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肺癌醣化酵素基因異常調控之研究

Dysregulation of Glycosyltransferase Genes in Lung Cancer

計畫編號：NSC91-2314-B-002-268

執行期限：91 年 8 月 1 日至 92 年 7 月 31 日

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一、中文摘要

黏液素被認為與癌細胞之局部侵犯與遠隔轉移有關，由於癌細胞之黏液素基因表現及其醣化過程異於正常細胞，有利其脫離局部病灶，並逃避宿主之免疫系統攻擊。在吾人先前之研究中,已證實肺癌會發生黏液素基因之變異，而此變異與異常醣化過程（涎黏液素之產生）均與肺癌病患術後之預後有關。同時並發現涎黏液素之發生與致癌基因 *neu/erbB-2* 有明顯相關性。吾人先前利用 multiplex PCR 探討肺癌之醣化酵素基因表現（包括 sialyltransferase，STs；與 fucosyltransferase，FucTs），侵犯力高易轉移的細胞株（CL_{1-5}）其 STs 與 FucTs 之表現量高於侵犯力弱的母細胞株（CL_{1-0}、CL_{1-1}）。臨床上，超過 50%肺癌檢體有 STs 與 FucTs 表現量增加之現象，而表現高量 ST3GalIII 與數種 FucTs（FucTIII、IV、VI、VII）之肺癌患者其臨床預後明顯較差，術後復發高與長期存活率低。本三年研究計劃之目的為：(1)廣泛而完整的探討醣化酵素基因（core1-4GnTs、β-1-4GalT I~VI、GlcNAcT I-VI）在肺癌之表現情形以及與肺癌預後及臨床表現之相關聯性；(2)對於由(1)所選出具臨床重要性之醣化酵素基因，探討基因表現之調控機轉。於第一年研究中，我們探討 N-acetylglucosaminyl-transferase (MGAT) I~V 在肺癌之表現量與臨床特徵及預後之相關性。

關鍵詞：肺癌、醣化酵素基因

Abstract

Mucins and mucin associated antigens are believed to play an important role in both invasion and metastasis of cancer cells. The altered expression of mucin peptides in cancer cells, as well as glycosylation, facilitates tumor cell invasion into blood stream, attachment to endothelial cells and escape from host immuno-surveillance.

Our previous studies had demonstrated the prognostic implication of mucin expression for lung cancer. Lung cancers overexpressing sialomucins tend to have higher chance of recurrence and metastasis. The expression of sialomucin is correlated with the overexpression of an oncoprotein, *erbB-2/neu*, and at least one mucin gene (*MUC5AC* apomucin). We are than interested about the altered expression of glycosyltransferase genes in lung cancer. Preliminarily, using multiplex PCR to evaluate the expression of sialyltransferase(ST) and fucosyltransferase (FucT) gene families in lung cancer (in >50% of patients), we have demonstrated that overexpression of STs and FucTs are common in lung cancer tissues and cell lines. Daughter cell line (CL_{1-5}) with high invasiveness and metastasis ability expressed more STs and FucTs than parent cell line (CL_{1-0} and CL_{1-1}). Clinically, patients bearing tumors with overexpression of ST3GalIII and FucTIII、IV、VI、VII are liable to cancer recurrence and death. We thus propose a three-year study to comprehensively study the role of glycosyltransferase genes expression in lung cancer. Is up-regulation or down-regulation of certain glycosyl -transferase which leads to expression of cancer-associated sialylated antigens occurs preferentially in lung cancer? Can the altered expression of glycosyltransferase genes serve as a prognostic marker? Specific glycosyltransferase genes considered to be important in lung cancers will be cloned and subjected to further study. In the first year, we evaluated the expression of N-acetyl -glucosaminyltransferase (MGAT) I~V in lung cancer and the association of MGAT expression with clinical features and prognosis of lung cancer.

Keywords: Lung cancer, glycosyltransferase

二、緣由與目的

Aberrant glycosylation of mucins

Mucin glycoprotein consists of a protein backbone with many carbohydrate side chains of varying lengths, sequences, compositions and anomeric linkages. They have a very large molecular weight (400 to > 1000 kDa), many O-glycosidically linked carbohydrate side chains which may constitite 50-85% of the total molecular weight, a high content of serine, threonine and proline in the protein backbone structure. The carbohydrate moieties of mucin glycoprotein may provide important biological functions of cells. These include receptor function for growth factors, hormones, toxins, bacteria, and virus lectins, growth regulation, cellular differentiation, homotypic and heterotypic, cell-cell interaction, cell-substratum or cell-basement membrane interactions and various immunological functions.[1] Experimentally, mucins had been demonstrated to promote tumor cell invasion,

metastasis and modulate the immune recognition phenomenon of cancer cells.[2] The aberrant expression of mucins and mucin related antigens are poor survival factors in carcinomas arising from various organs, such as colon and breast cancer. Our previous studies had demonstrated the prognostic implication of mucins for lung cancer. [3-5] Lung cancers overexpressing sialomucins (highly sialylated mucins not so heavily expressed in normal airway) tend to have higher chance of recurrence and metastasis. The overexpression of sialomucin correlated with *erbB-2/neu* oncoprotein overexpression and at least one mucin core peptide (*MUC5AC* apomucin).

Aberrant glycosylation of mucins is common in cancer cells. Among cancer-associated antigens, sialylated Lewis antigens such as sialyl *Lewis x* (sLe^x) and sialyl *Lewis a* (sLe^a) have been well characterized. It is well known that the sLe^x and sLe^a epitopes produced in cancer cells are mainly carried on mucin O-glycans. The augmented expression of sLe^x and sLe^a antigens is frequently observed in some cancerous tissues, including lung cancer. It has been reported that sialyl Le^x and sialyl Le^a are involved in the process of metastasis, because these compounds serve as ligands for P- and L-selectin expressed on the surface of vascular endothelial cells, and mediate the adhesion of malignant cells to the vascular endothelium[6] Clinical reports also recognized these compounds to be poor prognostic factors for lung cancer and colon cancer.[7,8]

Glycosyltransferase and mucin glycosylation

Glycosyltransferases are enzymes arrayed in Golgi apparatus.. These enzymes transfer glycosyl residues from nucleotide-activated sugar molecules to other carbohydrates or aglycans (peptides or lipids) in a highly efficient and specific way. They work like sequential part of an assembly line with “cooperative sequential specificity”, that the product of one glycosyltransferase becomes the acceptor substrate for the next glycosyltransferase. By estimation, there are more than 250 glycosyltransferases, and the assembly of these enzymes on Golgi may be tissue specific, i.e. different tissue may have different glycosylation process on same peptide backbone.[9,10]

Mucin-type glycoproteins are unique in having clusters of large numbers of O-glycans. These O-glycans contain N-acetylgalactosamine residues at reducing ends, which are linked to serine or threonine in a polypeptide. The initiation of O-linked glycosylation occurs through the action of UDP-Gal-Nac: polypeptide N-acetylgalactosaminyltransferase (ppGaN-Tase), which catalyzes the transfer of GalNAc from the nucleotide sugar UDP-GalNAc to the hydroxyl group of either serine or threonine. [11]

These attached O-glycans can be classified into several different groups according to the core structures subsequently added on GalNAc [12]. In many cells, core 1, Galβ1→3GalNAc, is the major constitute of O-glycans, core 1 oligosaccharides are converted to core 2 oligosaccharides, Galβ1→3 (GalNAcβ1 →6) GalNAc when core 2 β1,6-N-acetylglucos aminyltransferase (C2GnT) is present. [13] In the gastrointestinal tract and breast tissue, oligosaccharides with core 3, GlcNAcβ1→3 GalNAc, can be frequently found. These tissues also contain core 4, Galβ1→4Glc β1→6(GlcNAcβ1 →3)GalNAc. Core 4 is formed from core 3 by core 4 β1,6-N-acetyl glucosaminyltransferase (C4GnT). It has been reported that the amount of core 4 oligosaccharides is reduced in cancer cells, while the amount of core 2 oligosaccharides is maintained or increased.[14] More recently, the increase in the transcript of C2GnT was found to be associated with the progression of colonic carcinoma.[15]

A set of glycosyltransferases are required for the synthesis of cancer-associated sialylated antigens, they are: β1,3 N-acetylglucosaminyltransferase (β1,3GnT), β1,4-galactosyltransferase (β1,4GalT), α2,3-sialyltransferase (ST3Gal), and α1,3-fucosyltransferase (α1,3Fuc-T) for sLe^x synthesis, and β1,3GnT, β1,3 GalT, ST3Gal, and α1,4Fuc-T for sLe^a synthesis. The genes encoding β1,3GalT and β1,3GnT, which are required for the synthesis of type-1 (Le^a and Le^b) and type-2 chains (Le^x and Le^y), have recently been cloned. The genes encoding β1,4 GalTs(I-VI), ST3GalTs (I-IV), α1,3Fuc-T (III-VI), α1,4FucT (FucTIII) and Core 1-4GnTs had been cloned.

Glycotransferase gene expression and cancer

Detailed mechanisms involved in regulation of glycosyltransferase genes are largely unclear. Most of the glcosyltransferase genes ("glyco-genes") are expressed in an organ- and tissue-specific manner [16]. For example, the 5' untranslated region of GlcNAc-TV mRNA from various cells showed multiple sequences depending on the cell types [17]. Analysis of the 5' untranslated region revealed the presence of consensus motifs for transcription factors such as TATA box, AP-1/Ets, AP-2, HNF-1, and HP-1. The gene employs a multiple promoter system for transcription, and gene expression may then be regulated in a tissue-specific fashion. More over, multiple GlcNAc-TV transcripts were reported in various cell lines and tissues [18]. Disruption of the GlcNAc-TV gene in mice results in the loss of any detectable GlcNAc-TV activity, as well as β1-6 GlcNAc branching glycans in all tissue examined [19]. GlcNAc-TV homozygous null mice are viable and fertile, but showed enhanced rates of T cell aggregation and cell proliferation. Enhanced adhesion of cells (leukocytes, epithelial cells) to substratum, but reduced cell motility

was also noted [20]. GlcNAc-TV catalyzes the attachment of a β1-6GlcNAc residue, producing branched N-glycans. The high degree of branching of N-glycans appears to be related to the malignant potential of tumor cells. In particular, β1-6 branching of N-glycans is directly linked to tumor metastasis. Transfection of mammary carcinoma cell lines with a GlcNAc-TV expression vector increased metastasis by 4-40 folds [21]. The expression of GlcNAc-TV gene could also be induced by oncogene (e.g., *ras*-signaling pathway) or viral (e.g., *v-src*) transfections during hepato-carcinogenesis. In addition, the GlcNAc-TV activity is also augmented by phorbol esters and TGF-β1. Recently, *erbB-2/neu* has been shown to be able to stimulate the transcription of GlcNAc-TV through *Ras-Raf-Ets* signal transduction pathway [22].

Unlike GlcNAc-TV, the molecular basis of most human glycogenes remains elusive. As the genomic regions containing the promoter were isolated in only few genes, and complete nucleotide sequence of many genes had only recently become available.

Rationale of study design

Our previous studies had demonstrated the prognostic implication of mucin expression for lung cancer. Lung cancers overexpressing sialomucins tend to have higher chance of recurrence and metastasis. The expression of sialomucin is correlated with the overexpression of an oncoprotein, *erbB-2/neu*, and at least one mucin gene (*MUC5AC* apomucin). We are than interested about the altered expression of glycosyltransferase genes in lung cancer. Preliminarily, using multiplex PCR to evaluate the expression of sialyltransferase(ST) and fucosyltransferase (FucT) gene families in lung cancer (in >50% of patients), we have demonstrated that overexpression of STs and FucTs are common in lung cancer tissues and cell lines. Daughter cell line (CL_{1-5}) with high invasiveness and metastasis ability expressed more STs and FucTs than parent cell line (CL_{1-0} and CL_{1-1}). Clinically, patients bearing tumors with overexpression of ST3GalIII and FucTIII、IV、VI、VII are liable to cancer recurrence and death.

Expression of the above glyco-genes has also been studied in many other types of cancer, and also demonstrated tissue-specificity. For example, downregulation of FucT VII and ST3GalIII was noted in colon cancer and cervical cancer [23,24], while the opposite was shown in gastric cancer [25] and lung cancer (as in our preliminary data). Based on the PCR analyzing the CL cell lines, we noticed a successive increased expression of FucTIII, FucTVI and ST3Gal III from cell line with low invasiveness(CL_{1-0}) to high invasiveness(CL_{1-5}). Since the expression of glycosyltransferases is mainly regulated at the level of transcription, enzyme activity

of glycosyltransferases is strongly correlated with mRNA expression, measured by either Northern blot or in situ hybridization [26]. We hypothesize that increase expression (activity) of FucT III, FucT VI and ST3Gal III play important role in lung carcinogenesis.

Both STs (ST3s and ST6s) and FucTs (FucT-III-VII) are key enzymes participating in synthesizing Lewis and sialyl-Lewis antigens (Le^a, Le^b, Le^x, Le^y, sLe^x, sLe^a). Lewis and sialyl-Lewis antigens are involved in the process of metastasis, these sialylated and fucosylated compounds serve as the ligands of E-selectin or P-selectin expressed on the surface of vascular endothelial cells. ST3GalIII preferentially synthesize sLe^a, FucTIII-VII are enzymes catalyzing the last step of synthesis of Lewis and sialyl-Lewis antigens. FucT III is the Lewis enzyme and ubiquitously expressed in the epithelial cells of aerodigestive tracts, and is the only FucT with both α1,3 and α1,4 fucosylation activity to synthesize both α1,3-fucosyl-containing Le^x and sLe^x and α1,4-fucosyl-containing sLe^a. FucT VI and FucT VII preferentially synthesize Le^x, Le^y, and sLe^x. FucT VI possesses the strongest activity in synthesizing sLe^x antigen.

ST3GalIII and FucT III are identified as genes of interest. Therefore, we are interested in studying whether the abnormal activation of ST3GalIII and FucT III may contribute to the metastatic phenotype during the progression of cancers. We will clone ST3GalIII and FucT III genes and establish inducible expression system of ST3GalIII and FucT III in transfected cell lines. We propose to transfect ST3GalIII and FucT III sense cDNA into CL_{1-0} and anti-sense cDNA to CL_{1-5}. Biochemical or functional change of cells with overexpressed or down-translated enzymes will be evaluated by flow cytometry (formation of specific glycans, i.e. Le^x ,sLe^x and sLe^a), migration/ invasion/metastasis assay, anchorage-independent growth assay, *in vivo* tumorigenicity and metastasis. Catalytic activity of glycosyltansferases in tranfected cell lines will also be measured.

Besides ST3s and FucTs, several classes of glycosyltansfease genes may have contribution to tumorigenesis. These genes include core1-4GnTs、β-1-4GalT I~VI、GlcNAcTs (I-VI, especially III and V) [16,29]. Unlike STs and FucTs which modify terminal glycosylation, coreGnTs、β-1-4GalTs and GlcNAcTs synthesize the core glycan and elongate backbone glycans. Altered expression of these genes may affect cell surface receptors (e.g. cytokine and adhesion receptors) and influence cellular growth and proliferation. To identify candidate genes related to lung carcinogenesis, multiplex RTp-PCR of coreGnTs、β-1-4GalTs and GlcNAcTs will be performed in CL cell lines and in clinical lung cancer specimens.

三、材料與方法

Patients and tumor tissues

From 1992 to 1996, we have collected 309 pairs of lung tumor/nontumor lung tissues. Among them, 235 had the pathology of non-small cell lung cancer. Seventy tissue pairs from patients with detailed clinical data and follow-up history were chosen.

Surgical specimens of tumors and the adjacent uninvolved lung tissue will be obtained from patients with lung cancer at the time of resection. All patients should have non-small cell lung cancer confirmed by histological diagnosis. Preoperative staging work-up included chest radiograph, fiberoptic bronchoscopy with brushing/washing cytology and biopsy, sputum cytology, computed tomography of thorax and abdomen, and bone scanning. Computed tomography of the brain is not a routine staging procedure. All patients have been judged preoperatively to have resectable disease, and all undergo a complete resection of the tumor. The resection will be judged complete if all known tumorous tissue is completely removed, resection margins are microscopically free of the tumor, and the area or nodes proximal to the involved area or nodes is microscopically free of tumor. After excision, tumor samples and the uninvolved lung tissues were collected immediately, snap frozen in isopentane at –60°C placed in sterile jar and stored at –70°C until processed. Specimens used for formalin fixation and for OCT embedding are collected separately from specimens used for RNA isolation. The resected lung and lymph nodes were subjected to routine surgical pathological examination. Representative sections required for staging and histologic classification were generously taken. Sections of 4μm thickness were routinely stained with hematoxylin-eosin. Histologic classification was based on World Health Organization criteria. The final staging of each patient was pathologic, according to the international staging system for lung tumors.

Laser capture microdissection and RNA extraction

For microdissection, a laser microscope system (PixCell II™ LCM System, Arcturus Engineering, Inc., Mountain View, CA) is used. Areas containing tumor cells or cells from normal lung tissues for control are identified on unmounted H&E-stained section and are visualized through video monitor. CapSure™ film carriers are positioned over the cells of interest and capture the cells after a short pulse of low power infrared laser. The CapSure™ film carriers, with the attached cells, are lifted and placed into Eppendorf tubes and subjected for RNA extraction.

Multiplex PCR for glyco-genes

The expression of selected glyco-genes or gene family in tissues will be evaluated using multiplex PCR [31]. CDNA is synthesized from the isolated total RNA by

reverse transcription in 20 μl reactions containing 0.5μg of random primers, 200 U of superscript RTse, 2μg of total RNA, 4 μl of 5X RT buffer, 0.1mM each dNTP, 20 units Rnasin, and 6.5 μl DEPCed water. Each tube is incubated at room temperature for 10 min and then at 42°C for 45min, heated to 90°C for 10 mink, and then quick-chilled on ice. CDNA clones of each genes will be used as positive control. The PCR primers are designed based on the cDNA sequence of specific gene, and are selected with Primer 3 (a web-based primer designed, Whitehead Institute for Biomedical Research). In designing the primers for multiplex PCR, the possibility of primer dimerization is minimized by cross-examination of the mismatches in the sequences of each possible paur of all the primers chosen. Primers with optimal annealing temperatures of 56-60°C are chosen to prevent mismatches, and primers with higher temperatures are chosen for shorter PCR products. Each PCR is performed in a 100-μl reaction mixtuer containing 10 μl RT reaction mixture, 1X PCR buffer, 0.1mM of each dNTP, 2.5 units of *Taq* polymerase, adjusted concentration of each primers, and subjected to 28-30 cycles of PCR reaction. The PCR reaction products are then electrophoresed in a 3% agarose gel and stained with ethidium bromide, and the size of each cDNA product is determined by comparison to size marker (100bp DNA ladder).

Quantitaiton of multiplex RT-PCR products

After amplification, the PCR products were electrophoresed in 3% agarose gel, stained in 5 μg/ml ethidium bromide solution for 30 min, and then destained. The signal intensity of amplified native and mutated products was directly measured and digitized by IS-1000 digital imaging system (Alpha Innotech Incorp., San Leandro, CA). To evaluate the relative levels of expression of the target genes in multiplex RT-PCRs, the value of the internal standards (β2-microglobulin, GAPDH, or β-actin) in each test tube is used as the baseline gene expression of that sample, and the relative value is calculated for each of the target genes amplified in that reaction. These values are then used to compare expression across samples tested.

四、結果

The expression of N-acetylglucosamin- ylransferase (MGAT, or GnT) I~V was evaluated in 49 paired tissues of lung cancer patients. The enzymes have broad acceptor activity for both N- and “mucin-type” O-glycan branching activity. There were 30 adenocarcinoma, and 19 squamous cell carcinoma. Stage distribution was: 20 stage I, 10 stage II, 17 stage III; 9 T1, 36 T2, 2 T3, 2 T4; 24 N0, 10N1,14 N2, 1 N3.

Overexpression of MGAT(GnT)s in lung cancer is not rare: MGAT I (57.1%), MGAT II (69.4%), MGATIV (40.8%), MGAT V (36.7%).

The correlation between MGATs expression and the clinical features or outcome of lung cancer patients reveals that only the expression MGAT1 has correlation with histology (78.9% of squamous cell carcinoma overexpressed MGAT1, vs 43.3% of adenocarcinoma, P=0.014). Patients bearing tumors with MGAT1 overexpression tended to have earlier disease relapse and cancer death. (Fig 1A and 1B).

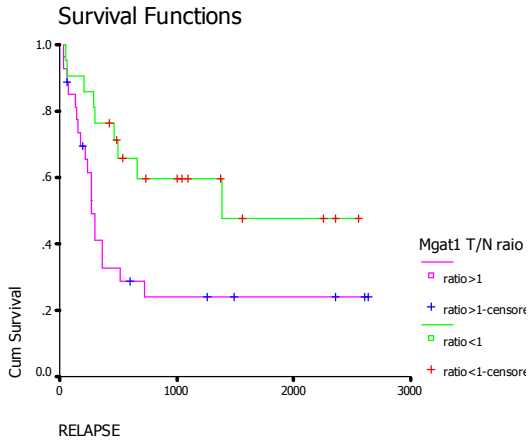
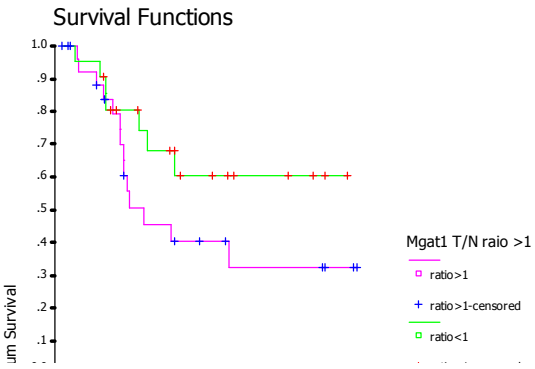


Fig 1A Kaplan-Meier curve of tumor relapse



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