Significance of *Integrin α5* Gene Expression as a Prognostic Factor in Node-negative Non-Small Cell Lung Cancer[1]

Masashi Adachi, Toshihiko Taki, Masahiko Higashiyama, Nobuoki Kohno, Haruhiko Inufusa, and Masayuki Miyake[2]

Department of Thoracic Surgery and Department V of Oncology, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Kita-ku, Osaka 530-8480 [M. A., T. T., M. M.]; Department of Surgery, The Center for Adult Diseases of Osaka, Osaka 537-0025 [M. H.]; Second Department of Internal Medicine, Ehime University School of Medicine, Ehime 791-0295 [N. K.]; and First Department of Surgery, Kinki University School of Medicine, Osaka 589-8511 [H. I.], Japan

ABSTRACT

The integrin family plays a major role in complex biological events such as differentiation, development, wound healing, and the altered adhesive and invasive properties of tumor cells. *Integrin α5β1* is a classical fibronectin receptor, and it has been known as a tumor suppressor gene because tumor cells overexpressing *α5β1* are less tumorigenic than their parent cells. However, this finding conflicts with some recent data that suggests that the emergence of α5β1 expression correlates with the tumor progression. We, therefore, investigated the expression of α5β1 integrin in 20 lung cancer cell lines by flow cytometric analysis and in 88 node-negative non-small cell lung cancers (NSCLCs) by RT-PCR and immunohistochemical assays to determine the significance of this prognostic factor. In the 20 lung cancer cell lines, 8 (40.0%) cell lines strongly expressed integrin α5, 3 (15.0%) cell lines had moderate or weak α5 expression, and the remaining 9 (45.0%) cell lines expressed no integrin α5. In the 88 node-negative NSCLC patients, 44 samples (50.0%) were evaluated as having integrin α5 overexpression, and the integrin α5 expression was significantly associated with the status of differentiation and the age of the patients (P = 0.0379 and 0.0312, respectively). In the node-negative patients, the overall survival rate for patients with integrin α5 overexpressed tumors was significantly worse than for those individuals whose tumors had normal integrin α5 expression (*P* = 0.016).

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[2] To whom requests for reprints should be addressed, at Kitano Hospital, Tazuke Kofukai Medical Research Institute, 13-3, Kamiyama-cho, Kita-ku, Osaka 530-8480, Japan. Phone: 81-6-6312-1221; Fax: 81-6-6312-8816.

INTRODUCTION

Tumor spread and invasion are the result of a series of several steps: (*a*) the loss of intracellular adhesion within the primary tumor; (*b*) entry into the lymphatic or blood vessels; (*c*) circulation of the tumor cells singly and in clumps; and (*d*) adherence of the cells to the surface of the luminal endothelium (1). After invading through the basement membrane via local proteolysis associated with the breakdown of the basement components, the tumor cells migrate through the defect in the extracellular matrix from the circulation and can initiate a metastatic colony. During these processes, the tumor cell may undergo the accumulation of a number of gene alterations (2). Thus, the major challenge to investigators who study cancer metastasis is: (*a*) to identify those gene products that might serve as markers to help identify metastatic cells; (*b*) to predict the aggressiveness of the disease; and (*c*) to locate and eradicate metastases.

Many of the steps in the metastatic sequence involve cell/cell and cell/extracellular matrix interactions mediated by specific cell surface molecules. Extracellular matrix receptors known as integrins have been postulated to play an important role in this process (3). Integrins are heterodimeric glycoproteins composed of distinct α and β subunits that function as cell adhesion receptors (4). Their functional properties are highly versatile; they anchor cells by mediating cell adhesion, provide traction for cell migration, transmit signals out of the cell, and help assemble the extracellular matrix (5). It has also been reported that signal transduction can flow in the reverse direction through integrins in that their ligand-binding activity is regulated from inside of the cell (6).

α5β1 integrin is a well-characterized receptor for fibronectin. The interaction of this receptor with its ligand results in signal transduction with multiple outcomes, including the regulation of cell adhesion and migration, matrix assembly, cytoskeletal organization, and the induction of collagenase and stromelysin gene expression (7, 8). The α5β1 receptor may also be regulated by other integrins, such as the αvβ3 vitronectin receptor (9). Autocrine transforming growth factor β1 modulates the expression of integrin α5β1 (10). Several studies have demonstrated that high levels of α5β1 integrin expression are negatively correlated with transformation and tumor expression (11, 12). Transformed Chinese hamster ovary cells or subclones overexpressing α5β1 were less tumorigenic than their counterparts expressing low levels of α5β1 (13). In a model of multistage carcinogenesis in mouse skin, the expression of α5β1 was downregulated in the progression from benign to malignant skin tumors (14). However, recently, several reports have contradicted these data (15–20). In the lung, the integrin α5 is generally not found in the normal tissue, but it is expressed in a considerable fraction of lung cancers (21). Studies on integrin expression in a variety of human and animal tumors have shown

Table 1 Integrin α5 expression on the lung cancer cell lines by flow cytometric analysis

Non-small cell lines		Small cell lines	
A549	S[a]	Lawson	N
ABC1	N	Lu135	N
AOI	S	Lu139	N
EBC1	N	MOA1P23	S
HAL8	S	N417	W
HAL24	S	SBC1	N
LC-MS	S	SBC2	N
LC-TK	M	SBC3	M
LK-1	S	SBC5	S
MAC10	N	QG90	N

[a] N, negative (<10%); W, weak (11–20%); M, moderate (21–60%); S, strong (>61%).

broad heterogeneity in their pattern of integrin expression, which was apparently also dependent on the cell type (22).

Therefore, we focused our interest on the expression of α5β1 in NSCLCs.[3] In this study, we used flow cytometric analysis, RT-PCR, and immunohistochemical assays for detecting the levels of α5β1 in cell lines and tumor tissues, and we investigated the usefulness of α5β1 in predicting the clinical behavior of NSCLC.

MATERIALS AND METHODS

Lung Cancer Cell Lines and Flow Cytometric Analysis. Twenty lung cancer cell lines including 10 NSCLC cell lines and 10 small cell lung cancer cell lines were analyzed in this study (Table 1). These cell lines were cultured in RPMI 1640 supplemented with 10% FCS. The cells were harvested at a semiconfluent stage and immunostained with a monoclonal antibody directed against integrin α5 (MAb1986; Chemicon International Inc., Temecula, CA). The cells were then immunostained with 1:200 dilution of FITC-conjugated goat antimouse IgG and analyzed by FACScan. The level of expression of integrin α5 was evaluated as follows: negative, <10%; weak, 11–20%; moderate, 21–60%; and strong, >61%.

Tissue Specimens. Tumor specimens were obtained from 88 node-negative NSCLC patients (63 males and 25 females) who had undergone surgeries from January 1991 to December 1993. The mean age at the time of operation was 62.5 years. To ascertain the presence of cancer cells, one-half of the tissue samples was immediately embedded in OCT compound (Miles Laboratories, Kankakee, IL), and was frozen and stored at −80°C until 6-μm sections were cut to use a cryostat. The histological examination was performed on H&E-stained tissue sections. One-half of a given tumor specimen containing mostly cancer cells was used for RT-PCR. The tumors were graded and staged according to the criteria of the Tumor-Node-Metastasis system (23). The salient clinical characteristics of the patients are presented in Table 2.

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-PCR; MAb, monoclonal antibody.

Table 2 Correlation between integrin α5 gene expression and clinicopathological factors in 88 node-negative NSCLC patients

		α5 status		
	Total	Overexpression	Normal expression	*P*
Age				
≦60	24	7	17	0.0312
>60	64	27	37	
Gender				
Female	25	10	15	NS[a]
Male	63	34	29	
Tumor size				
≦3 cm	31	16	15	NS
>3 cm	57	28	29	
Histology				
AD	53	25	28	NS
SQ	30	16	14	
LA	5	3	2	
Differentiation				
Well	22	6	16	0.0379
Moderately	44	24	20	
Poorly	22	14	8	
Total	88	44	44	

[a] NS, not significant; AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large cell carcinoma.

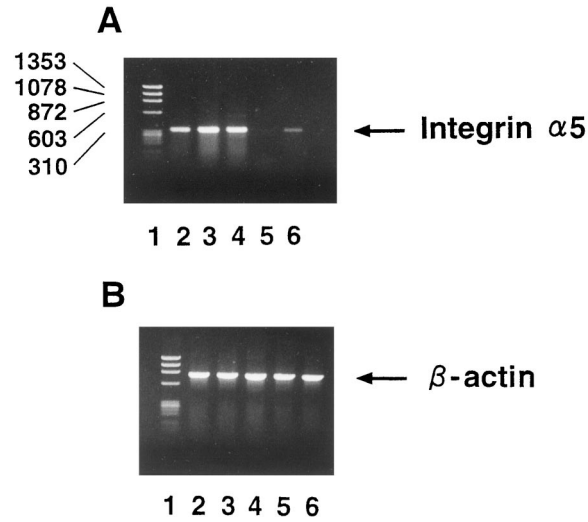

Fig. 1 *A*, agarose gel electrophoresis of RT-PCR-amplified 320 bp integrin α5 DNA. *Lane 1*, size marker; *Lane 2*, lung cancer cell line LK-1 (positive control); *Lanes 3* and *4*, integrin α5 overexpression group; *Lanes 5* and *6*, integrin α5 normal expression group. *B*, agarose gel electrophoresis of RT-PCR-amplified β-actin DNA (internal control) from each specimen.

Reverse Transcription of RNA followed by PCR Analysis (RT-PCR). Total RNA was isolated from the frozen tumor tissues by the acid guanidinium-thiocyanate procedure (24). Preparations of the integrin α5-positive lung cancer cell line LK-1 were used as positive controls. The total RNA (5 μg) was used for cDNA synthesis, and the first-strand cDNA solution (0.5 μl) was then used for the PCR, with primers designed to amplify a 320-bp sequence (sense primer sequence: 5′-CATTTCCGAGTCTGGGCCAA; antisense primer sequence:

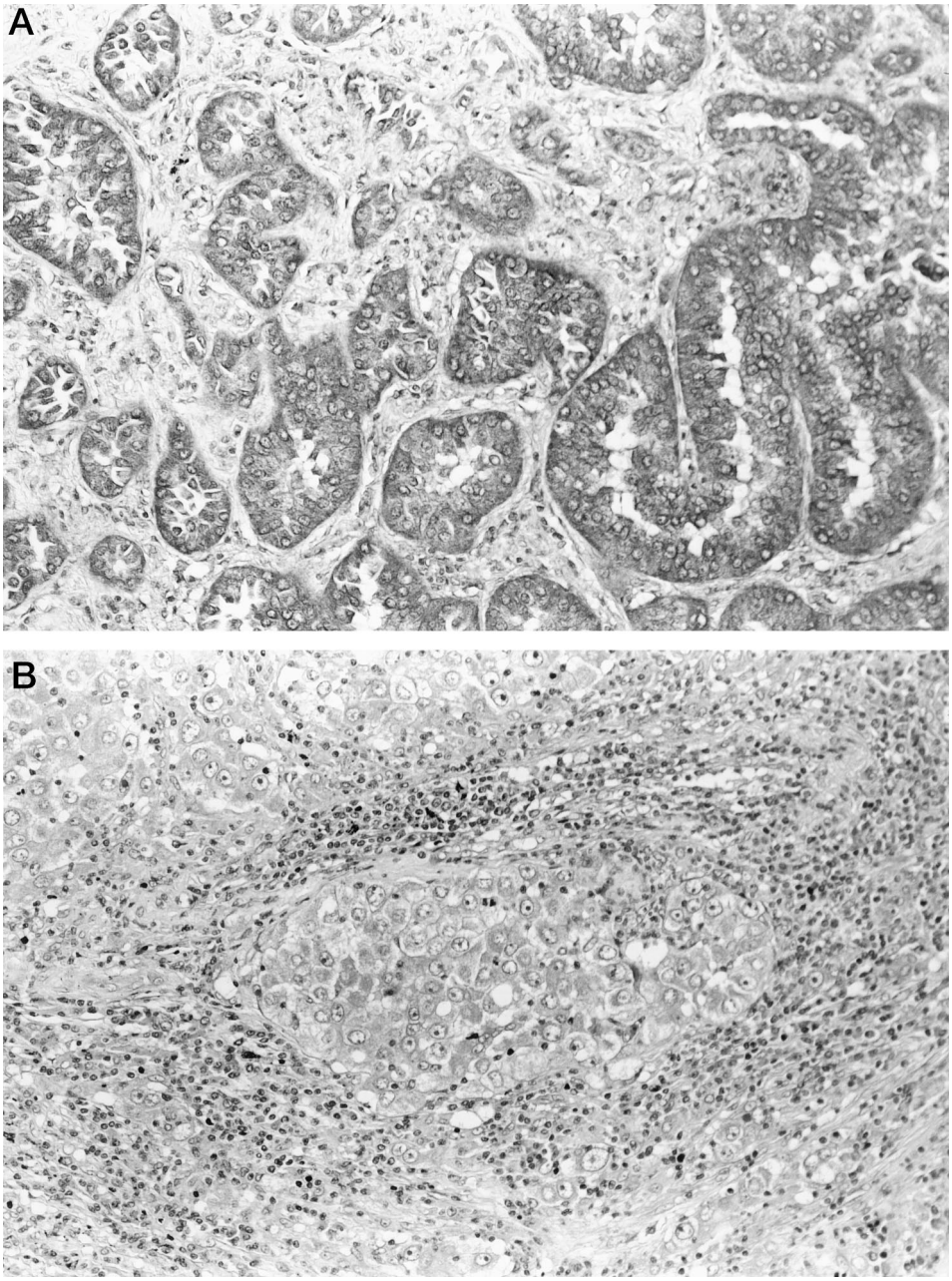

Fig. 2 Immunohistochemical staining of NSCLC tissues with anti-integrin α5-MAb. *A*, Positive immunostaining of an adenocarcinoma. *B*, negative immunostaining of an adenocarcinoma.

5′-TGGAGGCTTGAGCTGAGCTT; Ref. 25). Thirty cycles of 1 min denaturation at 94°C, 1 min annealing at 65°C, and a 1-min extension at 72°C were then performed. β-actin cDNA amplification using the same temperature profile for 30 cycles served as the internal control (26); the sense and antisense primers for the β-actin cDNA amplification were 5′-GAGAAGATGACCCAGATCATGT and 5′-ACTCCATGCCCAGGAAGGAAGG. To quantify the integrin α5 mRNA levels, 4 μl of PCR-amplified cDNA was electrophoresed on a 1% agarose gel, and the bands were visualized with ethidium bromide and photographed with a Polaroid camera (Fig. 1). Densitometric analysis of the photographic negatives was then used for band quantification.

Table 3 Relationship between RT-PCR results and immunohistochemical results of integrin α5 expression[a]

	RT-PCR		
Immunohistochemistry	α5-over-expression	α5-normal expression	Total
α5 positive	40	8	48
α5 negative	4	36	40
Total	44	44	88

[a] $P < 0.001$ (χ^2 test).

Table 4 Overall survival in node-negative NSCLC according to *integrin α5* gene expression

	α5 status (*n*)		5-yr survival rate (%)		
	Overexpression	Normal expression	Overexpression	Normal expression	*P*
Age					
≦60	7	17	69.7	85.7	0.330
>60	27	37	59.3	81.1	0.018
Gender					
Female	10	15	70.0	73.3	0.482
Male	34	29	61.3	86.2	0.017
Tumor size					
≦3 cm	16	15	81.2	86.7	0.642
>3 cm	28	29	52.8	79.3	0.014
Histology					
AD	25	28	60.0	78.6	0.092
SQ	16	14	68.8	85.7	0.176
LA	3	2	66.7	100	0.456
Differentiation					
Well	6	16	66.7	87.5	0.263
Moderately	24	20	66.2	70.0	0.596
Poorly	14	8	57.1	100.0	0.026
Total patients	44	44	63.2	81.8	0.016

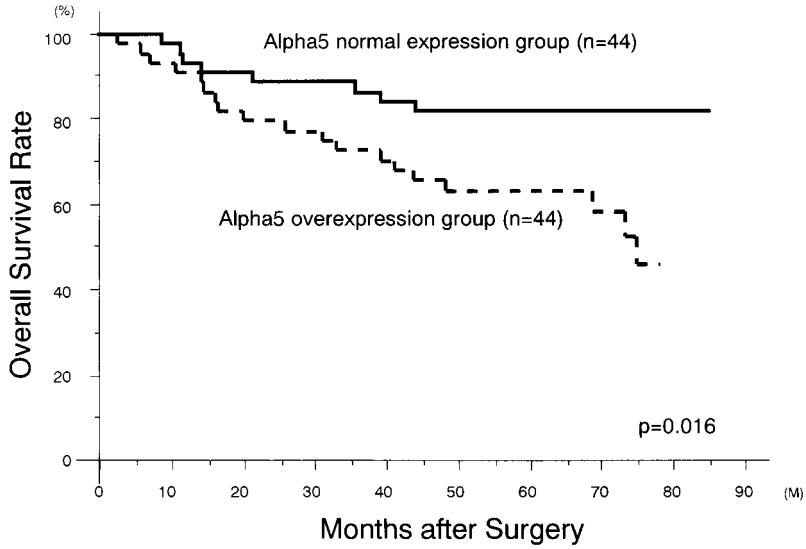

Fig. 3 Overall survival of the 88 node-negative patients in relation to their integrin α5 gene expression status.

Specimen Classification Based on RT-PCR Results. The densitometric value obtained for integrin α5 from the band of a given tumor tissue sample was divided by that of the corresponding β-actin band and was referred to as the integrin α5 expression ratio. The expression ratio of the tumor was then divided by that of LK-1 to obtain the integrin α5 conservation rate. When the conservation rate of a given specimen was >1.0, it was considered to indicate overexpression of the *integrin α5* gene (overexpression group), and if the rate was ≤1.0, it was defined as nonoverexpression (normal expression group).

Immunohistochemical Assay. Integrin α5 was immunostained using formalin-fixed, paraffin-embedded tissues as described previously (18). The sections were immersed for 20 min in 0.3% H_2O_2 in absolute methanol and then treated with 5% normal horse serum. Overnight incubation with the anti-integrin α5 MAb (MAb1986; Chemicon International Inc., Temecula, CA) was followed by incubation with biotinylated horse anti-mouse IgG (Vector, Burlingame, CA) and the avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector). The peroxidase reaction used 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.05% H_2O_2. Sections incubated with mouse myeloma SP_2 supernatant served as a negative reaction control.

Specimen Classification Based on Immunohistochemical Results. All of the immunostained sections were examined by two pathologists (Shinji Sawada and Tadashi Obayashi). Slides were examined under low power (×4) to identify regions that contained tumor cells. The proportion of high- and low-staining tumor cells in each of five randomly selected fields was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per ×40 field.

When ≥50% of the carcinoma cells in a given specimen stained positively, the sample was classified as positive, and when less than 50% were stained, the sample was classified as negative.

Statistical Analysis. The overall cancer-specific survival was defined from the date of surgery to the date of death due to cancer. The statistical significance between the incidence of the integrin α5 expression and several clinical and pathological parameters was assessed by the χ^2 test. The Kaplan-Meier method was used to estimate the probability of overall survival as a function of time (27) and was compared using the log-rank test (28). All of the *P*s are based on two-tailed statistical analysis; and a $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Expression of Integrin α5 in Lung Cancer Cell Lines. Of the 20 lung cancer cell lines examined, 8 cell lines (40.0%) expressed integrin α5 strongly, 3 cell lines (15.0%) had moderate or weak α5 expression, and no α5 was detected in the remaining 9 cell lines (45.0%; Table 1).

Detection of Integrin α5 using RT-PCR and Immunohistochemical Assay in NSCLC Tissues. Using RT-PCR, we found that the ratio of integrin α5/β-actin expression ranged from 0 to 3.2 (mean, 1.0) in the tumor specimens. There were 44 (50.0%) integrin α5-overexpression tumors. Of NSCLCs studied using the immunohistochemical method, 48 (54.5%) were classified as integrin α5-positive. In these cases, immunostaining was intense and seen uniformely at the cell-surface membrane and cytoplasm (Fig. 2). There were 40 cases (45.5%) with negative integrin α5 expression, and the immunostaining of most of these tumors was heterogeneous. Immunohistochemical results agreed well with those of the RT-PCR, and 86.4% of cases had no discrepancy (Table 3). In case of discrepancy, the results of RT-PCR were used in specimen classification.

Association of *Integrin α5* Gene Expression with the Overall Survival of NSCLC Patients. The integrin α5 expression was significantly associated with the state of differentiation and the age of the patients ($P = 0.0379$ and 0.0312, respectively; Table 2). In stratifying the survival of the 88 patients with node-negative NSCLCs according to their integrin α5 expression status, the 5-year survival rate of patients with α5 overexpressed tumors was significantly worse than that of individuals whose tumors had normal α5 expression (63.2% *versus* 81.8%; $P = 0.016$; Table 4; Fig. 3).

DISCUSSION

Integrin α5β1 is a fibronectin receptor, and several studies have demonstrated that high levels of α5β1 integrin expression are inversely correlated with transformation and tumor expression (11). For example, Giancotti and Ruoslahti (11) reported that increasing the expression of α5β1 integrin by gene transfer decreased the formation of tumors in Chinese hamster ovary cells, which suggests that the presence of α5β1 on the tumor cells may be a disadvantage for their proliferation. Indeed, after the transfection, the cells showed less migration and lost their ability to form tumors when injected s.c. into nude mice. Several other studies have also confirmed the correlation between low α5β1 expression and malignant transformation or higher malignant potential (12–14).

However, several recent reports, including our present study, showed that the overexpression of integrin α5 may indicate a more malignant phenotype. In malignant melanomas, the emergence of α5β1 expression was associated with melanocytic tumor progression (15, 16), and the expression of α5β1 integrin was up-regulated during the development of spindle cell carcinomas (17). In other kinds of tumors, the overexpression of α5β1 was also associated with a more malignant phenotype (18–20). These data seem to be in conflict with the previous data that α5β1 integrin plays an important role in tumor suppression. There may be several reasons for this conflict:

(*a*) the α5β1 expression may have an effect on signal transduction. Varner *et al.* (29) showed that α5β1 integrin expression in the absence of attachment to fibronectin activates a signal pathway leading to decreased cellular proliferation; and the binding of this receptor to fibronectin reverses this signal, thereby, contributing to the proliferation of transformed cells. They suggested that α5β1 integrin may generate different signals depending on whether it is bound to fibronectin;

(*b*) several observations have suggested that the α5β1 suppresses apoptosis and up-regulates Bcl-2 expression by adhesion to a substrate and by serum deprivation (30, 31). Bcl-2 overexpression mediated by α5β1 integrin may increase tumor survival and give the tumors resistance against chemotherapy and radiotherapy; and

(*c*) integrin α5β1 expression and tumor adhesion seem to be related. It has been reported that synthetic peptides containing the core sequences of fibronectin, which is the ligand for integrin α5β1, inhibited cell adhesion (32, 33). Thus, during the process of tumor cell adhesion to the endothelium, the overexpression of α5β1 integrin may, therefore, facilitate metastasis (34) and may be a significant factor for a poor prognosis.

In NSCLCs, the lymph node status is one of the most important prognostic factors. However, about 40% of the node-negative patients die of cancer recurrences, which suggests that they had systemic diseases at the time of surgery (23). Hence, patients with integrin α5 overexpression may be prone to metastasis and may have micrometastases even though they don't show any lymph node involvement at surgery. Thus, in the node-negative patients, integrin α5 overexpression may serve as a marker of micrometastasis and can be evaluated as a prognostic factor. In conclusion, we have demonstrated that integrin α5β1 overexpression is a significant predictive factor for a poor prognosis in the node-negative patients with NSCLCs.

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