Prognostic significance of expression of nm23-H1 and focal adhesion kinase in non-small cell lung cancer

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Abstract. nm23-H1, a nucleoside diphosphate kinase (NDPK), enhances drug sensitivity and has antimetastatic activity, whereas focal adhesion kinase (FAK) is closely associated with cell migration and tumour spreading. The relationship between these two proteins, however, is not well elucidated. In this study, we investigate their correlation in patients with non-small cell lung cancer (NSCLC). Expressions of nm23-H1 and FAK were examined by reverse transcription-polymerase chain reaction and immunoblotting in surgical resections. The relationship between these two genes was assessed statistically. Patients were classified into four groups according to the expression of nm23-H1 and FAK by immunohistochemistry: FAK-negative/nm23-H1-positive, FAK-negative/nm23-H1-negative, FAK-positive/nm23-H1-positive and FAK-positive/nm23-H1-negative. Although the causal correlation is still uncertain, our results showed that protein expression of nm23-H1 was inversely correlated with that of FAK. The combined analysis of nm23-H1 and FAK protein expression in the same tumour specimens revealed that patients with FAK-negative/nm23-H1-postive tumours survived the longest, 56 months, among those with nm23-H1 and FAK features ($P<0.001$). Our data indicate that expressions of nm23-H1 and FAK are inversely correlated. These results suggest that the status of nm23-H1 and FAK protein expression may help in predicting the aggressive behavior of NSCLC. However, further studies are warranted to clarify the impact of FAK on the function of nm23-H1 as an anti-metastatic gene.

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Introduction

Following two decades of extensive cancer prevention programs, lung cancer remains as one of the leading causes of cancer death worldwide. The condition will become worse if the smoking population keeps increased (1,2). In Taiwan, the annual death rate of lung cancer is more than 5,500 people (3), and most of the patients died as they were at the late stage of disease at diagnosis (4). However, some patients who were diagnosed at the early stage, and underwent adequate surgery still died of cancer because of the early recurrence, metastasis and disease-associated complications (5).

Recently, after identification of dihydrodiol dehydrogenase (DDH) overexpression in non-small cell lung cancer (NSCLC), we have proposed that DDH may take part in drug resistance (6). Although a subsequent study had demonstrated that DDH was evidently associated with cisplatin resistance in cancer cells (7), the detailed mechanism of how DDH affect drug sensitivity was elusive. Moreover, increased evidence indicated that drug resistance of cancer cells might also be affected by other genes, which were involved in cell proliferation and metastasis (8-11). Among these, antimetastatic gene nm23-H1 and metastasis-associated focal adhesion kinase (FAK) are two obvious targets (12,13).

The *nm23-H1*, a nucleoside diphosphate kinase (NDPK) gene, which was originally identified by differential screening of cDNA libraries between metastatic and non-metastatic murine melanoma cells, was shown to have antimetastatic effect (13-15). Currently, eight human members of nm23, including *nm23-H2*, DR-*nm23*, *nm23-H4* and *nm23-H5*, have been identified. Although these genes hade been shown to play significant roles in cell proliferation and differentiation, they were also closely associated with carcinogenesis and metastasis (16-21). It is worth noting that upon determining the mechanism of cisplatin resistance in human ovarian cancer cells by using ouabain inhibition, evidence was obtained that down-regulation of nm23-H1 reduced cisplatin sensitivity, and ectopic nm23-H1 expression could enhance cisplatin-mediated DNA damage in cancer cells further indicating that nm23-H1 expression was directly associated with drug sensitivity (22).

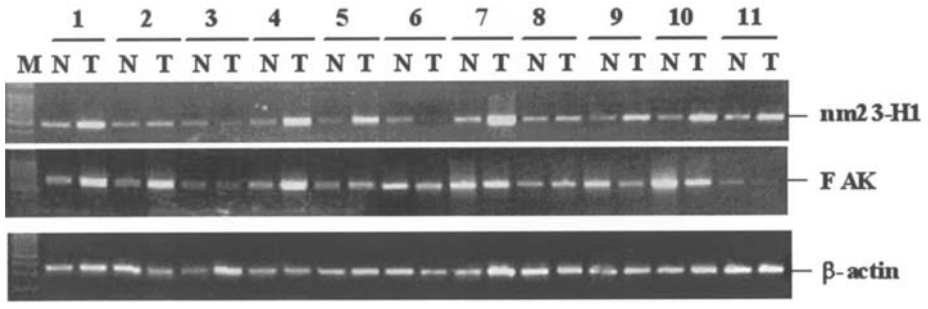

Figure 1. Expression of nm23-H1 and FAK in NSCLC as detected by RT-PCR.

Interestingly, FAK, a 125-kDa integrin-associated cytoplasmic tyrosine kinase, was also found correlated with drug sensitivity (12,23). Biochemically, FAK transduces a signal from extracellular matrices, cytokines and growth factors, and then relays the message via AKT and extracellular signal-regulated kinases (Erk) to protect cells from irradiation- and drug-induced cell injury. Although several elegant studies have respectively examined the expression pattern of nm23-H1 or FAK in NSCLC (13,16-23), correlation and clinicopathological significance of these two factors are not defined.

In this study, we used reverse transcription-polymerase chain reaction (RT-PCR), immunoblotting and immunohistochemistry to examine expression of FAK and nm23-H1 in surgical specimens of NSCLC. The relationship between FAK and nm23-H1, and their respective prognostic significance were evaluated by statistical analysis.

Materials and methods

Patients. From August 1986 to November 2003, tissue specimens from 381 consecutive patients with newly diagnosed lung cancer were collected. All patients had pathologically confirmed NSCLC. Stage of disease progression was classified according to the International System for staging lung cancer that has been adopted by the American Joint Committee on Cancer and the International Union Against Cancer (24). All patients had undergone surgical resection and radical N2 lymph node dissection. Tumour size, lymph node number, differentiation and vascular invasion were evaluated. Patient with lymph node involvement and patients with locoregional recurrence received irradiation at the afflicted areas. After treatment, patients were routinely followed every 3-6 months in the Outpatients. Tumour recurrence and metastasis were identified when blood examination, biochemical studies, chest radiography, abdominal sonography, whole body bone scan and computerized tomography scans of chest showed any suspected evidence of the disease.

RNA extraction and signal amplification using RT-PCR. Expression of nm23-H1 and FAK mRNA in NSCLC was determined by RT-PCR. Briefly, total-RNA was extracted from the resection using SNAP RNA column (Invitrogen Corp., San Diego, CA). Following spectrophotometric determination of RNA yield, cDNA was synthesized by oligo dT primer and AMV reverse transcriptase. An aliquot of cDNA was then subjected to 35 cycles of PCR using a standard procedure denaturing at 94˚C for 1 min, hybridizing at 55˚C for 30 sec, and elongating at 72˚C for 45 sec. The amplified products were resolved in a 2.5% agarose gel and visualized with ethidium bromide staining. Specificity of the amplified fragments was confirmed by DNA sequencing (ABI PRISM, Perkin-Elmer, Foster City, CA). A constitutively expressed gene, ß-actin, was used as an internal control.

Immunoblotting and immunohistochemical staining. Procedure for immunoblotting has been described previously (6). Briefly, proteins were separated in a 10% polyacrylamide gel with 4.5% stacking gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then incubated with antigen-specific antibodies. The signal was amplified by biotin-labelled goat anti-mouse IgG, and peroxidase-conjugated streptavidin. The protein band was visualized by exposing the membrane to X-Omat film (Eastman Kodak, Rochester, NY) with enhanced chemiluminescent reagent (NEN, Boston, MA). Immunohistochemical staining was performed by an immunoperoxidase method as previously described (6). Antibodies to FAK (BD, Franklin Lakes, NJ) and nm23-H1 (Santa Cruz, CA) were used respectively for immunohistochemistry and immunoblotting.

Slide evaluation. In each case, normal lung tissue served as internal negative control. Slides were read by three independent observers without clinicopathological knowledge. A specimen was considered positive when >10% of cancer cells were positively stained; and negative when <10% of the cells were positive (6,20,21).

Statistical analysis. Correlations between the expression of proteins were analyzed by χ^2 test. To calculate a coefficient of rank correlation between various proteins, Spearman rank correlation was used. The Spearman rank correlation coefficient ranges in value from -1 to 1. Survival curves were plotted with method of Kaplan-Meier (25). Statistical difference of survivals between different groups was compared by the log-rank test (26). Statistical analysis was performed using GraphPad Prism4 statistical software (San Diego, CA). Statistical significance was set at $P<0.05$.

Results

Expression of nm23-H1 and FAK in lung cancer cells. Overexpression of nm23 mRNA was detected in 7/11 (64%) lung

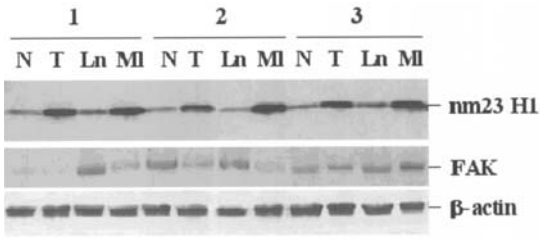

Figure 2. Expression of nm23-H1 and FAK in NSCLC as detected by immunoblotting analysis. M, DNA marker; N, non-tumour counterpart of lung cancer; T, tumour fraction of surgical resection; Ln, normal lymph node; Ml, metastatic lymph node.

Table I. Correlation of nm23-H1 expression with FAK.

Biological factors	nm23-H1 expression High (n=239)	Low (n=142)	P-value	Spearman's correlation
FAK				
+ (n=153)	62	91	<0.01[a]	-0.376
- (n=228)	177	51		

[a]P-value determined by the χ^2 test.

cancer specimens by RT-PCR. FAK overexpression, however, was only identified in 3/11 (27%) specimens (Fig. 1), and expression patterns between FAK and nm23 were inversely correlated (Spearman coefficient -0.31). By immunoblotting, nm23 was detected in both tumour fraction and the metastatic lymph node in RT-PCR-positive patients, FAK expression, on the other hand, was heterogeneous (Fig. 2).

Immunohistochemically, nm23-H1 was located in tumour cells both in tumour fraction and the metastatic lymph node (Fig. 3A and B). FAK was detected in tumour nests (Fig. 3C) and metastatic lymph node (Fig. 3D). The respective expression rate of 381 pathological sections was 62.7% (n=239) for nm23-H1, and 40.1% (n=153) for FAK, However, nm23-H1 expression was inversely correlated with that of FAK (P<0.01; Spearman's coefficient -0.38) (Table I).

Correlation with clinical outcomes. Clinically, the median follow-up was 43 months (range 2.0-86 months), and the mean age was 63.0 years (range 42-75 years). Among 381 patients, 304 men and 77 women enrolled, 275 patients (72.2%) were smokers. The combined analysis of nm23-H1 and FAK protein expression in the same tumour specimens revealed that patients (n=177) with FAK-negative/ nm23-H1-positive tumours survived the longest, 56.0 months, in comparison to other patients with nm23-H1 and FAK features: FAK-negative/nm23-H1-negative (n=51, 30.0 months), FAK-positive/nm23-H1-positive (n=62, 17.5 months), and FAK-positive/nm23-H1-negative (n=91, 15.0 months) (P<0.001) (Fig. 4).

Discussion

The results presented above indicated that expression of FAK was inversely correlated with that of nm23-H1 in NSCLC. Patients with FAK expression usually had worse prognosis, which was frequently associated with higher incidence of the early tumour recurrence and distant metastasis and the consequent shorter survival. Moreover, concurrently decreased expression of nm23-H1 was also noted in these patients.

As noted above, following its discovery by Steeg *et al* (15), several studies had suggested that antimetastatic potential of nm23-H1 could be associated with NDPK, geranyl and farnesyl pyrophosphate kinase, serine protein kinase or

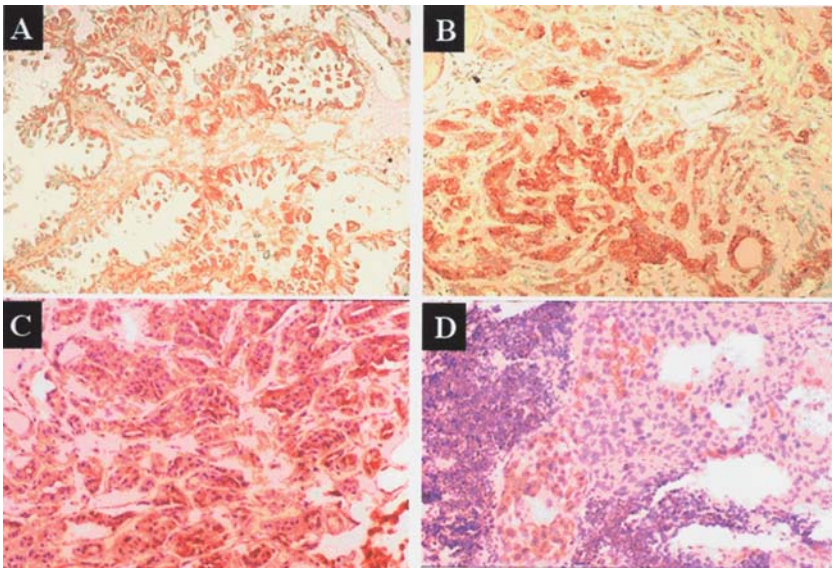

Figure 3. Immunohistochemical staining for nm23-H1 and FAK protein in NSCLC. (A) Representative nm23-H1-positive case, which demonstrates intense nm23-H1 immunoreactivity in the tumour cells (original magnification x200). (B) Representative nm23-H1-positive case of a metastatic lymph node in which nm23-H1 expression is similar to that of the lung tumour (original magnification x200). (C) FAK-positive case, in which FAK immunoreactivity is detected in the tumour cells (original magnification x200). (D) FAK expression in a metastatic lymph node (original magnification x200).

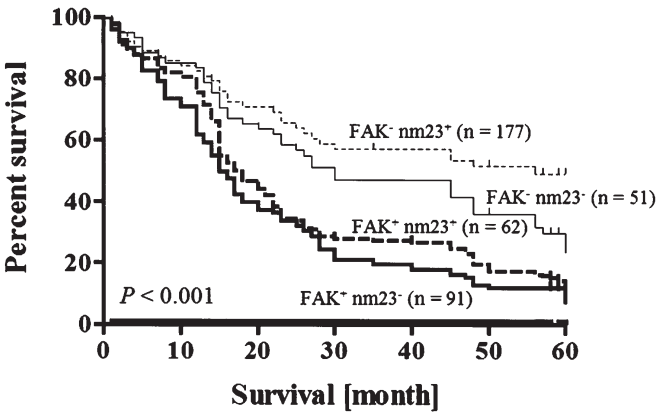

Figure 4. Cumulative survival curves of NSCLC patients that were divided according to the expression of nm23-H1 and FAK by immunohistochemistry: FAK-negative/nm23-H1-postive (FAK^-nm23^+), FAK-negative/nm23-H1-negative (FAK^-nm23^-), FAK-positive/nm23-H1-positive (FAK^+nm23^+), and FAK-positive/nm23-H1-negative (FAK^+nm23^-). Survival curves were plotted with method of Kaplan and Meier. Statistical difference of survival between two groups was compared by the log-rank test (P<0.001).

possibly histidine protein kinase activity of the protein (27). When expressed in a 'two component system' of *Escherichia coli*, nm23 reacted as a histidine sensor kinase that could forward the phosphate residue from autophosphorylation site to an aspartate of the response regulator protein, CTR1, to repress mitogen-activated protein kinase (MAPK) function. Interestingly, CTR1 shares significant homology to the kinase suppressor of Ras (KSR) (28). It is worth noting that nm23, as an NDPK, might be involved in the determination of GTP pool and G-protein reactivity of the cell (29). If so, the accelerated replication of nuclear DNA in rapidly proliferated cells could outrun that of mitochondria genome, and the incoherent replication rates between nucleus and mitochondria might tip the balance of GTP pool and G-protein reactivity to abrogate apoptosis of tumour cells and to increase tumour growth. The relatively heterogeneous expressions of nm23-H1 among different studies provided another line of evidence to support such anticipation (20,21,30,31). In particular, in patients at late stage of the disease, the complex interaction among cancer cells, microenvironment and local inflammatory reaction, could enforce tumour cells to evade immune surveillance and the effect of anticancer drugs (6,7,20,31). Recently, Chen *et al* found that reduction of both E-cadherin and nm23 mRNA expression remarkably correlated with low histological differentiation, increasing stage as well as lymph node metastases (P<0.05) (32).

Gautam *et al* found that overexpression of RRM1 in human and mouse lung cancer cell lines induced PTEN expression, reduced phosphorylation of FAK, suppressed migration, invasion, and metastasis formation, and increased survival in an animal model (33). Furthermore, in an elegant study with v-*src* transfection, Masumoto *et al* found that only activated src, but not ras, was able to induce cisplatin resistance in human gallbladder adenocarcinoma cells (34). They suggested that *src*-related drug resistance might be associated with increased DNA repair, of which the signal transduction could be different from that of Ras, phosphatidylinositol 3'-kinase (PI3K) and protein kinase C (PKC). Interaction between src and FAK is closely associated, showing that FAK activation could modulate nm23-H1 activity and increase cisplatin resistance.

Although the causal correlation is still uncertain, our results showed that protein expression of nm23-H1 was inversely correlated with that of FAK, pathophysiological balance between expression of FAK and nm23-H1 demands more detailed study. The combined analysis of nm23-H1 and FAK protein expression in the same tumour specimens revealed that patients with FAK-negative/nm23-H1-positive tumours survived the longest in comparison to other patients with nm23-H1 and FAK features (P<0.001). These results suggest that the status of nm23-H1 and FAK protein expression may help in predicting the aggressive behavior of non-small cell lung. However, further studies are warranted to clarify the impact of FAK on the function of nm23-H1 as an anti-metastatic gene.

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