapeutic and diagnostic potentials *in vitro* and *in vivo* as anticipated.

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequent form of primary liver cancer in adults, accounting for approximately 600,000 new cases per year worldwide (El-Serag and Rudolph, 2007). Historically, human cancers are treated mostly through radiotherapy, chemotherapy, and surgery. However, these cancer therapeutic options, especially chemotherapy and radiotherapy are usually associated with many adverse effects (Maor and Malnick, 2013). The HCC in particular has shown poor prognosis, low response rate to treatment, severe toxicity and high recurrence rates (Belnoue et al., 2004; Levin and Amos, 1995). Small and localized tumors are usually curable with surgery, however, most patients usually report with advanced condition at diagnosis, therefore surgery is not possible due to the underlying cirrhosis (Bruix and Sherman, 2005; Lorenz et al., 2000; Matar et al., 2009). This development therefore calls for novel strategies such as immunotherapy to address the limitations associated with chemotherapy and radiotherapy, knowing very well that surgery can not be relied upon always.

The immune system is known to play crucial role in the control of cancer and has essentially opened new therapeutic option which works by manipulating the immune system to eliminate malignant cancers (Berzofsky et al., 2004; Gilboa, 2004; Matar et al., 2009). Studies elsewhere on clinical trials have demonstrated clearly that immunotherapy is a potent option in the treatment of patients with HCC (Butterfield et al., 2007; Gao et al., 2007; Kobayashi et al., 2007; Peng et al., 2005). An aspect of immunotherapy referred to as targeted therapy, which include monoclonal antibodies and small molecule inhibitors have improved significantly the treatment of malignancies in recent times, providing

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; HRP, horeradishperoxidase; IgG, immunoglobulin G; mAb, monoclonal antibody; scFv, single-chain antibody fragment.

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potent alternative to the traditional cytotoxic chemotherapy and radiotherapy (Gerber, 2008). The main challenge to this therapy option is the difficulty in finding tumor-specific targets (Mitchell, 2011).

One promising target is Cluster of differentiation 24 (CD24), which is a glycosylphosphatidylinositol-linked cell surface glycoprotein encoded in human by the CD24 gene (Hough et al., 1994; Soave et al., 2013; Weichert et al., 2005). It has been identified as a P-selectin ligand and adhesion receptor for platelets and activated endothelial cells (Kristiansen et al., 2004; Lim, 2005; Soave et al., 2013). CD24 is over-expressed in many human tumors, and it is known to be over-expressed on HCC (Huang and Hsu, 1995), providing prospect for HCC targeted therapy. Studies on the use of CD24 over-expressed in prognosis, diagnosis, and targeted therapy on various cancerous conditions have captured the attention of scientists in recent times due to the prospects (Deng et al., 2012; Karimi-Busheri et al., 2013; Liu et al., 2013; Schostak et al., 2006).

In this current study, we developed CD24 targeted monoclonal antibody G7mAb through hybridoma technology and further generated single-chain antibody fragment (scFv) against CD24 (G7S) based on G7mAb, and investigated their diagnostic and targeted therapy features on HCC *in vitro* and *in vivo*. The antibodies exhibited both tumor targeted therapeutic and diagnostic potentials *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and animals

The murine myeloma cell line Sp2/0-Ag14 preserved in our lab was cultured in DMEM medium (high glucose), supplemented with 10% (v/v) fetal bovine serum (FBS). The human hepatoma cell line Huh7 and the human colon cancer cell line HCT116 preserved in our lab were cultured in RPMI 1640 medium containing 10% (v/v) FBS. The human colon cancer cell line HT29 preserved in our lab was cultured in McCoy'5A medium containing 10% (v/v) FBS. Cell culture media and supplements used in this study were purchased from Life technologies (Basel, Switzerland). The animals used in this study were purchased from Yangzhou University.

2.2. Construction of the expression system E. coli *BL21 (DE3)/pGEX-CD24*

The appropriate cDNA of the CD24 was obtained from the GENEBANK (accession number: 100133941). The expression system *Escherichia coli* DH5α/pGEX-CD24 was constructed by SANGON (Shanghai, China). The expression system *E. coli* BL21 (DE3)/pGEX-CD24 was then constructed by extracting the recombinant vector (Plasmid Minipreparation Kit, Biomiga, USA) and transforming into a more suitable *E. coli* strain, BL21 (DE3). The positive clones were then selected from Luria–Bertani (LB) plates containing appropriate anbiotics (100 μg/ml Ampicillin) and confirmed with colony PCR and sequencing (SANGON, Shanghai, China).

2.3. Expression and purification of the CD24

Positive clone of CD24 was selected and cultured in 10 ml of liquid LB medium containing 100 μg/ml ampicillin overnight at 37 °C. It was then subcultured (1:100) in 500 ml fresh medium and incubated for about 2.5 h at 37 °C. The expression of CD24 was then induced with 0.1 mM isopropyl-thio-b-D-galactoside (IPTG) and incubated for further 6 h at 37 °C. The bacteria was harvested and suspended in PBS (w(g)/v(ml) = 1/20; pH 7.4). It was then treated with lysozyme (1 mg/ml) (incubated 30 min on ice), 10 ml 0.2% Triton X-100, 5 μg/ml DNase (incubated at 4 °C for 10 min). The supernatant was then collected (4 °C, 3000 g, 30 min) and treated with DTT (1 mmol/L final concentration). The target protein GST-CD24 was verified by western blot assay, then purified with affinity chromatography employing GSTrapFF (1 ml) column (GE Healthcare, Buckingghamshire, UK). A successful purification was verified with SDS-PAGE.

2.4. Preparation of murine anti-CD24 antibody G7mAb

50 μg GST-CD24 and an equal volume of Quick Antibody adjuvant (Biodragon-Immunotech, Beijing, China) were intramuscularly injected into 6-week-old female BALB/c mice. Subsequencely, a booster immunization was conducted after 3 weeks in the same manner. Serum antibody titers were measured by indirect ELISA and the mouse with highest serum antibody titer was selected to be spleen donors for hybridoma generation. A final injection of 20 μg GST-CD24 and an equal volume of Quick Antibody adjuvant was given to the donor mouse 3 days before being sacrificed and the spleen removed. 5×10^8 spleenocytes were fused with 10^8 Sp2/0-Ag14 myolema cells using the PEG method and hyrodoma cells were selected in HAT medium (DMEM supplemented with 20% FBS, 10 mM sodium hypoxanthanine, 40 mM aminopterin, and 1.6 mM thymidine). Positive clones were selected using an indirect ELISA. Stable hybridoma cell line G7 secrecting anti-CD24 antibody G7mAb was obtained after three cycles of subcloning. The isotype of resoulting G7mAb was determined to be IgG1κ isotype using the isotyping kit (Proteintech Group Inc., China).

To produce anti-CD24 antibody G7mAb, hybidoma cells were injected intraperitoneally into liquid paraffin pretreated 8-week-old female mice with 10^6 cells per mouse. Approximately 7 days later, ascites were collected and applied onto protein G affinity chromatography cloum (GE Healthcare, Buckingghamshire, UK). A successful production and purification of G7mAb was confirmed with SDS-PAGE and western blot assay (HRP conjugated goat anti-mouse IgG (H + L); Millipore, USA).

2.5. Immunoblotting with G7mAb

Recombinant human CD24-Fc chemera (Sino Biological Inc., China) and recombinant human Fc (Sino Biological Inc., China) were loaded into different wells on two 15% (w/v) SDS–PAGE for electrophoresis and transferred onto two different polyvinylidenedifluoride (PVDF) membranes (Millipore, USA). Blotted membranes were placed in TBS buffer with 5% (w/v) skim milk at 37 °C for 2 h. One of the blotted membranes was incubated with G7mAb whereas the other was incubated with anti-Fc antibody (Sino Biological Inc., China) at 4 °C overnight. After washing with TBST and TBS, the membrane was incubated with HRP conjugated goat anti-mouse IgG (Millipore, USA) at room temperature for 1.5 h. After successive washing as described previously, the blots were treated with enhanced chemiluminescence (ECL) solution (Millipore, USA) and exposed in gel imaging systems (Bio-rad).

2.6. Friguet (Indirect ELISA) determination of G7mAb affinity

50 μL nine different concentrations of antigen (0, 3.19×10^{-11}, 2.55×10^{-10}, 2.05×10^{-9}, 1.62×10^{-8}, 1.30×10^{-7}, 1.04×10^{-6}, 8.32×10^{-6}, and 6.65×10^{-5} M) were mixed individually with 50 μl 10 pmol/L G7mAb, and incubated at 4 °C overnight. The mixtures were transferred and incubated for 1.5 h at 37 °C into the wells of a microtitration plate previously coated with CD24 peptide (SANGON, Shanghai, China). After washing with PBST and PBS, the bound immunoglublins were detected by adding HRP conjugated goat anti-mouse IgG (Millipore, USA). Visualization was achieved with TMB peroxidase substrate (BBI) and the absorbance at 450 nm with