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P^{53} FUSION PROTEIN EXPRESSION IN PROKARYOTE AND PREPARATION OF MONOCLONAL ANTIBODY TO P^{53}

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Objective: Conventional immunohistochemistry (IHC) is available to assess P^{53} mutations, and expensive imported anti-P^{53} monoclonal antibody has been used in China, it is necessary to study a new monoclonal antibody. Methods: The P^{53} DNA fragment enconding N-terminal 180 amiao acide was obtained by PCR and was cloned into P^{GEX}-2T plasmid expressing glutathione S-transferase (GST). The P^{53}-GST fusion protein expressed by JM109 was used for immunizing BALB/C mice. We have raised one hybridoma strain secreting McAb to human P^{53} (named M126). Results: The IHC analysis of 52 paraffin-embedded sections from human breast cancer with M126 and $P_{AB}1801$ (Zymed Co.) has showed that the positive immunoreactions were 25 cases (48%) and 22 cases (42.3%) respectively. The staining of M126 was stronger and preferable to $P_{AB}1801$. Conclusion: M126 can be instead of $P_{AB}1801$ for studying immunohistochemical analysis on P^{53} protein.

Key words: P^{53} fusion protein, Monoclonal antibody, Immunohistochemistry

The wild-type P^{53} gene, a tumor suppressor gene,[1] encoding a 53KD nuclear phosphoprotein involves in the negative regulation of cell growth. However, mutations of P^{53} plays an important role in the development of many human malignancies.[2] *In vitro* and *in vivo* analyses have shown that P^{53} mutation can lead to resistance to chemo- and radiation therapy,[3] DNA repair reduction,[4] genomic instability[5] and poorer prognosis.[6] This indicated that inactivation of P^{53} can cause progression of the tumor development. Several studies have reported P^{53} mutation to be an independent marker for poor prognosis in breast cancer.[3,7] Normal P^{53} protein has a very short half-life and thus the protein level is too low to be identificated immunohistochemically. However, most mutant P^{53} protein have a longer half-life[7] and are easily detected by IHC. Quick and simple IHC can provide strong evidence of such mutations. In present paper to the P^{53} cDNA fragment amplifi-cation, P^{53} and P^{GEX}-2T recombination, P^{53}-GST fusion protein expression, production and characterization of a new mouse monoclonal antibodies to human P^{53} are described, and it would be suitable for immuno-histochemical analysis.

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MATERIALS AND METHODS

Animal and Agent

Six-week-old female BALB/C mice purchased from Beijing Medical University for immunizing and ascites production; Positive control McAb $P_{AB}1801$, Bio-anti-mouse IgG, Strepavdin/horseradish peroxidase (Zymed Co.)

Recombinant Plasmid Construction and Fusion Protein Expression

Human P^{53} cDNA fragment encoding N-terminal 180 amino acids was amplified by PCR. The corresponding cDNA digested by restriction endonuclease BamHI and EcoRI was cloned into P^{GEX}-2T plasmid expressing glutathione S-transferase (GST). (Figure 1).

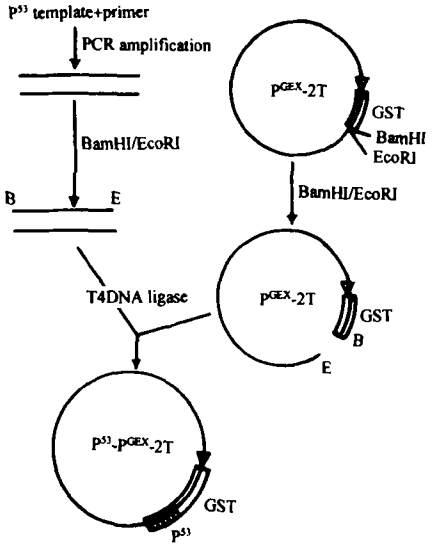

Fig. 1. The amplification by PCR and the cloning of P^{53} cDNA fragment.

50ng recombinant plasmid P^{53}- P^{GEX}-2T was added to 100 μl freshly prepared competent JM109 on ice for 45 minutes. The tube was incubated in a 42℃ water bath for 2 minutes, then chill briefly on ice, immediately transfer the transformed cells to a 1.5 Eppendorf tube containing 9000μl of LB medium (prewarm to 37℃) and incubated for 1 hour at 37℃ with shaking (250rpm). 100μl of the transformed cells were inoculated into LBA plates. Negative control was made in parallel. Incubated the plates at 37 ℃ overnight, picked several colonies into separate tubes containing 2 ml LBA. The cultures were grown to an OD_{600} of 0.6—0.8 with vigorous agitation at 37℃. The fusion protein expression was induced by adding 2μl of 100mM IPTG (fin. con. 0.1mM) and continuely incubated for an additional 1—2 hours. Centrifuged the sample and discarded the supernatant, added 100μl bacterial lysis solutiion containing 1mM PMSF and 1% Triton X-100 on ice for 20 minutes, then sonicated on ice, centrifuged at 10,000rpm for 10 minutes at 4℃. Saved the aliquots of the supernatant and the cell debris pellet for analysis by SDS-PAGE. These samples can be used to identify the localization of fusion protein. Purification of large-scal bacterial sonicates was performed by SDS-PAGE, staining with cold 0.1 MKC1, cutting the fusiion protein band, electroelution, concentrating and dialysing.

Production of A New Monoclonal Antibody to P^{53}

Mice were immunized with purified P^{53}-GST by traditional methods. The immunization dose was 50μg P^{53}-GST/mouse/time. Three days after the final immunization, one mouse was sacrificed and its spleen was removed aseptically. The spleen lymphocytes were separated and fused with SP2/0 myeloma cells in 50% PEG. The fused cells were suspended in 100ml DMEM Medium supplemented with HAT; about 10 days later culture supernantants were added to ELISA plates coated with P^{53}-GST and P^{21}-GST, respectively. The specificity of McAbs which were positive to P^{53}-GST and negative to P^{21}-GST was confirmed by IHC with paraffin sections from human brest cancer, which reacted with P_{AB}1801. Cells from IHC positive wells were cloned by limiting dilution and tested McAbs repeatedly to obtain stable hybridoma.

Comparison between M126 and P_{AB}1801 by IHC

In the light of SP method, paraffin sections were dewaxed for 15 minutes in two changes of xylene and rehydrated in graded alcohol. Endogeneous peroxidase was blocked by immersing the sections for 25 min. in 0.3 percent hydrogen peroxide in absolute methanol. Non specific binding was blocked by incubating the slids in 0.1% human serum albumin in phosphate-buffered salin (PBS) for 30 min. The sections were first incubated overnight at 4℃ with a primary McAb P_{AB}1801 and M126, the PBS was taken as negative control. Followed by a secondary bio-tinylated anti-mouse antibody at a concentration of 1:500 for 30 min, the slides were treated in strepavidin/horseradish peroxidase complex at 1:1000 dilution for 20 min. Careful rinses and several changes of PBS between each stage of the procedure were done. The colour was developed with 0.5mg/ml diamino-benzidine (DAB) prepared in PBS containing 0.03% H_2O_2, whereafter the slides were lightly counterstained with haematoxylin, dehydrated and mounted. Only nuclear staining was interpreted to be positive. The degree of P^{53} positive was graded using Sreenan methods.[9]

RESULTS

Recombinant Plasmid Construction and Fusion Protein Expression

Figure 2 has shown that there is one more band at about 50KD in bacterial lysate inducing by IPTG than that without induction. It accorded with the estimated P^{53}-GST molecular weight. DNA plasmids extracted from the bacterium expressing P53-GST were treated with restriction endonucleases BamHI/EcoRI, an about 550bp fragment of inserted DNA was found. This demonstrated that PCR product has been inserted into P^{GEX}-2T vector and have expressed P^{53}-GST fusion protein in the transformed JM109 cells. From Figure 2. It also was found that P^{53}-GST fusion protein was mainly located in supernatant and little in sediment. The pure proportion of fusion protein was over 95% after SDS PAGE and electroelusion.

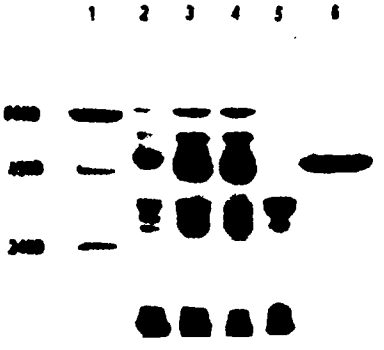

Fig. 2. SDS-PAGE analysis of P^{53}-GST in bacterial lysates. Samples were run on a 10% gel, gel was stained with coomassie blue; lanel. molecular weight standard; lane 2-3. Bacterial lysates without or with IPTG induction, respectively, lane 4-5. The supernatant and the sediment of induced bacterial lysates; lane 6. purified fusion protein P^{53}-GST.

A New McAb M126 Preparation

A hybridoma was selected from one fusion experiment on the basis of both ELISA and IHC. After several cloning and retesting, strong nuclear staining was observed on paraffin sections from human breast cancer which also was recognized by positive control anti-P^{53} McAb $P_{AB}1801$. To asses the value of the selected new anti-P^{53} McAb M126 in immuno-histochemical detection of P^{53} in paraffin-embedded clinical tumor material, 52 specimens were examined by M126, and the staining was compared with that obtained with $P_{AB}1801$. In general, the anti-P^{53} antibodies showed fine granular nuclear staining, of the 52 cases, 22 were positive and 27 negative. The discrepancy noticed between the two types of reagents were 3 cases in which the M126 stained tumor nuclei with stronger intensity while $P_{AB}1801$ appeared to be negative. In Figure 3 A, B indicated the immunostainings of paraffin-embedded from human breast cancer with M126 and $P_{AB}1801$.

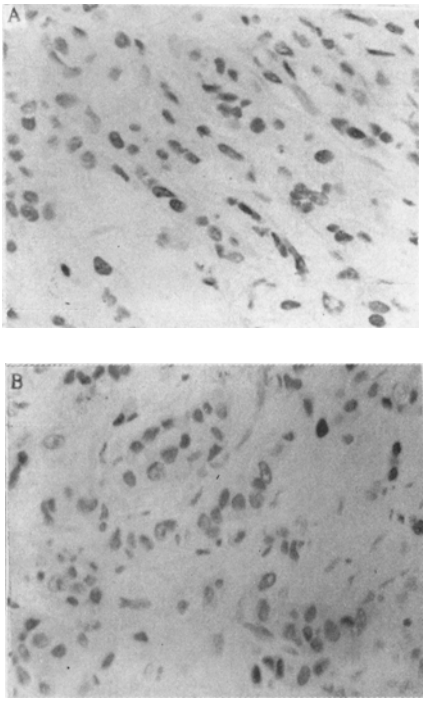

Fig. 3. IHC of P^{53} on paraffin sections from human breast cancer. 500× The stainings were performed by SP and counter- stained using hematoxylin, A and B were stained with McAb M126 and McAb $P_{AB}1801$, respectively.

DISCUSSION

The molecular genetic analysis of P^{53} in human tumors and cell lines have demonstrated that the P^{53} gene is frequently mutated in a wide range of human malignancies.[9] The comparisons of IHC and DNA sequencing data from several laboratories have established a close correlation of an aberrant P^{53} protein accumulation in tumor cells with the presence of point misssense mutations altering the amino acid

residues in the phylogenetically conserved central region of the P^{53} molecule.[10,11] Quick and simple immunostaining method can provide strong evidence of such mutations. P^{53} immunostaining permits not only the identification of tumors with aberrant accumulation of the protein, but also the subclassification of P^{53} positive lesions according to their characteristic staining patterns (nuclear versus cytoplasmic) and the proportion of positive tumor cells within each lesion.[10-12] But the limitation of immunohistochemical identification of the P^{53} aberrations includes the lack of anti-P^{53} reagents applicable on paraffin sections. Import anti-P^{53} McAbs are so costly that the application of P^{53} antibodies are hampered in China. We first obtained one hybridoma secreting anti-P^{53} monoclonal antibody, immunohistochemical analysis of 52 cases paraffin sections from human breast cancer with M126 and $P_{AB}1801$ showed that 25 cases and 22 cases were positive, respectively. The discrapancy noticed between the two types of reagents were 3 cases in which M126 stained tumor nuclei with stronger intensity while $P_{AB}1801$ appeared to be negative. This is possible that the epitope recognized by $P_{AB}1801$is unstable during fixing the tissue in formalin.[13] The different P^{53} antibodies[14] and fixatives[15] can result in different observed frequency of abnormal protein staining. The P^{53} staining patterns on frozen sections from huamn breast cancer and paraffin-embedded sections from human blader carcinoma were also investigated with M126 in order to facilitate future studies of the P^{53} oncoprotein in clinical materials. The results of this immunohistochemical study confirmed the applicability of M126 on paraffin embedded clinical tumor materials, demonstrated its specificity for P^{53} and suggested that the target epitope is mostly denaturation-resistant and that M126 is potentially applicable in immunohistochemical analysis.

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