

Preparation and Functional Verification of LAPTM4B Monoclonal Antibody

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Abstract: Lysosomal-associated transmembrane protein 4 beta (LAPTM4B) is a lysosomal transmembrane protein that is highly expressed in a variety of malignant tumors. In order to prepare LAPTM4B monoclonal antibody with strong specificity and high sensitivity, BALB / c mice were immunized with the target protein, and the spleen of the mice was fused with SP2 / 0. The hybridoma cells that can stably secrete antibodies were screened by indirect Elisa and Western blot. Ascites was prepared and purified to obtain antibodies. The specificity, sensitivity and scope of application of the antibody were verified by Western blot and IHC. Results: Thirty-six hybridoma cells stably secreting antibodies were successfully screened, and nine monoclonal antibodies were obtained. One of them had good characteristics in Western blot and IHC, which laid a foundation for further study of the biological characteristics of LAPTM4 B and the development of blocking antibodies for tumor therapy.

Keywords: LAPTM4B; Monoclonal Antibody; Immunoblotting; Immunohistochemistry.

1. Introduction

Lysosomal-associated transmembrane protein 4 beta (LAPTM4B) is a lysosomal transmembrane protein, which is abnormally highly expressed in various malignant tumors such as liver cancer[1], breast cancer[2, 3], gastric cancer[4], ovarian cancer[5] and lung cancer[6]. It is closely related to the proliferation, invasion, drug resistance and poor prognosis of tumor cells. Blocking its expression can not only inhibit the proliferation and migration of tumor cells, but also enhance the sensitivity of chemotherapy drugs[7, 8].

At present, the research on monoclonal antibodies against LAPTM4B is still in its early stage. Almost all the existing antibodies on the market are polyclonal antibodies. The only monoclonal antibodies are also developed by foreign companies, and the polyclonal antibodies have problems such as low specificity and large differences between batches, so it is difficult to meet the needs of precision medicine and antibody drug development. Therefore, this study aims to develop monoclonal antibodies with strong specificity, high sensitivity and wide applicability for LAPTM4B, which provides an important tool for the functional study of LAPTM4B, the mechanism of participation in disease, and the feasibility of targeted therapy, and also lays a foundation for potential tumor diagnosis.

2. Materials and Methods

2.1. Main Test Materials

LAPTM4B recombinant protein, HRP-Goat Anti-Mouse IgG (H + L), hybridoma growth factor mixture, PR40009 adjuvant, IHC kit were purchased from Proteintech. RPMI 1640 and DMEM basic medium were purchased from Vicente. Adult bovine serum was purchased from Sijiqing; pEG1450 purchased from Ruixi Biologicals; purified filler was purchased from Tiandirenhe; female BALB / c mice were purchased from Vital River, with production license SCXK (Beijing) 2021-000; the SP2 / 0 mouse myeloma cell line was preserved in Proteintech; the laboratory animal license is

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2.2. Animal Immunization

The LAPTM4B recombinant protein was mixed with PR40009 adjuvant at a ratio of 1 : 1. Three female BALB / c mice aged 6 to 8 weeks were selected for immunization, with a total of 4 immunizations, 40ug / mouse in the first three times and 20ug / mouse in the fourth time, with an interval of 2 weeks. One week after the third immunization, blood was collected from the tail vein of the mice, and the serum was separated. The serum antibody titer was detected by indirect ELISA.

2.3. Preparation of Monoclonal Antibody

2.3.1. Cell Fusion

The spleen of the mouse with the highest titer was fused with SP2 / 0 at a ratio of 1 : 3, and then cultured in HAT complete medium containing a mixture of hybridoma growth factors. One week later, RPMI 1640 complete medium containing a mixture of hybridoma growth factors was used for medium exchange.

2.3.2. Screening of Hybridoma Cells

Hybridoma cells were preliminarily screened, sub-cloned (two rounds), picked sub-cloned, and expanded culture using indirect Elisa. Since the antigen Ag29148 used in this experiment was expressed by pET-28a with a 6 × His tag, the unrelated protein (6 × His-GST) expressed in pET-28a was used as a negative control to screen hybridoma cells with good growth status, stable specificity, and high concentration of secreted antibodies.

2.4. Induction of Mouse Monoclonal Antibody Ascites

The selected hybridoma cell supernatant was used for Western blot (WB) experiments. According to the results, excellent cell lines were selected to prepare ascites by intraperitoneal injection of BALB / c mice. Observe the state of the mice every day. After the ascites of the mice is enlarged, the skin of the lower abdomen is wiped with iodine wine, and

the ascites is collected by gently piercing the abdominal cavity with a 5 mL syringe. The collected ascites was added to the centrifuge tube and centrifuged at 8000 r / min for 10 min. The supernatant was collected as ascites and packed into a 1.5 mL centrifuge tube and stored at -80 °C.

2.5. Subtype Identification

The subtype and titer of ascites in mice were determined by indirect Elisa method. The primary antibody was mouse ascites diluted 1000 times, and the secondary antibody was HRP-labeled goat anti-mouse secondary antibodies IgG1, IgG2a, IgG2b, IgG2c, IgG3, IgM, IgA and IgE.

2.6. Purification of Mouse Ascites

After subtype determination, Protein G was used for IgG1 subtype antibodies, and Protein A was used for IgG2a, IgG2b, IgG2c, and IgG3 subtype antibodies.

2.7. Identification of Monoclonal Antibodies

2.7.1. Identification of Specificity and Sensitivity of Monoclonal Antibody

The purified monoclonal antibody was used as the first antibody, the working concentration was 0.1 μ g / ml, HRP-Goat Anti-Mouse IgG (H + L) was used as the second antibody, K-562, rat retina, brain, heart, lung, liver, colon and spleen lysates were used as sample proteins for WB experiments to identify the specificity and sensitivity of monoclonal antibodies (monoclonal antibodies).

2.7.2. Applicability Identification of Monoclonal Antibodies

In order to determine the applicability of the prepared monoclonal antibody in IHC, the purified monoclonal antibody was used as the first antibody, and the working concentration was 0.1 μ g / ml. The brain, heart, lung and spleen tissues of rats were selected for IHC experiments.

3. Analysis of Results

3.1. Screening of Positive Hybridoma Cells

Through indirect Elisa, the false positive holes were screened for many times, and the positive holes with high OD450nm value were selected for preliminary screening, two subclones and pick subclones. A total of 36 monoclonal antibody cell lines that can stably secrete LAPT M4B protein were screened.

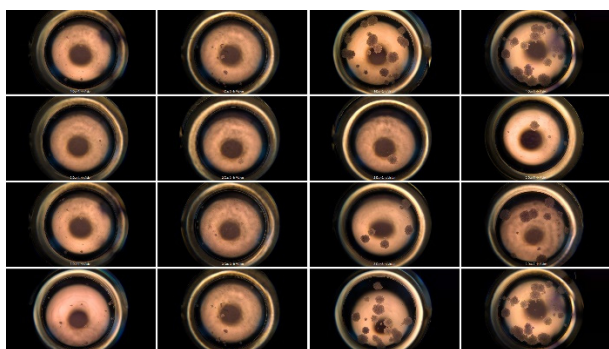


Figure 1. 4x field of view to observe the growth of hybridoma cells for a week

3.2. Western Blot Results of Cell Supernatant

After the hybridoma cells were expanded and cultured, the cell growth state was observed by microscopy. When appropriate, the supernatant was taken for WB detection. The

sample protein was selected from Hela lysate, and the sample was 60 μ g per well. As shown in the Figure 2 diagram, almost all cells have detection signals around 35-50 kDa. Thus, hybridoma cells that can stably secrete LAPT M4B monoclonal antibody and have high antibody sensitivity were successfully cloned.

3.3. Subtype Identification Results

The positive cell lines detected by Western blot in the supernatant of hybridoma cells were injected into the abdomen of mice, and the ascites of 9 mice were successfully collected. The subtypes were detected by indirect Elisa. The results are shown in Table 1. The subtypes of 1A4D8, 1C9E5, 1D11C2, H4F7D, 1H9D12 and 2A7F7D were IgG2a.

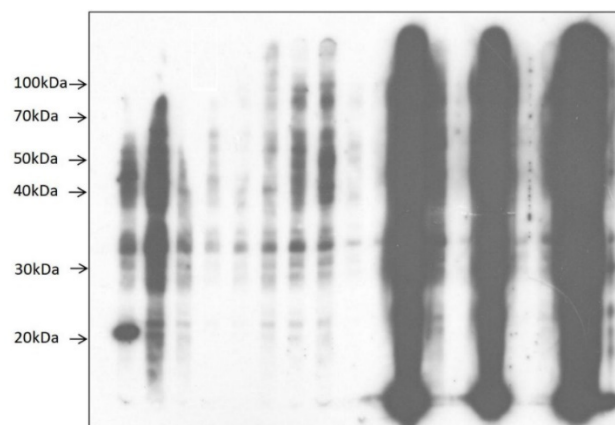


Figure 2. WB results with the supernatant of hybridoma cell lines

3.4. Ascites purification results

Protein A and Protein G fillers were used to purify the ascites that had been subtype-detected. Each ascites was purified by 1ml.

Table 1. Subtype identification and purification results

Cell line number	Subtype type	concentration μ g/ml
1A4D8	IgG1	1014.6
1C9E5	IgG1	563.3
1D11C2	IgG1	791.1
1H4F7D	IgG1	947.5
1H9D12	IgG1	863.2
2A7F7	IgG1	354.9
2F4C2	IgG2a	2384.5
2G9E3	IgG2a	2398.1
2H3G11	IgG2a	2411.6

3.5. Identification Results of Specificity and Sensitivity of Monoclonal Antibody

WB experiments were performed on the 9 monoclonal antibodies obtained. Except for 3 monoclonal antibodies of 2H3G11, 2G9E3 and 2F4C2, the target bands were not obtained, and the remaining 6 monoclonal antibodies were consistent and excellent in specificity. A clear band was generated at 35-50 kDa, but the sensitivity was significantly different. Some monoclonal antibodies can efficiently detect low-abundance proteins within 1 minute, while some rely on longer development time. Among them, the antibody with the best performance was 2A7F7. A clear and single band was detected in K-562 cell line samples and most tissue samples, and the signal was observed at 1 s and 10 s short development time. The antibody has excellent specificity and high sensitivity. The PVDF membrane used in the detection of 2A7F7 monoclonal antibody was verified by internal reference protein GAPDH. The results showed that a band of

nearly 35 kDa was detected in all samples on the 2A7F7 PVDF membrane using the internal reference protein GAPDH, and the signal was stable at 1 s and 10 s development time, indicating that GAPDH has a relatively stable expression level in these samples and can be used as an internal reference for the standardization of experimental data.

3.6. Applicability Identification Results of Monoclonal Antibodies

LAPTM4B is mainly localized in lysosomes, late endosomes, and cell membranes. The results of immunohistochemistry showed that 2A7F7 monoclonal antibody had specific staining in rat brain, heart, lung and spleen. The color of rat brain, heart and lung was the darkest, and the color of spleen was the lightest. 2A7F7 monoclonal antibody [9] is suitable for IHC detection.

4. Discussions

Recent studies have shown that LAPTM4B is abnormally highly expressed in various malignant tumors such as liver cancer, breast cancer, gastric cancer, ovarian cancer and lung cancer, and is closely related to cell proliferation, invasion, drug resistance and poor prognosis. Especially in liver cancer, LAPTM4B promotes the survival and proliferation of tumor cells by activating PI3K / Akt / mTOR and MAPK / ERK signaling pathways[7]. In addition, LAPTM4B can also regulate ABC transporters (such as P-gp) and reduce the sensitivity of tumor cells to chemotherapeutic drugs, making it an important participant in chemotherapeutic resistance. Therefore, LAPTM4B is considered as a new target for cancer diagnosis and treatment. It is of great significance to develop monoclonal antibodies with high specificity, sensitivity and wide application range for in-depth analysis of the molecular mechanism of LAPTM4B in tumorigenesis, metastasis and drug resistance, and clinical treatment of diseases. At the same time, the preparation of LAPTM4B monoclonal antibodies can also provide key reagents for clinical diagnosis (such as immunohistochemical markers) and targeted therapy (such as antibody-conjugated drugs or immunotherapy synergists).

In this experiment, BALB / c mice were immunized with LAPTM4B recombinant protein. PR40009 adjuvant was used to avoid the influence of immunogen emulsification on mice. The spleen of the highest titer mouse was fused with SP2 / 0 cells. By adding a mixture of hybridoma growth factors instead of feeder cells, the workload was reduced and the risk of contamination during cell culture was greatly reduced. Subsequently, 36 cell lines capable of stably secreting LAPTM4B monoclonal antibodies were successfully

screened by indirect Elisa and WB. These 36 cells were injected into mice, and 9 cases of ascites were successfully obtained. The subtype detection of ascites showed that 1A4D8,1C9E5,1D11C2, H4F7D, 1H9D12, and 2A7F7D subtypes were IgG2a, and 2F4C2,2G9E3, and 2H3G11 subtypes were IgG1. After purification, the above 9 monoclonal antibodies were obtained. The specificity of 9 monoclonal antibodies was verified by WB. Among them, 2A7F7 monoclonal antibody has high specificity and sensitivity, and is suitable for IHC experiments and has a wide range of use.

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