Optimised expression and purification of RBP4 and preparation of anti-RBP4 monoclonal antibody

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Retinol-binding protein 4 (RBP4) is a member of the lipocalin super family and is mainly secreted by the liver. RBP4 is secreted into the blood to combine with retinol, playing a crucial role in the transport of retinol and vitamin A [1]. In 2005, QIN Y et al. found that RBP4 acted as an adipose factor and participated in insulin resistance. Moreover, many studies have revealed that RBP4 is closely related to insulin-resistant type 2 diabetes [2], cardiovascular disease [3], metabolic syndrome [4,5] and other diseases. In recent years, studies have reported that RBP4 is associated with cancer, and it has a remarkable effect in promoting the migration and proliferation of ovarian cancer cells. The underlying molecular mechanism of RBP4 involves the activation of the RhoA/Rock1 pathway and the expression of CyclinD1. It may represent a potential target for the treatment of obese cancer patients [6]. High serum RBP4 levels are associated

Abbreviations
DAB, diaminobenzidine; DE3, E. coli BL21; ECL, enhanced chemiluminescence; HCC, hepatocellular carcinoma; iELISA, indirect enzyme-linked immunosorbent sandwich assay; IHC, immunohistochemistry; IOD, integrated optical density; IPTG, isopropyl-β-d-thiogalactoside; KEGG, kyoto encyclopedia of genes and genomes; NEK2-His, never in mitosis gene-A-related expressed kinase 2-His; RBP4, retinol-binding protein 4; SAA4-His, serum amyloid A-4-His; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; SHBG-His, sex hormone-binding globulin-His; TCGA, the cancer genome atlas.
with an increased risk of colon adenoma and pancreatic cancer [7,8]. The RBP4-STRAR6 pathway drives the maintenance of cancer stem cells and mediates the occurrence of colon cancer induced by a high-fat diet [9]. With the deepening of research, RBP4 has been shown to be involved in the occurrence and development of liver diseases [10]. Relevant studies have indicated that serum RBP4 concentration is negatively correlated with the severity of disease in early HCV patients, and as the serum RBP4 concentration decreases, the degree of liver fibrosis increases proportionally [11,12]. Moritoshi et al. [13] found that the low expression of RBP4 in HCC tissues was closely related to the development of HCC. In the early stage of this study, transcriptomics sequencing and proteomics technology analysis demonstrated that RBP4 had low expression in the serum of patients with HCC, suggesting that it may become a diagnostic marker for HCC. Researchers have constructed an enzyme-linked immunosorbent double antibody sandwich assay kit based on anti-RBP4 mouse IgA mAb to facilitate the detection of RBP4 [14]. However, reports on the production process of optimised expression and purification of RBP4 protein or the production of high-specificity and high-affinity anti-RBP4 mAb are few. In addition, commercial RBP4 antibodies and kits vary widely amongst different manufacturers and different batches; thus, the production of high specificity anti-RBP4 mAb is required.

We expressed the RBP4 recombinant protein using the prokaryotic expression system. After purification, the recombinant protein was immunised to BALB/c mice to prepare anti-RBP4 mAb. Anti-RBP4 mAb could be used to detect the RBP4 expression level by immunohistochemistry (IHC) on the basis of the expression level of RBP4 in HCC tissues found on The Cancer Genome Atlas (TCGA) database. Therefore, the proposed mAb has advantages of high affinity and specificity for RBP4 protein, thereby providing insights in advancing auxiliary examination of related diseases.

Materials and methods

Cell lines, plasmid, experimental animals, main reagents and instruments

Normal human liver cell line HL-7702, HCC cell hep3B, HCC cell huh7 and SP2/0 mouse myeloma cells were purchased from the Shanghai Academy of Biological Sciences and Cell Collection and Chinese Academy of Sciences. Strain DH5α competent cells and E. coli BL21 (DE3) were purchased from Beijing Quanshijin Company. The plasmid pET-30a was donated by the School of Life Science and Technology of Guangxi University. SPF-grade 4-week-old female BALB/c mice, weighing 14 g-17 g, were sourced from Shanghai Slack Laboratory Animal Co., Ltd. [SCXK (Shanghai) 2017-0005], with certificate number: 20170005000441. All mice were housed in specific pathogen-free facilities and cared for in Laboratory Animal Center of Guangxi Medical University [SCXK (Gui) 2020-003]. Sterile surgery was conducted in the Experimental Animal Centre of Guangxi Medical University. All animal experiments had been approved by the Laboratory Animal Welfare and Ethics Committee of Guangxi Medical University (NO. 201911011) and performed in accordance with national ethics regulations. In the experimental design, we had achieved the minimum necessary amount by optimising the experimental scheme. In the process of the experiment, we used a relatively mild injection technique and appropriate amount of adjuvant to reduce the serious side effects of animals and reduce the pain of the animals. Other materials purchased include the following: total RNA extraction and transcription kit, 10× T4 DNA ligase (Takara, Japan), RBP4 gene primers (Beijing Ruiboxingke, China), plasmid extraction kit (Omega Bio-Tek, USA), Hind III and BamHI enzymes (Promega, USA), 2× Fast Pfu PCR enzyme (Beijing full gold, China), Ni-Agarose Resin (China Kangwei Century, China), SDS/PAGE protein gel reagents and BCA kits (Beyotime biological company, China), Freund’s complete adjuvant, Freund’s incomplete adjuvant, HRP-labelled goat anti-mouse secondary antibody (Thermo, USA), Bovine Serum Albumin (BSA) (Solebold, China), gradient RT-PCR instrument (Bio-Rad, USA), gel image analyser (BIO-BAD, USA), ultrasonic cell disruptor (Ningbo Xinzhi Company, China), western blot electrophoresis imaging system (Thermo, USA), EVOS FL automatic cell imager (Thermo, USA) and HCC tissue sample chip (Shanghai Xinchao, China).

Cell culture

HL-7702 and Huh7 were cultured in DMEM containing 10% FBS, and Hep3B was cultured in MEM containing 10% FBS. Mouse myeloma cell line SP2/0 was cultured in RPMI-1640 medium containing 10% FBS, hybridoma cell lines were cultured in RPMI-1640 medium containing 20% FBS and 20% HAT, and monoclonal hybridoma cell lines were cultured in RPMI-1640 medium containing 20% FBS and 20% HT. All the cells were incubated at 37 °C in 5% CO2.

Construction of recombinant plasmid

The amino acid sequence of RBP4 protein was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the position of its signal peptide was analysed by SignalP5.0. The antigenic index of RBP4 protein was predicted by DNAstart software, and the appropriate sequence was selected for expression. The RBP4 primer
sequences were AAAGATCCGAGCGC GACTGCCGA GTGAG (forward) and GGGAACCTTCTACAAAG GTTTCTTTCTGT (reverse). Total RNA of a normal human liver cell line HL-7702 was extracted as raw material, and RBP4 gene was obtained by RT-PCR amplification. The reaction conditions are as follows: 95 °C 3 min, 95 °C 30 s, 61 °C 30 s, 72 °C 90 s, and 35 cycles; 4 ºC infinite cycle. A small amount of pET-30a plasmid was extracted with the kit, and the pET-30a plasmid and RBP4 gene were digested with BamHI enzyme and HindIII enzyme, respectively. The ligated vector was transformed into competent DH5α and plated onto LB kanamycin plates. The next day, a single colony was picked from the plate and cultured in LB, and the recombinant plasmid was identified by restriction enzyme digestion with BamHI and HindIII.

Recombinant protein expression, condition optimisation and purification

The pET30a-RBP4 plasmid and pET30a empty plasmid were divided into two groups, namely the IPTG-induced group and the uninduced group. The IPTG-induced group was added with isopropyl-β-d-thiogalactoside (IPTG) to a final concentration of 1.0 mmol·L⁻¹, and the expression was induced at 37 °C for 4 h. The bacterial pellet was broken by ultrasonic disruption, and the bacterial supernatants and the precipitates were collected separately. The expression form of the recombinant protein was identified by 12% sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE). According to these protein expression steps, different induction temperatures (18, 28, 37 and 42 °C) and different induction IPTG concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1 mm) were considered to determine the optimal expression conditions. The supernatant protein solution was added to a chromatography column containing 2 mL Ni agarose gel packing and incubated overnight at 4 °C on a shaker to purify the recombinant protein. Proteins were eluted with 150 mM imidazole elution concentration, which was increased from 50 mM to 200 mM to determine the optimal elution condition, and the elution peaks containing a large amount of RBP4 protein were collected. This solution was purified by 250 mmol·L⁻¹ KCl staining. Finally, the RBP4 recombinant protein containing His tag was confirmed by western blot using anti-His-tag antibody.

Mice immunisation and serum titre detection by indirect enzyme-linked immunosorbent sandwich assay (iELISA)

The first dose was mixed with the same volume of complete Freund’s adjuvant at a dose of 150 μg·mouse⁻¹, and the BALB/c female mice were immunised with multiple subcutaneous injections at an interval of 14 days, using recombinant RBP4 protein at a concentration of 1 μg·μL⁻¹ as the immunogen. For the second immunisation, the dose of 80 μg·mouse⁻¹ was mixed with the same volume of Freund's incomplete adjuvant, and the interval was 2 weeks. The dose of the third and fourth immunisations, identical to the second, was given at 2-week interval. Seven days after the fourth immunisation, the iELISA was used to detect the serum titre of mouse tail vein blood. In summary, the 96-well microplate was coated with 100 μL RBP4 recombinant protein (2 μg·mL⁻¹) at 4 °C overnight. The positive serum and negative serum samples were diluted in a threefold ratio, from 1 : 1000 to 1 : 729,000, and the 0.1% BSA solution was used as a blank control. The HRP-IgG goat anti-mouse (1 : 10,000) was added and incubated at 37 °C for 30 min. Under dark conditions, the substrate colour solution was added with 100 μL·well⁻¹. Finally, the reaction was stopped by 2 M sulphuric acid, and the OD450nm value was measured. The serum dilution corresponding to positive serum (P)/negative serum (N) value ≥ 2.1 was used as the serum titre of immunised mice. In addition, immunised mice with serum titre greater than 1 : 10,000 were selected for the next cell fusion.

Cell fusion

Mice with serum titre greater than 1 : 10,000 were selected for cell fusion. Three days after booster immunisation of mice, spleen cells and SP2/0 cells are fused at a ratio of 2 : 10 under the concentration of 50% PEG. After fusion, the hybridoma cells were cultured in 1/3 HAT selection medium. After 9 days, the hybridoma cell supernatants were determined by iELISA. The positive hybridoma cell lines were selected and subjected to three subclonal screening by the limiting dilution method.

mAb preparation and identification

BALB/c mice were sensitised intraperitoneally with 500 μL of paraffin. After 7 days, the 1 x 10⁷ monoclonal hybridoma cells were injected into the mice abdominal cavity to prepare ascites. The monoclonal antibodies were purified by the rProtein G affinity chromatography column. The mouse mAb isotyping (IgA, IgM, IgG1, IgG2a, IgG2b and IgG3) detection kit was used to detect mAb isotype. The iELISA method was used to determine the antibody affinity constant. According to the checkerboard square method, the RBP4 recombinant protein was coated on the 96-well microplate at the concentration of 4, 2, 1 and 0.5 μg·mL⁻¹, and the selected mAb was serially diluted with a double dilution ratio (1 : 1000-1 : 1,024,000). The calculation method of affinity constant Ka was the same as that reported in the previous study [15]. Recombinant RBP4-His protein, recombinant sex hormone-binding globulin-His (SHBG-His) protein, recombinant serum amyloid A-4-His (SAA4-His) protein, recombinant never in mitosis.
gene-A related expressed kinase 2-His (NEK2-His) protein and BSA albumin were coated on the 96-well microplate at a concentration of 2 μg·mL⁻¹, and the sample diluent was used as a negative control. According to the iELISA method, the anti-RBP4 mAb was diluted at 1 : 20,000 and then tested. HCC cell line hep3B, HCC cell huh7 total protein, SHBG-His recombinant protein, RBP4-His recombinant protein and RBP4 protein standard were electrophoresed in 12% SDS/PAGE gel and transferred into the PVDF membrane. The membrane was incubated with purified anti-RBP4 mAb (1 : 10,000) overnight at 4 °C. HRP-IgG goat anti-mouse (1 : 1500) for 60 min at room temperature, the membrane was washed with TBST buffer for three times and then developed with enhanced chemiluminescence (ECL).

Immunohistochemical assays
The tissue chip contains tissue samples from 10 hepatocellular carcinoma (HCC) patients and the corresponding adjacent tissues. In accordance with the improved nuclear grading scheme of the Edmondson and Steiner system, all tissue samples were pathologically diagnosed as hepatocellular carcinoma, and the histological grade was categorised as grade I ~ III (n = 1), II (n = 4), III (n = 2) and III (n = 3). A summary of the pathological characteristics and the patient characteristics is presented in Table 1. The expression of RBP4 was analysed in 10 pairs of HCC tissues and adjacent non-tumorous liver tissues based on the standard immunohistochemistry procedures. The sample was incubated with the anti-RBP4 mAb (1 : 1500) for 60 min at 37 °C. The staining of the sample was performed using the dianinobenzidine (DAB) solution at room temperature for 3–5 min and then counterstained with haematoxylin. Other immunohistochemical assay procedures were the same as that reported in the study [16]. Image-Pro Plus 6.0 software was used for semi-quantification of the staining. In summary, three areas of each sample were captured randomly at 40× the magnification of EVOS FL automatic cell imager, and the measurement parameter was the integrated optical density (IOD). The parameter setting referred to the method of Li Gang et al. [17], and then, the values of log IOD were counted.

Table 1. Relationships between RBP4 expression and clinicopathological variables of HCC. NA, No value.

| Characteristics          | RBP4 (lg IOD) | n | Mean ± SD | P-value |
|--------------------------|--------------|---|-----------|---------|
| Age (years)              |              |   |           |         |
| ≤ 50                     | 5            |   | 1.67 ± 1.72 | 0.552  |
| > 50                     | 5            |   | 0.91 ± 1.94 |         |
| Edmondson grade          |              |   |           |         |
| I-II                     | 5            |   | 1.19 ± 1.88 | 0.857  |
| III                      | 5            |   | 1.41 ± 1.87 |         |
| Liver metastasis         |              |   |           |         |
| Yes                      | 0            |   | NA        |         |
| No                       | 10           |   | 1.30 ± 1.77 | 0.001  |
| Tissues                  |              |   |           |         |
| HCC tissue               | 10           |   | 1.30 ± 1.77 | 0.001  |
| Adjacent tissue          | 10           |   | 3.99 ± 0.77 |         |

**RBP4 mRNA expression and survival analysis in UALCAN**

The mRNA expression levels of *RBP4* between the HCC tissue and normal liver tissue were analysed using UALCAN database. UALCAN (http://ualcan.path.uab.edu/index.html) was a web-based tool, which allowed researchers to perform analyses of TCGA gene expression data [18]. In addition to gene expression analysis, we performed survival analysis in UALCAN. The HCC patients were divided into high and low expression groups according to the expression of RBP4. The overall survival of HCC patients was analysed using a Kaplan–Meier plot. Log rank P value was calculated and displayed on the web page.

**Statistical analysis**

ImageJ image software was used to evaluate the grey value of western blot bands and Coomassie brilliant blue staining band. IBM SPSS Statistics 22.0 (IBM Corp, Armonk, NY, USA) was used for statistical analysis. The differential expression of RBP4 between HCC and adjacent non-tumorous liver tissues was determined using the two-tailed paired Student’s t-test. One-way ANOVA was used to analyse the differences amongst multiple groups. P < 0.05 was considered statistically significant. Origin 9.1 was used to calculate the antibody concentration corresponding to the EC50 of the mAb.

**Results**

**RBP4 gene cloning and recombination**

According to the prediction of DNA start software, the surface possibility and flexibility regions of RBP4 protein were moderate, indicating a higher possibility of forming B-cell epitope and easy to bind to antibodies. The index score of 19-201aa sequence RBP4 protein antigen, as an immunogen, was moderate, and the local hydrophilicity was favourable, thereby producing a stronger immune response (Fig. 1A). SignalP5.0 analysis showed that 1-18aa was the signal peptide sequence. The 19-201aa fragment was selected for RBP4 expression to increase the expression level. At the same time, pET30a was selected as the expression vector (Fig. 1B), and the *RBP4* gene was cloned into pET30a. Specific primers were used to amplify the *RBP4* gene fragment with a size of ~ 600 bp, and the
extracted cDNA of human normal liver cell line HL-7702 was used as a template (Fig. 1C). Five recombinant strains were selected for RT-PCR amplification during the transformation to obtain the RBP4 gene fragment, and electrophoresis showed a 600 bp target sequence fragment (Fig. 1D). After the recombinant plasmid pET30a-RBP4 was successfully transferred into DH5α competent cells, it was digested with BamH I and Hind III enzymes. Finally, pET30a plasmid at 5400 bp and RBP4 gene fragment at 600 bp were obtained (Fig. 1E). The pET30a-RBP4 clonal bacterial solution was sent for sequencing, and the result was consistent with the gene coding sequence published by NCBI (Fig. 1F). This finding indicates that the vector had been successfully constructed and can be used for subsequent experiments.

Expression and purification of RBP4 recombinant protein

After conditional exploration, the RBP4 recombinant protein was highly expressed in the precipitated inclusion body, and bands at approximately 29 kDa (RBP4-His) were evident in lanes 1 and 2, indicating that the E. coli BL21 was successfully induced to express the RBP4 recombinant protein (Fig. 2A). The proportion of RBP4 recombinant protein to total protein was 73.99%. The results of induction of recombinant protein expression at different induction temperatures (18, 28, 37 and 42 °C) showed that the optimal induction temperature for RBP4 recombinant protein was 28 °C (P < 0.000) (Fig. 2B). Furthermore, the expression level of RBP4 recombinant protein was the highest when IPTG was 1.0 mM (P < 0.000) (Fig. 2C). Under optimal induction conditions, RBP4 recombinant protein accounted for 92.06% of the total protein. The RBP4 recombinant protein was preliminarily purified by the Ni column. According to the elution results, the RBP4 recombinant protein was gradually eluted with imidazole at concentrations of 100, 150 and 200 mM, and a higher concentration of RBP4 recombinant protein was found in imidazole with 150 and 200 mM (Fig. 2D). The RBP4 recombinant protein was purified by the Ni column, followed by the KCl gel-cutting recovery method. The RBP4 recombinant protein was obtained with a purity of 98% by SDS/PAGE electrophoresis, and the protein size was consistent with the expectation (Fig. 2E). The purified RBP4 protein was transferred to PVDF membrane, and the anti-His antibody was used as the primary antibody. Western blot analysis showed that the RBP4 recombinant protein with His tag could specifically bind to the anti-His antibody, and the band deepened with the increase in the sample loading amount of RBP4 protein (Fig. 2F).

Preparation and purification of the RBP4 mAb

After immunising the mice with purified RBP4 recombinant protein as the immunogen, the serum titre was determined by iELISA. The polyclonal antibody serum titre of three mice could reach 1 : 729,000 (Fig. 3A). The average P/N of the anti-RBP4 polyclonal antibody was 3.9 (ratio > 2.1), which indicated that the purified RBP4 recombinant protein obtained by this method still had an ideal immunogenicity and could be used for the next step of mAb preparation. After fusion of mouse spleen cells and myeloma cells, hybridoma cells were selected, considering the medium containing 1/3 HAT (Fig. 3B). After two cell fusion experiments, the cell fusion rates were 14.79% and 25.37%. However, no positive hybridoma strains were screened out by iELISA. Amongst nine 96-well plates, the cell fusion rate of the third sample was 90%, and 32 positive strains were screened out by determining the titre of hybridoma cell supernatant, and the positive rate was 5.00%. The hybridoma cells were subcloned by the limiting dilution method, and the hybridoma cells after subcloning for 8 days showed a single-cell cluster growth morphology (Fig. 3C). Then, the hybridoma cells with more vigorous growth and single-cell cluster growth morphology were selected for the second subcloning. After subcloning for three times, all cell lines in the 96-well plate were positive, and the test result of the hybridoma cell 1D2 is shown in Table 2. Finally, five monoclonal hybridoma cell lines stably secreting anti-RBP4 mAb, namely 1D2, 1G2, 2F4, 10G2 and 10B10, were obtained. The five monoclonal hybridoma cell lines were expanded in large quantities by ascites induction method. The antibody was purified according to the experimental method of rProtein G affinity chromatography column, and the purity of the antibody was tested by SDS/PAGE gel electrophoresis. The results showed that the antibodies of the five monoclonal hybridoma cell lines showed evident bands at 55 kDa (heavy chain) and 25 kDa (light chain). In addition, no other miscellaneous bands were found on the gel. This finding showed that the mAb obtained in this experiment has high purity (Fig. 3D).

Identification of RBP4 mAb

The antibodies of the five purified monoclonal hybridoma cell lines were identified according to the experimental method of the subtype kit. The results showed that the five monoclonal antibodies belonged to different subtypes of IgG (Fig. 3E). Western blot was used to distinguish the specificity of monoclonal antibodies secreted by the five monoclonal strains. The results
Preparation of anti-RBP4 monoclonal antibody
showed that the 1D2 mAb could react with the recombinant RBP4-His protein expressed in prokaryotic expression system and the natural RBP4 protein expressed by HCC cells Hep3B and Huh7. However, no cross-reaction was observed with the recombinant SHBG-His control. Other monoclonal strains could cross-react with the recombinant SHBG-His control (Fig. 4A), indicating that the 1D2 mAb had a higher specificity against RBP4. Therefore, 1D2 was selected for further experiments. The affinity constant of purified 1D2 mAb was determined by iELISA. The EC50 of the antibody with the concentration of 0.5, 1, 2 and 4 µg·mL⁻¹ RBP4 recombinant protein was 0.197, 0.154, 0.166 and 0.122 µg·mL⁻¹, respectively (Fig. 4B). Therefore, the average affinity constant of anti-RBP4 mAb was 3.98 x 10⁸ L·mol⁻¹. Recombinant RBP4-His protein, recombinant SHBG-His protein, recombinant NEK2-His protein, BSA albumin and diluent negative control were coated on 96-well ELISA plate, and the specificity of the anti-RBP4 mAb was identified by iELISA. The anti-RBP4 mAb only specifically reacted with recombinant RBP4-His protein, and it did not cross-react with other recombinant proteins containing His tag, BSA albumin or diluent negative control.
The western blot result also showed that the anti-RBP4 mAb interacted with natural RBP4 protein expressed by HCC cells Hep3B and Huh7 and illustrated the same position as the standard RBP4 protein (R&D). However, the recombinant SHBG-His protein did not cross-react, indicating that the anti-RBP4 mAb only and specifically bound to the RBP4 protein (Fig. 4D).

**Lower expression of RBP4 in HCC tissues by immunohistochemical detection with anti-RBP4 mAb**

The expression of RBP4 in 10 pairs of HCC tissues and adjacent non-tumorous liver tissues was detected by immunohistochemical method with anti-RBP4 mAb to explore the value of anti-RBP4 mAb in the HCC. The result indicated that anti-RBP4 mAb detected the expression of RBP4 protein in HCC tissues and adjacent tissues of different pathological grades (Fig. 5A). IMAGE-PRO Plus 6.0 digital image analysis software was used to semi-quantitatively analyse the positive reaction of brown colour. We found that RBP4 expression was strongly positively stained on adjacent non-tumorous liver tissues and weakly positively stained on HCC tissues. The RBP4 expression in HCC tissues was significantly lower than in adjacent non-tumorous liver tissues \((P = 0.001)\) (Fig. 5B). This finding was consistent with the results of The Cancer Genome Atlas (TCGA) database (Fig. 5C). At the same time, to evaluate the clinical significance of RBP4 expression in HCC patients, Kaplan–Meier survival analysis of the expression level of RBP4 in HCC
patients from TCGA database was conducted based on UALCAN. The results showed that HCC patients with lower RBP4 expression had poorer prognosis (log-rank test, \( P = 0.017 \)) (Fig. 5D).

**Discussion**

RBP4 protein, as a secreted protein of the retinol family, is widely distributed in blood, urine and other body fluids. The normal concentration of human serum RBP4 protein is 25–70 mg/L, and the urine concentration is 0.7 mg/L. Serum RBP4 transports retinoid proteins to target organs and reabsorbs them back into the blood through renal tubules. Therefore, RBP4 is often used as a clinical indicator for early diagnosis and efficacy evaluation of renal diseases [19]. Liver is the main organ for RBP4 protein synthesis; thus, its health status greatly affects the expression level of RBP4 [20]. Some studies have shown that the serum RBP4 expression level of patients with chronic hepatitis B infection is lower than that of healthy people, and it is negatively correlated with the severity of the disease [21]. The detection of serum retinol and prealbumin levels in patients with liver cirrhosis secondary to hepatitis may represent a sensitive indicator of acute liver damage.

RBP4, as an adipokine, is closely related to the severity of liver disease in patients with alcoholic cirrhosis [22]. Researches have found that the concentration of serum RBP4 is negatively correlated with the disease severity in patients with early HCV liver fibrosis, and its concentration decreases as the degree of liver fibrosis increases [12]. The negative correlation between RBP4 and the severity of liver fibrosis might be due to the decreased level of RBP4, which participated in the activation of hepatic stellate cell overexpression and the deposition of type I collagen in the liver, that promoted the progression of liver fibrosis [12,23], which eventually developed into liver cirrhosis or HCC. In summary, changing the concentration of serum RBP4 might represent a sensitive indicator of liver function injury.

In the clinical testing, antibodies are the commonly used tools. At present, reports on the preparation of monoclonal antibodies against RBP4 are few. The RBP4 protein was analysed by bioinformatics analysis, and the N-terminal amino acid sequence (1-18aa) of the RBP4 protein was found to be the secretion signal peptide sequence. Thus, the amino acid sequence with favourable epitope specificity (19-201aa) was selected for the protein expression. *E. coli* was selected as a protein expression tool for expressing RBP4.

![Identification of RBP4 mAb](image)

Fig. 4. Identification of RBP4 mAb. (A) Determination of monoclonal strains specificity. (B) mAb affinity determination. (C) iELISA for mAb specificity determination, data were described as means ± SD of three experiments. (D) Western blot for mAb specificity determination.
recombinant protein, considering its characteristics of prokaryotic expression system with clear genetic background, strong operability, mature and stable technology, large yield and low cost [24,25]. After reasonable IPTG inducers and optimised temperature expression conditions, the proportion of RBP4 recombinant protein in the total protein was increased, and the yield of RBP4 expressed in Escherichia coli was effectively increased. The inclusion body recombinant protein should be renatured with a certain concentration of urea because the RBP4 recombinant protein existed in the form of precipitated inclusion bodies. Thus, the inactive recombinant protein should be renatured with a certain concentration of urea because the RBP4 recombinant protein existed in the form of precipitated inclusion bodies. Thus, the inactive recombinant protein could be folded accurately to restore its activity and maintain appropriate antigen activity [26,27]. The findings indicated that the imidazole concentration of 150 mM was beneficial to the efficient recovery of the target protein, but still a small amount of other protein was found, probably affecting the effect of RBP4 protein as an immunogen. After the initial purification by the Ni affinity chromatography column, RBP4 recombinant protein was further purified by KCl silver-stained gum recovery to obtain a higher purity RBP4 recombinant protein. During the gum recovery process, the imidazole, urea and salt ions in the RBP4 recombinant protein solution were also removed, reducing the loss of the recombinant protein in the final dialysis process, which was conducive to obtaining a large amount of high-purity recombinant protein. The RBP4 recombinant protein was successfully identified by western blot, indicating that the recombinant protein expressed in the form of inclusion bodies maintained the integrity of the linear epitope and had compelling antigenicity after renaturation.

The results of serum titre showed that the serum antibody titres of mice could reach more than 1 : 729,000, which was much higher than the currently
specificity. When the affinity constant was between $10^7$ and $10^9$ L·mol$^{-1}$, it belonged to the high-affinity antibody [30], and the high-affinity mAb had a wide range of application value. At the same time, the specificity of anti-RBP4 mAb was determined by iELISA and western blot; it indicated that the anti-RBP4 mAb did not produce nonspecific reaction. The recombinant RBP4 protein expressed in the prokaryotic expression system, RBP4 protein standard (R&D) expressed in the eukaryotic expression system and natural RBP4 protein secreted by HCC cell lines could all react specifically and were more conducive to the accurate detection of RBP4, effectively avoiding false-positive results. The TCGA database contains comprehensive cancer genome data, including mutations, copy number variations, mRNA expression, miRNA expression and methylation data [31]. TCGA is widely recognised amongst most researchers and has been widely used to verify the consistency of experimental data [32]. The immunohistochemical analysis showed that the expression level of RBP4 protein in HCC tissues was significantly lower than the adjacent tissues, and it decreased with the increase in HCC pathological grade. These results were highly consistent with the analysis results of the TCGA database. In addition, Kaplan–Meier survival curve analysis showed that low RBP4 expression in HCC patients had a poor prognosis, suggesting that RBP4 may represent a potential prognostic factor in HCC patients. Therefore, anti-RBP4 mAb has potential application value for immunohistochemical detection.

The liver is the main secretion site of RBP4 protein. When it is damaged, it affects the synthesis of RBP4, resulting in the decrease in serum RBP4 concentration, accompanied by the aggravation of liver damage. With the deepening of studies, researchers found that the expression level of RBP4 was closely related to cancers. In head and neck cancer [33], breast cancer [34], hepatocellular carcinoma [11] and other cancers, serum RBP4 levels were significantly reduced, and the decrease might be caused by methylation [35]. Therefore, the decrease in RBP4 expression level in cancer tissues might be a new indicator for early detection of cancers in the clinic. This finding indicates that detecting the expression level of RBP4 in liver tissue might have diagnostic significance for early detection of HCC.

However, the current study also had several shortcomings in the process of preparing monoclonal antibodies. For example, only two types of antibodies secreted by the newly prepared five monoclonal hybridoma cell lines could be used for detection by western blot and immunohistochemistry simultaneously, and the three remaining strains could only be used for immunohistochemical detection. This finding might be because the anti-RBP4 monoclonal antibodies secreted by the three strains specifically recognised the internal conformational epitopes of natural RBP4 protein, rather than the linear epitopes on the protein surface. As for the application of anti-RBP4 mAb in immunohistochemical detection, we will consider to expand the sample size to detect HCC patients at different pathological grades in the future. In addition, the application value of anti-RBP4 mAb in the diagnosis of HCC will be evaluated.

**Conclusions**

In conclusion, the recombinant RBP4 protein with high concentration, high purity (98%) and outstanding antigenic activity was successfully expressed and...
obtained. The expression and purification method had important reference value for the development of a large-scale standardised production process of RBP4 recombinant protein. The anti-RBP4 mAb was prepared; it had high affinity and strong specificity and could specifically bind to the natural RBP4 protein and suitable for immunohistochemical analysis.

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Conflict of interest
The authors declare no conflict of interest.

Data accessibility
All data in our study are available from the corresponding author on reasonable request.

Author contributions
HL performed the experiment, analysed data and wrote the original draft. WS, LCY, QLC, SPH performed the animal experiment. XH, YSL, FJW analysed the data and revised the manuscript. MH designed the study, supervised experiments, MH and XH supported the study. All authors reviewed the final manuscript.

References
1 Miettinen M, Lasota J, Sobin LH. Gastrointestinal stromal tumors of the stomach in children and young adults: a clinicopathologic, immunohistochemical, and molecular genetic study of 44 cases with long-term follow-up and review of the literature. Am J Surg Pathol. 2005;29:1373–81.
2 Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabotolny JM, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature. 2005;436:556–62.
3 Li F, Xia K, Sheikh SA, Cheng J, Li C, Yang T. Involvement of RBP4 in hyperinsulinism-induced vascular smooth muscle cell proliferation. Endocrine. 2015;48:472–82.
4 Li GE, Sangbedo IC, Xu L, Fu J, Li L, Feng D, et al. Childhood retinol-binding protein 4 (RBP4) levels predicting the 10-year risk of insulin resistance and metabolic syndrome: the BCAMS study. Cardiovasc Diabetol. 2018;17:69.
5 Majerczyk M, Kocelak P, Chorea P, Arabzada H, Owczarek AJ, Bożentowicz-Wikarek M, et al. Components of metabolic syndrome in relation to plasma levels of retinol binding protein 4 (RBP4) in a cohort of people aged 65 years and older. J Endocrinol Invest. 2018;41:1211–9.
6 Wang Y, Wang Y, Zhang Z. Adipokine RBP4 drives ovarian cancer cell migration. J Ovarian Res. 2018;11:29.
7 Abola MV, Thompson CL, Chen Z, Chak A, Berger NA, Kirwan JP, et al. Serum levels of retinol-binding protein 4 and risk of colon adenoma. Endocr Relat Cancer. 2015;22:L1–4.
8 El-Mesallamy HO, Hamdy NM, Zaghloul AS, Sallam AM. Serum retinol binding protein-4 and neutrophil gelatinase-associated lipocalin are interrelated in pancreatic cancer patients. Scand J Clin Lab Invest. 2012;72:602–7.
9 Karunanithi S, Levi L, DeVecchio J, Karagkounis G, Reizes O, Lathia JD, et al. RBP4-STR A6 pathway drives cancer stem cell maintenance and mediates high-fat diet-induced colon carcinogenesis. Stem Cell Rep. 2017;9:438–50.
10 Chen X, Shen T, Li Q, Chen X, Li Y, Li D, et al. Retinol binding protein-4 levels and non-alcoholic fatty liver disease: a community-based cross-sectional study. Sci Rep. 2017;7:45100.
11 Kataria Y, Deaton RJ, Enk E, Jin M, Petrauskaite M, Dong L, et al. Retinoid and carotenoid status in serum and liver among patients at high-risk for liver cancer. BMC Gastroenterol. 2016;16:30.
12 Fayed HM, Mahmoud HS, Ali EM. The utility of retinol-binding protein 4 in predicting liver fibrosis in chronic hepatitis C patients in response to direct-acting antivirals. Clin Exp Gastroenterol. 2020;13:53–63.
13 Kinoshita M, Miyata M. Underexpression of mRNA in human hepatocellular carcinoma focusing on eight loci. Hepatology. 2002;36:433–8.
14 Lee NS, Kim HS, Park SE, Blüher M, Park CY, Youn BS. Development of a mouse IgG monoclonal antibody-based enzyme-linked immunosorbent sandwich assay for the analyses of RBP4. Sci Rep. 2018;8:2578.
15 Chen C, Li X, Gu M, Di R, Kang N, Wang L, et al. Biological characterisation and application of human MTH1 and monoclonal antibody preparation. Oncot Rep. 2019;41:1851–62.
16 Zhang D, Xie C, Wang R, Yang Q, Chen H, Ling S, et al. Effective preparation of a monoclonal antibody against human chromogranin A for immunohistochemical diagnosis. BMC Biotechnol. 2018;18:25.
17 Li G, Zhong Y, Shen Q, Zhou Y, Deng X, Li C, et al. NEK2 serves as a prognostic biomarker for hepatocellular carcinoma. *Int J Oncol.* 2017;50:405–13.
18 Chandrashekar DS, Bashel B, Balasubramanyam SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia.* 2017;19:649–58.
19 Jing J, Isoherranen N, Robinson-Cohen C, Petrie I, Kestenbaum BR, Yeung CK. Chronic kidney disease alters vitamin A homeostasis via effects on hepatic RBP4 protein expression and metabolic enzymes. *Clin Transl Sci.* 2016;9:207–15.
20 Welles JE, Toro AL, Sunilkumar S, Stevens SA, Purnell CJ, Kimball SR, et al. Retinol-binding protein 4 mRNA translation in hepatocytes is enhanced by activation of mTORC1. *Am J Physiol Endocrinol Metab.* 2021;320:E306–15.
21 Kwon JH, Park ST, Kim GD, You CR, Kim JD, Woo HY, et al. The value of serum retinol-binding protein 4 levels for determining disease severity in patients with chronic liver disease. *Korean J Hepatol.* 2009;15:59–69.
22 Kalafateli M, Triantos C, Tschochatzis E, Michalaki M, Koutroumpakis E, Thomopoulos K, et al. Adipokines levels are associated with the severity of liver disease in patients with alcoholic cirrhosis. *World J Gastroenterol.* 2015;21:3020–9.
23 Chayanupatkul M, Honsawek S, Chongsrisawat V, Vimolket L, Poovorawan Y. Serum retinol binding protein 4 and clinical outcome in postoperative biliary atresia. *Hepatol Int.* 2011;5:906–12.
24 Rosano GL, Ceccarelli EA. Recombinant protein expression in Escherichia coli: advances and challenges. *Front Microbiol.* 2014;5:172.
25 Gileadi O. Recombinant protein expression in E. coli: a historical perspective. *Methods Mol Biol.* 2017;1586:3–10.
26 Singh A, Upadhyay V, Panda AK. Solubilization and refolding of inclusion body proteins. *Methods Mol Biol.* 2015;1258:283–91.
27 Singh A, Upadhyay V, Upadhyay AK, Singh SM, Panda AK. Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. *Microb Cell Fact.* 2015;14:41.
28 Xie C, Wang R, Saeed A, Yang Q, Chen H, Ling S, et al. Preparation of anti-human podoplanin monoclonal antibody and its application in immunohistochemical diagnosis. *Sci Rep.* 2018;8:10162.
29 Xi Y, Zhang Y, Fang J, Whittaker K, Luo S, Huang RP. Prokaryotic expression of hepatitis C Virus-NS3 protein and preparation of a monoclonal antibody. *Monoclon Antib Immunodiagn Immunother.* 2017;36:251–8.
30 Maragos CM, Busman M, Plattner RD. Development of monoclonal antibodies for the fusarin mycotoxins. *Food Addit Contam.* 2008;25:105–14.
31 Tomczak K, Czerwińska P, Wiznerowicz M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. *Contemp Oncol.* 2015;19:A68–77.
32 Ke RS, Zhang K, Lv LZ, Dong YP, Pan F, Yang F, et al. Prognostic value and oncogene function of heterogeneous nuclear ribonucleoprotein A1 overexpression in HBV-related hepatocellular carcinoma. *Int J Biol Macromol.* 2019;129:140–51.
33 Chen KC, Hsueh WT, Ou CY, Huang CC, Lee WT, Fang SY, et al. Alcohol drinking obliterates the inverse association between serum retinol and risk of head and neck cancer. *Medicine.* 2015;94:e1064.
34 Merdad A, Karim S, Schulten HJ, Jayapal M, Dallol A, Buhmeida A, et al. Transcriptomics profiling study of breast cancer from Kingdom of Saudi Arabia revealed altered expression of Adiponectin and Fatty Acid Binding Protein 4: Is lipid metabolism associated with breast cancer? *BMC Genom.* 2015;16(Suppl 1):S11.
35 Abe M, Yamashita S, Mori Y, Abe T, Saijo H, Hoshi K, et al. High-risk oral leukoplakia is associated with aberrant promoter methylation of multiple genes. *BMC Cancer.* 2016;16:350.