Preparation of Anti-Human Podoplanin Monoclonal Antibody and its application in Immunohistochemical Diagnosis

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Podoplanin (PDPN), a 38 kDa transmembrane sialoglycoprotein from human, is expressed in lymphatic endothelial cells but not in vascular endothelial cells, and has been considered as a specific marker of lymph. In this study, the gene encoding the extracellular part of PDPN (ePDPN) was synthesized and used to expressed fusion protein ePDPN-His and GST-ePDPN, respectively, in E.coli. The purified GST-ePDPN fusion protein was mixed with QuickAntibody-Mouse5W adjuvant to immune mice, and the antiserum titer was determined by indirect ELISA. A stable cell line named 5B3 generating anti-PDPN monoclonal antibody (mAb) was obtained by hybridoma technology. The isotype of 5B3 cell line was IgG2b, and the chromosome number was 102 ± 4. The 5B3 mAb was purified successfully from ascites fluid through Protein G column, and its affinity constant was $2.94 \times 10^8$ L/mol. Besides, excellent specificity of the 5B3 mAb was further demonstrated in ELISA, western blot and immunohistochemistry experiments, suggesting that 5B3 mAb displays similar application value to D2-40, a commercial available antibody. Hence, the current study provides conclusive guidelines for preparation of other mAbs and their applications in immunohistochemistry diagnosis.

Podoplanin (PDPN) is an O-linked transmembrane sialoglycoprotein that consists of 162 amino acids with molecular weight 38 kDa1–3. Previous studies have shown that PDPN is a specific marker of lymphatic endothelial, and it is highly expressed in lymphatic endothelial cells but not in vascular endothelial cells4. PDPN can also be found in other tissues types, including follicular dendritic cells, reticular cells, mesothelial cells, testicular germ cells and ovarian cells5. Furthermore, PDPN has also been reported in lymphangiomas, Kaposi sarcomas, seminomas and epithelioid mesotheliomas hemangioblastomas6. To date, PDPN contributes to the diagnosis of lymphatic endothelial cell-derived tumors and to the judgement of lymphatic invasion and metastasis in other tumor tissues by labeling the lymphatic endothelium7. Therefore, it is important to develop methodologies for early diagnosis of lymphatic-related tumors using PDPN as a biomarker.

Currently, IHC detection has been recommended as gold standard for tumor diagnosis by the World Health Organization (WHO). Hence, the major challenge in the present study is generation of a monoclonal antibody with high affinity and specificity against PDPN for IHC diagnosis. To date, several commercial mAbs against PDPN have been used, including D2-40, NZ-1, 18H5, LpMab-9 and LpMab-108–10. Among these mAbs, only D2-40 has been used for pathological diagnosis. However, dysgerminoma tissue as an antigen for producing the D2-40 faced challenges such as weak immune response by low concentration of PDPN protein, and many non-specific antibodies generated by numerous substances with the method11. In fact, it's difficult for researchers to obtain carcinoma in situ like dysgerminoma tissue or to obtain cancer cell lines to immune mice. Furthermore, it's challenging to select a stable eukaryotic cell line expressing PDPN since the cells can be contaminated during an extended cell culturing process. In view to the current situation, it is urgent to establish a rapid, easy, cost-effective, feasible and reliable method such as expressing the PDPN antigen in E. coli, and producing a novel

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anti-PDPN mAb based on the resulted antigen to diagnose related tumors by IHC. The present study provides insights into establishing new methodologies for rapid preparation of such novel mAbs.

Materials and Methods

Reagents. All the strains used in this study were from Fujian Agriculture and Forestry University (Fujian, China). The anti-His 6 tag mAb, anti-GST tag mAb and horseradish peroxidase (HRP) labeled goat anti-mouse IgG were purchased from ZSGB-Bio Co., Ltd (Beijing, China). D2-40 antibody and all paraffin-fixed tissue sections were supported by Fuzhou Maxin Biotech Co., Ltd (Fuzhou, China). Hypersensitivity ECL chemiluminescence detection kit was purchased from Wuhan Sanying Co., Ltd (Wuhan, China). Hypoxanthine, aminopterin and thymidine supplement (HAT), hypoxanthine and thymidine supplement (HT) and polyethylene glycol 1450 solution (PEG 1450) were purchased from Sigma. RPMI medium 1640 powder and fetal bovine serum (FBS) were purchased from Gibco.

Mice and QuickAntibody-Mouse5W adjuvant. Female Balb/c mice (6 to 8 weeks old) were obtained from Wushi Animal Laboratory (Shanghai, China). All animal experiments were performed according to the protocols approved by the Animal Ethics Committee of the Fujian Agriculture and Forestry University. QuickAntibody-Mouse5W adjuvant (Quickadjuvant) was purchased from Beijing Biodragon Immunotechnologies Co., Ltd (Beijing, China).

Cells. Murine myeloma cell line SP2/0 from our laboratory was cultured in RPMI-1640 medium with 10% FBS. Hybridoma cells were cultured in RPMI-1640 medium with 20% FBS containing HAT. After 10 d of cell fusion, hybridoma cells were cultured in RPMI-1640 medium with 20% FBS containing HT, and screened by indirect ELISA (iELISA) to obtain a stable cell line that stably secreting mAb against PDPN. The selected hybridoma cells were further expanded in RPMI-1640 medium with 10% FBS.

Codon optimization and synthesis of ePDPN gene. The identifier of PDPN in the Uniprot is Q86YL7. According to the database, the amino acids of 23–131 is noted as the extracellular part of PDPN (ePDPN) and was selected for codon optimization for expression in E. coli Transetta (DE3) and evaluated by graphical codon usage analyser (http://gcua.schoedl.de)12. The optimized DNA sequence of ePDPN was synthesized by Shanghai BioSune Biotech Co., Ltd (Shanghai, China).

Expression and purification of ePDPN antigen. After DNA amplification by PCR, the resulted products were digested by Xho I/Xho I and EcoR I/Not I, respectively, and inserted into plasmids of pET-28a and pGEX-6P1 digested with corresponding enzymes 13. More information about primers was detailed in Table 1. The ligated DNA mixture was transformed into the E. coli Transetta (DE3) competent cells by calcium chloride transformation. After verification by PCR and sequence alignment, target protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG).

The cell culture was harvested at 4 °C by centrifugation at 6,000 r/min for 5 min. The pellets were resuspended with buffer A (0.01 mol/L imidazole buffer, pH 8.0) and the cells were broken by sonication. The supernatant after centrifugation at 12,000 r/min for 10 min was collected and incubated for 30 min at 4 °C with Ni2+ affinity resin in a buffer A. Pre-chilled 0.02 mol/L imidazole buffer (pH 8.0) was used to wash the resin to remove substances that bound non-specifically. The target protein was gradually eluted by adding pre-cooled 0.25 mol/L imidazole buffer (pH 8.0).

The procedure for GST-ePDPN purification was similar to that of purification of ePDPN-His protein. GST protein fusion purification resin was used to incubate with collected supernatant after sonication containing the GST-ePDPN. Phosphate-buffered saline (PBS, 0.01 mol/L) was used to wash the resin to reduce binding of non-specific proteins. After washing, the target protein GST-ePDPN was eluted by GST elution buffer (0.01 mol/L reduced glutathione dissolved in 0.05 mol/L Tris-cl solution, pH 8.0). The concentration of ePDPN-His and GST-ePDPN fusion proteins was determined by BCA method and Nanodrop (Thermo), respectively 14.

Identification of fusion ePDPN antigen. Both purified recombinant ePDPN fusion proteins were identified by western blot 15. Protein loading buffer was added into tubes containing protein sample and the mixture was incubated in boiling water for 10 min. The sample was centrifuged at 12,000 r/min for 5 min, and the supernatant was collected for running SDS-PAGE. The proteins on gel were transferred to a PVDF membrane and blocked by PBS with 5% milk (5% PBSM) at room temperature for 1 h. After washing three times with PBS, anti-His 6 tag mAb and anti-GST tag mAb (at dilution of 1:8000) were added into the reaction and incubated at room temperature for 1 h followed by washing of the PVDF membranes three times with PBS containing tween-20 (PBST) and three times of PBS. Subsequently, HRP-labeled goat anti-mouse IgG (diluted at 1:8000 with 5% PBSM) was incubated with the reaction mixture at room temperature for 1 h. After washing three times with PBST and PBS.

Table 1. Primers for PCR.

| Name       | Primer Sequence |
|------------|-----------------|
| Nco I      | 5'-TTACCATGGCGAGCAGCGCAAC-3' |
| Xho I      | 5'-AACTCGAGCGCGTTACGGTACTCAGG-3' |
| EcoR I     | 5'-TCCGATATCTGGGACACCGCCAAAC-3' |
| Not I      | 5'-GTTGGCGCGCCGTTACTCGAGCGAGGTTACG-3' |

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both PVDF membranes were incubated with mixture of ECL chemiluminescence reagents followed by imaging in fully functional multicolor fluorescence imaging instrument (Bio-Rad).

Animal immunization and titer analysis by iELISA. Three female Balb/c mice (6 to 8 weeks old) were injected intra-muscularly with 60 µg of GST-ePDPN fusion protein appropriately mixed with 150 µL Quickadjuvant (final volume adjusted to 300 µL with 0.9% saline solution) as first immunization. Subsequent injections were given at intervals of 20 days, respectively. Blood was extracted from the tail vein of each mouse after third immunization, and the titer was determined by iELISA16. Briefly, 100 µL of ePDPN-His fusion protein as coating antigen diluted to 5 µg/mL with carbonate/bicarbonate coating buffer was added into each well of the ELISA plate, and incubated at 37 °C for 2 h. Plate was washed and blocked with 5% PBSM (200 µL/well), and incubated at 37 °C for 2 h. Then, 100 µL of serially diluted anti-PDPN antiserum was added into each well followed by incubation at 37 °C for 1 h. After washing properly, HRP-labeled goat anti-mouse IgG (diluted at 1:8000 in 5% PBSM, 100 µL/well) was added and incubated at 37 °C for 1 h. Plate was washed and 100 µL substrate solution was added into each well and incubated at 37 °C for 10 min. Reaction was stopped by adding 2 mol/L H2SO4 (50 µL/well), and the optical density (OD) value at 450 nm was determined by micro-plate reader (Thermo).

Cell fusion and hybridoma screening. The mouse that showed highest titer was injected intraperitoneally with 20 µg GST-ePDPN antigen mixed with 100 µL 0.9% saline solution to boost immunization. Three days later, splenocytes from the immunized mouse were mixed with murine SP2/0 myeloma cells at a ratio of 10:1 in RPMI-1640 mixed with 20% FBS/HAT medium containing hybridoma cells was equally distributed (200 µL/well) into 96-well micro-titer cell culture plates in which feeder cells have been added a day before cell fusion, and incubated at 37 °C with 5% CO2. After 5 d, half of the supernatant in each well of micro-titer plates was substituted with fresh medium. Ten days after cell fusion, the titer of the supernatant of culture medium was determined by iELISA for anti-PDPN mAb activity. A stable cell line was obtained by subsequent limiting dilution method until the positive percentage reached 100%.

Characterization of positive hybridoma cells against PDPN. To analyze the mAb isotype, ELISA was performed following the instruction of mouse mAb Isotyping (IgA, IgM, IgG1, IgG2a, IgG2b, IgG3) Kit18. Chromosome analysis was carried out according to previously published protocols from our lab19. Hybridoma cells on glass slides were stained with Giemsa staining solution, and the number of chromosomes in the hybridoma cell with distinct dispersion was counted under microscope.

Production of anti-PDPN mAb. To obtain large amount of mAbs and to reduce possibilities of cell contamination, the ascites method was selected. Balb/c mouse was injected intraperitoneally with 0.5 mL paraffin oil. One week later, about 1 × 106 positive hybridoma cells were injected into mouse intraperitoneally. The ascites fluid was collected through a needle after at least one week. After centrifugation, the middle layer of the ascites fluid containing mAb was carefully transferred into a new tube followed by purification with Protein G, and the purified mAb was analyzed by SDS-PAGE20. The concentration of the purified mAb was determined under the manufacturer instruction of BCA protein assay. The titer of the mAb was measured by iELISA with similar protocol used in the experiment of titer analysis described above, and negative control was ascites from negative mouse.

Affinity determination of mAb. The affinity of mAb against PDPN was determined followed procedures previously published21. After coating with different concentrations of ePDPN-His (4, 1.5 and 0.5 µg/mL), wells were added with serially diluted anti-PDPN mAb followed by adding goat anti-mouse HRP-IgG and substrate. The OD value of each well was measured at 450 nm by micro-plate reader after the reaction was stopped. A curve diagram based on equation (1) was developed to show the relationship between concentration of the antibody as the abscissa and the value of absorption as the ordinate. Relative affinity of anti-PDPN mAb was measured in pairs according to formula (2), where [Ab] or [Ab]t was from x0 of Table 222. The average of their affinity was used as the final result.

| Concentration (ng/mL) | Parameter | Value | Standard Error | Adj. R-Square |
|-----------------------|-----------|-------|----------------|---------------|
| 4000                  | x0        | 6.51544 | 9.47014        | 0.99147       |
|                       | A1        | −2.64599 | 3.63223        |               |
|                       | A2        | 3.62507  | 0.20052        |               |
|                       | p         | 0.73334  | 0.25921        |               |
| 1500                  | x0        | 14287.891 | 5.81974E9      | 0.975456      |
|                       | A1        | −3.38706 | 41.95306       |               |
|                       | A2        | 21.54778 | 744.10623      |               |
|                       | p         | 0.11967  | 1.71155        |               |
| 500                   | x0        | 224.28169 | 34.01063       | 0.99544       |
|                       | A1        | 0.07755  | 0.03482        |               |
|                       | A2        | 1.62498  | 0.12436        |               |
|                       | p         | 1.21719  | 0.17486        |               |

Table 2. Parameters of fitting curve based on equation (1).
E. coli expression, and Fig. 1B showed comparison between original and optimized sequences, both of which encode exactly same protein sequence (Fig. S1). The optimized codon was identified by graphical codon usage analyzer (http://gcua.schoedl.de) that the relative adaptiveness of all codons was more than 20% meaning all the codons are suitable for expression in E. coli. Since PDPN is a transmembrane protein, the extracellular domain is the main part for generating antibodies for diagnostic or other applications. Also, considering the likelihood of solubility of antigen expressed in E. coli, the ePDPN which has a better chance of being soluble than the intact membrane protein was used for antigen generation. The human ePDPN DNA sequence needs to be codon optimized for E. coli expression, and Fig. 1B showed comparison between original and optimized sequences, both of which encode exactly same protein sequence (Fig. S1). The optimized codon was identified by graphical codon usage analyzer (http://gcua.schoedl.de) that the relative adaptiveness of all codons was more than 20% meaning all the codons should be suitable for expression in E. coli Transetta (DE3) (Fig. 1C). The optimized DNA sequence of ePDPN was synthesized by Shanghai BioSune Biotech Co., Ltd (Shanghai, China).

Expression and identification of ePDPN antigen. The amplified DNA fragment of ePDPN was inserted into vectors pET-28a and pGEX-6P1, respectively, for expression in E. coli Transetta (DE3). After purification, the obtained fusion proteins of ePDPN-His and GST-ePDPN were identified by SDS-PAGE and western blot. Compared to empty vector, specific distinct bands were observed at 24 kDa (ePDPN-His) and at 50 kDa (GST-ePDPN) after IPTG induction and affinity chromatography (lane 1–4 in Fig. 2). The concentration of ePDPN-His and GST-ePDPN was determined to 1.7 mg/mL and 0.3 mg/mL, respectively. Both of target proteins were recognized by their respective labeled antibodies (lane 5 in Fig. 2), and further indicating that the production of ePDPN was successful.

Immunization and screening of positive hybridoma clones. GST-ePDPN recombinant protein was injected to immune female Balb/c mice, and ePDPN-His protein was used as the coating antigen. After immunizations four times, the titer of antisem from mouse were determined by ELISA. The ELISA showed that the titer from immunized mice were significantly higher than that from the negative control (Fig. 3A). The results further indicated that the immunization of mouse with GST-ePDPN fusion protein successfully induced immunogenicity for subsequent production of mAb against PDPN. Therefore, the mouse was selected for cell fusion. Spleen cells from the above immunized mouse were harvested aseptically and fused with SP2/0 myeloma cells at the ratio of 10:1 in the presence of PEG 1450 as the fusion reagent. After the fusion reaction, feeder cells, spleen

\[ y = A_2 + \frac{A_1 - A_2}{1 + \left( \frac{[Ag]}{[Ab]} \right)^n} \]

\[ K_a = \frac{n - 1}{2 \times (n[Ab]_t - [Ab])} \]

[Ab] is the concentration of antibody at the 50% inhibition of control values (IC\(_{50}\)) when the concentration of antigen is [Ag]; [Ab], is the concentration of antibody at IC\(_{50}\) when the concentration of antigen is [Ag], and n represents the ratio of [Ag] to [Ab].

Stability analysis of mAb by IHC. To analyze stability of the antibody, the anti-PDPN mAb was incubated at 37 °C or 4 °C for several days followed by analysis by IHC method. After dewaxing and hydration, sections from paraffin-fixed normal rectal tissues were heated under high pressure with 10 mM citrate buffer (pH 6.0) to repair antigen. Then, sections were washed three times with PBS. After blocking by 3% H\(_2\)O\(_2\), for 10 min and washing by PBS, the mAb was added into the sections and incubated for 1 h followed by washing. MaxVisionTM/HRP reagent was introduced into the sections and incubated for 25 min. After washing by PBS, the 3,3-diaminobenzidine (DAB) chromogenic liquid was added into the sections and incubated for 10 min followed by stopping with water. After redyeing with hematoxylin for 25 s and returning to blue for 30 s, the sections were dehydrated by gradient of ethanol, and treated by xylene for 3 min. Finally, the sections were sealed by neutral gum and checked under microscope.

Determination of specificity of mAb. The specificity of mAb against PDPN was determined by three methods described as follow. In the experiment of iELISA, ePDPN-His, His-B-cell lymphoma 6 protein (His-Bcl6), His-Chromograin A (His-CgA), Albumin Human (HSA) and Interferon-gamma (IFN-γ) from human as coating antigens were diluted to 5 μg/mL and added into the micro-titer plates (100 μL/well), respectively. After anti-PDPN mAb (at a dilution of 1:8000) was added, goat anti-mouse HRP conjugate and substrate were added subsequently, the reaction was stopped and the absorbance of each well was measured for analysis. The second method to identify the specificity of the mAb was western blot. After respectively running in 15% and 10% SDS-PAGE gel, ePDPN-His and GST-ePDPN fusion proteins were transferred into the PVDF membrane and subsequently blocked. The membrane was incubated with the mAb at a dilution of 1:8000 and then goat anti-mouse HRP-IgG. ECL chemiluminescence detection reagents were mixed and added, and imaging was carried out in fully functional multicolor fluorescence imaging instrument. The protocol of IHC was the same with that of stability analysis of mAb against PDPN described above. After sections from lung tissue, mesothelioma, seminoma, submucosal lymphatic vessels and thyroid were interacted with anti-PDPN mAb, MaxVisionTM/HRP reagent and the DAB chromogenic liquid were added subsequently. The sections were treated with a series of chemicals followed by observation and analysis.

Results
Verification and synthesis of the PDPN sequence after optimization. Molecular structure of PDPN was divided into four parts according to the Uniprot, including signal peptide, extracellular part, transmembrane part and intracellular part (Fig. 1A). Since PDPN is a transmembrane protein, the extracellular domain is the main part for generating antibodies for diagnostic or other applications. Also, considering the likelihood of solubility of antigen expressed in E. coli, the ePDPN which has a better chance of being soluble than the intact membrane protein was used for antigen generation. The human ePDPN DNA sequence needs to be codon optimized for E. coli expression, and Fig. 1B showed comparison between original and optimized sequences, both of which encode exactly same protein sequence (Fig. S1). The optimized codon was identified by graphical codon usage analyzer (http://gcua.schoedl.de) that the relative adaptiveness of all codons was more than 20% meaning all the codons should be suitable for expression in E. coli Transetta (DE3) (Fig. 1C). The optimized DNA sequence of ePDPN was synthesized by Shanghai BioSune Biotech Co., Ltd (Shanghai, China).

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cells, unfused myeloma cells and fused cells originated from only myeloma cells or from only spleen cells could not grow in HAT selection medium. Whereas, hybridoma cells formed by fusion of spleen cells and myeloma cells should grow successfully in the medium. On the first day of fusion, all types of cells were observed under inverted microscope. Three days later, few small hybridoma cell clones appeared to grow in the medium. As the cells proliferated, the scale of the growing cell clones became larger and larger, and they reached to a confluence covering 70% of the area at the bottom of the 96-well plates after 9 d of fusion (Fig. 3B). In this study, hybridoma cell clones from 9 wells out of 672 wells showed positive activity of anti-PDPN mAb determined by iELISA with the positive clone rate of 1.3%. The nine hybridoma cell clones were named 2F9, 3E1, 4D1, 4E12, 4F12, 5B3, 7C5, 7A11 and 7D11, respectively. Among them, the 5B3 clone showed highly significant specificity in ELISA and IHC from the initial screening. More information is described later in this article, and the 5B3 hybridoma cell line was selected for further experiments.

Identification of hybridoma cells. To identify antibody isotype of the anti-PDPN mAb produced from the 5B3 cell line, a capture ELISA method was performed by using isotyping mAb kit. The results showed that the
The isotype of 5B3 mAb is IgG 2b (Fig. 4A), the isotype that can be purified by Protein G column and with the longest half-life of about 23 days among all the immunoglobulins. The 5B3 cell line was also used for the follow-up tests.

To analyze chromosomes, the 5B3 cells were fixed on a glass slide, stained by Giemsa cell staining solution, and observed under inverted microscope. The results showed that the chromosome number of 5B3 cell line was 102 ± 4 (Fig. 4B), suggesting that the chromosome number of the hybridoma cell 5B3 was approximately equal to the total chromosome number of spleen cell (39 ± 1) and myeloma cell SP2/0 (66 ± 4). The results further demonstrated that the 5B3 cell line was produced by the successful fusion of spleen cells and myeloma cells.

**Purification of monoclonal antibody from ascites fluid.** The 5B3 hybridoma cells were injected into the peritoneal cavity of pristine-primed Balb/c mice for producing ascites fluid, and the resulted ascites fluid was collected and purified by affinity chromatography using Protein G columns. SDS-PAGE was performed to analyze the purified mAb. The results showed two distinct bands with the heavy chain at 50 kDa and the light chain at 26 kDa (Fig. 5A), indicating that 5B3 mAb was successfully purified. After determination by BCA protein assay, concentration of the purified 5B3 mAb reached 0.6 mg/mL. The titer of the mAb was determined by iELISA and the results showed the titer of anti-PDPN mAb reached 1.28 × 10⁵ (Fig. 5B), demonstrating that the mAb remained active after purification and could be used for further characterization.

**Characterization of the anti-PDPN monoclonal antibody.** The affinity analysis of 5B3 mAb was performed by iELISA, and the parameters of the fitting curves (Fig. 6A) based on equation (1) was shown in Table 2. The results showed the affinity of 5B3 mAb was 2.94 × 10⁸ L/mol, suggesting that the 5B3 mAb was sensitive to PDNP. To further analyze the stability, the 5B3 mAb was incubated at 37 °C and 4 °C then dropped into sections from normal rectal tissues at different days, respectively. The results showed that the mAb remained active after purification and could be used for further characterization.
to ePDPN-His and no cross-reactivity to the other related antigens was observed (Fig. 6C). Western blot results exhibited two distinct bands, GST-ePDPN in lane 1 and ePDPN-His in lane 2, 3 (Fig. 6D), and other bands were multimers of His-ePDPN and degradants of the protein trimer or tetramer, suggesting that the 5B3 mAb specifically recognized the target proteins GST-ePDPN and ePDPN-His. The results further demonstrated that the 5B3 mAb is highly specific to PDPN.

Figure 5. Analysis of purified mAb. (A) SDS-PAGE analysis of the purified mAb. Lane M: Marker, lane 1: ascites fluid, lane 2: the purified 5B3 mAb. (B) Titer of 5B3 mAb was determined by iELISA. The minimum OD value greater than 1 is at a titer of $1.28 \times 10^5$ in this figure, indicating that the antibody remained active after purification. Negative control was ascites from negative mouse. Two replicates were performed for each test.

Figure 6. Characterization of purified 5B3 mAb. (A) Affinity was analyzed by iELISA. Different concentrations (4, 1.5 and 0.5 $\mu$g/mL) of coating antigen (ePDPN-His) were used to determine $[\text{Ab}]$ and $[\text{Ab}]_t$ in the formula that were from $x_0$ in Table 2, and the average affinity was $2.94 \times 10^8$ L/mol. Two replicates were performed in each test. (B) Stability of the 5B3 mAb against PDPN was determined by IHC. The results demonstrated that the mAb maintained good stability in tested temperature. “3” on the Y axis indicated strong positive, “2” on the Y axis indicated normal positive, and “1” on the Y axis indicated weak positive. Each test was performed two replicates. (C) The specificity of the 5B3 mAb was determined by iELISA. Two replicates were performed in each test. (D) The specificity of the 5B3 mAb was determined by western blot. Lane M: Protein Marker, Lane 1: GST-ePDPN, Lane 2, 3: ePDPN-His.
Tissue sections detection for the monoclonal antibody against PDPN. All the staining results of tissue sections dropped 5B3 mAb were highly similar to the positive control dropped D2-40 (Fig. 7). 5B3 mAb specifically recognized the lymphatic vessels and did not recognize any blood vessels in the lung tissue (Fig. 7A) and submucosal lymphatic vessels (not shown). The mesothelioma cells were stained in the membrane, and the staining degree in experimental group was much stronger than that of the positive group (Fig. 7B). Tumor cells were also stained in the sections of spermatogonia (Fig. 7C), suggesting that PDPN was expressed in normal cells of spermatogonia. However, extremely weak non-specific staining of smooth muscle was observed, although the whole staining was same to the positive control in thyroid (Fig. 7D). These results suggested that the specificity of the 5B3 mAb is highly similar to D2-40 except in smooth muscle. The results also demonstrated that our approach to prepare an IHC antibody is feasible and reliable.

Discussion
Although some mAbs against human podoplanin have been produced and are now commercially available, the method and process of McAb preparation described in this study were different with traditional methods. Procurement of the encoding gene of PDPN was the first problem we encountered. Currently, several strategies are available, such as extracting DNA from paraffin wax, extracting RNA from cells, and gene synthesis. Expressing genes in eukaryotic cells needs introns, and may usually face difficulties of obtaining tumor tissues and cell lines that profoundly expressing PDPN. Gene synthesis method has many advantages such as cost-effectiveness, time efficient and sequence accuracy. Therefore, gene synthesis was selected. Meanwhile, it's important for researchers to consider the codon bias between homo sapiens and E. coli for successful expression of the transmembrane protein. The ePDPN was selected for further immune response generator since it is exposed physiologically for antibody access. Graphical codon usage analyser was used for the identification after codon optimization, and the relative adaptiveness of all codon was more than 20%, indicating the optimized codons are likely to facilitate expression of ePDPN in E.coli.

It has been reported that the human ciliary neurotrophic factor with 6-His tag showed soluble expression after optimization under all test conditions21. In fact, it's the first report regarding using recombinant GST-fused PDPN with codon optimization to prepare a monoclonal antibody against PDPN for immunohistochemical diagnosis, and the method used in this study is more efficient than those traditional methods22. Compared to the dysgerminoma tissue immunization, the highly pure PDPN antigen used in this study possess better chances of triggering an immune response, and reducing non-specific antibodies generation, further enhancing the efficiency of screening of monoclonal antibody with high affinity and specificity. Previously published paper showed that the GST-fused recombinant proteins were expressed successfully at high soluble levels in E.coli after codon optimization, including VP1, VP2, and VP3 of Chinese sacbrood virus. High antibody levels and lymphocyte proliferation were induced and promoted via immunization with the purified GST-tag fusion protein24. Our results are consistent with the findings described in references above.

The observation that the ePDPN exhibits different oligomeric states (multimer/monomer) may indicate that the natural PDPN protein may perform biological functions in multimer forms. Dimer may be the most...
stable structure for PDPN, since it is resistant to dissociation under SDS treatment (Fig. 6D). Similar phenomenon was also observed in our previous experiments (data not shown). Another lighter band showed on Fig. 6D may be multimer of His-ePDPN or degradation products from unstable trimer or tetramer of His-ePDPN. For GST-ePDPN, aggregation of the ePDPN protein may be prevented by GST protein fusion, or by folding effects driven by fusion with GST protein.

In the early stage of the study, Freund's adjuvant was used to mix with GST-PDPN fusion protein to immunize Balb/c mice for immunogenic response. However, several hybridoma cells produced by fusing spleen cells from the mice and myeloma cells could not recognize PDPN in sections from paraffin-fixed normal rectal tissues even after six times of cell fusion. Unlike Freund's adjuvant, Quickadjuvant is less likely to destroy the structure of antigen during mixing, and required no emulsification. Furthermore, antibody that recognizes a conformational epitope could be easily obtained from mice immunized with Quickadjuvant. It has been previously reported that mixing of Alteplase antigen with Quickadjuvant produced a specific antibody against recombinant human plasminogen activator. Therefore, Quickadjuvant was used to inject three mice in the study, and mAb cell line 5B3 was produced from these mice after cell fusion for three times. The antibody showed specific binding with PDPN.

Compared to other commercial antibodies, only D2-40 antibody against PDPN has been used for IHC diagnosis in the market to date. In this study, the positive stable hybridoma cell line 5B3 secreting mAb against PDPN was obtained, and the anti-PDPN mAb was purified from ascites with the titer of 1.28 × 10^7. In addition, the 5B3 mAb kept a similarly high stability within 31 days after incubation at 4 °C and 37 °C. Moreover, the isotype of 5B3 mAb is IgG3, a stable and widely used isotype for mAb. The affinity of 5B3 mAb is 2.94 × 10^9 L/mol, while the affinity of D2–40 was not disclosed, suggesting that 5B3 mAb is a high affinity mAb. 5B3 mAb showed great opportunities for wider applications as it exhibited affinity range from 10^9 to 10^11 L/mol. The specificity of the 5B3 mAb was analyzed by iELISA, western blot and IHC. The results showed that 5B3 mAb has an exceptional recognition for PDPN, especially in sections from lung tissue, mesothelioma, seminoma and submucosal lymphatic vessels. The results are in consistency with the IHC results of reported organization tissue sections previously.

In the current study, we have successfully demonstrated a rapid, easy, feasible and reliable method to prepare mAb for IHC diagnosis by expressing part of protein after optimization in E. coli and immunization with Quickadjuvant.

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**Author Contributions**
S.W., Q.Y., and C.X. designed this study. C.X., S.L. and H.C. carried out the experiments with the help of R.W., S.X., and L.Z. C.X. and A.S. drafted the manuscript. A.S. and S.W. contributed to the revision of the manuscript. All the authors read and approved the final version of the manuscript.

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