行政院國家科學委員會專題研究計畫　期中進度報告

第二型環氧酵素(COX-2)基因對於非小細胞肺癌之醣化酵素基因與 Lewis 涎化抗原表現之調控(1/3)

計畫類別： 個別型計畫
計畫編號： NSC92-2314-B-002-294-
執行期間： 92 年 08 月 01 日至 93 年 07 月 31 日
執行單位： 國立臺灣大學醫學院內科

計畫主持人： 余忠仁

報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中　華　民　國 93 年 6 月 21 日

行政院國家科學委員會補助專題研究計畫 **□成 果 報 告**
■期中進度報告

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計畫參與人員：

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執行單位：國立台灣大學醫學院內科

中　華　民　國　九十三　年　六　月　十六　日

一、中文摘要

關鍵詞：肺癌、醣化酵素基因、Lewis 涎化抗原、第二型環氧酵素

癌細胞之醣化過程異於正常細胞，特殊之醣化抗原(如 Lewis 涎化抗原)表現有利其脫離源發病灶，入侵血管，並逃避宿主之免疫系統攻擊。在吾人先前之研究中,已證實肺癌之異常醣化過程（Lewis 涎化抗原之產生）均與肺癌病患術後之預後有關，而利用 multiplex PCR 探討肺癌之醣化酵素基因表現（包括 sialyltransferase，STs；與 fucosyltransferase，FucTs），ST3GalIII、FucTIII、IV、VI、VII 之表現量與 Lewis 涎化抗原之產生及肺癌之遠處轉移、術後存活有明顯相關。細胞株之研究亦顯示侵犯力高易轉移的細胞株（CL_{1-5}，CL_{1-5} F4），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}）。而此一系列之細胞株呈現次第增加的第二型環氧酵素表現量。第二型環氧酵素被認為與腫瘤生長、反細胞凋亡、血管生成、細胞移動與侵犯能力有關，有初步證據證實再大腸癌細胞，第二型環氧酵素基因可以藉由調控某些醣化酵素基因而表現出 Lewis 涎化抗原，促進癌細胞轉移。其調控機轉至今仍無相關文獻探討。本三年研究計劃之目的為：(1)廣泛而完整的探討第二型環氧酵素基因與其產物（前列腺素，尤其是 PGE2，PGI2）之受體（EP1-4；PPARα，γ，δ）在肺癌之表現情形以及與醣化酵素基因、以及 Lewis 涎化抗原表現之相關聯性；(2)對於由(1)所選出具臨床意義之 EP，PPAR 受體與醣化酵素基因，本研究將著手探討第二型環氧酵素對這些醣化酵素基因表現之訊息傳遞調控機轉。

英文摘要

Keywords：Lung cancer, glycosyltransferase, sialyl Lewis antigen, cyclooxygenase-2

Products of anerrant glycosylation may facilitate 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 sialyl Lewis antigens (sLe^x and sLe^a) for lung cancer. Lung cancers overexpressing sLe antigens tend to have higher chance of recurrence and metastasis. Using multiplex PCR to evaluate the expression of sialyltransferase(ST) and fucosyltransferase(FucT) gene families in lung cancer, we have demonstrated that tumors with overexpression of ST3GalIII and FucTIII、IV、VI、VII are closely related to expression of sialyl Lewis antigens, and to cancer recurrence and postoperative death. Cell line study revealed that cell lines (CL_{1-5} , CL_{1-5} F4) with high invasiveness and metastasis ability expressed more STs and FucTs than parent cell line (CL_{1-0} and CL_{1-1}). This panel of cell lines posses incremental expression of cyclooxygenase-2 (COX-2), positively correlate with the degree of invasiveness. COX-2 involves many aspects of cancer cell biology, including cell growth, antiapoptosis, angiogenesis, cell motility and invasion. Evidence has been demonstrated in colon cancer cell lines that, COX-2 can promote cancer metastasis through expression of certain glycosyltransferase genes and sialyl Lewis antigens. Up to now, the mechanisms of how COX-2 regulate the expression of GT genes has never been reported. We thus propose a three-year study to comprehensively study the expression of COX-2, the receptors of COX-2 products (i.e., prostglandins, PG), EP1-4 and PPARα，γ，δ in lung cancer. And correlate with expression status of GT genes and sLe antigens completed in our previous work. The study will identify specific glycosyltransferase genes, and, probably sepcific EP or PPAR related to COX-2 expression. The regulatory mechanism of COX-2 on these GT genes will then be explored.

二、緣由與目的

Cyclooxygenase is the enzyme catalyzing the rate-limiting step in prostaglandin synthesis, converting arachidonic acid(AA) into prostaglandin(PG) H2. Two isoforms of this enzyme have been identified. Cyclooxygenase-1 (COX-1) is constitutively expressed in many tissues and plays roles in tissue homeostasis. In most cells and tissues, cyclooxygenase-2 (COX-2) is an inducible isoform whose expression is stimulated by growth factors, cytokines, and tumor promoters (1-3).

Mounting evidence documents elevated expression of COX-2 in a variety of malignancies including colon, gastric, esophageal, prostate, pancreatic, breast and lung carcinomas (4-13) Overexpression of tumor COX-2 may be important in tumor invasion (14,15), angiogenesis (16-19), resistance to apoptosis (20-22), and suppression of host immunity (13, 23). Recently, the importance of COX-s expression in lung cancer had been demonstrated by a variety of reports (24-29).

Elevated expression of COX-2 has been shown to increase tumor invasiveness and enhance metastatic potential (4, 30). The complex events associated with tumor cell invasion include the active movement of cells across the extracellular matrix and spread to distant organ sites (31). Overexpression of COX-2 in cancer cells increased cell adhesion to the extracellular proteins laminin and Matrigel (20, 30). The biochemical changes associated with these altered cellular dynamics include increased expression and activation of maetalloproteinase-2 (MMP2), and reduced expression of E-cadherin (20, 30). In coherence, COX-2 inhibitors have been found to reduce tumor cell migration, cell adhesion, and tumor invasiveness in in vivo and in vitro experimental systems. These effects were observed in various cancer cells including liver, prostate, colon and breast (32-35). A recent study in non-small cell lung cancer cell lines concluded that cell invasiveness promoted by COX-2 is mediated by the cell surface receptor for hyaluronate, CD44 (36). Overexpression of COX-2 caused cell invasiveness and also increased CD44 expression. Moreover, cell invasion was significantly reduced in cells overexpressing COX-2, but where CD44 was specifically blocked. In colorectal carcinoma cells, the phosphatidylinositol-3-kinase/ Akt kinase/protein kinase B pathway was recently found to mediate PGE2-promoted cell motility (37). Based on these results, there are likely to be multiple, cell-type dependent mediating pathways for COX-2 promoted cell invasion.

More recently, investigators had demonstrated the interaction between between COX-2 activity and the expression of sialyl Lewis antigens, the cancer-associated carbohydrate antigens.closely related to hematogeneous metastasis of colon, breast and lung cancer, and the expression of enzymes synthesizing sialyl Lewis antigens (15). Effects of COX-2 activity and prostaglandin E2 on cell adhesion, expression of sialyl Lewis antigens, and glycosyltransferase(GT) genes were determined in Caco-2-m cells (a colon cancer cell line expressed COX-2 low level), Caco-2-COX-2 (Caco-2 programmed to overexpress COX-2), and HT-29 cells (a colon cancer cell line with high level of COX-2). Caco-2-COX-2 cells had increased SPan-1 levels and increased adherence to endothelial cells via SPan-1 compared with Caco-2-m cells. HT-29 cells expressed sialyl Lewis a (sLe^a) and adhered to endothelial cells via sLe^a. Treatment with a COX-2 inhibitor, celecoxib, decreased SPan-1 and sialyl Lewis a expression and adherence to endothelial cells. Caco-2-COX-2 and HT-29 cells metastasized to the liver, whereas Caco-2-m cells did not. Pretreatment with celecoxib reduced the metastatic potential, and inhibit the

expression of three GTs synthesizing sLe^a antigen, ß3Gal-T5 and ST3Gal III and IV. The study indicate a direct link between COX-2 and enhanced adhesion of carcinoma cells to endothelial cells, and enhanced liver metastatic potential via accelerated production of sialyl Lewis antigens. COX-2 inhibitors may suppress metastasis. However, they didn't establish a similar correlation or consequences in clinical settings.

Despite tremendous progress made in understanding the functional role of COX-2 in cell growth, cell death, cell motlity, and in cancer, the complete picture of downstream signaling pathways for the various effects of COX-2 is not yet understood. Prostaglandins (PGs) have been shown to play roles in the wide spectrum of biological processes related to COX-2. PGH2 is an unstable endoperoxide intermediate, which subsequently is converted to one of several structurally related eicosanoids, including PGD2, PGF2α, PGI2, and thromboxane A2, by the activity of specific cellular PG synthases.

PGE2 is constitutively produced by many cancers, including nons-small cell lung cancer (38). PGE2 elicits its effects on target cells through interaction with prostaglandin E series receptors (cell surface G-protein-coupled eicosanoid receptors) of four distinct subtypes, designated EP1, EP2, EP3, and EP4 (39). Signaling downstream from the different EP receptors varies, probably because each receptor interacts with different G proteins. EP1 causes influx of Ca2+ and activation of protein kinase C; receptors EP2 and EP4 activate adenylate cyclase, which increases cellular cyclic AMP levels and activates protein kinase A; and EP3 signals primarily through an inhibitory G protein to decrease intracellular cyclic AMP levels. Growth-promoting effects are signaled via EP receptors in several cell types. In primary keratinocytes, growth promotion was signaled via an EP2 and/or EP4 receptor-cAMP coupled response (40). As mentioned beforehand, in a recent study in colorectal carcinoma cells, PGE2 promoted cell growth and motility via the EP4 receptor by activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt/PKB) pathway (37). Similarly, the effect of PGE2 in mediating the expression of CD44 and MMP2 in lung cancer was recently shown to work through the EP4 receptor (41). Interestingly, EP2 and EP3 were also expressed in the colon and lung carcinoma cells (41, 42) and their binding affinities to PGE2 are similar to EP4. It is currently not understood how growth and motility signaling was selectively directed to the EP4 receptor.

The classical signaling pathway of PGI2 uses a G-protein-coupled cell surface receptor termed IP (43). Researches had demonstrated PGI2-IP signaling is indeed important in preventing thrombosis and is involoved in inflammatory responses (44), but nothing related to tumorigenesis. Peroxisomal proliferator-activated receptors (PPARs), PPARα, PPARγ, PPARδ, though initially cloned as a family of orphan receptors, are now known to mediate downstream signaling of nuclear PG, including PGI2. PPARs bind to sequence-specific DNA response elements (PPRE) as a heterodimer with the retinoic acid receptor (RXR) (45). Although the identity of definitive high-affinity natural ligands for PPARs is lacking, there is evidence that AA metabolites can serve as activating ligands for PPARs. In particular, the PGD2 metabolite, 15-deoxy Δ 12,14 PGJ2 is a potent activator of the PPARγ isoform (46, 47), whereas a stable analog of PGI2, carbaprostacyclin (cPGI), has been shown to activate PPARδ and to a much lesser extent, PPARα.(48,49) Research on PPARs has revealed that the PPARα and PPARγ isoforms play fundamental roles in such diverse physiological processes as lipid metabolism, immunity, and

cellular differentiation (50,51). There also has been a great deal of interest in PPARs and cancer. Activators of PPARα will induce the formation of hepatocellular carcinomas in rodents (52), whereas ligands for PPARγ have been shown to induce cellular changes consistent with differentiation and reversal of the neoplastic phenotype in liposarcoma (53), breast (54), and colon carcinoma cells (55). In addition, inactivating mutations in PPARγ recently were identified in a subset of colorectal tumors, strongly suggesting that this isoform has a tumor suppressive role during colorectal carcinogenesis (56) Recently, PPARδ was shown to be aberrantly expressed in colorectal cancers, and that endogenous PPARδ is transcriptionally responsive to PGI2 (57). It is also interesting to note that co-overexpression of COX-2 and PPARδ has also been reported in endometrial adenocarcinoma (58) However, the functional consequence of PPARδ activation in carcinogenesis still needs to be determined. So far, all reseraches investigating PPARs expression in lung cancer have focused on PPARγ and clearly suggested PPARγ plays a tumor suppressor role in lung cancer (59-61). No reports had been made regarding the expression of the other PPARs (PPARα, and PPARδ) and their association with COX-2 gene expression in lung cancer.

The following experiments will be carried out to establish the potential relationship between the expression of COX-2 and the expression of specific GT genes and to elucidate the regulatory mechanism of COX-2 on specific GT gene.

1. Real-time quantitative RT-PCR to measure the expression of COX-2, EP (EP1, EP2, EP3, EP4) and PPAR (PPARα, γ, δ) genes in tissues of non-small cell lung cancer, as well as in CL cell lines. The data will be matched with the expression status of GT genes and sLe antigens available from our previous study. This will demonstrated the association among COX-2, its specific receptors (especially EP4 and PPAR-δ) and GTs (especially FucT III-VII, and ST3GalIII) genes and sLe antigens.
2. We will establish cell line from CL_{1-0} with stable transfection of human COX-2 cDNA with inducible overexpression system for studying COX-2 signaling pathway related to GT genes expression in lung cancer cells. The pharmacological effect of COX-2 inhibitor, PGE2, EP agonist/antagonist, PPAR agonist/antagonist on cell migration, invasion, and adhesion to HUVEC and ECM, GT expression, sialyl Lewis antigens expression will be performed.

三、材料與方法

Lung cancer tissues

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. All patients have been judged preoperatively to have resectable disease, and all undergo a complete resection of the 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. The 74 pairs of lung tumor/nontumor lung tissues used in previous studies were applied to investigation. 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.

Cell lines

A panel of human lung adenocarcinoma cell lines, CL_{1-0}, CL_{1-1}, CL_{1-5}, $CL_{1-5}F4$, in ascending order of invasiveness and metastasis, was established previously [30]. CL_{1-0} is the parent cell line, while CL_{1-1}, CL_{1-5} and $CL_{1-5}F4$ are sublines that were selected from CL_{1-0} cultured with a polycarbonate membrane coated with Matrigel in Transwell invasion chamber. The cells that migrated through the membrane were harvested. The sublines from the first, and fifth rounds of selection were assigned CL_{1-1}, CL_{1-5} and a further fourth round of selection of CL_{1-5} was assigned $CL_{1-5}F4$. A549 cell lines stably transfected with COX-2 and anti-sense COX-2, as well as vector alone were given by Dr. Stewart Dubinnett from UCLA.

Real-time Quantitative RT-PCR for COX-2

The expression of COX-2 in tissues and cell lines, and GT genes in CL_{1-0} or $CL_{1-5}F4$ were evaluated with real-time PCR. RNA samples were reverse transcribed using MgCl2 (5.5 mM), dNTPs (0.5 mM each), random hexamers (1.25 μM), oligodeoxythymidylic acid (1.25 μM), RNase inhibitor (0.4 unit/μl), and multiscribe reverse transcriptase (1.25 units/μl), all from PE Biosystems (Warrington, United Kingdom). The mix was aliquoted into individual tubes (16 μl/tube), and template RNA was added (4 μl/tube of 250 ng/μl RNA). Samples were incubated for 60 min at 25°C, 45 min at 48°C, and then 5 min at 95°C. A reaction mix was made containing Taqman buffer (5.5 mM MgCl2, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP); TBP (TATA Box binding protein, as internal control) forward and reverse primers and probe (all at 50 nM); forward and reverse primers for COX-2 (300 nM); probe (200 nM); AmpErase UNG (0.01 unit/μl); and AmpliTaq Gold DNA Polymerase (0.025 unit/μl), all from PE Biosystems. A volume of 48 μl of reaction mix was aliquoted into separate tubes for each cDNA sample and 2 μl/replicate of cDNA were added. After mixing, 23 μl of sample were added to the wells on a PCR plate. Each sample was added in duplicate. A no-template control (containing water) was included in triplicate. Wells were sealed with optical caps, and the PCR reaction was run on an ABI Prism 7700 using standard conditions. COX-2 primers and probe for quantitative PCR were designed using the PRIMER express program (PE Biosystems). The sequences of the COX-2 primers and probe were as follows. Forward: 5'-ATGGAATTACCCAGTTTGTTGAATC-3';

reverse: 5'-TGGAAGCCTGTGATACTTTCTGTACT-3'; probe (FAM labeled): 5'-TCCTACCACCAGCAACCCTGCCAT-3'.

Quantitaiton of multiplex RT-PCR of GT gnees products

The expression of selected glyco-genes or gene family in tissues will be evaluated using multiplex PCR, which has been established in our previous study. 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). 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, or GAPDH) 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.

Detection of Lewis antigens by flow cytometry

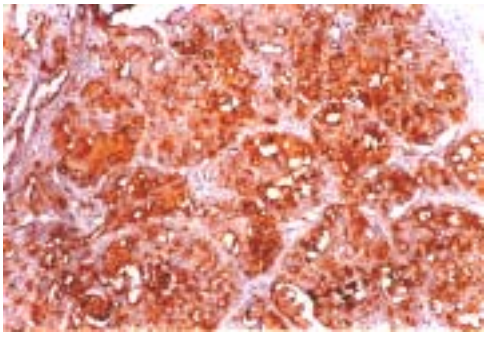
Cells ($1x10^6$) are detached with 2 mmol/L EDTA, washed and resuspended in NaCl/Pi containing 1% BSA, the incubated with different mAbs to Lewis antigens (1:50 dilution) for 30 min at 4C. After two washes, the cells are incubated for 45 min at 4C with a 1:200 dilution of FITC labeled goat anti-Igs. Then the cells are subjected to fluorescence analysis performed on a FACScan.

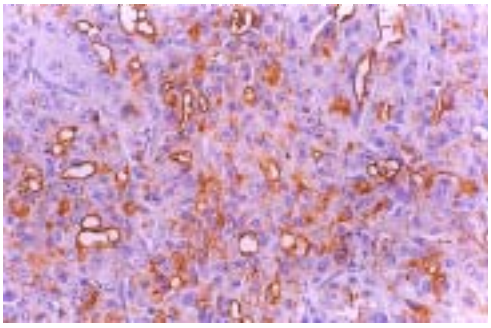
四、結果

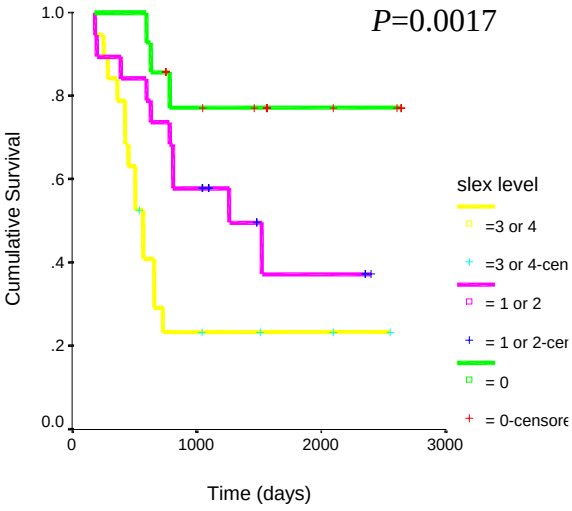
Previously, we have studied the expression of sialyl Lewis antigens (sialyl Lewis X, sLe^x and sialyl Lewis A, sLe^a) and the glycosyltransferases responsible for synthesizing both antigens in 74 non-small cell lung cancer tissues. The results revealed that Survival analysis demonstrated that patients bearing tumors with expression of sLe^a and sLe^x antigens tended to have shorter disease-free-survival and overall survival (Fig 1A, 1B, 1C, 1D).

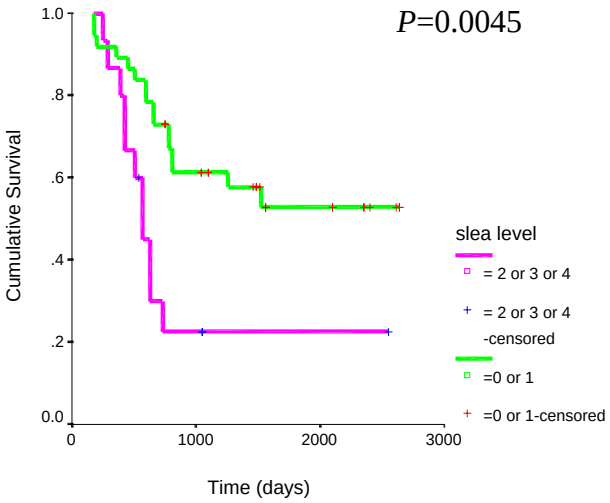
Fig 1A. sLe^x staining

Fig 1B. sLe^a staining





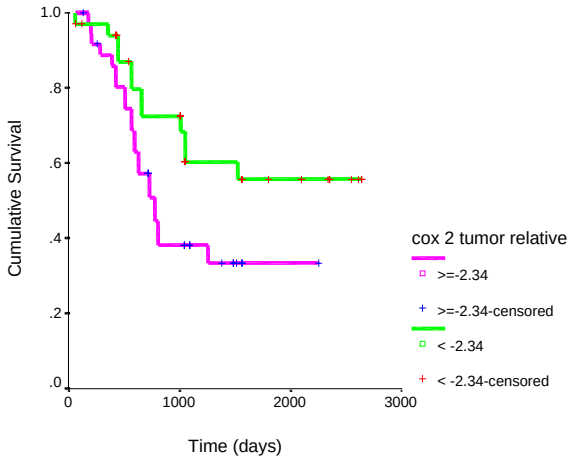




Tumors with higher level of either sLe^a or sLe^x expression tend to have higher chance of brain metastasis (P=0.048 for 3+ sLe^x, and P=0.017 for 2+ and 3+ sLe^a), while high level of sLe^x was also correlated with lung metastasis (P=0.038).

While using multiplex PCR to quantify the mRNA expression of glycosytlransferase genes (Fuc-T III, IV, V, VI, VII and ST3GalI, II, III, IV, V, VI), overexpression (defined as the expression ratio of specific gene is 1.5 fold higher than that in paired non-tumor lung tissue) of glycosyltransferase genes could be demonstrated in about half of lung cancer regardless of the histology. Correlation can be established between high level of sialyl Lewis antigen expression and overexpression of specific glcysoytransferase genes. High level of sLe^a antigen expression in lung cancer tissue correlated with overexpression of ST3Gal III (P=0.028), but not with FucT-III (P=0.097). High level of sLe^x antigen expression in lung cancer tissue correlated with overexpression of three fucosyltransferase genes: FucT-III (P=0.009), and FucT-VI (P<0.001). When comparing the expression of various glcosyltransferase 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).

By measuring the expression of COX-2 RNA in lung cancer, we have demonstrated that NSCLC with high level of COX-2 expression tended to have a worse prognosis (Fig.2), and the expression of COX-2 RNA was associated with the overexpression of FucT-VII (P=0.003).



cox 2 tumor relative to median * f7 ratio Crosstabulation

Count

		f7 ratio		
		<1	>=1	Total
cox 2 tumor relative to median	< -2.34	22	12	34
	>=-2.34	11	26	37
Total		33	38	71

Fig. 2 COX-2 expression and survival of NSCLC

However, using COX-2 stable transfection with A549 cells (Fig. 3), the expression of sialyl Lewis antigens did not show significant change when compared with parent lines (Fig. 4 & Fig. 5).

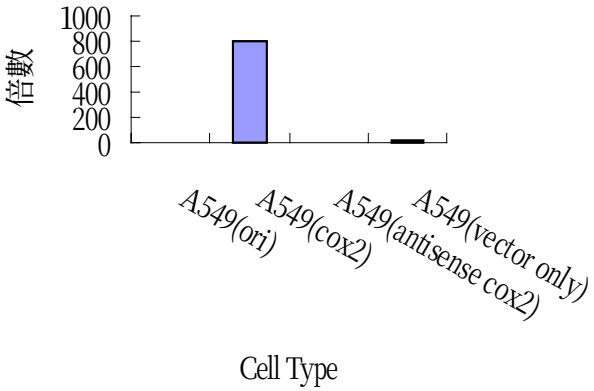


Fig. 3 COX-2 RTQ-PCR in A549 cell lines

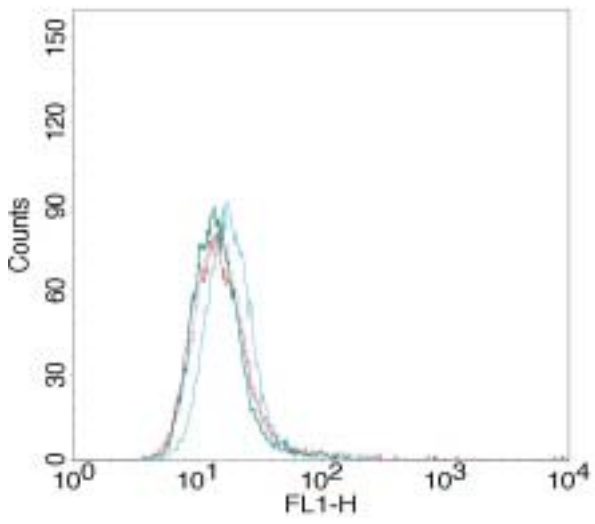


Fig. 4 SLe^a expression in transfected cell lines

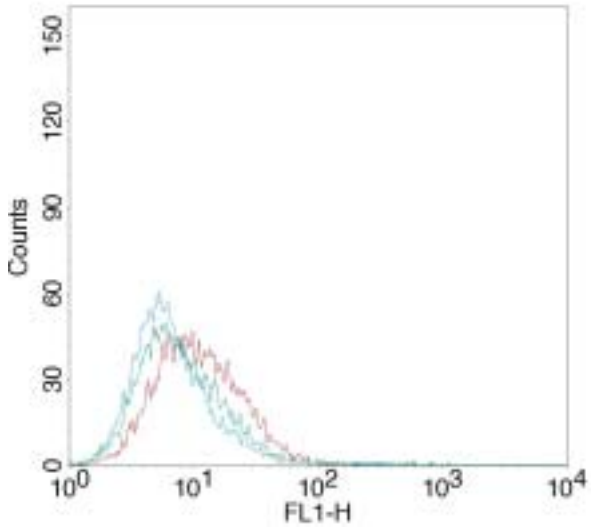


Fig. 5 SLe^x expression in transfected cell lines

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