Expression of *Periostin*, Homologous with an Insect Cell Adhesion Molecule, as a Prognostic Marker in Non-small Cell Lung Cancers

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We used our palindromic polymerase chain reaction (PCR)-driven cDNA differential display technique to identify and isolate a gene, designated *periostin*, from cancer tissues and found it to be overexpressed in several human tumors. We attempted to determine the influence of *periostin* expression on clinical outcome in patients with non-small cell lung cancer (NSCLC) by reverse transcriptase (RT)-PCR analysis. *Periostin* gene was highly expressed at the tumor periphery of lung cancer tissue but not within the tumor by *in situ* RNA hybridization, suggesting that expression of *periostin* may be involved in the process of tumor invasion. *Periostin* transcripts were detected in 50 (49.0%) of the tumor samples, although some paired normal lung samples showed weak expression. There was no relationship between *periostin* gene expression and gender, N- or T-status. The NSCLC patients with *periostin* expression had significantly poorer survival than the patients without *periostin* expression (*P*=0.0338).

Lung cancer is a major cause of death from malignant diseases, due to its high incidence and malignant behavior, and the lack of major advances in treatment strategy.[1] Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan, with more than 15 000 patients undergoing surgical operation at Japanese institutions.[2] The clinical behavior of non-small lung cancer (NSCLC) is largely associated with its stage.[3] However, a significant number of tumors in patients with early-stage NSCLC show aggressive behavior and a tendency to relapse. Moreover, the prognosis for patients with operable NSCLC remains gloomy in comparison with that observed in operable gastric, colon, or breast cancer.[4] A variety of clinicopathologic characteristics may affect prognosis.[5] Despite steadily accumulating evidence that numerous genetic markers influence the biological behavior of NSCLC, the intrinsic nature of the gene deregulation that leads small tumors to metastasize remains highly elusive.[6]

The abnormal expression of certain genes in cancer cells is closely related to various aspects of tumor progression, including tumor growth, invasion and metastasis. Recently, we used the palindromic polymerase chain reaction (PCR)-driven cDNA differential display technique developed in our laboratory[7] to identify and isolate *periostin* cDNA, and found it to be overexpressed in several human tumors including primary colon cancer, metastatic liver cancer from colon cancer and breast cancer (Bao *et al.*, manuscript in preparation). The periostin protein shares structural and sequence homology with fasciclin I, which is an insect adhesion molecule.[8–10] It was reported that cellular adhesion molecules play an important role in the process of metastasis.[11]

As available prognostic markers leave much to be desired for NSCLC, we investigated *periostin* transcript in patients with NSCLC by means of a reverse transcriptase PCR (RT-PCR) and *in situ* hybridization analysis, and we assessed the prognostic value of *periostin* expression in these patients.

MATERIALS AND METHODS

Patients The study groups included 102 NSCLC patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between January 1997 and December 1999. Lung cancers were classified according to the general rule for clinical and pathological record of lung cancer.[12] All tumor and normal lung samples were collected at resection and immediately frozen in liquid nitrogen. Written consent was obtained from all patients.

Group characteristics The clinical and pathological characteristics of the 102 NSCLC patients are shown in Table I; these included 42 cases at stage I, 16 at stage II, 40 at stage III and 4 at stage IV. The mean age was 64.5 years (range, 42–88). Among the 102 NSCLC patients, 24 (23.5%) were women and 78 (76.5%) were men; 73 were lymph node metastasis-negative and 29 were positive. Of

90 patients, 29 were squamous cell carcinoma and 61 were adenocarcinoma (Table I).

***In situ* RNA hybridization** Sections of human squamous cell lung cancer tissues were purchased from Novagen Co. (Madison, WI). The paraffin-embedded slides were deparaffinized by incubation in xylene and dehydrated in graded ethanol-water solutions. *In situ* RNA hybridization was performed as described previously.[13] Human lung cDNA, synthesized from polyA$^+$ RNA from normal lung tissues, was purchased from Clontech Co. (Palo Alto, CA). Human *periostin* PCR was performed and the specificity of the PCR products of periostin were confirmed by use of a PCR II vector kit (Invitrogen Co., Carlsbad, CA) and an autosequencer. A 392-bp fragment corresponding to the N-terminus of human *periostin* was excised using *Bam*HI and *Eco*RI from human *periostin* cDNA in PCR II vector and then cloned in pBluescript (Stratagene, La Jolla, CA). Probes were generated with T7 and T3 RNA polymerase in the presence of [^{35}S]UTP.

RT-PCR assays for *periostin* Total RNA was isolated from tumor and adjacent histologically normal lung using an Isogen kit (Nippon Gene, Toyama) according to the

manufacturer's instructions. RNA concentration was determined by spectrophotometry and adjusted to a concentration of 200 ng/ml. RNA (1 μg) was reverse-transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 mg of oligo(dT)$_{16}$. The reaction mixture was incubated at 42°C for 50 min, followed by incubation at 72°C for 15 min. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for *periostin* gene were as follows: the forward primer, 5-ATGATTCCCTTTTTACCCATGTTTTCTCTA-3 (positions 1–30) and the reverse primer, 5-GAAGGAATAATC-ATGCCATTTTTTAAGTCC-3 (positions 531–560). The cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 24 cycles at 94°C for 45 s, 49°C for 45 s, 72°C for 45 s, and finally, 72°C for 5 min. Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized with ethidium bromide and photographed under ultraviolet transillumination. The intensities of the *periostin* and GAPDH bands were quan-

Table I. Clinico-pathological Data of 102 Non-small Cell Lung Cancer Patients

Factors		*Periostin* expression		
		Positive group (%)	Negative group (%)	*P* value (χ^2 method)
Mean age (years)	64.5±9.7	50 (49)	52 (51)	
Age				
<60		33 (44.6)	41 (55.4)	0.2182
≥60		17 (60.7)	11 (39.3)	
Gender				
Male		37 (47.4)	41 (52.6)	0.7313
Female		13 (54.2)	11 (45.8)	
Tumor status				
T1, T2		26 (45.6)	31 (54.4)	0.5654
T3, T4		24 (53.3)	21 (46.7)	
Lymph node metastasis				
Negative		31 (42.5)	42 (57.5)	0.0599
Positive		19 (65.5)	10 (34.5)	
Stage				
Stage I+II		26 (44.8)	32 (55.2)	0.4399
Stage III+IV		24 (54.5)	20 (45.5)	
Histology				
SCC		15 (51.7)	14 (48.3)	0.7710
Adeno		28 (45.9)	33 (54.1)	
Histology				
SCC		15 (51.7)	14 (48.3)	0.9007
Non-SCC		35 (47.9)	38 (52.1)	
Prognosis				
Alive/censored		38 (44.7)	47 (55.3)	0.0924
Dead		12 (70.6)	5 (29.4)	

SCC, squamous cell carcinoma; Adeno, adenocarcinoma.

tified using NIH Image and the ratio of the *periostin/GAPDH* gene expressions from each sample was calculated. Tumor/normal ratio>2.0 were considered as positive expression.

Statistical methods Statistical analysis was done using the Stat-View software package (Abacus Concepts Inc., Berkeley, CA). Differences among the means of age, gender, N- and T-status and pathological subtypes in the patients with NSCLC were examined using the χ^2 method. The overall survival of NSCLC patients was examined by the Kaplan-Meier method and survival characteristics were compared using log-rank tests. A difference was considered significant when the *P* value was less than 0.05.

RESULTS

Expression of *periostin* by *in situ* RNA hybridization for lung cancer *Periostin* mRNA could be detected by *in situ* RNA hybridization. High expression of the *periostin* gene was observed in the stroma cells just surrounding squamous cell lung carcinoma, whereas very little expression was found in cancer cells. Strong staining was observed in the advancing margin of lung cancer as opposed to the central area of the tumor (Fig. 1). However, detection with the sense probe was unsuccessful (data not shown).

***Periostin* mRNA expression by RT-PCR assay** Among the samples from 102 NSCLC patients studied, 50 (49.0%)
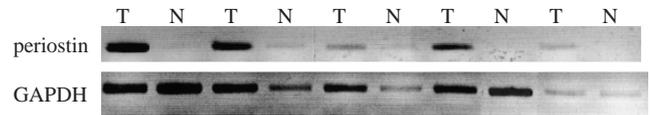

Fig. 2. *Periostin* mRNA expression in non-small cell lung cancer (T) and adjacent normal lung (N).

of the tumor samples had *periostin* transcript. Some of the paired normal lung samples weakly expressed *periostin* (Fig. 2). The *periostin*-positive tumors appeared in males (37/78, 47.4%) and in females (13/24, 54.2%), and the difference was not significant (*P*=0.7313). There was no difference of *periostin* expression in T-status (T1−2 26/57, 45.6%; T3−4 24/45, 53.3%, *P*=0.5654). The *periostin*-positive tumors appeared to be more frequent in lymph node metastasis-positive patients (19/29, 65.5%) than in lymph node metastasis-negative patients (31/73, 42.5%), but the difference was not significant (*P*=0.0599). Patients were further stratified according to pathological factors. There was no significant difference between histological subtype (squamous cell carcinoma vs. adenocarcinoma and squamous vs. non-squamous cell carcinoma) and *periostin* gene expression: 15/29 (51.7%) squamous cell carcinomas and 28/61 (45.9%) adenocarcinomas had *periostin* transcript (*P*=0.7710) (Table I). Compared to squamous cell carcinomas, 35/73 (47.9%) non-squamous cell carcinomas had *periostin* transcript (*P*=0.9007).
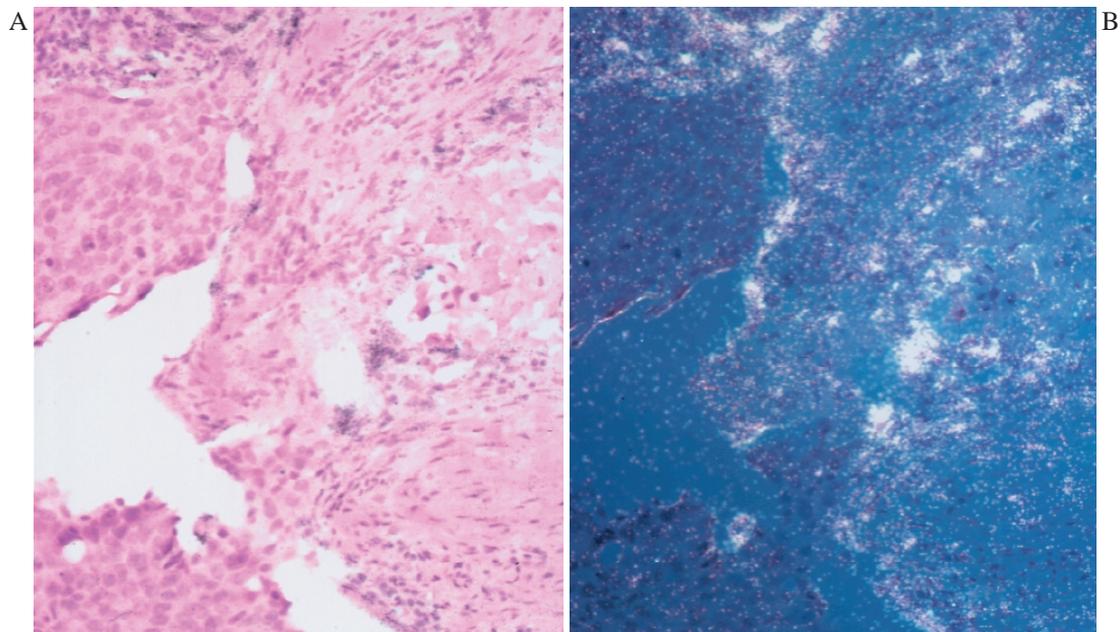

Fig. 1. *In situ* RNA hybridization of *periostin* for squamous cell lung cancer tissue. A positive signal was seen in the surrounding stroma cells, but not within the tumor cells. A, bright field (black dots are positive); B, dark field (white spots are positive).
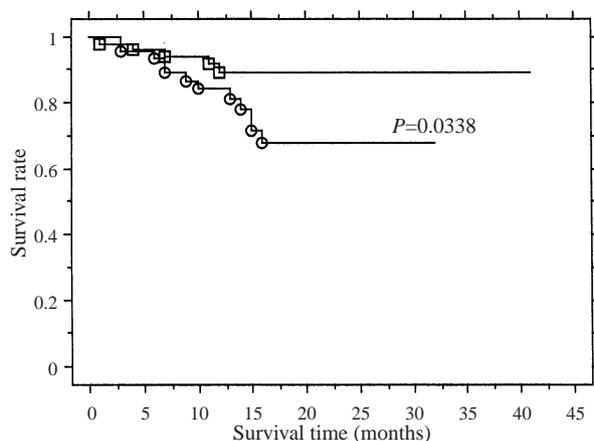
Fig. 3. Correlation between expression of *periostin* and survival rate in non-small cell lung cancer. ○ *periostin* mRNA overexpression; □ *periostin* mRNA no or normal expression (*P*=0.0338).

Relationship between clinical course of patients with NSCLC and *periostin* expression The overall survival of 102 NSCLC patients was determined in relation to the expression of *periostin*: 47/52 (90.4%) patients who did not have *periostin* transcript were alive, while 38 of 50 (76.0%) who had *periostin* transcript were alive (Table I). The NSCLC patients with *periostin* expression had significantly poorer survival than the patients without *periostin* expression (*P*=0.0338, log-rank test) (Fig. 3).

DISCUSSION

In the present study, the basal level of *periostin* mRNA was analyzed for the first time in tumor samples from patients with NSCLC who had clinical follow-up. *Periostin* mRNA was expressed in half of NSCLC and was preferentially expressed in NSCLC that was lymph node metastasis-positive, although the difference was not significant (*P*=0.0599). It had been shown by SAGE analysis (serial analysis of gene expression) that the human *periostin* gene is expressed in several human tumors, including carcinomas of ovary, breast and brain tumors.[14] It was reported that *periostin* is overexpressed in both ovarian tumors and *in vitro*-expanded Cedars-Sinai Ovarian Cancer cell line.[15] Independently, the *periostin* gene has been cloned from colon cancer tissues (Bao *et al.*, manuscript in preparation).

Periostin protein is highly homologous to βigH3, a molecule induced by TGF-β1 (transforming growth factor β1), that promotes the adhesion and spreading of fibroblasts[16, 17] as well as to fasciclin I, an insect adhesion molecule.[8–10] It has been shown that mouse recombinant *periostin* supports

the attachment and spreading of MC3T3-E1 cells in a solid-phase binding assay.[9] This effect was impaired by an anti-mouse *periostin* antibody.[9] These *in vitro* studies suggested that *periostin* might be involved in cell adhesion and invasion. Cellular adhesion molecules play an important role in the process of metastasis.[18] Positive and negative regulation of cell adhesion will influence the breakaway of cells from the primary tumor, transport in the circulation and adhesion to cellular and extracellular matrix elements in particular secondary sites. Several families of cell adhesion molecules have been identified, together with specific aberrations in malignant disease. Our results suggest that the *periostin* gene may be associated with metastatic potential, or alternatively with the degree of malignancy.

Periostin, formerly named osteoblast-specific factor-2, was cloned from a mouse osteoblast cell line (MC3T3-E1) cDNA library.[8] To avoid confusion of osteoblast-specific factor-2 with the Osteoblast-Specific transcription Factor-2, OSF-2 (also called *Cbfa1*), the group that originally cloned it redesignated this gene *periostin*.[9] Subsequently, human *periostin* was cloned from human osteosarcoma and human placenta cDNA libraries.[8] Immunohistochemistry revealed that mouse *periostin* is preferentially expressed in the periosteum, indicating a potential role in bone formation and maintenance of structure.[9] On the other hand, it was reported that micrometastatic cancer cells were present in the bone marrow of patients with resectable NSCLC,[19] which indicates that micro bone metastases are involved even in early-stage lung cancer. The prevalence of bone metastases from lung cancer was reported to range from 32% to 40% at autopsy.[20] Thus, the *periostin* gene may be associated with bone metastatic potential of lung cancers.

Our results show that the overall survival rates were different between the groups with and without *periostin* expression. Because there were no significant differences in the distribution of other possible prognostic factors such as T- and N-status, *periostin* transcript may be a novel prognostic marker in NSCLC. However, *periostin* mRNA expression, as well as other possible prognostic factors such as age, gender, stage, did not affect the prognosis of NSCLC using Cox's proportional-hazards regression model (data not shown). Longer follow-up seems to be warranted to assess the role of *periostin* in the progression of cancer.

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