CHARACTER OF TUMOR ASSOCIATED PROTEIN RECOGNIZED BY MONOCLONAL ANTIBODY AGAINST YUNNAN GEJIU LUNG CANCER

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ABSTRACT

Objectives: To identify and characterize lung cancer associated protein N35 and attempt to learn the prospective possibility of the clinical application of the protein N35. Methods: Immunoprecipitation, immunoblotting, differential centrifigation and subcellular assay, immunohistochemistry, N-glycanase digestion, mitotic cell immunoflourescence and multiple methods of affinity chromatography have been used with the monoclonal antibody N-35 to detect the distribution of the protein N35 among the various cancer cell lines and normal human tissue, the relationship between the protein N35 and glycoprotein, the location of the subcellular structure and chromosomal domain of the protein N35,the most effective way of purification of tumor associated protein N35. Results: The protein N35 is a glycoprotein, distributes to the human lung cancer cell line GLC-82, human cervical cancer cell line Hela, human hepatic cancer cell line HepG-2 and human breast cancer cell line PMC with different relative molecular mass(Mr), but no expression of the protein ingredient in normal human fresh tissue; concentrates at the nuclei significantly ,much more than at the mitochondrail and membrane, locates at the centriole of the chromosomal domain. Conclusions: The lung cancer associated protein N35 might be expressed only by the cancer cells and related with the proliferation of cancer cells as a role of tumor cell growth regulator.

Keywords: Lung cancer cells, Tumor associated protein, Glycoprotein, Centriole

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The detection and characterization of the cancer antigens by the monoclonal antibodies as the immunoprobes has recently been carried out well [1,2,3]. In this study, A novel protein molecule (named protein N35 temporarily) of lung cancer cells had been identified and characterized by immumnohistochemistry, immunoprecipitation, immunoblotting, differential centrifugation and subcellular assay, N-glycanase digestion and mitotic cell immunoflourescence with the monoclonal antibody N-35 which was used as an immunoprobe to detect the distribution of the protein N35 in various cancer cell lines and normal human tissues; the relationship between the protein N35 and glycoprotein; the location at the subcellular structure and chromosomal domain of the protein N35. This protein has been purified and the different affinity chromatographies were optimized to get the most effective purification of the associated protein N35. All of above showed the protein molecule N35 was distributed in the lung cancer cell line GLC-82, human cervical cancer cell line Hela, human hepatic cancer cell line HepG-2 and human breast cancer cell line PMC-42 with different relative molecular mass(Mr), but no expression of the protein ingredient in the normal human heart and lung fresh tissues. The Mr of the associated protein N35 was 75×10^3~130×10^3 and determined as a glycoprotein by N-glycanase digestion with 3 fragments of Mr being 65×10^3,84×10^3 and 86×10^3. The differential centrifugation and subcellular assay showed that most associated protein N35 distributed in the nuclei comparing with that in mitochondrail and membrane and the result was confirmed by immumnohistochemistry. The results also showed by immunoflourescence staining of the mitotic cells that associated protein N35 was located on the centriole of the chromosomal domain at the S-G2 phage. The associated protein N35 might only be expressed by the cancer cells

and related with the proliferation of cancer cells as a role of tumor cell growth regulator.

MATERIALS AND METHODS

Cancer Cell Lines, Tissues and Monoclonal-antibody

Yunnan Gejiu human pneumo-adenocarcinoma cell line GLC-82 and monoclonal antibody N-35 were established in our Institute. The human cervical cancer cell line Hela, human hepatic cancer cell line HepG-2 and human breast cancer cell line PMC- 42 were from Shanghai Institute of Cell Biology. The fresh tissue of normal human heart and lung were from the local hospital.

Immunoprecipitation Assay

Immunoprecipitation assay was used to determine the relationship of lung cancer cells and the monoclonal antibody. Briefly, the cultured cells ($>10^6$) of Yunnan Gejiu human pneumoadeno-carcinoma cell line GLC-82 were harvested and lysed by NP40 at 4℃ for 1h. The lysate was treated with protein-A-shepharose-4B beads to previously deleted unrelated proteins. The supernatant was reacted with supernatant of N-35 at the concentration of 1:100, 4℃ overnight. SDS-PAGE was carried out with the pellet above for the Westernblot assay using N-35 at the concentration of 1:100. The X -ray photos were exposed to view the positive bands activated by chemoluminescebce reagent (ECL).

Immunoblotting Assay

Immunoblotting assay was applied to detect the distribution of associated protein among the cancer cells and normal tissues. The cells ($>10^6$) of human lung cancer cell GLC-82, human cervical cancer Hela, human hepatic cancer HepG-2 and human breast cancer PMC- 42, fresh normal cells from sample of operation section were lysed by NP40 at 4℃ for 1h. The lysate was treated seoerately with SDS-PAGE, then transferred to NC membrane for the Westernblot assay using N-35 at the concentration of 1:100. The X -ray photos were exposed to view the positive bands activated by chemoluminescebce reagent (ECL).

Differential Centrifugation and Subcellular Assay

Differential centrifugation and subcellular assay were used to detect the associated protein and its distribution. The cultured cells ($>10^7$) of GLC-82 were lysed with PMSF buffer at 4℃ for 1h. The initial lysate was centrifuged at 1900 rpm (600g), 10min, 4℃ to get the pellet A, saved pellet A at 4℃ with PMSF. The supernatant from the first centrifugation was centrifuged at 10,000 rpm (10,000g), 20min, 4℃ to get the pellet B, saved pellet B at 4℃ with PMSF. The supernatant from the second centrifugation was centrifuged at 50,000 rpm (26,000g), 20min, 4℃ to get the pellet C, saved pellet C at 4℃ with PMSF. The samples of initial lysate, supernatant of A and C, the pellet of A, B and C were assayed by immunoblotting with the McAb N-35 as the immunoprobe.

Immumnohistochemistry Assay

Immumnohistochemistry assay was used to determine the location of the subcellular structure of associated protei. Avidin-biotin staining (ABC Kit) assay was performed with lung cancer parafine sections were stored at 56℃ for 8 hours before the assay to delete parafine and xylol to water. Digesting 30 min at 37℃ with 0.1% trypsin. After washing with PBS-T, the sections were covered with 1% BSA 30 min at 37℃. McAb N-35 was incubated consequently with tissue at 37℃,for 60 min, biodinconjuhated goat-anti-mouse IgG, at 37℃,for 30 min, at 37℃,for 30 min. The section sample was stained with DAB assay.

Mitotic Cell Immunoflourescence Assay

Live cell immunoflourescence assay was used to detect the localization of associated protein at the chromosomal domain of the mitotic cells. The cells of GLC-82 were cultured on the glass coverslips and harvested after 24 h during their log phase. The live cells on the coverslips were treated consequently with 2.5 % paraformaldehyde, 5 min, 20℃; 1% NP40 10min, 20℃; 0.02% azide 5 min, 20℃; McAb N-35 30min, 37℃; S α M-FICT 20 min, 20℃; 33342 DNA Dye 1 min, 20℃. The coverslips were enveloped with the coverglasses by the mounting media. The photos were taken under the flourescence microscope with the different light sources.

N-glycanase Digestion

Digestion of the associated protein by N-glycanase was carried out with the cultured GLC-

82 cells (>10^7,and lysed with 1% NP40, 30 min 4 ℃. The initial lysate was centrifuged at 15000 rpm,10', 4℃. 75μl of supernatant was mixed with 25μl of N-Glycanase. The mixture was incubated at 37℃, 2h (or 4℃, overnight). The samples of initial lysate and the digested supernatant were detected with McAb N-35 by SDS-PAGE and Westernblot.

Immunoaffinity Chromatographies

Purification of associated protein with immunoaffinity chromatography .The Protein-A-Sepharose-4B beads were sunk in Tris buffer (pH8.6), 4℃, rising for overnight and washed with PBS buffer for 3 times. The supernatant of McAb N-35 was absorbed with treated protein-A-Sepharose-4B column. (3~5 cycles) until any protein can be detected from the residue. The column banded with McAb was eluted with glycine buffer (pH2.3). The eluate was dialysed with 0.05M Tris buffer (pH8.1), 4 ℃ , rising for overnight till to pH7.2. SDS-PAGE and immunoblot were carried out for the assay of the purified McAb N-35. The purified McAb N-35 was dialysed with Coupling buffer, 4 ℃ , rising for overnight, and mixed with the Protein-A-Sepharose-4B beads, 4 ℃ , rising for overnight. GLC-82 cell lysate was absorbed by the column of Protein-A-Sepharose-4B-N-35, SDS-PAGE and immunoblot were carried out for the assay of the purified associated protein N35. One more method of purification with Wheatgerm-lectin-Sepharose-6MB was carried out to purify associated protein N35 and got the same results.

RESULTS

Immunoprecipitation Assay for The Relationship of Yunnan Gejiu Lung Cancer Cells and The Monoclonal Antibody

Antigen-antibody complex of McAb and associated protein N35 was showed by immunoblot. The bands of relative molecular mass (Mr)=150 x 10^3 and 50 x 10^3 were separately the light chain and heavy chain of IgG of the mouse. The band of Mr=75 x 10^3~130 x 10^3 was the purpose band of protein N35.The relationship was confirmed between them for further detection of the associated protein N35. (Figure 1.)

Immunoblotting Assay for The Distribution of Associated Protein N35 Among the Cancer Cells And Normal Tissues

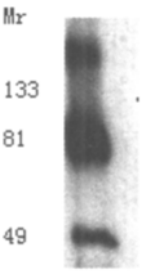

Fig 1. Confirming the relationship between mAB N-35 and protein N35 by Immunoprecipitation.

The associated protein N35 was recognized by McAb N-35 in the lung cancer cell line GLC-82, human cervical cancer cell line Hela, human hepatic cancer cell line HepG-2 and human breast cancer cell line PMC-42 with different relative molecular mass (Mr), but no expression of the protein ingredient in the normal human heart and lung fresh tissues. The Mr of the associated protein N35 was 75×10^3~130×10^3. (Figure 2)

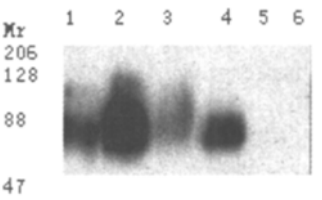

Fig 2. The distribution of protein N35 in various tissues and cells by Western blot.

The lines of 1,2,3,4,5 and 6 were separately: Hela,GLC-82, HepeG-2, PMC, normal human heart and lung tissues. The protein N35 detected by mAB N35 distributed in GLC-82 (Mr=75 x 10^3~130 x 10^3), Hela (Mr=75 x 10^3~130 x 10^3) , HepG-2 (Mr=75 x 10^3~130 x 10^3) and PMC (Mr=80 x 10^3~95 x 10^3) with different Mr, but no expression in the protein ingredient of normal human lung and heart tissue.

Differentail Centrifigation and Subcellular Assay of The Associated Protein N35

The subcellular structures were segregated by differential centrifugation.The immunoblot assay the associated protein N35 distributed on the nuclei significantly much more than at the mitochondrail and membrane, as the same result as by immumnohistochemistry. (Figure 3)

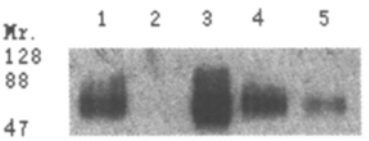

Fig 3. The lines of 1, 2, 3, 4 and 5 were separately: cell residue, supernatant from last altracentrifugation, nuclei, mitochondria and membrane of the cell line GLC-82 that harvested from the differential centrifugation. Westenblot subcellular assay (volume:100ul/well or protein amount:10ug/well) showed the associated protein N35 distributed on the nuclei significantly, much more than that on the mitochondrail and membrane.

Immumnohistochemistry Assay for Location of the Subcellular Structure of Associated Protein N35.

The parafine section of lung cancer biopsy was treated with Avidin-biotin staining (ABC Kit) by McAb N-35 as the first antibody. The positive grains in brown were significantly distributed in the nuclei, less were beyond nuclei in the cells. The cell membrane was staining weakly, as the same results as by weaternblot assay (Figure 4)

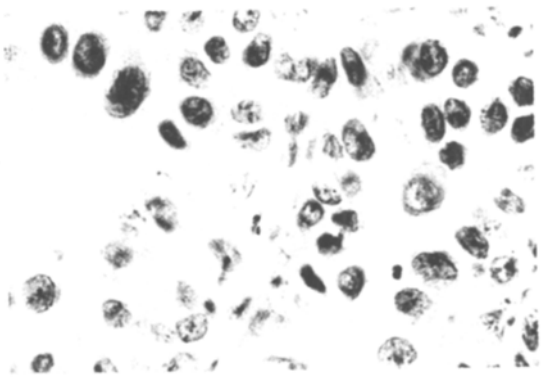

Fig.4 Immumnohistochemistry assay for location of the subcellular structure of associated protein N-35 (× 400)

Live Cell Immunoflourescence Assay for Origination of Associated Protein N35 at The Chromosomal Domain of The Mitotic Cells

Immunofluoresence assay for live cells at log phase of GLC-82 was carried out with McAb N-35 as an immunoprobe for the origination of associated protein N35. The photos were exactly taken from the same field one by one with different light sources. The staining of DNA dyeing showed DNA structure in blue, such as the equatorial plate. FITC dyeing showed the positive staining of associated protein N35 in green at the centrioles which were originated symmetrically on the both sides of the equatorial plate of the mitotic cells at the S~G_2 phage. (Figure 5)

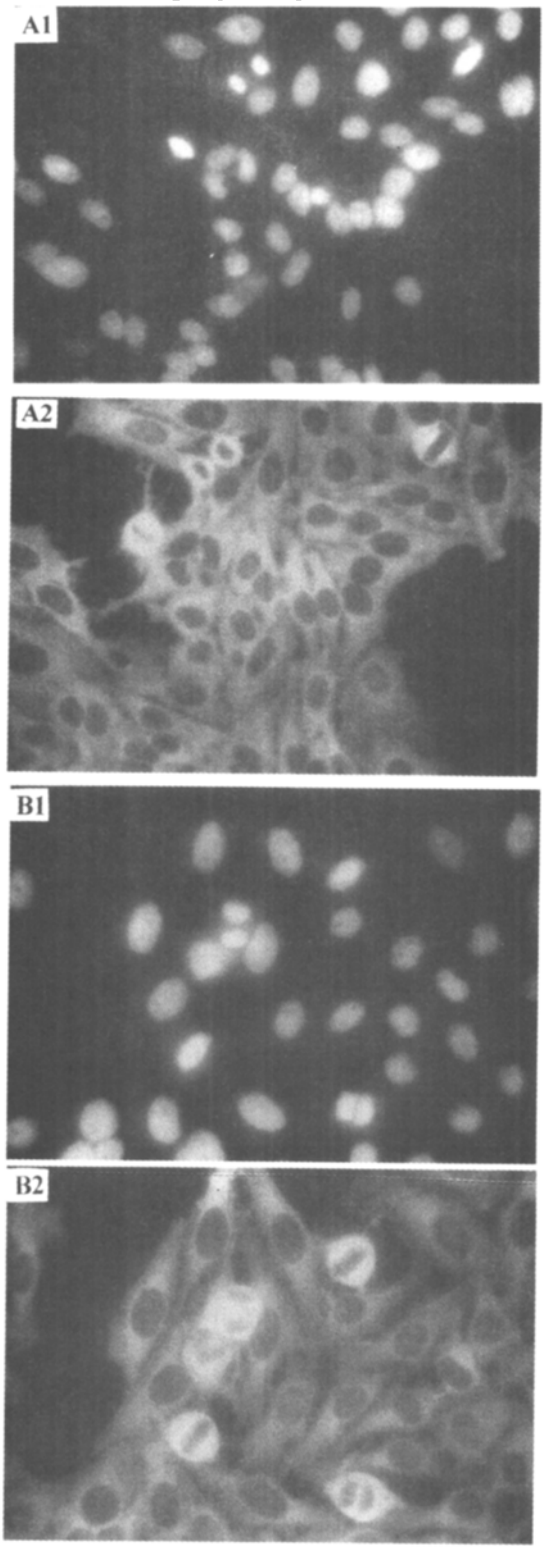

Fig. 5 The origination of associated protein N35 at the mitotic cells by immunoflourescence staining of the mitotic cells that associated protein N35 was located on the centriole of the chromosomal domain at the S-G_2 phage. (× 400)

Fig.5. 2 of photos of 5A or 5B were exposed from the same field exactly with different luorescence source. Fig 5A1 and 5B1 showed the DNA(equatorial plate) staining in blue. Fig 5A2 and 5B2 showed the special flourescence staining of the protein N35 at the centrioles symmetrically on the both sides of the equatorial plate.

Digestion of the Associated Protein N35 by N-Glycanase

The lysate of GLC-82 was treated with N-glycanase. Both lysate and digested product were detected by immunoblot with McAb N-35.The lysate kept its band with Mr of 75×10^3~130×10^3 and the digested product was changed into 3 bands with Mr of 75×10^3~130×10^3. The associated protein N35 was obviously confirmed as a glycoprotein. (Figure 6)

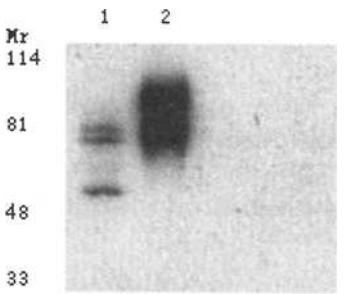

Fig 6. The assay of enzyme digestion of protein N35 by N-Glycanase and Westernblot Line 1 showed 3 bands of 65×10^3、84×10^3 and 86×10^3 from the digested lysate of GLC-82 cells by N-Glycanase. Line 2 showed 1 band of 75×10^3~130×10^3 the lysate before digestion.

Immunoaffinity Chromatography for Purification of Associated Protein N35

For further characterization of associated protein N35, both immunoaffinity chromatography methods of the Protein-A-Sepharose-4B-N-35 and Wheatgerm-lectin-Sepharose-6MB were carried out successfully. (Figure 7)

DISCUSSION

The monoclonal antibodies to cancer are

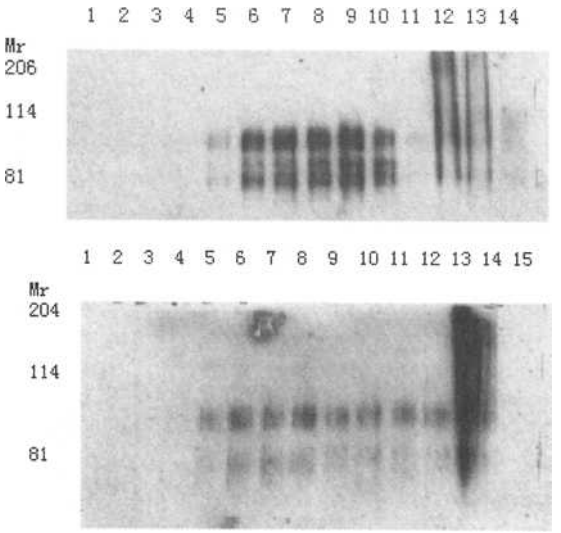

Fig. 7. Purification of associated protein N35

Fig. 7A. The purification of protein N35 form the cell lysate of GLC-82 by the mABN-35- Sepharose - 4B column. The lines of 1~ 12 were the samples of the elution from the mABN-35- Sepharose - 4B column. The protein N35 was detected from line 5 to line 12. The Lines 13 ~ 15 were the control system

Fig. 7B. The purification of protein N35 form the cell lysate of GLC-82 by the Wheatgerm-Lectin-Sepharose-6MB column. The lines of 1~ 10 were the samples of the elution from the mABN-35- Sepharose - 4B column. The protein N35 was detected from line 5 to line 10. The Lines 11 ~ 14 were the control system.

recently playing more important roles in the researches of tumor cell and molecular immunology[1,2,3], which will lead to the more understanding of associated proteins and their coding genes as the targets in the new stages for cancer biotherapy and gene therapy.[4] Among the associated protein molecules, the factors related with the proliferation of cancer cells are more detected significantly than others[5]. In this research, a novel protein molecular (named N35 temporarily) of lung cancer cells has been identified and characterized by immumno-histochemistry, immunoprecipitation, immuno-blotting, differential centrifugation and subcellular assay; N-glycanase digestion and mitotic cell immunoflourescence with the monoclonal antibody N-35 as an immunoprobe to detect the distribution of the protein N35 among the various cancer cell lines and normal human tissues; the relationship between the protein N35 and glycoprotein; the location of the subcellular structure and chromosomal domain of the protein N35. It also has been purified by the different affinity chromatographies to get the most effective way for

the purification of the associated protein N35. The results showed the protein molecule N35 was distributed in the lung cancer cell line GLC-82, human cervical cancer cell line Hela, human hepatic cancer cell line HepG-2 and human breast cancer cell line PMC with different relative molecular mass(Mr), but no expression of the protein ingredient in the normal human heart and lung fresh tissues. Although the immunoreaction of antigenic protein should usually be affected by degrading of its three-dimensional structure[6], but we found the associated protein N35 still remained its stable immunoreactive affinity with the monoclonal antibody N-35 after being treated at 100℃ for 1~2 min during the SDS-PAGE and immunoblotting. It suggested that the immunoreaction of associated protein N35 not dependent upon the more complex structure of the protein with folded chain units, and work as the thread molecule of the protein. We proposed the associated protein N35 be an important tumor associated antigen recognized by the monoclonal antibody N-35 with different Mr among the various cancer cells with the different mechanism of transcription or glycosylation of the protein express, but there would be the same antigenic determinants allogenically among the various cancer cells. The glycoprotein moleculei expressed by eukaryotic cells usually play the roles of membrane protein or cross membrane protein functionally as the receptor and channel [7]. After digestion with N-glycanase, the associated protein N35 with Mr 75×10^3~130×10^3 was changed into 3 fragments of 65×10^3, 84×10^3 and 86×10^3, and absolutely determined as a glycoprotein. It supported the same observation of Laferte S [8]. The result from differential centrifugation and subcellary assay showed the associated protein N35 distributed on the nuclei significantly much more than at the mitochondrail and membrane, as the same result as by immumnohistochemistry. This did not support that the glycoprotein N35 was the membrane protein. The centriole, without DNA structure and prepared template, related closely with activities of mitotic poles, and determines the formation of centrosomes during the cell cycles, plays the very important roles in the activities of the mitosis [9]. The centrioles and centrosome play an important role in maintenance of cell polarity and in progression through the cell cycle by determining the number, polarity, and organization of interphase and mitotic microtubules [10].By the immunoflourescence staining of the mitotic cells, we also found that associated protein N35 was originated at the centriole of the chromosomal domain at the S-G2 phase. This result suggested the associated protein N35 not only further be the nuclei protein, but also be relative with the proliferation of cancer cells. We proposed that associated protein N35 might be an important protein molecule as a role of tumor cell growth regulator.

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