行政院國家科學委員會補助專題研究計畫成果報告

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※ **Glycosyltransferase genes expression in lung cancer** ※
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計畫主持人：余 忠 仁
共同主持人：
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- □赴國外出差或研習心得報告一份
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- □出席國際學術會議心得報告及發表之論文各一份
- □國際合作研究計畫國外研究報告書一份

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中 華 民 國 九十一 年 十二 月 二十五 日

行政院國家科學委員會專題研究計畫成果報告

肺癌之醣化酵素基因表現

Glycosyltransferase genes expression in lung cancer

計畫編號：NSC90-2314-B-002-281-M54

執行期限：自民國90年08月01日起至民國91年07月31日

主持人：余忠仁

執行機構及單位名稱：台大醫學院內科

一、中文摘要

肺癌之研究眾多，至今有許多因子被發現與肺癌之形成或預後有關，黏液素及其相關聯醣基乃為其中之一。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。本研究計劃之目的為探討與涎黏液素形成有關之醣化酵素基因在肺癌之表現情形：我們利用核酸聚合酵素鏈鎖反應法，分析醣化酵素基因在肺癌組織及細胞株之表現情形，及其與涎黏液素及涎化抗原（如 sialyl Le^x 與 sialyl Le^a）之相關聯性。主要發現為 sLe^x，sLe^a，與參與涎化抗原形成之醣化酵素基因之表現與肺癌之數後再發、遠處轉移、及癌症死亡有明顯相關。

關鍵詞：肺癌、黏液素、醣化酵素基因

計畫英文摘要

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, can facilitate the 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 underlying mechanisms for sialomucin expression is unknown. Altered expression of glycosyltransferase genes may be part of the answer. We then propose a three year study in order to characterize the expression pattern of glycosyltransferase (GT) genes in lung cancer. Is up-regulation or down-regulation of certain GTs which leads to expression of cancer-associated sialylated antigens occurs preferentially in lung cancer? Can the altered expression of GT genes serve as a prognostic marker? Multiplex PCR is chosen to evaluate their transcript amounts. Keywords : Lung cancer, mucin, glycosyltransferase,

二、緣由與目的

Many molecular events involve in the carcinogenic and metastatic process of lung cancer, including mucins and mucin-associated carbohydrate antigens, [1]

Mucin glycoprotein have a very large molecular weight (400 to > 1000 kDa), many O-glycosidically linked carbohydrate side chains which may constitute 50-85% of the total molecular weight, a high content of serine, theronine and proline in the protein backbone structure. The carbohydrate moieties of mucin glycoprotein 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.[2] Experimentally, mucins had been demonstrated to promote tumor cell invasion, metastasis and modulate the immune recognition phenomenon of cancer cells.[3]

The aberrant expression of mucins and mucin related antigens are noted to be poor survival factors in carcinomas arising from various organs, including lung cancer. We previously demonstrated the prognostic implication of mucins for lung cancer. [4-6] Lung cancers overexpressing sialomucins tend to have higher chance of recurrence and metastasis.

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. Those sialyl Le^x and sulfated sialyl Le^x in O-glycnas have been shown to be preferential ligands for P- and L-selectin.[7] The augmented expression of sLe^x and sLe^a antigens is frequently observed in some cancerous tissues, including lung cancer, and had been recognized to be poor prognostic factors for lung cancer and colon cancer.[8,9]

The aberrant glycosylation of mucins includes: (1) changes in the expression of core region carbohydrates, due to incomplete synthesis, (2) change of backbone region and peripheral region carbohydrates which occurs mainly due to elongation and modification of existing structures. 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 GT becomes the acceptor substrate for the next GT. By estimation, there are more than 100 glycosyltransferases, and the assembly of these enzymes on Golgi may be tissue specific. [10,11] Mucin-type glycoproteins are unique in having clusters of large numbers of O-glycans. These O-glycans contain N-acetyl- galactosamine residues at reducing ends, which are linked to serine or threonine in a polypeptide. [12] These attached O-glycans can be classified into several different groups according to the core structures subsequently added on GalNAc.[13]

The synthesis of cancer-associated sialylated antigens then requires a set of several GT: 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 current study is try to characterize whether the expression pattern of GT genes in lung cance will lead to expression of cancer-associated sialylated antigens occurs preferentially in lung cancer. Can the alteration of GT gene expression serve as a prognostic marker, like sialylated antigens? Enzymes related to synthesis of sialyl *Lewis x* and sialyl *Lewis a* antigens are selected as genes of interest. Multiplex PCR is performed to evaluate their transcript amounts.

三、材料與方法

Patients and tumor tissues

Surgical specimens of tumors and the adjacent uninvolved lung tissue will be obtained from patients at the time of resection. After excision, tumor samples and the uninvolved lung tissues were collected immediately, snap frozen in isopentane at -60℃ placed in sterile jar and stored at -70 ℃ until processed. Specimens used for formalin fixation are collected separately from specimens used for RNA isolation. 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.

RNA extraction

Total cellular RNA were extracted from peripheral blood leukocytes, tissues and cell lines using the phenol-chloroform and guanidinium thiocyanate-phenol-chloroform extraction method, respectively. After verification of specimen histology by cryostat sectioning, the frozen tissue specimens were subjected to DNA and RNA extraction.

Immunohistochemistry Analysis

After deparaffinization and rehydration of the paraffin sections, the sections of non-cancerous and cancerous tissue are treated with 0.3% (v/v) H_2O_2 , and are incubated for 20 minutes with 0.5% normal goat serum in PBS at room temperature. Next, mAbs against sLe^x and sLe^a were diluted and added to sections, and allowed to react for 12 hours at room temperature. Specific binding of these mAbs to the tissues is determined by the streptavidin-biotin technique.

Multiplex PCR of GT genes mRNA expression

Polymerase chain reactions for α1,3 and 1,4 FucTs (FucT-III,-IV,-V,-VI, and –VII), α2,3-STs (ST3Gal-I, -II, -III, -IV, -V) were established by using β2-microglobulin and GAPDH genes as internal controls. Two μl of template cDNA was added into PCR master mix containing multiple primer pairs and reaction buffer. The PCR products were electrophoresed in 2% agarose, stained in EtBr, and UV enlightened. The polaroid image of the gel was captured by computerized digital imaging scanning and densitometry performed using the NIH image program. The specific GT signal values were divided by the values of the internal control genes.

Statistical Analyses

All the data were analyzed with SPSS software (SPSS version 10.1, SPSS Inc., Chicago). Where appropriate, the data were expressed as mean ± standard deviation (continuous variables) or as a percentage of the group from which they were derived (categorical variables). Continuous variables were compared using the Student's *t*-test. *Chi*-square or Fisher's exact tests were used to compare the clinicopathologic characteristics of patients (and tumors) with the expression of sLe^x, sLe^a, and glycosyltransferase genes. A difference between the expression of the 11GTs was searched using a nonparametric one-way ANOVA (Kruskal-Wallis test). Overall survival and relapse-free survival curves were calculated using the Kaplan and Meier method. Comparison between curves was carried out by the log rank test. In multivariate analysis of survival, the Cox proportion hazards regression model was used to study the effects of different variables. Patients who survived during follow-up or died of causes other than lung cancer were censored. All statistical tests were two-sided. Difference was considered significant when the P value was <0.05.

四、結果與討論

Patients and tumor tissues

There were 42 adenocarcinomas, 26 squamous cell carcinomas, 3 large cell carcinomas, and 3 adenosquamous carcinoma. The gender distribution was: 48 men and 26 women. The mean age was 61.3 ± 11.4 years old, range from 24.1 to 78.2 years old. Cigarette smoking was more prevalent in male patients (37 patients, 71.2%) than in female patients (4 patients, 15.4%). The distribution of pathological stage was: 37 stage I disease (11 stage IA and 26 stage IB), 17 stage II (2 stage IIA and 15 stage IIB), and 20 stage IIIA. T stage distribution was: 14 T1, 50 T2, 10 T3. N stage distribution was: 43 N0, 15N1, 16N2. Tumor recurrence developed in 44 patients in a mean duration of 11.8 ± 11.7 months (range from 2 to 50 months, median 9 months), 32 had recurrence due to distant metastasis (17 brain, 13 bone, 7 contralateral lung, 5 liver, and 2 pericardium). Thirty-three patients died 11.8 ± 19 months (range from 5-51 months, median 19 months) after operation. The follow-up period for the survived patients was 41.1 ± 29.3 months (median, 35 months), lasted till October 31, 2002.

Expression of sLe^x and sLe^a antigens and clinicopathologic features in lung cancer

Thirty-three (44.6%) tissue sections of 74 studied subjects were found to be positive for expression of sLe^a, 18 (24,3%) were defined as higher level of expression (2+ and 3+, positive stain in > 25% of tumor cells, Fig 1A).

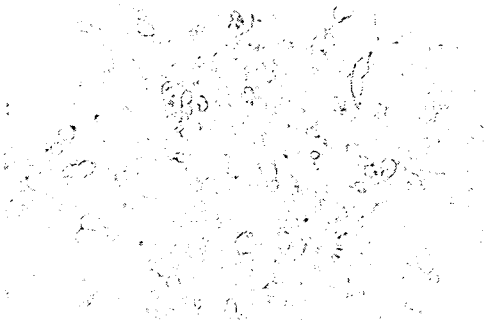

Fig 1A

Fifty-three (71.6%) were positive for expression of sLe^x, 24 (32.4%) were defined to have higher level of expression (3+, positive stain in > 50% of tumor cells, Fig 1B).

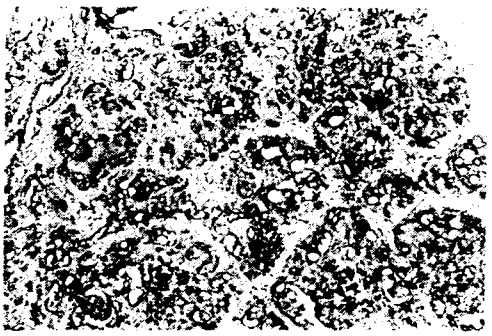

Fig 1B

No correlation could be established between age (< or >= 60 years old), gender, smoking status or histology (adenocarcinoma vs. non adenocarcinoma) and the expression of these two sialyl Lewis antigens. None of the 14 T1 tumor expressed high level of sLe^a antigens, versus 18 out of 60 T2 or T3 tumors (P=0.015). Immunoreactivity to sLe^a or sLe^x in N0, N1 or N2 groups was not significantly different. site of distant metastases, patients bearing tumors with higher level of either sLe^a or sLe^x expression tend to have higher chance of brain metastasis (P=0.048 for 3+ sLe^x, and P=0.017 for 2+ and 3+ sLe^a), while high level of sLe^x correlated with lung metastasis (P=0.038).

Glycosyltransferase expression and clinico-pathologic features

Overexpression (defined as the expression ratio of specific gene is 1.5 fold higher than that in paired non-tumor lung tissue) of GT genes could be demonstrated in about half of lung cancer regardless of the histology. ST3Gal VI and FucT IV genes were overexpressed in less than half (45.8% and 44.9%, respectively) of lung cancer. The others genes were overexpressed in more

than half of cancer tissues (FucT III, 62.8%, FucT V, 57.7%, FucT IV 51.3%, FucT VII, 52.6%, ST3GalI, 77.8%, ST3GalII 56.6%, S3GalIII 60.3%, ST3GalIV, 57.9%, ST3GalV, 57.9%). Using Kruskal-Wallis test, the level of overexpression was significantly higher in ST3Gal I, ST3Gal III and ST3Gal VI than other ST genes (P<0.001; Fig 1B). The expression of GT genes was not related to gender or smoking status, Progressive increment of the fraction of FucT III overexpression was associated with advanced T stage (T1, 5/14, 35.7%; T2, 32/50, 64%; T3, 9/10, 90%; P= 0.018), ST3Gal V (decrement, 0.010). N stage: ST3Gal IV (N2 vs N1, increment, 0.05), ST3Gal II (decrement, N0 vs N1+2, 0.013). When comparing the expression of various GT genes with the pattern of tumor relapse, distant metastasis was related to overexpression of ST3Gal III (P=0.048), FucT-V (P=0.037), and FucT–VI (P=0.01). Brain metastasis was related to overexpression of FucT-IV (P=0.007), FucT-V (P=0.008), and FucT-VI (P=0.009).

Relationship between Sialyl Lewis antigen staining and overexpression of GT genes

Correlation can be established between high level of sialyl Lewis antigen expression and overexpression of specific GT genes. High level of sLe^a antigen expression in lung cancer tissue correlated with overexpression of ST3Gal III (P=0.028). High level of sLe^x antigen expression in lung cancer tissue correlated with overexpression of three FucT genes: FucT-III (P=0.009), FucT-V (P=0.049), and FucT-VI (P<0.001).

Survival according to sLe^a and sLe^x staining and overexpression of glycosyltransferase genes

Survival analysis demonstrated that patients bearing tumors with expression of sLea and sLex antigens and overexpression of five GT genes (ST3GalIII and FucT III,IV,VI,VII) tended to have shorter disease-free-survival and overall survival (only survival curves were demonstrated in Fig. 2A,2B,2C,2D and 2E).

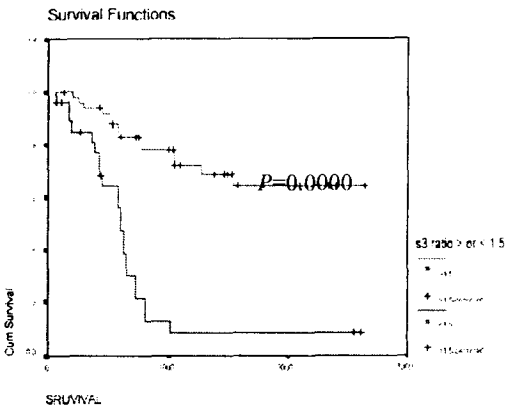

Fig 2A. Overexpression of ST3GalIII is related to poor survival outcome in non-small cell lung cancer.

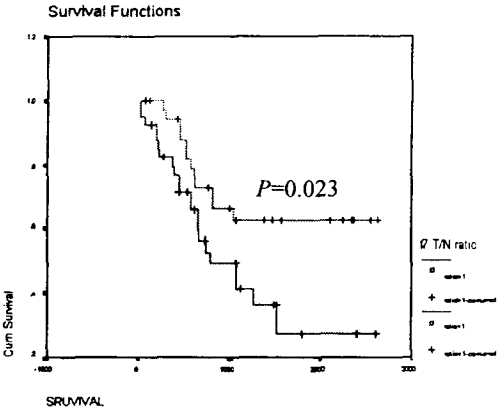

Fig 2B Overexpression of FucT VII is related to poor survival outcome in non-small cell lung cancer.

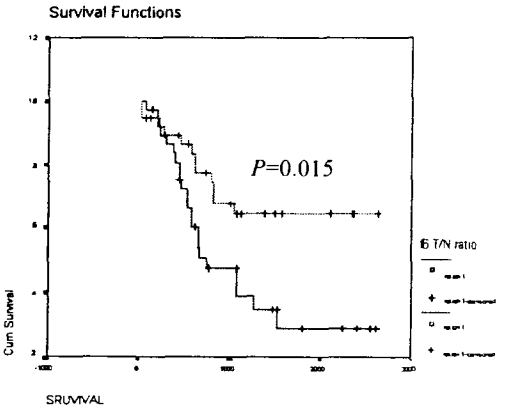

Fig 2C Overexpression of FucT VI is related to poor survival outcome in non-small cell lung cancer.

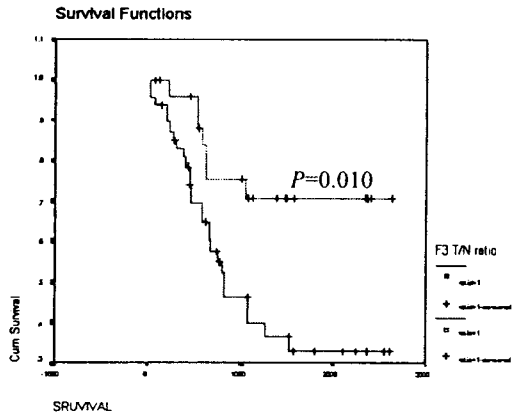

Fig 2D Overexpression of FucT III is related to poor survival outcome in non-small cell lung cancer

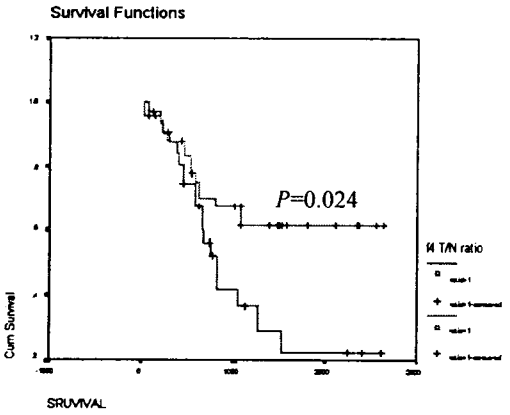

Fig 2E Overexpression of FucT IV is related to poor survival outcome in non-small cell lung cancer

Multivariate analysis of survival

The relative hazard ratios among the variables of pathological stage, high level expression of sLea and sLex antigens, overexpression of ST3Gal III, FucT III, FucT IV, FucT VI, and FucT VII were analyzed by the Cox regression model. The multivariate logistic regression proved that stage (OR=3.204, 95%CI=1.249-8.222, p=0.015), sLex high level expression (OR=5.425, 95%CI=1.345-21.877, p=0.017), and overexpression of ST3Gal III (OR=8.217, 95%CI=1.984-21.033, p=0.004) were independent factors affecting the survival.

Our results demonstrates that dysregulation of GTs is frequently detected in lung cancer. The expression of two α2,3 ST (ST3Gal- II and ST3Gal-IV) is related to advanced stage. The expression of four Fuc-Ts (Fuc-T III, IV, VI, VII) and one α2,3 ST (ST3GalIII) is related to cancer invasiveness, metastasis and cancer death. Again, the study proved the expression of sLe^x and sLe^a antignes in lung cancer indicates poor prognosis of patients, most probably due to distant metastasis, especially brain metastasis. The overexpression of certain GTs contributes to the expression of both sialyl antigens. Since the expression of GT is mainly regulated at the level of transcription, enzyme activity of GT is strongly correlated with mRNA expression, measured by either Northern blot or in situ hybridization. We hypothesize that increase expression (activity) of FucTs and ST3Gal III play important role in lung carcinogenesis.

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