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Molecular Cloning of a Tumor-Associated Antigen Recognized by Monoclonal Antibody 3H11

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Monoclonal antibody (MAb) 3H11 can bind specifically to different cancer cells from different tissues. MAb 3H11 labeled with radioactive isotopes has been used clinically to detect primary cancer and metastatic cancer. Molecular cloning of the antigen recognized by MAb 3H11 is important in studying tumor occurrence and in developing new biotherapy for cancer. Using MAb 3H11, we screened cDNA library made from the human gastric cancer cell line MGC 803, which reacts with MAb 3H11, and isolated one positive clone specifically recognized by the antibody. The insert cDNA fragment was 0.5 kb. After recombining with glutathione-*S*-transferase expression vector pGEX-4T, the cDNA fragment could be expressed into a fusion protein that specifically reacted with MAb 3H11. Moreover, the fusion protein could competitively inhibit MAb 3H11 binding to MGC 803 cells. Based on the nucleotide sequence of the cDNA fragment, the full length of the cDNA (2156 bp) was obtained by Rapid-Amplification-cDNA-End (RACE) and nested PCR. Its reading frame was 1767 bp encoding a protein of 589 amino acids. Sequence analysis indicated that there is no highly homologous gene in the GenBank. Northern blot and RT-PCR showed that the mRNA of MAb 3H11 antigen was extensively distributed in embryonic tissue and in different cancerous tissues, but not in corresponding normal tissues. Moreover, in producing antibodies to the antigen expressed prokaryotically, we found that the immunogenicity of the antigen was low in mammalian. Thus we believe that this novel antigen acts as an expression regulator in embryo cells and regains expression in tumor cells. In addition, this antigen is characterized by low differentiation and high proliferation. Molecular function of the antigen needs to be investigated. © 2001 Academic Press

Key Words: **molecular cloning; monoclonal antibody; tumor associated antigen.**

Tumor markers play an important role in cancer diagnosis and early detection of recurrence. Because a tumor specific marker has not yet been found, tumor makers currently being used in clinical practice include certain enzymes, hormones, carcinoembryonic protein, monoclonal antibody-defined epitopes, adhesion molecules, angiogenesis, oncogenes, suppressor genes, and oncogenes and suppressor genes encoded protein products [1]. Many tumor markers defined by monoclonal antibodies have been successfully applied in clinical detection of different tumors [2, 3]. However, only a few of the genes for these markers have been cloned [4, 5], primarily because the epitopes are not protein or are not linear protein.

Monoclonal antibody (MAb) 3H11 was derived from mice immunized with different cancer cell lines. In immunohistochemical experiments MAb 3H11 can bind specifically to different tumors of such origins as liver, lung, bladder, breast, colon, stomach, and some tumor cell lines including gastric cancer cell line MGC 803 cells [6]. Furthermore, isotope-labeled MAb 3H11 has been used clinically as a radioimaging reagent to detect primary cancer and metastastic cancer [7]. The critical step in investigating the normal function of an antigen and its role in carcinogenesis is to isolate its cDNA.

Previous studies have shown that the epitope recognized by MAb 3H11 is a protein, not a carbohydrate group. However, MAb 3H11 could not immunoprecipitate any protein from a MGC 803 cell lysate, nor detect a distinct protein band in a Western blot. Purifying MAb 3H11 antigen from MGC 803 cell extracts by immuno-affinity chromatography and gel filtration is difficult, so oligonucleotide-probes for molecular cloning are unavailable. In the present study, we cloned a cDNA fragment of the antigen that binds MAb 3H11 by immunoscreening the MGC 803 cDNA expression library. Then, we cloned the cDNA full length of the antigen by "Rapid-Amplification-cDNA-End" (RACE) and nested PCR and studied the expression of the antigen in cancerous tissues.

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MATERIALS AND METHODS

cDNA library construction. mRNA purification and poly(A)+ mRNA enrichment were processed with a Messenger RNA Isolation Kit (Stratagene) from 5×10^7 MGC 803 cells. A cDNA library was prepared and packaged with ZAP Express cDNA Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer's instruction. The library quality was determined with its diversity and the average size of the inserted cDNA fragments.

Screening cDNA library. The non-amplified library was plated and transferred to nitrocellulose filters. Initial screening was performed with 1 μg/ml MAb 3H11 (diluted in phosphate-buffered saline containing 1% bovine serum albumin). The filters were then washed in phosphate-buffered saline and bound MAb 3H11 was detected with alkaline phophatase coupled sheep anti-mouse antibodies followed with the mixture solution of nitro blue tetrazolium and bromochloroindolylphosphate. The positive bacteriophages were identified and isolated through two additional rounds of screening. Unrelated antibodies were used as controls. Subsequently, the positive clones were transformed into phagemids under the assistance of ExAssist Helper phages. The phagemid DNA was extracted, and the inserted cDNA fragments were sequenced with T3 and T7 universal primers by Sequenase Version 2.0 DNA Sequencing Kit (USB).

Identification of the immunopositive clone. Inserts of the positive clones were subcloned into *Eco*RI/*Xho*I site of plasmids pGEX-4T-1, pGEX-4T-2, and pGEX-4T-3 respectively, and maintained in *E. coli.* JM109. Glutathione-*S*-transferase (GST) fusion proteins were expressed after induction and purified with Glutathione Sepharose 4B beads (Pharmacia). To determine the specific reactivity of the expressed fusion proteins, a Western blot was carried out with MAb 3H11. The expressed proteins were fractionated by SDS–polyacrylamide gel (10%) electrophoresis, transferred to nitrocellulose filters, and then immunoblotted with MAb 3H11. The fusion proteins that reacted with MAb 3H11 in an ELISA were quantitated and added to MGC 803 coated 96-well-plates. Then, we assessed the effects of the fusion proteins on binding of MAb 3H11 to MGC 803 cells.

Determining the size of MAb 3H11 antigen mRNA. The 20 μg total RNA that was extracted from MGC 803 cells with TRIZOL Reagent (Gibco) was electrophoresed using 1.1 M formaldehyde in 1.0% agarose gel. Then, the product was transferred to nitrocellulose filters and hybridized with ^{32}P-labeled cDNA probe from the insert of the positive clone. X-ray film was exposed and developed the next day.

Cloning the 3′-terminal of MAb 3H11 antigen. Two consecutive sense primers S1 (5′ ACA GCA GCA GTA CAG AGC TG) and S2 (5′ GAA AGT CTA GAG GAA GAA CG) were synthesized corresponding to the 3′-terminal sequence of the cloned cDNA fragment. PCR was performed with the phagemid DNA of MGC 803 cDNA library as template, and primer S1 and T7 as primers. Using 2 μl of the PCR product as template, we conducted nested PCR with S2 and T7. The nested PCR products were separated by electrophoresis, and specifically amplified bands were recovered and cloned into the pCRII TOPO vector (Invitrogen) for DNA sequence analysis.

Cloning the 5′-terminal of MAb 3H11 antigen. Two antisense primers R1 (5′ AAT GTC TAA CGT TGA CTG A) and R2 (5′ CTG AAT TTT CAT ATG AGT CTG) were designed according to the upstream sequence of the immuno-screened cDNA fragment. With MGC 803 cDNA library as template, the first PCR used primers R1 and T3. Using 2 μl of the first PCR product as template, we conducted nested PCR with primer R2 and T3. The amplified sequence was purified and sequenced. Consequently, antisense primer R3 (5′ CTG GTA GTC AAT AAT ATC CTG AC) was designed referring to the 5′-terminal sequence of the new cloned fragment. With Marathon-Ready cDNA (Clontech) from human lung carcinoma as template. Rapid Amplification of cDNA End (RACE) was done with primer R3 and AP1 (5′ CCATCCTAATACGACTCACTATAGGGC), and then R3 and AP2 (5′ ACTCACTATAGGGCTCGAGCGGC) according to the kit manual instruction. On the basis of overlapping sequence, the whole cDNA molecule was completely ligated.

Studying the distribution of MAb 3H11 antigen. A variety of human tumor tissues and corresponding normal tissues were obtained from surgical specimens after fresh resection. Cultured cells of different tumor cell lines were collected. Total RNA was purified with TRIZOL Reagent, and 20 μg of each was electrophoresed, transferred to nitrocellulose filters, and probed by ^{32}P-labeled MAb 3H11 antigen cDNA.

Preparing antibodies against MAb 3H11 antigen. MAb 3H11 antigen cDNA fragments of different sizes were subcloned into pGEX-4T. GST-fused proteins were expressed in *E. coli* JM109 and purified. Rabbits and mice were immunized, respectively. Polyclonal antibodies and monoclonal antibodies were produced with regular protocols. Immunoprecipitation and Western blot experiments were performed with the produced antibodies and MGC 803 cell lysates.

RESULTS

cDNA library construction and analysis. We obtained 5 μg poly(A)+ enriched mRNA for synthesizing the cDNA library. Double-stranded cDNA was generated by means of "nick-translation". *Eco*RI adapters were ligated to the double-stranded cDNA, which was then digested with *Xho*I and size selected. Only the cDNA molecules larger than 500 bp were collected and ligated into ZAP expression vectors. The diversity of the primary library was 1.2×10^6 pfu. The recombinant rate was 9/9, and the average size of cDNA inserts was about 2.0 kb.

Screening cDNA library. A total of 1.5×10^6 of non-amplified library clones was screened with MAb 3H11. We isolated one positive clone, which was specifically recognized by MAb 3H11, but not by other antibodies. The restriction map showed that it contained a 540 bp cDNA fragment. The DNA sequence indicated there was no highly homologous molecule in the GenBank, and one of its three reading frames can go through the full length.

Identifying the positive clones. To exclude false positive clones, which often happen in screening libraries with antibodies, the 540 bp fragment was cloned into pGEX-4T-1, pGEX-4T-2, and pGEX-4T-3, respectively to be expressed in different reading frames. Purified GST fusion proteins were fractionated and immunoblotted with MAb 3H11. The protein expressed from pGEX-4T-1, which was from the only reading frame going through the full length of the cDNA fragment, could be specifically recognized by MAb 3H11 (Fig. 1). Moreover this protein could competitively inhibit the binding between MGC 803 cells and MAb 3H11, but had no effect on the binding between MGC 803 cells and other antibodies (data not shown).

Determining the size of MAb 3H11 antigen mRNA. Total RNA preparations were subjected to Northern analysis with the random primer ^{32}P-labeled cDNA

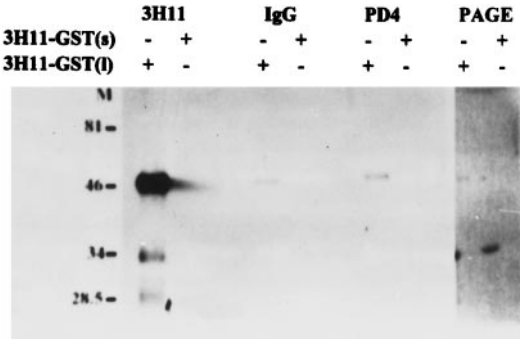

FIG. 1. Western blot: Equal amounts of 3H11-GST(1) (expressed in pGEX-4T-1) and 3H11-GST(s) (expressed in pGEX-4T-3) were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters to be immunoblotted with MAb 3H11, MAb PD4, and mouse IgG. MAb 3H11 specifically reacted with 3H11-GST (1). M, protein standards (KD).

probe. The only detected band was near the 18S RNA site, about 2.3 kb.

Full-length cloning of MAb 3H11 antigen. With the phagemid DNA of MGC 803 cDNA library as template, primer S1 and T7 as primers, the first PCR had no distinct amplified bands. With primary PCR product as the template, and S2 and T7 as primers, nested PCR showed a specifically amplified band. DNA of this band was recovered and sequenced. It was 643 bp and had an 87 bp overlap with the 3′-terminal of the immunoscreened cDNA fragment. According to the defined reading frame, the cloned 3′-terminal had a stop codon, a poly(A) signal and a poly(A) tail.

With MGC 803 cDNA library as template, R1 and T3 as primers, the first PCR had no specific products. Using the first PCR products as template, we conducted nested PCR with primer R2 and T3. The amplified DNA fragment was 0.8 kb, and had a 63 bp overlap with the 5′-terminal of the immunoscreened cDNA fragment. Its 5′-terminal was still open.

The further 5′-terminal was cloned by RACE with Marathon-Ready cDNA as template. It had a 57 bp overlap with the cloned sequence, and was no longer open at its 5′-terminal. The full length of the cDNA molecule (2156 bp) was ligated, and its reading frame was 1767 bp encoding a protein of 589 amino acids (Figs. 2 and 3). The GenBank Accession No. assigned to this sequence is AF317887. Its sequence data indicated there was no highly homologous gene in the GenBank before August of 2000.

Expression spectrum of MAb 3H11 antigen. As shown in Fig. 4, mRNA of MAb 3H11 defined antigen was extensively distributed in cancerous tissues, but was generally not detectable in corresponding normal tissues. PT-PCR indicated this antigen was also expressed in embryo tissue and placenta tissue. These results were consistent with the immunohistochemical results of MAb 3H11 (data not shown).

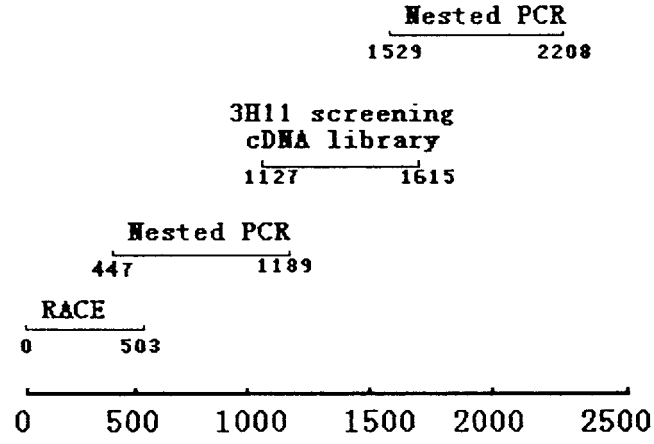

FIG. 2. Sketch of molecular cloning of MAb 3H11 antigen.

Antibodies against MAb 3H11 antigen. Different fragments of the antigen cDNA were subcloned into pGEX-4T and expressed in *E. coli* JM109. The fusion

gagattgaaatattaacaaaggaaatcaataaacttgaattgaagatcagtgatttccct
E I E I L T K E I N K L E L K I S D F P
gatgaaaatgaggcacttagagagcgtgtgggccttgaaccaaagacaatgattgattta
D E N E A L R E R V G L E P K T M I D L
actgaatttagaaatagcaaacacttaaaacagcagcagtacagagctgaaaaccagatt
T E F R N S K H L K Q Q Q Y R A E N Q I
cttttgaaagagattgaaagtctagaggaagaacgacttgatctgaaaaaaaaaattcgt
L L K E I E S L E E E R L D L K K K I R
caaatggctcaagaaagaggaaaaagaagtgcaacttcaggattaaccactgaggacctg
Q M A Q E R G K R S A T S G L T T E D L
aacctaactgaaaacatttctcaaggagatagaataagtgaaagaaaattggatttattg
N L T E N I S Q G D R I S E R K L D L L
agcctcaaaaatatgagtgaagcacaatcaaagaatgaatttctttcaagagaactaatt
S L K N M S E A Q S K N E F L S R E L I
gaaaaagaaagagatttagaaaggagtaggacagtgatagccaaatttcagaataaatgc
E K E R D L E R S R T V I A K F Q N K C
tatagaatcaaagaatgcagaaggaatctttgatgcgagtctgcatttgaatgcccaagt
Y R I K E C R R N L –
tgatcagcttaccggaagaaatgaagaattaagacaggagctcagggaatctcggaaaga
ggctataaattattcacagcagttggcaaaagctaatttaaagatagaccatcttgaaaa
agaaactagtcttttacgacaatcagaaggatcgaatgttgtttttaaaggaattgactt
acctgatgggatagcaccatctagtgccgatcattaattctcagaatgatatttaataca
tttgttacaggaactagaaaaaaaaaaaaaaaa

FIG. 3. Nucleotide sequence and derived amino acid sequence of the antigen recognized by MAb 3H11. The deduced reading frame is 1767 bp, and the encoded protein is 589 aa. The start and stop codons are indicated in bold.

proteins were purified to immunize Balb/c mice and rabbits. Although monoclonal antibodies could specifically react with MAb 3H11 antigen expressed in bacteria, none of them react with MGC 803 cells with ELISA. Polyclonal antibodies from rabbits and total proteins of MGC 803 were used for immunoprecipitation. No distinct protein band was obtained on SDS–PAGE. A specific band, about 70 KD was detected on Western blot, although the signal was not very strong (Fig. 5). The molecular size of the band was consistent with the cDNA sequence information.

DISCUSSION

MAb 3H11 can specifically bind to different tumor tissues of many origins. Labeled with isotope, it has been used as a radioimaging reagent to detect primary and metastatic cancer in patients. However, the antigen that binds MAb 3H11 has not been identified. Positive results have not been obtained in immunoblot and immunoprecipitation assays with MAb 3H11, so purifying this antigen has been difficult. The cloning of the antigen by immunoscreening the cDNA expression library initiated the study of this important molecule. To get the desired antibodies, different regions of the cloned cDNA were expressed in a prokaryotic system, and the GST fusion proteins were used to immunize mice. We found that the immunogenicity of the antigen was quite low in mammalian, indicating that this gene may be highly conserved in mammalian species.

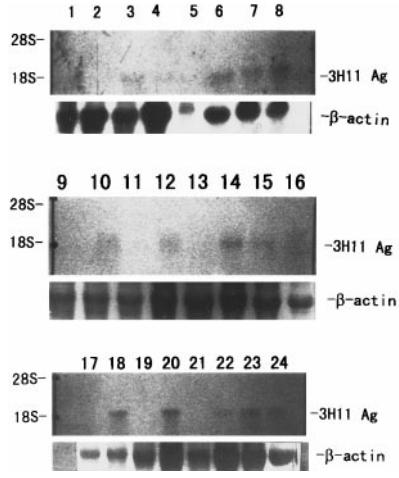

FIG. 4. Northern blot: 20 μg of total RNA of each samples was electrophoresed, transferred to nitrocellulose filters, and probed with ^{32}P-labeled MAb 3H11 antigen cDNA. mRNA of MAb 3H11 defined antigen was extensively distributed in cancerous tissues such as colon (3, 18), ovary (4, 24), cardiac (10), rectum (12), lung (14), breast (15, 23), esophagus (20), and the following malignant cell lines: leukemia K562 (6), cervical cancer Hela (7), gastric cancer MGC 803 (8, 16), and bladder cancer B10 (22). Then antigen was generally not detected in corresponding normal tissues: colon (2, 17), cardiac (9), rectum (11), esophagus (19), and endothelial cells. No expression was detected in (1) lung cancer tissue and (13) kidney carcinoma. mRNA quality of the fleshy tumor tissue (5) was not good enough.

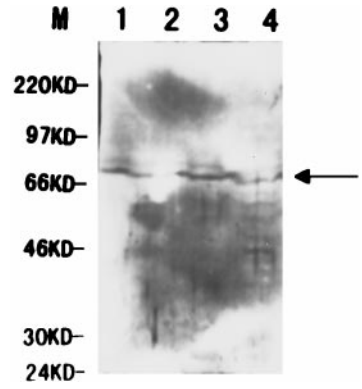

FIG. 5. Western blot developed with ECL reagents: Polyclonal antibodies against MAb 3H11 antigen can detect a specific 70 KD protein band in the membrane of leukemia K562 cells (1), MGC 803 cell membrane (2), raw extracts of MGC 803 membrane protein (3), and total protein of MGC 803 (4). M, protein markers (KD).

The mRNA of MAb 3H11 antigen is extensively distributed in cancerous tissues but not in corresponding normal tissues, and the immunogenicity of the antigen is low in mammalian; therefore, we believe that the antigen may act as an expression regulator in embryo cells and may regain expression in tumor cells. The antigen is also associated with low differentiation and high proliferation.

Because no homologous sequence was found in the GenBank, we could not obtain information about the function of this antigen. Intensive work is now underway to identify the function of this molecule in normal and cancerous cells.

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