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STUDY ON METASTASIS ASSOCIATED GENE SCREENED BY MONOCLONAL ANTIBODY HIL

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The cDNA expression libraries derived from a highly metastatic cell subline Anip-973 and from its parental cell line, low metastatic AGZY-83a were screened by monoclonal antibodies (MoAbs) seperately against these two cell lines. A positive clone (H_4-D) from the Anip-973 cDNA library was isolated and its nucleotide sequence was determined. This clone contained 978 bp with an open reading frame of 318 bp encoding a polypeptide consisting of 106 amino acids. The H_4-D cDNA sequence showed 85% homology with a human propionyl-CoA carboxylase α-chain. In Western bloting analysis, the MoAb H_4 recognized 2 bands (15 KDa and 27 KDa) of Anip-973 cell membrane protein. The mRNA expression of H_4-D was higher in Anip-973 cells than that in AGZY-83a cells. The metastatic potential of Anip-973 cells was markedly decreased after being pretreated with MoAb H_4. The above findings indicated that H_4-D has a certain relationship with the metastatic phenotype of Anip-973 cells.

Key words: Neoplasm metastasis, Gene, Monoclonal antibody, cDNA cloning.

Tumor metastasis is a very complex process. It is presumed that metastasis of tumor cell is under the positive and negative control of a set of genes. In a broad sense, the genes controlling and regulating tumor cell metastasis may include: 1. the genes encoding proteins that directly function at a particular step or a few steps of the metastatic cascade, such as cell adhesion molecules, matrix-degrading proteinases and their inhibitors, autocrine motility factor (AMF), growth factors and their receptors; 2. certain oncogenes and tumor suppressor genes, such as *ras*, *myc*, p53 etc., which have been proved in some cases to be involved in tumor invasion and metastasis and 3. metastasis associated genes and metastasis suppressor genes. The latter is more important and interesting to scientists, because they may include some regulatory genes playing critical roles in metastatic process at several steps. The discovery of metastasis suppressor gene nm23 has been inspiring scientists to search for novel genes involved in metastasis. It is also the aim of this study which was based on the former work of our lab.

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MATERIALS, METHODS AND RESULTS

Cells and Antibodies

Low metastatic human lung adenocarcinoma cell line AGZY-83a was obtained from Angang Tumor Institute. Highly metastatic cell subline Anip-973 was selected from its parental cell line AGZY-83a in our lab.[1] MoAbs (HIL-H_4 against Anip-973, HIL-L_5

and -L_{10} against AGZY-83a) have been described.[2]

Screening of cDNA Libraries

λZAPII cDNA expression libraries produced from AGZY-83a and Anip-973 cells were kindly provided by Dr. Xu Mingxu (at the Tumor Institute, Harbin Medical University). The AGZY-83a and Anip-973 cDNA libraries were screened separately with a pool of the three MoAbs. The recombinant λ phages of expression library were plated with E.coli XL_1-Blue host cells and then grown at 42 ℃ for 4 h. Nitrocellulose (NC) filters, previously soaked in 10 mM isopropylthio-β-D-galactoside (IPTG), were placed onto the XL_1-Blue lawn and the plates were incubated at 37 ℃ for 4–5 h. The filters were removed from the plates and washed in TNT (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20). All subsequent steps were performed as described.[3] Mouse IgG primary MoAb was detected by binding an alkline phosphatase (AP) conjugated goat anti-mouse IgG secondary antibody. The AP accumulated at the site of the primary MoAb was exposed to BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate) and NBT (Nitro Blue Tetrazolium) for 15 min. and then terminated with distilled water. Screening of 2×10^6 recombinant phages resulted in the identification of a single reactive plaque produced by the phage from Anip-973 cDNA library. The plaque was replated and rescreened until a homogeneous population of immunopositive recombinant phages is obtained. The positive clone was then incubated separately with each MoAb. The results showed that only MoAb H_4 was able to react with it.

cDNA Cloning and Subcloning

The bacteriophage λ from positive plaque was purified as described.[4] After digested with restriction endonucleases *Eco*RI and *Xho*I, two insert fragments (H_4–D_2 of 0.4 kb and H_4–D_2 of 0.6 kb) were detected by 1% agarose gel electrophoresis. These two fragments were subcloned into vector PUC18 by using T_4 DNA ligase (Figure 1). The ligation products were used to transform competent JM109 cells and the cells were then spread on agarose medium containing ampicillin, IPTG and X-Gal. White clones were those contained inserts.

RNA Dot Blot Hybridization

For further identification of the specificity of this cDNA clone, total cellular RNAs of AGZY-83a and Anip-973 were extracted separately as described.[5] RNAs denatured in formaldehyde were spotted on NC filters equilibrated in 20×SSC (Standard Saline Citrate). The filters were prehybridized in 6×SSC, 5×Denhardt's solution, 0.5% SDS (Sodium Dodecyl Sulfate), 100 μg/ml salmon sperm DNA and 50% formamide for 6 h at 42 ℃. α-^{32}P-dCTP labeled H_4-D_2 cDNA probe was added directly to the prehybridization mix and then allowed to hybridize overnight at 42 ℃. The filters were washed under highly stringent condition followed by autoradiography with an intensifying screen at -70 ℃. The autoradiograph showed that the expression of H_4-D_2 fragment was higher in Anip-973 than that in AGZY-83a (Figure 2).

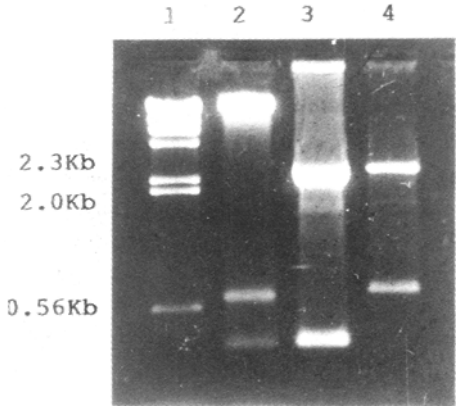

Fig. 1. Restriction digestion of obtained λDNA and recombinant plasmid PUC18. *Hind*III digested λDNA was used a molecular weight marker (Lang 1). Obtained λDNA containing H_4-D insert was digested with *Eco*RI and *Xho*I (Lane 2). Recombinant plasmid PUC18 containing H_4 -D_1 insert (Lane 3) and H_4 -D_2 insert (Lane 4) was digested with *Eco*RI and *Hind*III.

Western Blotting

The membrane proteins of AGZY-83a and Anip-973 cells were extracted as described.[6] The extracts were run on a 12% SDS-PAGE under reducing conditions. After separation, the proteins were electrophoretically transferred to PVDF filter for 4 h at 4 ℃. The filter was handled as described above for

screening of cDNA libraries. The results indicated that the MoAb H_4 recognized 2 bands (15 KDa and 27 KDa) of Anip-973 cell membrane proteins (Figure 3).

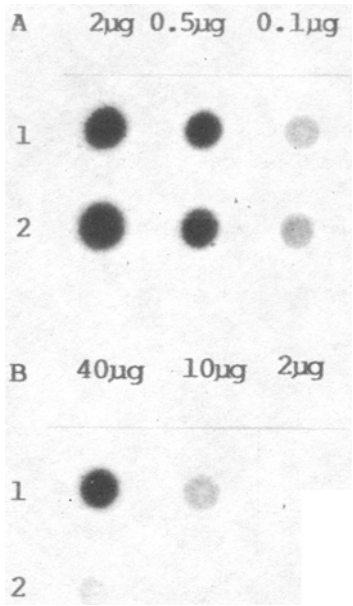

Fig. 2. RNA dot blot hybridization. (A) Total RNA was hybridized to ^{32}P-labeled human α-tubulin probe (as positive control). (B) Total RNA was hybridized to ^{32}P-labeled H_4-D_2 cDNA probe. 1. Total RNA from Anip-973 cells. 2. Total RNA from AGZY-83a cells.

Assay on the Inhibition of Experimental Metastases by MoAb H_4

Gently trypsinized Anip-973 cells were in-cubated with MoAb H_4 for 1 h at 37 ℃. The cells were centrifuged and suspended in PBS, and then 2×10^5 living cells were injected into the tail vein of nude mouse. The nude mice received Anip-973 cells without any pretreatment were used as control group. All mice were killed two months later. Macroscopic lung metastases were counted and other organs were examined histologically. The results showed that lung metastases were found in all 10 mice of control group (10/10), and widespread metastases to heart, brain, kidney, ovary, pancreas, skeleton muscle etc. were found in 3 mice of control group. In contrast, Anip-973 cells pretreated with MoAb H_4 metastasized only to lung and/or skeleton muscle in 6/10 of experimental group. There was statistically significant difference between the two groups ($P<0.05$).

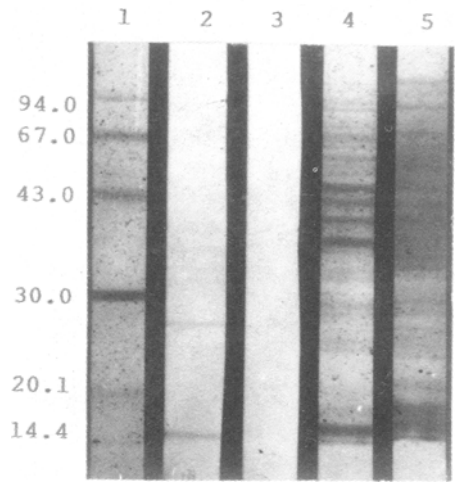

Fig. 3. Western blot analysis. Protein molecular weight standards weights in kilodalton (KDa) (Line 1). MoAb H_4 immunoprecipitated cell membrane extracts from Anip-973 and AGZY-83a cells (Lane2, 3, respectively). All bands of cell membrane extracts from Anip-973 cells (Lane 4) and AGZY-83a cells (Lane 5) were stained with amino black.

DNA Sequence Analysis

The template DNA of recombinant plasmid PUC18 was prepared by using a mini alkaline-lysis/PEG precipitation procedure.[7] Sequencing of the double stranded DNA with the dideoxy chain termination method was done according to T^7 Sequencing Kit protocol. DNA sequence analysis was performed on 370A DNA sequencer (Applied Biosystems, Inc.). The H_4-D cDNA sequence (Figure 4) reveals an open reading frame of 318 bp encoding a polypeptide consisting of 106 amino acids, and a 660 bp 3' untranslated region, which includes the consensus polyadenylatation signal (AATAAA) at position +935 followed 15 bases later by a poly (A) tail. Comparison with available sequence data revealed that H_4-D cDNA sequence has 85% homology with a human propionyl-CoA carboxylase α-chain.

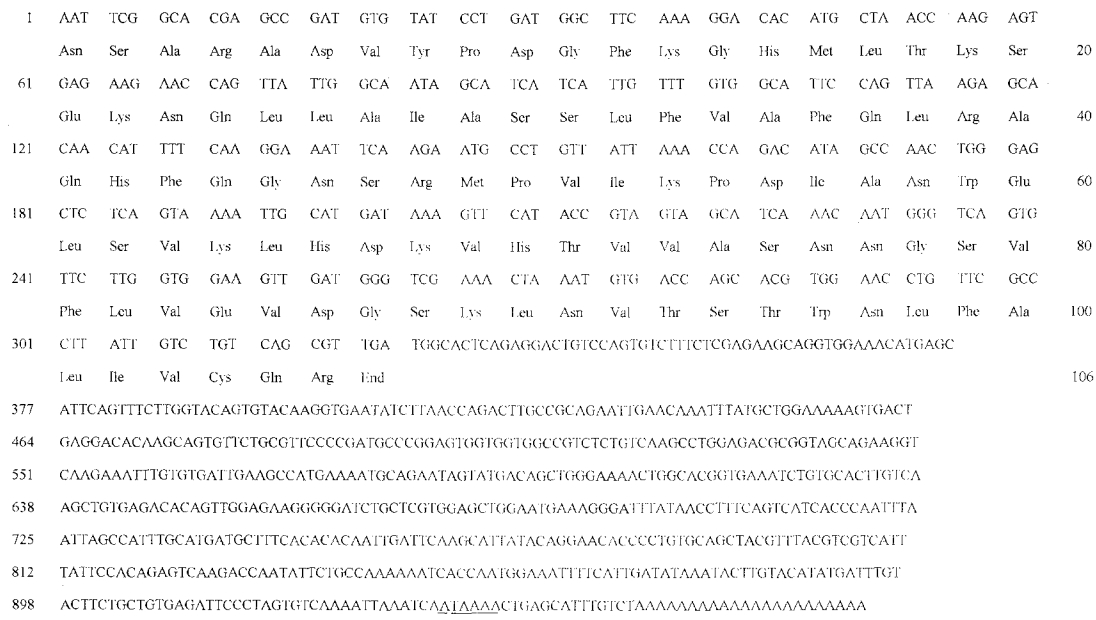

Fig. 4. Nucleotide and deduced amino acid sequence of H4-D cDNA clone

DISCUSSION

We isolated a positive cDNA clone H_4-D reacted with MoAb H_4 from the Anip-973 λ ZAPII cDNA library. H_4-D was preferentially expressed in highly metastatic cell subline Anip-973 and MoAb H_4 was found to inhibit experimental metastasis. The above results suggested that cDNA H_4-D is correlated with the metastatic phenotype of Anip-973 cells. The H_4-D cDNA sequence showed 85% homology with a human propionyl-CoA carboxylase α-chain. This enzyme involved in the degradation of some amino acids, fatty acids and other metabolites. Inherited deficiency of the enzyme results in propionic acidemia, an autosomal recessive disorder leading to death of baby in early infantile, which indicates that the enzyme is an important material in metabolism. Although the relationship of this enzyme with metastasis is unclear at the moment, we associated our result with the well known metastasis suppressor gene nm23, the products of which, NDP kinases, are also universally detected in normal tissue cells. NDP kinases, were speculated to have at least two major function in tumor invasion and metastasis: microtubule assembly/disassembly and signal transduction through G proteins. However, the role of nm23 as a metastasis suppressor gene is challenged by some studies. In some types of tumor nm23 expression is higher in more malignant variants and the protein products of nm23 may be a transcription factor involving in the activation of c-*myc* gene,[8] suggesting the pleiotropy of nm23 gene. The exact functions of nm23 gene inhibiting metastasis is actually unknown. By further studies the new functions of some known enzymes may be found and critical regulative mechanism in metastatic process may eventually be disclosed. The availability of the H_4-D cDNA fragment provide us with the possibility to test its function in various *in vitro* and *vivo* models.

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ADENOSQUAMOUS CARCINOMA OF THE NASOPHARYNX CONTAINING EB VIRUS, APROPOS OF A CASE

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According to the standards of WHO clasification, nasopharyngeal carcinoma (NPC) is originated from the lining epithelium of the nasopharyngeal mucosa and those carcinomas of the nasopharynx, including adenocarcinoma, adenosquamous carcinoma and others are not termed NPCs. NPCs are consistently associated with EB virus. Besides NPCs is there also an association of EB virus with adenosquamous carcinoma? This is unknown. The authors report a case of adenosquamous carcinoma of thenasopharynx herein which contained EB virus.

A 57-year old woman visited the out-patient department of our hospital because of haveing a stuffy nose, epistaxis and tinnitus of the left ear for two months on April end, 1995. Endoscopic examination revealed a cauliflower mass filling the nasopharyngeal cavity. Computed tomography showed that the left parapharyngeal space and carotid sheath had been invaded by tumor tissues, and the left sphenoid major wing, the internal plate and the basal part of the alar process as well as the basal part of the sphenoid bone were eroded. The left enlarged superior deep cervical lymph node, being 2 cm in diameter and movable, could be palpated. The titres of IgA against EB viral-capsid antigen (IgA/VCA) and early antigen (IgA/EA) were 1/160 and 1/80, respectively. Biopsies were taken both from the nasopharynx and the enlarged lymph node.

Microscopic observation of the nasopharyngeal biopsy showed that the carcinoma cells wee fusiform, cylindrical or irregular in shape and had obvious cell borders and distinct intercellular bridges in between. The carcinoma cells were arranged in glandular structures and trabeculae. Mucin which was confirmed by Alcian blue stain presented within the lumens. A quite number of dendritic cells (S-100 positive) and monocytes/macrophages (lysozyme positive) as well as a few T-cells (CD45RO positive) could be demonstrated with immunohistochemistry. EB virus DNA was detected using polymerase chain reaction (PCR) and southern blot analysis. EB virus early small RNAs (EBERs) were found in most of the carcinoma cells expressed EB virus LMP-1. ENNA-2 and ZEBRA positive cells could not be found.

The histopathological features of the metastatic lymph node were similar to what had been found in the primary growth. The metastatic deposit contain EB virus DNA, and the vast majority of carcinoma cells were EBERs positive too. A considerable number of squamous cells with intercellular bridges and some carcinoma cells containing mucin could also be found except to typical glands presented.

According to the above findings, a diagnosis of "adenosquamous carcinoma containing EB virus of the nasopharynx and metastatic lymph node" can be made.

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