

Significance of *Integrin* $\alpha 5$ Gene Expression as a Prognostic Factor in Node-negative Non-Small Cell Lung Cancer¹

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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* $\alpha 5\beta 1$ is a classical fibronectin receptor, and it has been known as a tumor suppressor gene because tumor cells overexpressing $\alpha 5\beta 1$ are less tumorigenic than their parent cells. However, this finding conflicts with some recent data that suggests that the emergence of $\alpha 5\beta 1$ expression correlates with the tumor progression. We, therefore, investigated the expression of $\alpha 5\beta 1$ 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 $\alpha 5$, 3 (15.0%) cell lines had moderate or weak $\alpha 5$ expression, and the remaining 9 (45.0%) cell lines expressed no integrin $\alpha 5$. In the 88 node-negative NSCLC patients, 44 samples (50.0%) were evaluated as having integrin $\alpha 5$ overexpression, and the integrin $\alpha 5$ 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 $\alpha 5$ overexpressed tumors was significantly worse than for those individuals whose tumors had normal integrin $\alpha 5$ expression ($P = 0.016$).

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).

$\alpha 5\beta 1$ 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 $\alpha 5\beta 1$ receptor may also be regulated by other integrins, such as the $\alpha v\beta 3$ vitronectin receptor (9). Autocrine transforming growth factor $\beta 1$ modulates the expression of integrin $\alpha 5\beta 1$ (10). Several studies have demonstrated that high levels of $\alpha 5\beta 1$ integrin expression are negatively correlated with transformation and tumor expression (11, 12). Transformed Chinese hamster ovary cells or subclones overexpressing $\alpha 5\beta 1$ were less tumorigenic than their counterparts expressing low levels of $\alpha 5\beta 1$ (13). In a model of multi-stage carcinogenesis in mouse skin, the expression of $\alpha 5\beta 1$ 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 $\alpha 5$ 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

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Table 1 Integrin $\alpha 5$ 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 $\alpha 5\beta 1$ in NSCLCs.³ In this study, we used flow cytometric analysis, RT-PCR, and immunohistochemical assays for detecting the levels of $\alpha 5\beta 1$ in cell lines and tumor tissues, and we investigated the usefulness of $\alpha 5\beta 1$ 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 $\alpha 5$ (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 $\alpha 5$ 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 $\alpha 5$ gene expression and clinicopathological factors in 88 node-negative NSCLC patients

	Total	$\alpha 5$ status		P
		Overexpression	Normal expression	
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.

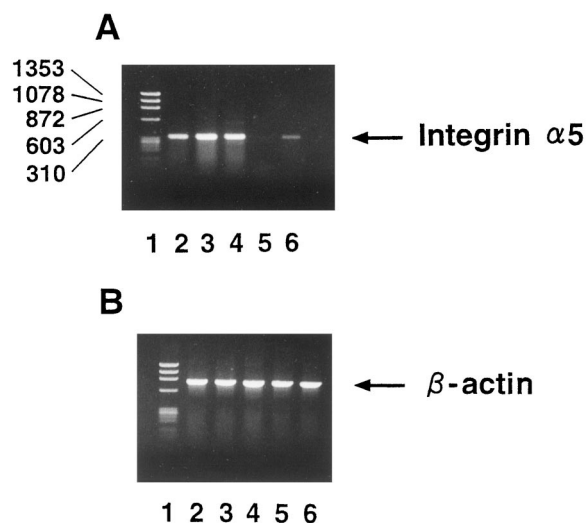


Fig. 1 A, agarose gel electrophoresis of RT-PCR-amplified 320 bp integrin $\alpha 5$ DNA. Lane 1, size marker; Lane 2, lung cancer cell line LK-1 (positive control); Lanes 3 and 4, integrin $\alpha 5$ overexpression group; Lanes 5 and 6, integrin $\alpha 5$ normal expression group. B, agarose gel electrophoresis 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 $\alpha 5$ -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'-CATTTCGAGTCTGGGCCAA; antisense primer sequence:

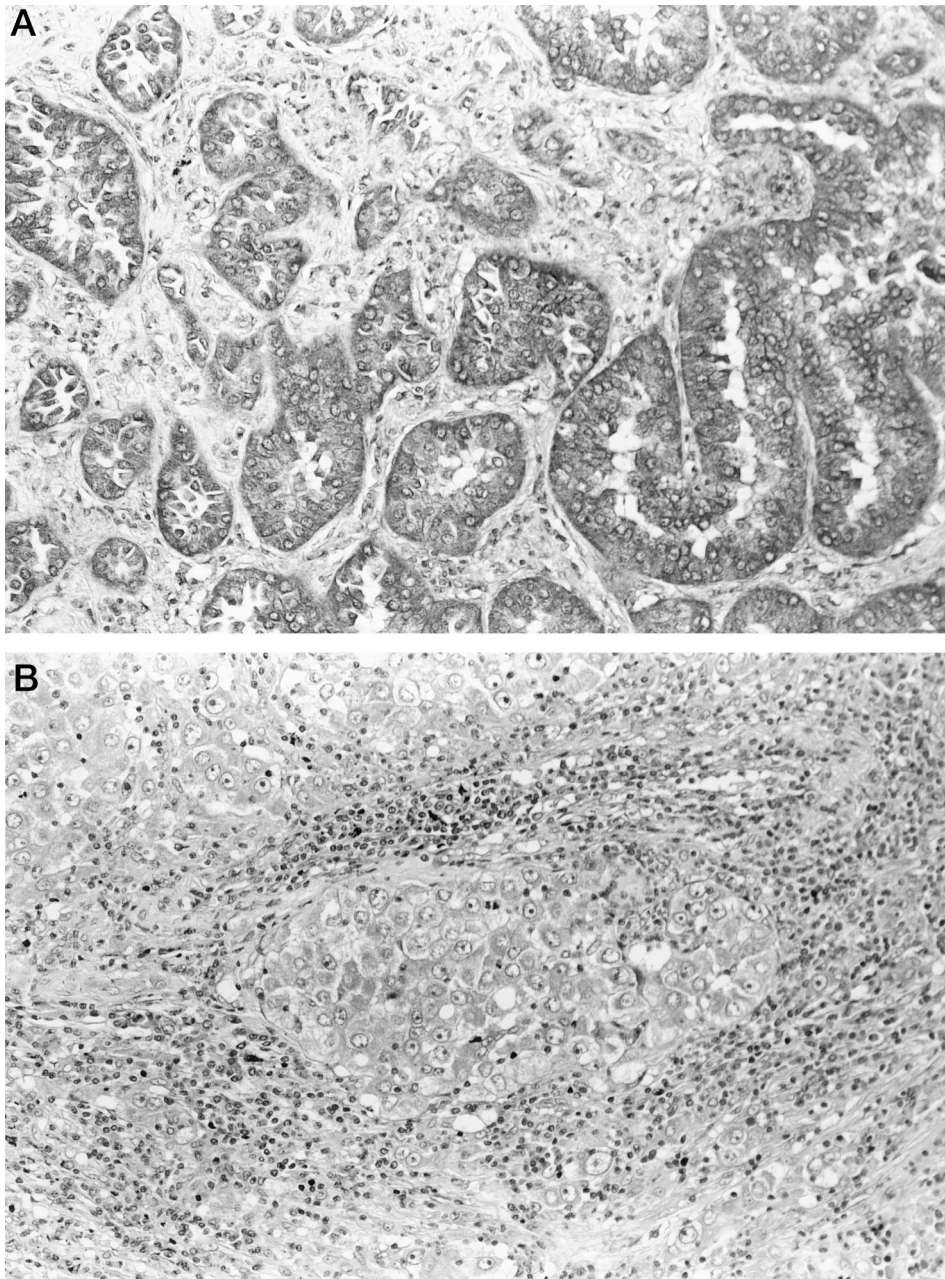


Fig. 2 Immunohistochemical staining of NSCLC tissues with anti-integrin $\alpha 5$ -MAb. A, Positive immunostaining of an adenocarcinoma. B, negative immunostaining of an adenocarcinoma.

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'-GAGAA-GATGACCCAGATCATGT and 5'-ACTCCATGCCAG-GAAGGAAGG. To quantify the integrin $\alpha 5$ mRNA levels, 4 μ l 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 $\alpha 5$ expression^a

Immunohistochemistry	RT-PCR		Total
	$\alpha 5$ -over-expression	$\alpha 5$ -normal expression	
$\alpha 5$ positive	40	8	48
$\alpha 5$ 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* $\alpha 5$ gene expression

	$\alpha 5$ status (n)		5-yr survival rate (%)		P
	Overexpression	Normal expression	Overexpression	Normal expression	
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

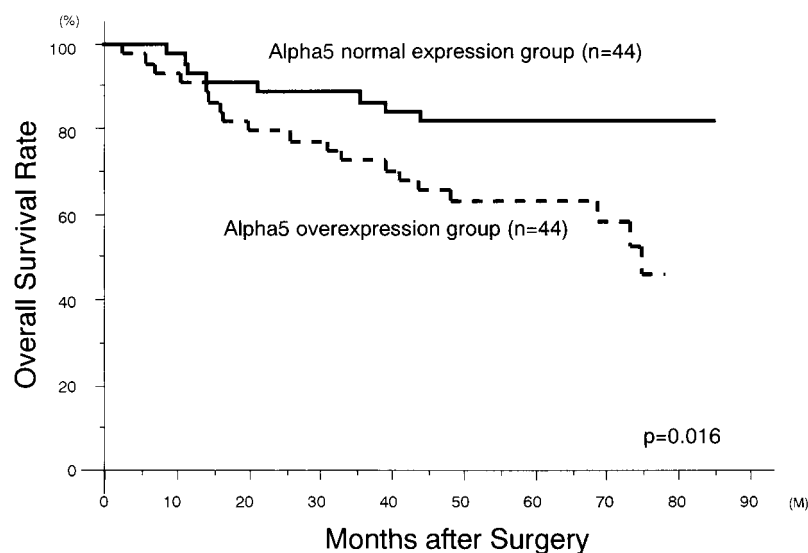


Fig. 3 Overall survival of the 88 node-negative patients in relation to their integrin $\alpha 5$ gene expression status.

Specimen Classification Based on RT-PCR Results.

The densitometric value obtained for integrin $\alpha 5$ 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 $\alpha 5$ expression ratio. The expression ratio of the tumor was then divided by that of LK-1 to obtain the integrin $\alpha 5$ conservation rate. When the conservation rate of a given specimen was > 1.0 , it was considered to indicate overexpression of the *integrin* $\alpha 5$ gene (overexpression group), and if the rate was ≤ 1.0 , it was defined as nonoverexpression (normal expression group).

Immunohistochemical Assay. Integrin $\alpha 5$ 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

$\alpha 5$ 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 SP₂ 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 ($\times 4$) 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 $\times 40$ field.

When $\geq 50\%$ 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 $\alpha 5$ 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 $\alpha 5$ in Lung Cancer Cell Lines.

Of the 20 lung cancer cell lines examined, 8 cell lines (40.0%) expressed integrin $\alpha 5$ strongly, 3 cell lines (15.0%) had moderate or weak $\alpha 5$ expression, and no $\alpha 5$ was detected in the remaining 9 cell lines (45.0%; Table 1).

Detection of Integrin $\alpha 5$ using RT-PCR and Immunohistochemical Assay in NSCLC Tissues. Using RT-PCR, we found that the ratio of integrin $\alpha 5/\beta$ -actin expression ranged from 0 to 3.2 (mean, 1.0) in the tumor specimens. There were 44 (50.0%) integrin $\alpha 5$ -overexpression tumors. Of NSCLCs studied using the immunohistochemical method, 48 (54.5%) were classified as integrin $\alpha 5$ -positive. In these cases, immunostaining was intense and seen uniformly at the cell-surface membrane and cytoplasm (Fig. 2). There were 40 cases (45.5%) with negative integrin $\alpha 5$ 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 $\alpha 5$ Gene Expression with the Overall Survival of NSCLC Patients. The integrin $\alpha 5$ 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 $\alpha 5$ expression status, the 5-year survival rate of patients with $\alpha 5$ overexpressed tumors was significantly worse than that of individuals whose tumors had normal $\alpha 5$ expression (63.2% versus 81.8%; *P* = 0.016; Table 4; Fig. 3).

DISCUSSION

Integrin $\alpha 5\beta 1$ is a fibronectin receptor, and several studies have demonstrated that high levels of $\alpha 5\beta 1$ integrin expression are inversely correlated with transformation and tumor expression (11). For example, Giancotti and Ruoslahti (11) reported that increasing the expression of $\alpha 5\beta 1$ integrin by gene transfer decreased the formation of tumors in Chinese hamster ovary cells, which suggests that the presence of $\alpha 5\beta 1$ 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

$\alpha 5\beta 1$ expression and malignant transformation or higher malignant potential (12–14).

However, several recent reports, including our present study, showed that the overexpression of integrin $\alpha 5$ may indicate a more malignant phenotype. In malignant melanomas, the emergence of $\alpha 5\beta 1$ expression was associated with melanocytic tumor progression (15, 16), and the expression of $\alpha 5\beta 1$ integrin was up-regulated during the development of spindle cell carcinomas (17). In other kinds of tumors, the overexpression of $\alpha 5\beta 1$ was also associated with a more malignant phenotype (18–20). These data seem to be in conflict with the previous data that $\alpha 5\beta 1$ integrin plays an important role in tumor suppression. There may be several reasons for this conflict:

(a) the $\alpha 5\beta 1$ expression may have an effect on signal transduction. Varner *et al.* (29) showed that $\alpha 5\beta 1$ 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 $\alpha 5\beta 1$ integrin may generate different signals depending on whether it is bound to fibronectin;

(b) several observations have suggested that the $\alpha 5\beta 1$ suppresses apoptosis and up-regulates Bcl-2 expression by adhesion to a substrate and by serum deprivation (30, 31). Bcl-2 overexpression mediated by $\alpha 5\beta 1$ integrin may increase tumor survival and give the tumors resistance against chemotherapy and radiotherapy; and

(c) integrin $\alpha 5\beta 1$ 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 $\alpha 5\beta 1$, inhibited cell adhesion (32, 33). Thus, during the process of tumor cell adhesion to the endothelium, the overexpression of $\alpha 5\beta 1$ 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 $\alpha 5$ 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 $\alpha 5$ overexpression may serve as a marker of micrometastasis and can be evaluated as a prognostic factor. In conclusion, we have demonstrated that integrin $\alpha 5\beta 1$ overexpression is a significant predictive factor for a poor prognosis in the node-negative patients with NSCLCs.

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