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Production and Characterization of a Monoclonal Antibody against an Antigen on the Surface of Non-Small Cell Carcinoma of the Lung

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ABSTRACT

Background: Lung carcinoma is a multiple type cancer comprising of small cell and non-small cell carcinomas (NSCLC). For therapeutic and diagnostic purposes, serum monoclonal antibodies have been produced against lung cancer. **Objective:** To characterize a murine monoclonal antibody (ME3D11) reactive with human NSCLC. **Methods:** A murine monoclonal antibody (ME3D11) reactive with human NSCLC was selected after immunization of BALB/c mice with a human large cell carcinoma with neuroendocrine differentiation, and was tested by immunofluorescence staining and Western blot analysis. **Results:** Our study showed that the antigen recognized by ME3D11 antibody was a cell surface antigen of 170kDa. This antigen is expressed on the cell surface of all NSCLC and a few carcinoma cell lines. In contrast, this antigen is neither expressed on the cell surface of human sarcoma, nor on the hematopoietic and normal cell lines. This antibody had no effect on spontaneous proliferation of Mehr-80 cell line in vitro. **Conclusion:** High degree of binding of this monoclonal antibody to NSCLC and some other carcinoma cells warrants further studies on its potential use in diagnosis and therapy of cancer by conjugation to drugs, toxins or radionuclides.

Keywords: NSCLC, ME3D11, Mehr-80

INTRODUCTION

Lung carcinomas represent a heterogeneous category of tumors with different prognosis and responses to various forms of therapy. From a clinical point of view they are simply classified into two main groups namely small cell lung cancer and non-small cell lung cancers (NSCLC). NSCLC are subdivided into three major types including adenocarcinoma, squamous cell carcinoma and large cell carcinoma.

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Their diagnosis and classification are based mainly on histopathological features (1). Monoclonal antibody technology has had great impact on the study of human lung cancer (2-4). A growing number of studies have reported the production of monoclonal antibodies against human lung carcinoma cell lines (5-7) for understanding the biology of lung cancer, and use in cancer diagnosis and therapy (8-13). Despite major advances in the diagnosis and therapy of human cancers, lung cancer is still the most common cause of death due to cancer in the world (14, 15). Therefore, there is a need for production of more specific monoclonal antibodies that could be used in the diagnosis and therapy of lung cancer.

In this report, we describe the production of monoclonal antibodies with specificity for non small cell lung cancer. For this purpose, a group of mice were immunized with a large cell carcinoma line (Mehr-80) with neuroendocrine differentiation established in our laboratory (16). The hybridomas were then screened against a wide range of human tumor cell lines. Using this strategy, we isolated, cloned, and selected a hybridoma cell line that secretes ME3D11 antibody. We also identified the antigen recognized by this antibody.

MATERIALS AND METHODS

Cell lines and Cell Culture. Mehr-80 cell line was established from a patient with large cell lung carcinoma in our laboratory as described previously (16). Other carcinoma cell lines were obtained from ATCC and ECCAC or were those used in previous studies (17). They included lung (A549, COR L 105, COR L23, Calu-6), prostate (DU145, LNCAP), breast (SKBR 3, MCF-7, MDA-MB-468), glioblastoma (A172), head and neck (HN5), nasopharyngeal (Hep2), hepatocarcinoma (hep G2), and colon carcinoma (CC1 218) cell lines. In addition to these cell lines we used fetal lung fibroblasts (HFIF-PI5), monkey kidney (vero), T cell leukemia (jurkat), Burkitts lymphoma (Raji), chronic myelogenous leukemia (K 562) and histiocytic leukemia (U937) cell lines.

With the exception of hematopoietic cell lines which were cultured in RPMI 1640 medium, all other cell lines were cultured routinely in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal calf serum and the antibiotics penicillin, streptomycin and neomycin. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂ and 95% air as described previously (18).

Generation of Monoclonal Antibodies. A group of BALB/c mice (6-8 weeks old) were immunized subcutaneously with 10⁷ Mehr-80 cells in complete Freund's adjuvant. Immunization was then repeated intraperitoneally and in the absence of adjuvant, five times weekly. Two to three days prior to fusion, the spleen cells were removed and fused with SP2/0 myeloma cell line. The hybridoma culture supernatants were tested for binding to Mehr-80 cell line using immunocytochemistry. Positive hybridomas were selected, cloned and purified for further studies (2-4).

Screening of Hybridoma Supernatants by Immunocytochemistry. Mehr-80 cells were grown on glass Petri dish (100 mm), fixed with 50:50 acetone/methanol for 5 minutes and left to dry at room temperature. Each petri dish had enough space for about 30 tests. About 15 µl of hybrid supernatant was added to the test area and incubated in a humid chamber for 1h followed by extensive washing with PBS. Then the petri dish was incubated with horse radish peroxidase (HRP) conjugated with goat anti-mouse IgG antisera (Sigma) for 1 h, washed with PBS and later treated with DAB-NICL2 chromogen-H₂O₂ substrate (Sigma) to initiate the peroxidase reaction. After about 5-15 minutes, black or brown spots appeared in certain locations. These spots were examined

under microscope for positive reactivity. Using this method, positive hybridomas were selected and cloned (18).

Determination of Antibody Isotype and Purification. The isotype of mouse monoclonal antibody was determined using the mouse antibody isotyping kit, according to the manufacturer's instructions (Roche, Germany).

About 3000 mL of hybrid supernatant were collected, filtered and treated with ammonium sulfate to a final saturation of 45%. After overnight incubation, the supernatant was removed and the remaining precipitate was centrifuged at 4000 rpm for 20 min at 4°C. The precipitate was dissolved in double distilled water and centrifuged again at 2000 rpm for 5 min. The supernatant was then purified by affinity chromatography on protein G column. The purified antibody was dialyzed extensively against PBS, filtered, sterilized, aliquoted and stored at -20°C for future studies.

Indirect Immunofluorescent Staining. Tumor cell lines were grown on circular cover slips in 24 well plates in DMEM/10% FCS. When cells became confluent, the supernatant was decanted and the cell monolayer was fixed with methanol for 5 minutes at 4°C. After washing with 1% BSA-PBS, the cell monolayer was incubated with 250 µl of hybridoma supernatant for 1 hour at room temperature. After 3 washes with 1% BSA-PBS, the cells were treated with FITC-conjugated goat anti-mouse Ig antisera (Serotec Ltd., Oxford, UK) for 45 minutes at 4°C. Following three more washes, the cover slips were transferred and mounted on slides using Vectashield containing DAPI mounting media (Vector laboratories Ltd., Peterborough, UK) and examined for fluorescence under a Zeiss Axiovert 100, Fluorescent microscope (Carl Zeiss Ltd, Germany).

ELISA Method. Hybridoma culture supernatants were tested for reactivity against a panel of human cell lines utilizing an ELISA method. Human tumor cell lines were grown to near confluence in 96-well plates. After removing the culture medium, cells were incubated with culture supernatant for 1 hour on ice. Following three washes, the cells were treated with HRP-linked goat anti-mouse Ig antiserum for 45 minutes, washed again and the end reaction was initiated by the addition of TMB substrate. Cells incubated with culture medium containing 2% FBS served as a negative control.

Flow Cytometry. About 1×10^6 tumor cells were shaken with 1 ml of hybridoma culture supernatant or control medium for 1 hour at 4°C as described previously (19). Tumor cells were then washed three times by centrifugation prior to incubation with FITC-conjugated anti-mouse secondary antibody (Serotec) for 45 minutes at 4°C on a rotator. Following three more washes with DMEM/2% FBS, the final cell pellet was resuspended in 1 ml FACS Flow™ buffer (Becton Dickinson UK) and then analyzed by a Beckman Coulter Epics XL flow cytometer (Becton Dickinson, UK) (18).

Western Blotting. Western blotting was performed as described previously (19). Briefly, Mehr-80 cells were grown to near confluence in 6-well tissue culture plates (Greiner Bio-One, UK) containing 5 ml of DMEM-10% FCS. Tumor cells were washed with PBS and then solubilized with 400 µl of LDS sample buffer (Invitrogen, UK) supplemented with 4 µl of protein inhibitor cocktail containing AEBSF, aprotinin, leupeptin, bestatin, pepstatin A, and E-64 (Sigma-Aldrich, UK). The cell lysates were heated at 72°C for 10 minutes and then equal amounts of cell lysate were separated on 4-12% Bis-Tris-gels (Invitrogen, UK) using the XCell II™ Surelock™ Mini-Cell system (Invitrogen, U.K). The proteins were transferred to PVDF membranes using the XCell II™ Mini-Cell Blot Module kit (Invitrogen, UK).

The PVDF membranes were incubated with primary antibody for one hour at room temperature and the specific signals were detected using the ECL detection system (Amersham, UK).

Growth Inhibition Assay. Confluent cultures of Mehr-80 cell line were treated with trypsin and about 5×10^3 cells in 100 μ l complete culture media were plated in a 96-well plate. After 4 hours at 37°C, 100 μ l aliquots of dilutions of mouse monoclonal antibody were added to triplicate wells and the cultures were incubated at 37°C for 3-7 days. Controls containing medium alone were also set up and incubated for the same period. When the cells in wells containing medium alone were almost confluent, all cells were fixed with 0.25% glutaraldehyde, washed in water, air dried and stained with 0.05% methylene blue (100 μ l well) for 15 minutes. After washing with tap water and air drying, 200 μ l of 0.33 N HCl were added to each well and the absorbance was measured at 620 nm by an ELISA reader. In order to determine the initial number of cells plated in each experiment, an extra plate was set up and the cells were harvested 4 hours after the start of incubation at 37°C. Growth as a percent of control was determined from the following formula (20):

$$\% \text{ Growth} = (B-A)/(C-A) \times 100$$

Where A = A_{620} at start of incubation.

B = A_{620} after treatment with antibody.

C = A_{620} after incubation in the medium alone.

RESULTS

Among monoclonal antibodies made by 10 hybridomas against a large cell carcinoma cell line (Mehr-80), ME3D11 reacted with all lung carcinoma cell lines tested in this study with different strengths; however it had the highest level of binding to Mehr-80. (Fig 1). It also showed positive reactivity with some other carcinoma including a prostate carcinomas, a head & neck carcinoma, a glioblastoma and nasopharyngeal carcinoma cell lines screened by ELISA and flow cytometric methods. In contrast, this antibody did not bind to sarcomatous (data not shown), malignant hematopoietic or non-malignant cell lines (Fig1, 2, 3). The isotype of monoclonal antibody was IgG1 with kappa light chains.

The results of indirect immunofluorescence assay presented in Figure 4 show that the antigenic determinant recognized by this antibody is located on the surface of the tumor cells. The effect of this antibody was then studied on the growth of human tumor cell lines. At concentrations of up to 200nM, this antibody did not show any inhibitory or even stimulatory effect on the growth of Mehr-80 cell line in culture (data not shown).

The results of Western blot analysis with ME3D11 antibody showed that the antigenic determinant recognized by ME3D11 has a molecular weight of about 170 KD (Fig5). Since EGFR is also a 170 KD membranous protein with high levels of expression in a wide range of epithelial tumors, we compared the reactivity of anti EGFR monoclonal antibody (ICR 62) and ME3D11 with Mehr-80 cell line. As shown in Fig 6, the level of binding of mAb ICR62 and mAb ME3D11 to Mehr Cell line is not similar.

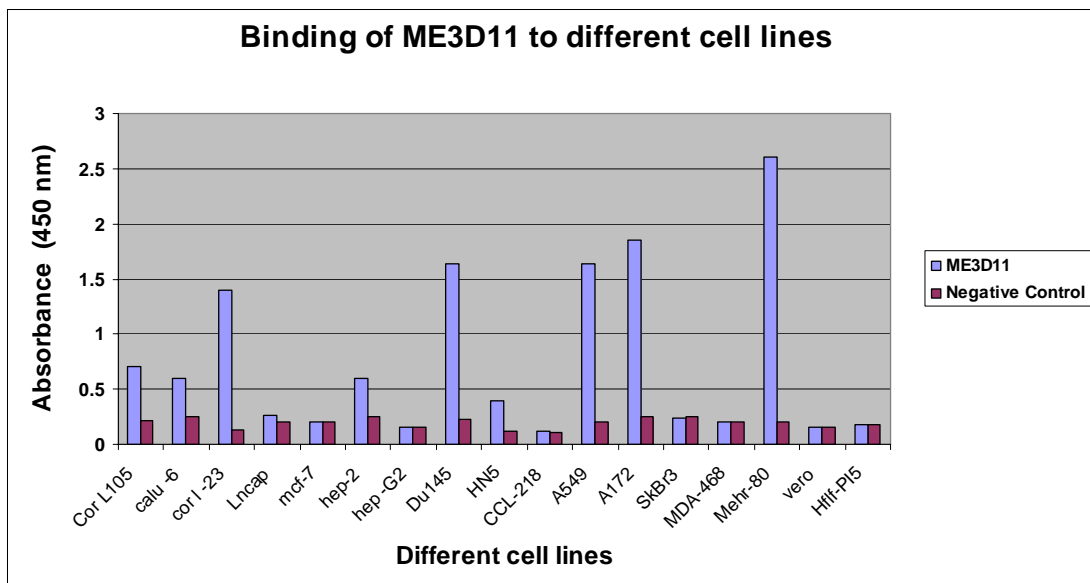
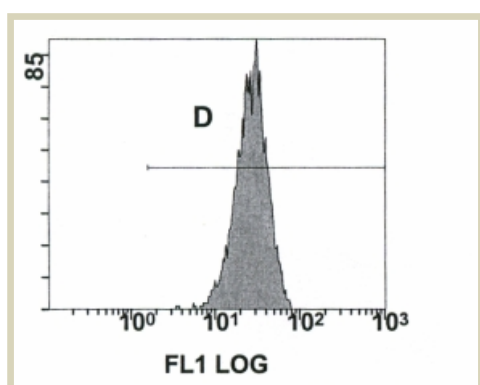
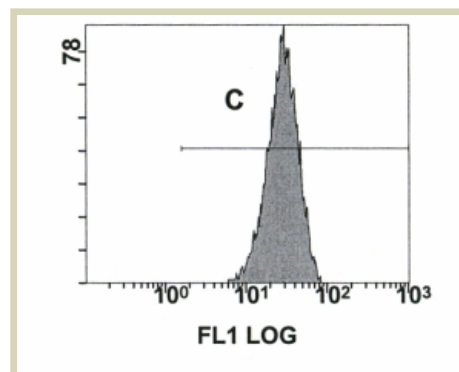


Figure1. Binding of ME3D11 to different cell lines
 Screening of a panel of cell lines by ME3D11 using Elisa assay shows reactivity of this antibody with non small cell lung carcinoma (NSCLC) cell lines with different strength and with several other carcinoma cell lines including Du145, A172, HN5, and Hep2

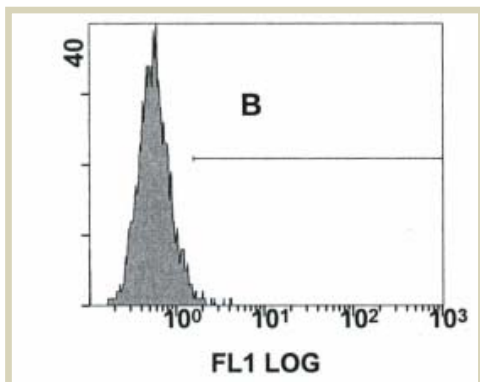
Figure2 (a)



Histogram of ME3D11 hybrid supernatant

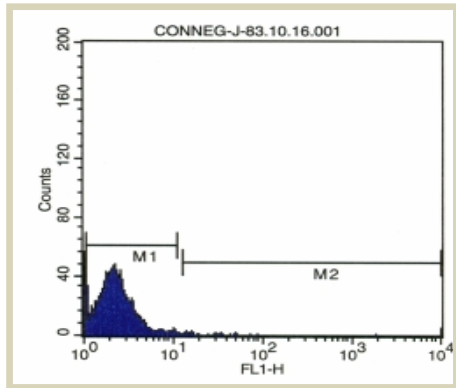


Histogram of purified ME3D11 antibody

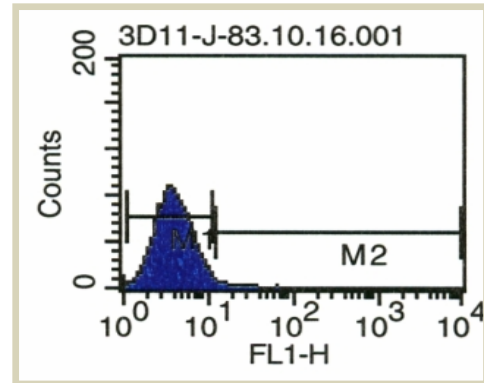


Histogram of negative control

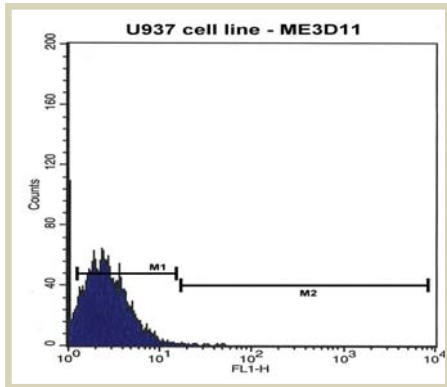
Figure 2(b)



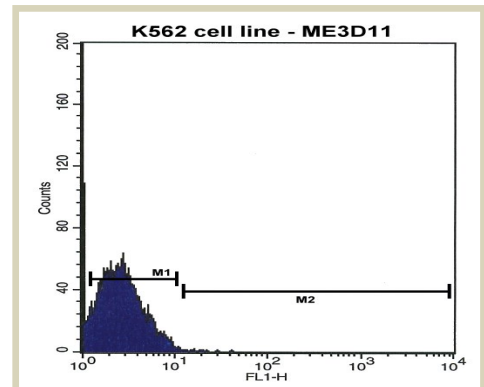
Histogram of negative control



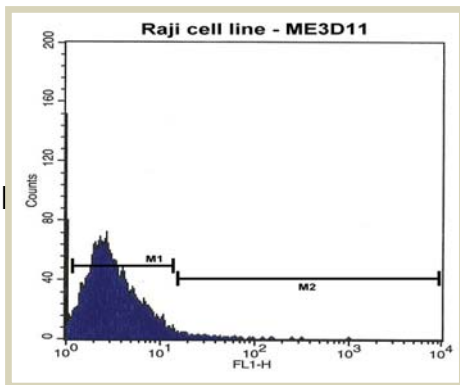
Histogram of ME3D11 with jurkat



Histogram of ME3D11 with u937



Histogram of ME3D11 with k562



Histogram of ME3D11 with Raji

Figure 2. Flowcytometric results of ME3D11 antibody reaction with Mehr-80 ,Jurkat, u937, k562 and Raji cell lines

Flowcytometric histograms showing no reactivity of ME 3D11 with hematopoitic cell lines and flourcent intensity in these cell lines is similar to negative control comparing to positive reaction of this antibody with Mehr -80cell line.

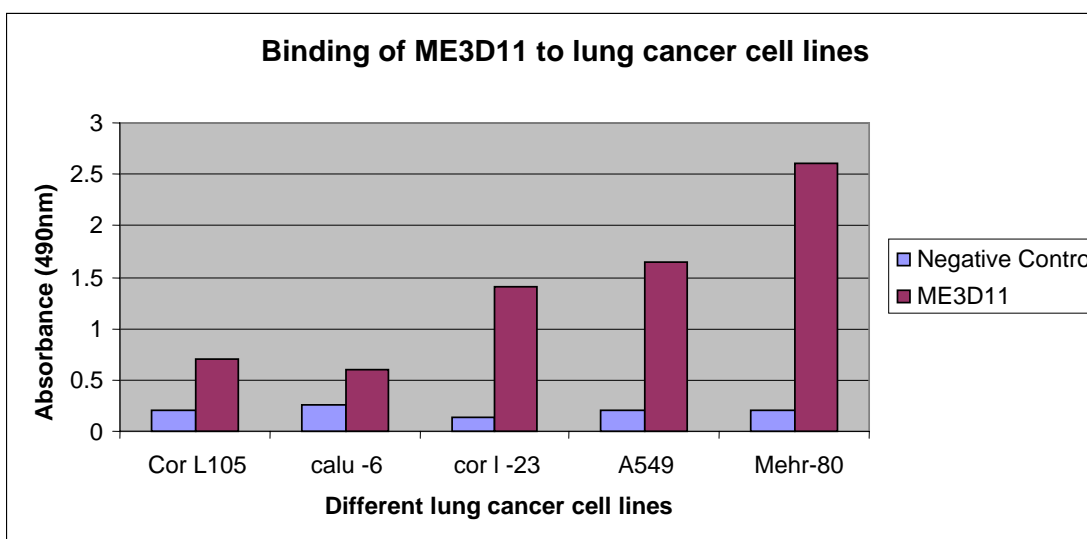


Figure 3. Binding of ME3D11 to lung cancer cell lines

Figure 4(a)

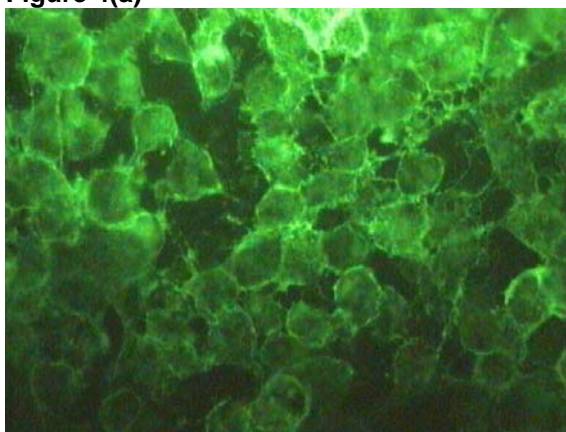


Figure 4(b)

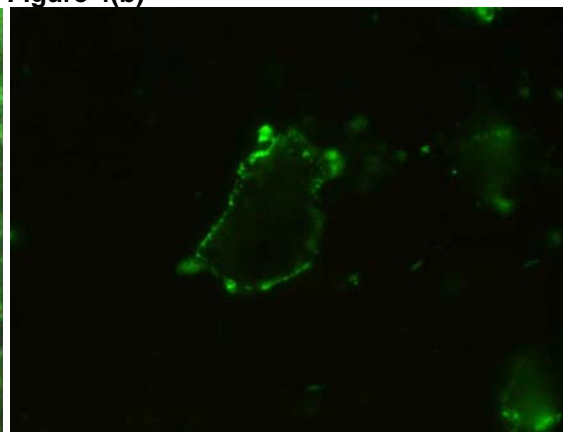


Figure 4. Indirect immunofluorescent assay results

The pattern of Mehr-80 cell line reactivity with ME 3D11 seems to be membranous

Molecular weight of 3D11



Figure 5. Result of immunoblotting using Mehr-80 cell lysate
The molecular weight of ME3D11 target antigen estimated to be 170 KD.

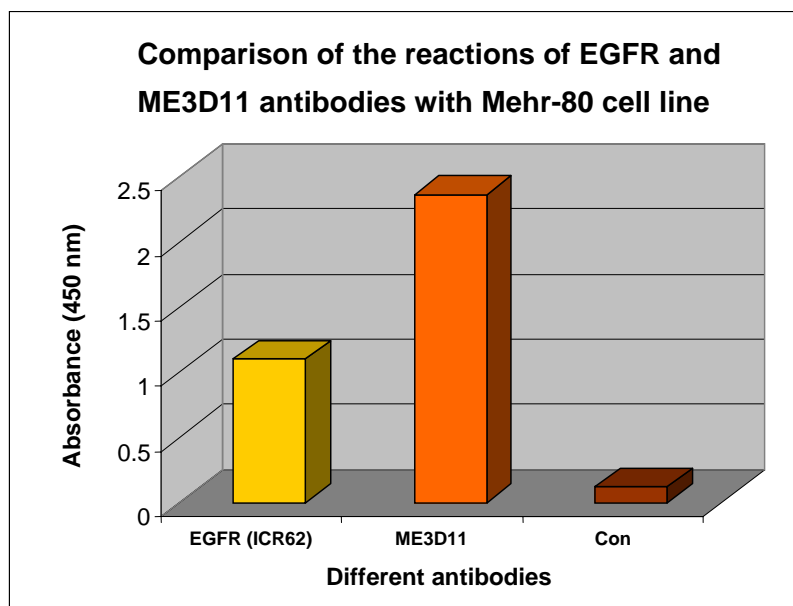


Figure 6. Elisa result of reactivity of ICR 62 and ME3D11 to Mehr 80 cell line

DISCUSSION

In this study, we have shown that ME3D11 is directed against an antigen which is present on all lung cancer cell lines (A 549, COR-L23, calu-6, CORL105) and some other carcinoma cell lines examined here including a prostate cell line (DU145), a glioblastoma cell line (A172), a head and neck cell line (HN5) and a nasopharyngeal carcinoma (Hep-2). However, it had no reactivity with sarcomatous or malignant hematopoietic cell lines of different origins (Raji, jurkat, K562, U937). Interestingly, ME3D11 antibody also failed to react with normal fatal lung fibroblasts (HFIF-PI5) and monkey kidney (vero) cell lines (Fig 1-3). The results of immunofluorescence staining suggest that the antigen recognized by ME3D11 is a cell surface antigen (Fig 4). Such antigens are ideal targets for monoclonal antibody-based therapy.

In reviewing the literature, we found that there are two 170 KD antigen determinants on the surface of some tumors including lung carcinoma. One of them is EGFR which is shown to have high levels of expression in a wide range of epithelial tumours and in some studies it was also associated with a poor prognosis in some of the patients (19, 21). However, in our study the reactivity of ME3D11 with Mehr-80 cells is more intense than the reactivity of anti EGFR with this cell line (Fig. 6). In addition, there was no reactivity with MDA-MB 468 and CCL-218 cell lines that express high numbers of EGFR on their surface suggesting that the antigen recognized by ME3D11 is not EGFR (Fig. 1). Another 170 KD determinant on the surface of some tumors including lung tumors is P-glycoprotein. It acts as an efflux pump to decrease the amount of drugs in tumor cells (22). The P-glycoprotein is present on normal tissues, but its expression increases in some tumors and correlates with chemo resistance (23). The similarity of P-glycoprotein and the antigen recognized by ME3D11 is probable. However, P-glycoprotein expression is not limited to carcinomas and our results presented here suggest that the antigen recognized by ME 3D11 is expressed exclusively in some carcino-

mas especially in all non small cell lung carcinoma (NSCLC) cell lines tested. Evaluation of a large number of human carcinoma cell lines and tumour specimens as well as normal tissues together with amino acid sequence analysis of the target antigens recognized by this antibody are warranted. The results of such investigations should unravel the full potential of this antibody in diagnosis and therapy of human cancers and determine the importance of this antigen in the biologic behavior of human cancers.

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REFERENCES

- 1 Virtanen C, Ishikawa Y, Honjoh D, Kimura M, Shimane M, Miyoshi T et al. Integrated classification of lung tumors and cell lines by expression profiling. *Proc Natl Acad Sci U S A*. 2002 ;99:12357-62.
- 2 Minna JD, Cuttitta F, Rosen S, Bunn PA Jr, Carney DN, Gazdar AF et al. methods for production of monoclonal antibodies with specificity for human lung cancer cell. *In Vitro*. 1981;17:1058-70.
- 3 Mulshine JL, Cuttitta F, Bibro M, Fedorko J, Fargion S, Little C et al .Monoclonal antibodies that distinguish non small cell from small cell lung cancer. *J Immunol*. 1983 ;131:497-502.
- 4 Cuttitta F, Rosen S, Gazdar AF, Minna JD. Monoclonal antibodies that demonstrate specificity for several types of human lung cancer. *Proc Natl Acad Sci U S A*. 1981 ;78:4591-5.
- 5 Stahel RA, O'Hara CJ, Waibel R, Martin A. Monoclonal antibodies against mesothelial membrane antigen discriminate between malignant mesothelioma and lung adenocarcinoma. *Int J Cancer*. 1988 ;41:218-23.
- 6 van Duijnhoven HL, Moors EH, Groeneveld A, Timmer ED, de Leij LF, Wagenaar SS et al. Monoclonal antibody Mon-114: detection of a marker for neuroendocrine differentiation in human lung cancer. *Cancer Lett*. 1992 ;63:61-6.
- 7 Kudo T, Kobayashi S, Murakami K, Takano R, Nose M, Sato N et al. A novel human monoclonal antibody directed to a tumor associated antigen. *Jpn J Cancer Res*. 1993 ;84:760-9.
- 8 Rusch V, Macapinlac H, Heelan R, Kramer E, Larson S, McCormack P et al. NR-LU-10 monoclonal antibody scanning. A helpful new adjunct to computed tomography in evaluating non small cell lung cancer. *J Thorac Cardiovasc Surg*. 1993 ;106:200-4.
- 9 Shitara K, Hanai N, Yoshida H. Detection of a novel lung adenocarcinoma associated serum antigen defined by two monoclonal antibodies, KM 432 and KM 227. *Anticancer Res*. 1993 ;13:579-86.
- 10 Hua ZX, Tanaka KE, Tazelaar HD, Myers J, Markowitz GS, Borczuk AC. Immunoreactivity for LN2 and LN3 distinguishes small cell carcinomas from non small cell carcinomas in the lung. *Hum Pathol*. 1998 ;29:1441-6.
- 11 Krueger P, Nitz C, Foster R, MacDonald C, Gelber O, Lalehzadeh G et al. A new small cell lung cancer (SCLC)-specific marker discovered through antigenic subtraction of neuroblastoma cells. *Cancer Immunol Immunother*. 2003 ;52:367-77.
- 12 Kamma H, Endo K, Horiguchi H, Iijima T , Ogata T. Lung cancer associated monoclonal antibody 15 that recognizes cell growth related membrane antigens gp 85/45 and its growth-inhibitory effect on human lung cancer cell in vitro. *Cancer Research*. 1989; 49: 5118-22.
- 13 Lai RS, Hsu HK, Lu JY, Ger LP, Lai NS. CYFRA 21-1 enzyme-linked immunosorbent assay. Evaluation as a tumor marker in non-small cell lung cancer. *Chest*. 1996 ;109:995-1000.
- 14 Rosell R, Felip E, Garcia-Campelo R, Balaña C. The biology of non small cell lung cancer: Identifying new targets for rational therapy. *Lung Cancer*. 2004 ;46:135-48.
- 15 Huber RM, Stratakis DF. Molecular oncology perspectives in lung cancer. *Lung Cancer*. 2004 ;45 :S209-13.
- 16 Sharifzadeh S, Owji SM, Pezeshki AM, Malek-Hoseini Z, Kumar PV, Ghayumi SM et al. establishment and characterization of a human large cell lung cancer cell line with neuroendocrine differentiation. *Pathol Oncol Res*. 2004;10:225-30.
- 17 Modjtahedi H, Dean C. Monoclonal antibody to the EGF receptor act as betacellulin antagonists. *Biochem Biophys Res Commun*. 1996 ;221:625-30.
- 18 Harlow, E., and Lane, D., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 174-195 (1988).
- 19 Cunningham MP, Thomas H, Fan Z, Modjtahedi H. Responses of human tumour cells to treatment with anti-epidermal growth factor receptor (EGFR) monoclonal antibody ICR62 used alone and in combination with the EGFR tyrosine kinase inhibitor gefitinib. *Cancer Res*. 2006; 66:7708-15.
- 20 Modjtahedi H, Styles J, Dean C. The growth response of human tumor cell line expressing the EGF receptor to treatment with EGF and/or M abs that block ligand binding. *Int j oncol*. 1993; 3:237-243.
- 21 Modjtahedi H, Styles JM, Dean CJ. The human EGF receptor as a target for cancer therapy : six new rat mAbs against the receptor on the breast carcinoma MDA-MB468. *Br J Cancer*. 1993 ;67:247-53.
- 22 Volm M, Bak M Jr, Efferth T, Lathan B, Mattern J. Immunocytochemical deletion of a resistance-associated glycoprotein in tissue culture cells, ascites tumours and human tumour Xenografts by Mab 265/F4. *Anticancer Res*. 1988; 8:531-5.
- 23 Pujol JL, Simony J, Gautier V, Marty-Ané C, Pujol H, Michel FB. immunohistochemical study of P-glycoprotein distribution in lung cancer. *Lung Cancer* 1993; 10:1-12.