Original Research Article

Preparation of monoclonal antibody against human KIAA0100 protein and Northern blot analysis of human KIAA0100 gene

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ABSTRACT

Monoclonal antibodies (MAbs) are important tools for the study of proteins’ function and structure. But there has been no report on the preparation of MAbs against human KIAA0100 protein up to date. Here, first, we generated the mouse MAb against human KIAA0100 protein using purified recombinant 6×Histidine (6×His)-tagged human KIAA0100 protein segment (1557–2234) as an antigen; then, the mRNA expression of human KIAA0100 gene was detected in U937 cells using Northern blot analysis. The results showed that the mouse MAb against human KIAA0100 protein could sensitively recognize the human KIAA0100 protein using Western blot analysis and immunocytochemistry analysis. Besides, Western blot analysis revealed that human KIAA0100 gene possibly encoded two different protein products (254 kDa and < 250 kDa) in U937 cells. Moreover, Northern blot analysis confirmed that human KIAA0100 gene might produce two different mRNA products (6000–10000 bp and 5000–6000 bp) in U937 cells. The results provide a basis for large-scale production of the MAb against human KIAA0100 protein, which will be useful for the study of human KIAA0100 protein’s function/structure and MAb-targeted drugs in the future.

1. Introduction

Since Köhler and Milstein [1] proposed the method of hybridoma technology and prepared mouse Monoclonal antibodies (MAbs), many antibodies specific to various antigens have been obtained [2]. MAbs have many advantages in comparison with polyclonal antibodies, such as high specificity, easy preparation, and good reproducibility. Nowadays, they are applied to the diagnosis and therapy of an array of human disorders, including cancer and infectious diseases [3]. Besides, MAbs are also important tools for the study of proteins’ function and structure.

Human KIAA0100 gene was identified from a cDNA library of human immature myeloid cell line KG-1 in 1995 [4]. It belongs to the HUGE database, which contains more than 2,400 members identified in the Kazusa cDNA sequencing project [5–8]. Northern blot analysis showed its mRNA was expressed in the brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, thymus, spleen, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, HeLa cells, and KG-1 cells [4]. However, human KIAA0100 protein’s function and structure have remained unknown so far. Moreover, there has been no report on the preparation of MAbs against human KIAA0100 protein up to date.

In this study, recombinant 6×His-tagged human KIAA0100 protein segment (1557–2234) was expressed by the prokaryotic expression vector pET-30a in vitro, and a hybridoma cell line secreting the mouse MAb against human KIAA0100 protein was successfully established. Then, the mRNA expression of human KIAA0100 gene was detected using Northern blot analysis in U937 cells.

2. Materials and methods

2.1. Reagents

Restriction enzymes (NdeI and HindIII), isopropyl b-D-thiogalactopyranoside (IPTG), RNA ladder and pMDTM18-T Vector cloning kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Escherichia coli (E.coli) BL21 (DE3), TIANprep Mini Plasmid Kit and DNA Gel Extraction Kit were purchased from TANGEN Biotechnology Co., Ltd. (Beijing, China). Mouse anti-6×His MAb was purchased from GenScript Co., Ltd. (Nanjing, China). Horseradish peroxidase (HRP)-
conjugated goat anti-mouse IgG antibody was purchased from Proteintech Group, Inc. (Chicago, USA). Ni²⁺ charged Immuno-diabetic AcidColumn (IDA) Sepharose and protein A sepharose were purchased from General Electric Company (Fairfield, USA). Bacitracin chloride (Bac) protein assay reagent kit, enhanced chemiluminescence (ECL) reagents, RevertAid™ First Strand cDNA Synthesis Kit, and RPMI-1640 medium were purchased from Thermo Fisher Scientific Inc. (Waltham, USA). Freund’s complete adjuvant, Freund’s incomplete adjuvant, propidium iodide (PI) and DAB-substrate system were purchased from Sigma-Aldrich Co. (Saint Louis, USA). Fetal bovine serum and Trizol were purchased from Invitrogen (Carlsbad, USA). Radio immunoprecipitation assay (RIPA) lysis buffer was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Immunohistochemical detection kit was purchased from Boster Biotechnology Co., Ltd. (Wuhan, China). Protease inhibitor, DIG High Prime DNA Labeling and Detection Starter Kit II, and DIG Immunohistochemical detection kit was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. Cell culture and extraction of the total protein from U937 cells

Mouse myeloma cells (SP2/0) and U937 cells were obtained from Department of Clinical Hematology, the Second Affiliated Hospital, Xi’an Jiaotong university (Xi’an, China). SP2/0 cells were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO₂. U937 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO₂. In all experiments, cells were used in the log-growth phase. The cultured U937 cells were washed twice with cold phosphate buffered saline (PBS) and lysed in cold RIPA lysis buffer and protease inhibitor for 30 min at 0 °C to extract the total cellular protein. The lysis product was finally centrifuged at 14,000×g for 20 min at 4 °C, and then the supernatant was collected and stored in ~80 °C refrigerator.

2.3. Construction and identification of recombinant plasmid

The nucleotide sequence encoding human KIAA0100 protein segment (1557–2234) was analyzed and optimized using the Optimum™ Codon software. The optimized nucleotide sequence was synthesized by GenScript Co., Ltd. and inserted into NdeI and HindIII restriction sites of prokaryotic expression vector pET-30a. The recombinant plasmid was named pET-30a-KIAA0100 (1557–2234) and transformed into the expression system of E.coli BL21(DE3) cells. The transformed cells were grown in LB agar containing 50 μg/mL kanamycin and incubated overnight at 37 °C. The positive bacterial colonies containing the recombinant plasmid were selected by means of PCR assay and sequencing. The PCR products of the positive bacterial colonies were further puriﬁed by DNA Gel Extraction Kit and sequenced by GenScript Co., Ltd. The positive recombinant E.coli BL21(DE3) cells were further grown in LB medium containing 50 μg/mL kanamycin overnight at 37 °C, and then the recombinant plasmid was extracted from E.coli BL21(DE3) cells using TIANprep Mini Plasmid Kit, further conﬁrmed by restriction endonuclease analysis.

2.4. Expression and puriﬁcation of recombinant protein

The positive recombinant E.coli BL21(DE3) cells were grown in LB medium with 50 μg/mL kanamycin, induced 16 h by 1 mM IPTG at 15 °C, and then the recombinant human KIAA0100 protein segment (1557–2234) was expressed as a fusion protein with C-terminal 6×His-tag. The induced bacteria were centrifuged at 8,000 rpm for 5 min at 4 °C, washed twice with PBS, resuspended in PBS and disrupted by sonication. Broken bacteria were centrifuged at 1,300g for 30 min at 4 °C. The recombinant protein in inclusion body was further dissolved in 8 mol/L urea buffer, and puriﬁed with Ni²⁺-IDA Sepharose aﬃnity column. The puriﬁed recombinant protein was ﬁnally analyzed using 10% sodium dodecysulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

2.5. Concentration and renaturation of puriﬁed recombinant protein

10 kDa ultraﬁltration cube was used to concentrate the puriﬁed recombinant protein. The concentration product was centrifuged at 6,000 rpm for 20 min at 4 °C, and the supernatant was collected. In order to renature the puriﬁed recombinant protein, the supernatant was added sodium lauryl sarcosine (final concentration: 0.5%) and then dialysed in 1 L dialysis buﬀer solution (1×PBS, 0.5% sodium lauryl sarcosine, pH 7.4) using 3.5 kDa dialysis bag for 3 h at 4 °C. The dialysis product was ﬁltered with 0.2 μm ﬁlter after repeating the above dialysis process. The purity and concentration of the renatured recombinant protein were ﬁnally detected by using 10% SDS-PAGE and BCA protein assay reagent kit, respectively.

2.6. Western blot analysis of recombinant protein

The puriﬁed recombinant protein was separated using 10% SDS-PAGE and transferred onto nitrocellulose ﬁlter membrane. The nitrocellulose ﬁlter membrane was blocked by 5% skim milk, further incubated with mouse anti-6×His Mab (dilution: 1:500) overnight at 4 °C, and then followed with HRP-conjugated goat anti-mouse IgG antibody (dilution: 1:3,000) at 27 °C for 1 h and 45 min. The signals were achieved with an ECL detection reagent, and speciﬁc protein bands were observed using GeneGnome chemiluminescence imaging system (Syngene, Britain).

2.7. Immunization of mice and establishment of hybridomas

Healthy BALB/c mice (female, 6–8 weeks old) were injected subcutaneously at different sites with 40 μg of puriﬁed recombinant protein mixed with an equal volume of Freund’s complete adjuvant. Booster injections of the same antigen mixed with an equal volume of Freund’s incomplete adjuvant were given twice subcutaneously at 2-week intervals, and a ﬁnal intravenous injection was given exactly 3 days before harvesting splenocytes. Following a previously published protocol [9], serum titers were detected with an indirect enzyme-linked immunosorbent assay (ELISA). The serum of non-immune and healthy BALB/c mice (female, 6–8 weeks old) was used as negative control. The splenocytes were ﬁnally harvested from the successfully immunized mice and fused with SP2/0 myeloma cells to generate hybridomas.

2.8. Screening hybridomas and Mab production

The titers of the hybridomas supernatants were determined with indirect ELISA, and the supernatants of SP2/0 myeloma cells were used as the negative control. The hybridomas that produced the Mab against KIAA0100 protein were subcloned by serial dilution. After two rounds of subcloning, a hybridoma cell line that secreted the Mab against human KIAA0100 protein was established. 5×10⁶ hybridoma cells were ﬁnally intraperitoneally injected into BALB/c mice to induce ascites containing the Mab against human KIAA0100 protein. The ascites was collected, and then puriﬁed by protein A column. The isotypes of Mab against human KIAA0100 protein were determined.
using Clonotyping System-HRP kit.

2.9. Western blot analysis of MAb against human KIAA0100 protein

The total protein (20 μg) of U937 cells was separated by 6% SDS-PAGE, and transferred onto nitrocellulose filter membrane. The membrane was blocked in 5% skim milk at room temperature for 2 h, incubated with primary antibody (the MAb against human KIAA0100 protein, 1 μg/ml) overnight at 4 °C, and then followed with HRP-conjugated goat anti-mouse IgG antibody (dilution: 1:5,000) at 27 °C for 1 h and 45 min. The signals were developed using ECL, and specific protein bands were observed using chemiluminescence imaging system (Syngene, Britain).

2.10. Immunocytochemistry analysis of MAb against human KIAA0100 protein

Immunocytochemistry analysis was performed using immunohistochemical detection kit according to the manufacturer’s instructions. For simplicity, the cultured U937 cells were washed twice in PBS, resuspended in PBS, dripped on silicon glass slides, and fixed in acetone for 20 min. Endogenous peroxidase was then denatured with 3% H₂O₂ in methanol for 30 min at room temperature. After washing twice with PBS, the cells were blocked with 5% bovine serum albumin (BSA) blocking buffer for 1 h at 37 °C, and then incubated with the primary antibody (the MAb against human KIAA0100 protein, 1 μg/ml) overnight at 4 °C. The cells were further washed twice in PBS and incubated with the secondary antibody for 30 min at 37 °C. After washing twice with PBS, the reaction products were visualized with dihydride 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 3 min at room temperature. The cells were finally washed twice in distilled water, counterstained with hematoxylin, dehydrated in ethanol, cleared and hardened in xylene. The result was observed with optical microscope.

2.11. Northern blot analysis

Trizol was applied to extract total RNA from the cultured U937 cells according to the manufacturer’s instructions. Concentration and quality of the total RNA were respectively evaluated by absorbance at 260 nm and the ratio of 260 nm/280 nm with NanoDrop2000 (Thermo Fisher Scientific Inc, USA). The total RNA integrity was detected by 1% agarose gel electrophoresis. First-strand cDNA was obtained by using the RevertAid™ First-Strand cDNA Synthesis kit according to the manufacturer’s instructions, and a DNA probe (833 bp) was generated by PCR. The PCR was done using the following conditions: pre-denaturation for 3 min at 95 °C; 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 45 s, extension at 72 °C for 150 s; and a final extension at 72 °C for 10 min. The sequences of PCR primers are listed below: 5′-GCAACTACGATTAAGCCA-3′ (forward); 5′-ACAGAGATACCCCCAACA-3′ (reverse). The PCR product was separated by 1.2% agarose gel electrophoresis, purified by using DNA Gel Extraction Kit and sequenced by GenScript Co., Ltd., and then labeled with DIG using DIG High Prime DNA Labeling and Detection Starter Kit II according to the manufacturer’s instructions. The RNA ladder was used as size markers. The total RNA (15 μg) of U937 cells was separated on 0.7% formaldehyde agarose gel electrophoresis and further transferred to nylon membrane filters. The membrane was finally hybridized with the DNA probe using DIG High Prime DNA Labeling and Detection Starter Kit II according to the manufacturer’s instructions. The signals were developed using the DIG detection kit, and the results were observed using chemiluminescence imaging system (Syngene, Britain).

3. Results

3.1. Identification of recombinant plasmid

The 2109 bp PCR product of the positive bacterial colony was observed by 2% agarose gel electrophoresis, which was in accordance with the expected values (Fig. 1A). Sequence determination showed that the fusion gene sequence was correct through the analysis using the Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information Search Database (NCBI). In addition, the restriction digestion analysis showed the two restriction digest products with the size of 2895 bp and 4419 bp were observed by 1.0% agarose gel electrophoresis, which was in accordance with the expected values (Fig. 1B).

3.2. Expression and purification of the recombinant protein

The E.coli BL21(DE3) cells with pET-30a-KIAA0100 (1557–2234) were induced by 1 mM IPTG at 15 °C for 16 h, broken by sonication, and then centrifuged at 1, 3000g for 30 min at 4 °C. SDS-PAGE analysis showed the recombinant protein mainly existed in the broken E.coli BL21(DE3) cells’ sedimentation after centrifugation as inclusion bodies; its molecular weight was 79 kDa, which was in accordance with the predicted value (Fig. 2A). The inclusion bodies were then dissolved in urea buffer, and successfully purified with Ni²⁺-IDA Sepharose column (Fig. 2B).

3.3. The purity and concentration assay of the renatured recombinant protein

The purity of the renatured recombinant protein was detected by 10% SDS-PAGE, and the optical density of the protein bands was analyzed by the BandScan software (The purity of the recombinant protein = the optical density of the band of the recombinant protein ×100% the sum of all bands). The optical density of the renatured recombinant protein was 85%. In addition, the concentration assay indicated the concentration of the renatured recombinant protein was 0.7 mg/mL, and the standard curve for the concentration assay was showed as Fig. 3B.

3.4. Western blot analysis of the recombinant protein

Western blot analysis suggested that the recombinant protein could be recognized by mouse anti-6×His MAb, and its molecular weight was 79 kDa in accordance with bioinformatic predicted value. The result showed that the recombinant protein was successfully obtained (Fig. 4).
3.5. The titer and isotype assay of MAb

Indirect ELISA was used to screen the titer of the mouse antiserum after the third immunization. The results showed that the antiserum's titer of No.5 mouse was the highest, so the No.5 mouse was used to generate the hybridoma cells (Fig. 5). The splenocytes were harvested from the immunized mice and fused with SP2/0 myeloma cells to generate hybridoma cells. The positive hybridoma clones that secreted MAb against human KIAA0100 protein were screened with indirect ELISA. After two rounds of subcloning and purification, a hybridoma cell line that secreted MAb against human KIAA0100 protein was established. 5×10⁵ hybridoma cells were finally intraperitoneally injected into BALB/c mice to induce ascites containing MAbs directly against human KIAA0100 protein. The ascites was finally purified by protein A column. Indirect ELISA showed the titer of the MAb against human KIAA0100 protein was 1:5120000 (Fig. 6). The results of isotype assay showed that the MAb against human KIAA0100 protein was identified as IgG2a.
3.6. MAb recognized the recombinant protein and human KIAA0100 protein

Western blot analysis showed that MAb against human KIAA0100 protein could sensitively recognize the recombinant protein and human KIAA0100 protein (Fig. 7). Notably, two protein bands (254 kDa and < 250 kDa) were observed in U937 cells using Western blot analysis, which indicated that human KIAA0100 gene possibly encoded two protein products in U937 cells.

3.7. Northern blot analysis

The results showed that concentration and absorbance at the ratio of 260 nm/280 nm of the total RNA of U937 cells were 0.8339 μg/μL and 1.94, respectively. The 1% agarose gel electrophoresis showed that the total RNA with an 28S:18S ratio of approximately 2:1 was considered acceptable for use (Fig. 9A). The 1.2% agarose gel electrophoresis indicated that the DNA probe of 833 bp was successfully obtained by PCR (Fig. 9B). The DNA probe sequencing suggested that the sequence of the DNA probe was correct. Moreover, Northern blot hybridization analysis confirmed that human KIAA0100 gene possibly produced two different mRNA products (6000–10000 bp and 5000–6000 bp) in U937 cells. This phenomenon might be due to alternative splicing or alternative initiation of transcription (Fig. 9C).

4. Discussion

Human KIAA0100 gene is contained within HUGE database, which is a database for human large proteins newly identified in the Kazusa cDNA project, the aim of which was to predict the primary structure of proteins from the sequences of human large cDNAs [5]. Over 2400 novel human genes have been isolated in this project from the cDNA libraries, which were derived from human fetal brain, adult whole brain, amygdale, hippocampus and cultured human immature myeloid cell line KG-1 [4,10–21]. Human KIAA0100 gene encodes a putative protein of 2235 amino acid residues with a calculated molecular weight of 254 kDa. Human KIAA0100 proteins’ function and structure have remained unknown so far. Breast cancer-overexpressed gene 1 (BCOX1) is an alternative splicing variant of human KIAA0100 gene, which encodes a putative protein of 222 amino acid residues with a calculated molecular weight of 24.920 kDa. Its mRNA expression was moderately elevated in ductal in situ carcinoma (DCIS), peaked in invasive breast carcinoma (IBC) and metastatic breast carcinoma cells (MET) whereas absent in benign ductal epithelial cells [22]. Besides, high levels of BCOX1 expression were associated with poor prognosis in patients with invasive ductal carcinomas of the breast [23]. Furthermore, microRNA-195 could...
suppress tumor cell proliferation and metastasis by directly targeting BCOX1 in prostate carcinoma [24].

MAB is a sensitive recognition biomolecule applied in chemical analysis [25], and an important tool for the study of proteins’ function and structure. It has not only fueled breakthrough discoveries in basic research, but has also been developed as clinical diagnostics, reagents for high throughput drug screening, and more importantly, life-saving medicines [26]. However, there has been no report on the preparation of MABs against human KIAA0100 protein up to date.

In this study, prokaryotic expression systems were used to express recombinant human KIAA0100 protein segment (1557–2234) in vitro, and then recombinant human KIAA0100 protein segment (1557–2234) was used as antigen for the development of MAB against human KIAA0100 protein. A hybridoma cell line stably secreting MAB against human KIAA0100 protein was successfully established. The results from Western blot analysis and immunocytochemistry analysis showed that MAB against human KIAA0100 protein could sensitively recognize human KIAA0100 protein. This is the first report on the preparation of MAB against human KIAA0100 protein in the world. Moreover, Western blot analysis showed that human KIAA0100 gene possibly encoded two different protein products (254 kDa and < 250 kDa) in U937 cells. In order to explain this phenomenon, Northern blot hybridization was performed to detect human KIAA0100 gene expression in mRNA level in U937 cells, and the result showed that human KIAA0100 gene might produced two different mRNA products (6000–10000 bp and 5000–6000 bp) in U937 cells. So we surmised that the another KIAA0100 protein product(< 250 kDa) was possibly produced by the alternative splicing or alternative initiation of transcription (5000–6000 bp) of human KIAA0100 gene. This indicated that human KIAA0100 gene’s function may be complicated. In conclusion, this paper lays a foundation for the study of human KIAA0100 protein’s function/structure and MAB-targeted drugs.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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