Differential Expression of CD34 and Smooth Muscle Actin in the Stroma of Small Lung Adenocarcinoma with Mixed Bronchioloalveolar and Invasive Components

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Background : Absence of CD34-positive fibroblasts was reported within the stroma associated with invasive carcinomas. Conversely, tumor-associated desmoplastic stroma is characterized by the presence of smooth muscle actin (SMA)-reactive myofibroblasts. The present study was undertaken in order to elucidate whether the different distributions of stromal CD34-positive fibroblasts and SMA-reactive myofibroblasts are sensitive or specific markers of tumor invasion in small lung adenocarcinomas. **Methods :** Immunohistochemical stainings for CD34 and SMA were done in 37 peripheral adenocarcinomas less than 3.0 cm in diameter, including 16 adenocarcinomas with bronchioloalveolar carcinoma (BAC) and invasive components (mixed), and 21 invasive adenocarcinomas without BAC components (invasive). **Results :** The fibroblasts within the BAC components of the mixed group were mainly CD34-positive (81.2%) and preferentially SMA-negative (56.3%). In contrast, the fibroblasts within the invasive components of the mixed group were mainly CD34-negative (75.0%) and SMA-positive (87.5%). The stromal cells of the invasive group were mostly negative for CD34 (90.5%) and positive for SMA (95.3%). **Conclusions :** The loss of CD34 and the acquisition of SMA in the stromal cells within the tumor were related to tumor invasion (p<0.05). Thus, expression patterns of CD34 and SMA can be used to detect small foci of early stromal invasion in adenocarcinomas of the lung.

Adenocarcinomas arising in the peripheral lung parenchyma are the most representative type of lung cancer. Asymptomatic, small-sized, lung adenocarcinomas are now more frequently detected due to the advance of diagnostic techniques, however, some of them display poor clinical outcome such as metastasis or recurrence. Since the histological and biological heterogeneities of adenocarcinomas often make it difficult to predict the outcome of operated patients, the clarification of the histopathologic prognostic factors of these tumors is important in the selection of an appropriate treatment.

Obviously, an excellent prognosis can be expected in a non-invasive bronchioloalveolar carcinoma (BAC), whereas the invasive features seen in an adenocarcinoma is indicative of poor prognosis, which worsens in proportion to stromal invasion,[1,2] especially in small adenocarcinomas.[3] Conversely, several reports have supposed that the interobserver concordance was poor for the pathological findings in lung adenocarcinomas.[4,5] Many conventional adenocarcinomas can have a major component resembling BAC, and the histomorphologic differential assessment of stromal invasion in BAC and in an invasive adenocarcinoma can be extremely difficult due to the presence of fibrosis and tangential sectioning.

Fibroblasts in tumor tissue are thought to directly and/or indirectly interact with tumor cells and to have important roles in tumor invasion and metastasis. The morphology of the proliferating fibroblasts in invasive cancer is different from that of the fibroblasts in normal tissue. Proliferating fibroblasts in invasive cancer have large nuclei and usually contain nucleoli.[6]

CD34 is a transmembrane glycoprotein that is thought to be involved in the modulation of cell adhesion and signal transduction.[7] The CD34-positive (CD34+) fibroblasts in many organs are thought to represent an uncommitted cell capable of multidirectional mesenchymal differentiation,[8] which suggests that there is an inverse relation between CD34 expression and smooth muscle actin (SMA)-reactive myofibroblastic differentiation.[9] The absence of CD34+ fibroblasts favors the diagnosis of an invasive cancer in several organs.[10-13] In contrast, the tumor-associated desmoplastic stroma is characterized by the presence of SMA-reactive myofibroblasts.[13]

The present study was undertaken in order to elucidate whether the different distribution patterns of stromal CD34+ fibroblasts and SMA-reactive myofibroblasts are sensitive or specific markers of invasion in lung adenocarcinomas.

MATERIALS AND METHODS

Materials

Thirty-seven cases of adenocarcinoma of the lung, having maximal tumor dimensions of 3 cm, were retrospectively selected from the surgical pathology records of the Department of Pathology at Dong-A University Medical Center, from 1994 to 2003. No preoperative chemotherapy or radiotherapy had been performed in any of these cases. Standard lobectomy and lymph node dissections were performed in every case. The clinical records and pathological reports were also obtained in the 37 cases. Informed consent had been obtained from all subjects at the time of surgery. The postoperative pathological staging was determined according to the guidelines of the American Joint Committee on Cancer.[14]

Pathological examination

The hematoxylin and eosin-stained slides were reviewed in each case to confirm the original diagnosis that was based on the WHO criteria.[15] The tumor was defined as BAC when the adenocarcinoma lesion had a pure bronchioloalveolar growth pattern, with no evidence of stromal, vascular or pleural invasion.

Histopathological "invasion" was defined when the tumor cells were arranged in an acinic/papillotubular structure or as solid nests in a fibroblastic stroma, often accompanied by collagenization, and when the alveolar structures were no longer discernible. Furthermore, all cases were subdivided into three groups: BAC, adenocarcinomas with BAC components and invasive carcinomas (mixed), and invasive adenocarcinomas without BAC components (invasive). These findings were independently investigated by three experienced pulmonary pathologists (M.S.R, C.H.L, B.K.C), and discrepancies were resolved by joint examination of the slides under a multi-headed microscope. The three pathologists were blinded with respect to the outcome at the time they carried out their classification.

Immunohistochemical evaluation

Immunohistochemical studies for CD34 and SMA were performed on formalin-fixed, paraffin-embedded, 4 m-thick tissue sections, using the avidin-biotin-peroxidase complex method. Primary antibodeis were mouse monoclonal antibodies against CD34 (Immunotech, Marseille, France) at a 1:400 dilution, and against SMA (Dako, Glostrup, Denmark) in a 1:300 dilution. Deparaffinization of all sections was performed through a series of xylene baths, with rehydration through a series of graded alcohol solutions. To enhance the immunoreactivity, microwave antigen retrieval was performed in pH6 citrate buffer at 750 W for 30 min. After blocking the endogenous peroxidase activity with 5% hydrogen peroxidase for 10 min, primary antibody incubation was performed for 30 min at room temperature. An Envision^{TM}Chem^{TM}Detection Kit (Dako Corporation, CA, USA) was used for the incubation of the secondary antibody for 30 min at room temperature. After washing the tissue samples in Tris buffered saline for 10 min, 3, 3′-diaminobenzidine was used as a chromogen, and Mayer's hematoxylin counterstain was then applied.

The fibroblast reactivities for CD34 and SMA were recorded as positive or negative, and as reduced when there was focal positive expression in less than 50% of total fibroblasts.

Statistical analysis

Statistical analysis was performed using the Statistical Package Service Solution software (SPSS for Windows Standard version 10.1, Chicago, USA). χ^2 tests were performed to assess the relation between the staining pattern of CD34 or SMA and the different histological features. The level of significance was set at $p<0.05$.

RESULTS

Clinicopathological characteristics

The median age of the 37 cases was 56 years, ranging from 40 to 75, and there were 19 men and 18 women. The tumor sizes ranged from 1 to 3 cm, with 8 cases with tumors ≤ 2 cm, and 29 with tumors >2 cm. Histologically, all the primary tumors were adenocarcinomas, showing a mixture of various histological subtypes, including acinar, papillary, solid and BAC. When the 37 cases were subdivided into the three groups, there were 0, 16 and 21 in the BAC, mixed and invasive groups, respectively. Lymphote metastasis was present in 24 cases out of 37 cases and lymphovascular tumor invasion was in 26 cases out of 37 cases.

Expression of CD34 and SMA in normal alveolar framework

The stroma of the normal alveolar framework adjacent to the tumor contained CD34+ fibroblasts. These cells showed slender elongated dendrite-like processes, featuring a bi- or multipolar arrangement. Their nuclei were small and inconspicuous. SMA was detected in the vessel walls, whereas SMA-reactive myofibroblasts were not detected in the stroma of the normal alveolar framework.

Expression of CD34 and SMA in the mixed group (Table 1, Fig. 1, 2)

Within the BAC components of the mixed group, the distribution of CD34+ fibroblasts was similar to that observed in the

Table 1. Distribution of CD34 positive fibroblasts and smooth muscle actin-reactive myofibroblasts in the stroma of small lung adenocarcinomas with mixed bronchioloalveolar and invasive components

Histologic group	No. of cases	CD34				SMA			
		Positive (%)	Reduced (%)	Negative (%)	p value	Positive (%)	Reduced (%)	Negative (%)	p value
Mixed	16				0.018				0.04
BAC components	16	13 (81.2)	2 (12.5)	1 (6.3)		3 (18.7)	4 (25.0)	9 (56.3)	
Invasive components	16	1 (6.3)	3 (18.7)	12 (75.0)		14 (87.5)	2 (12.5)	0 (0)	
Invasive	21	0 (0)	2 (9.5)	19 (90.5)		20 (95.3)	1 (4.7)	0 (0)	

SMA, smooth muscle actin; Mixed, adenocarcinoma with bronchioloalveolar and invasive components; BAC, bronchioloalveolar carcinoma; Invasive, invasive adenocarcinoma without bronchioloalveolar component.
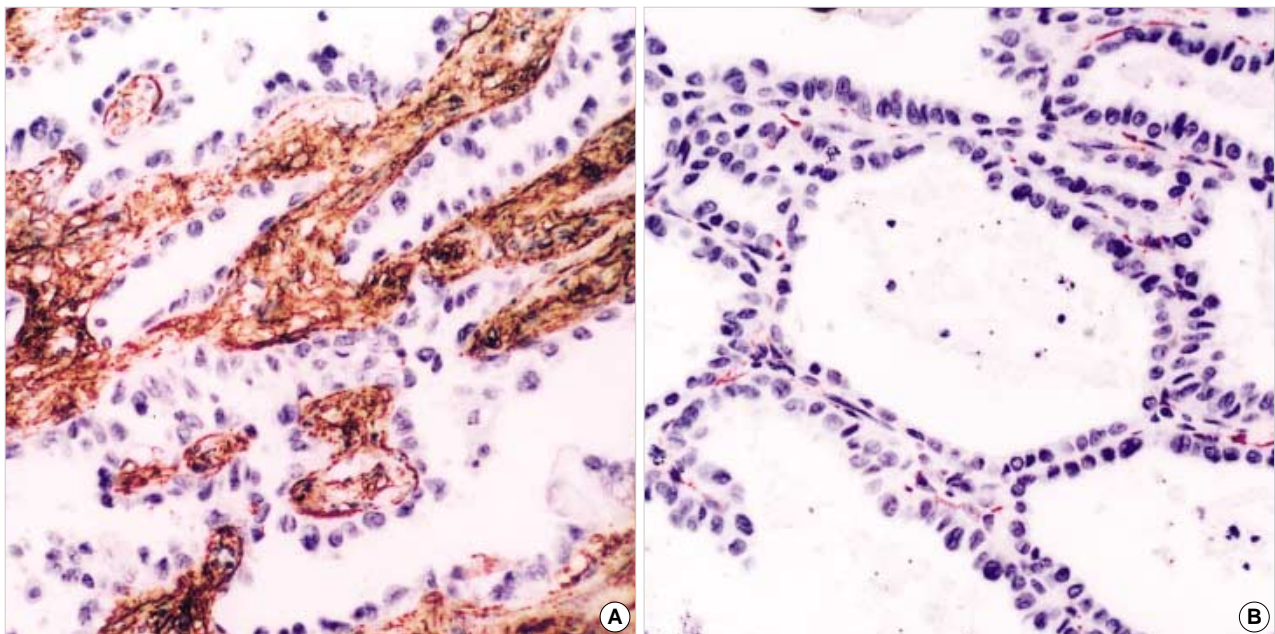

Fig. 1. In a cases of the mixed group, the bronchioloalveolar component shows CD34 positive stromal fibroblasts (A) and focal expression of smooth muscle actin (B).
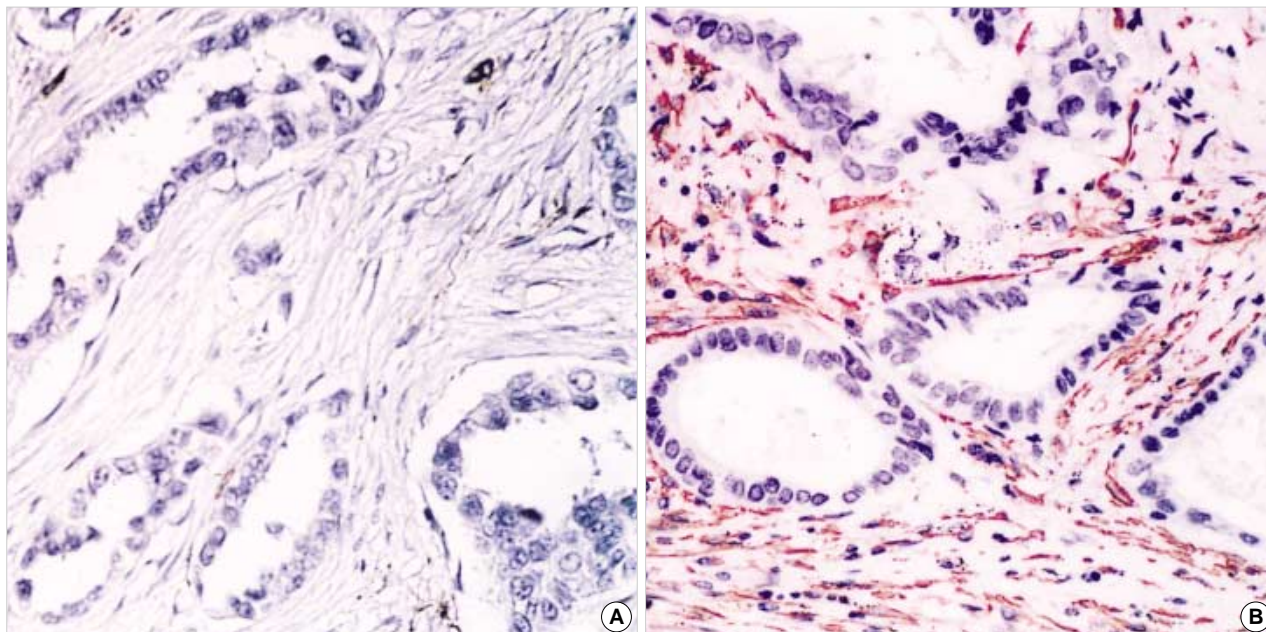
Fig. 2. In a case of the mixed group, the invasive component shows loss of CD34 expression (A) and smooth muscle actin-reactive myofibroblasts (B).
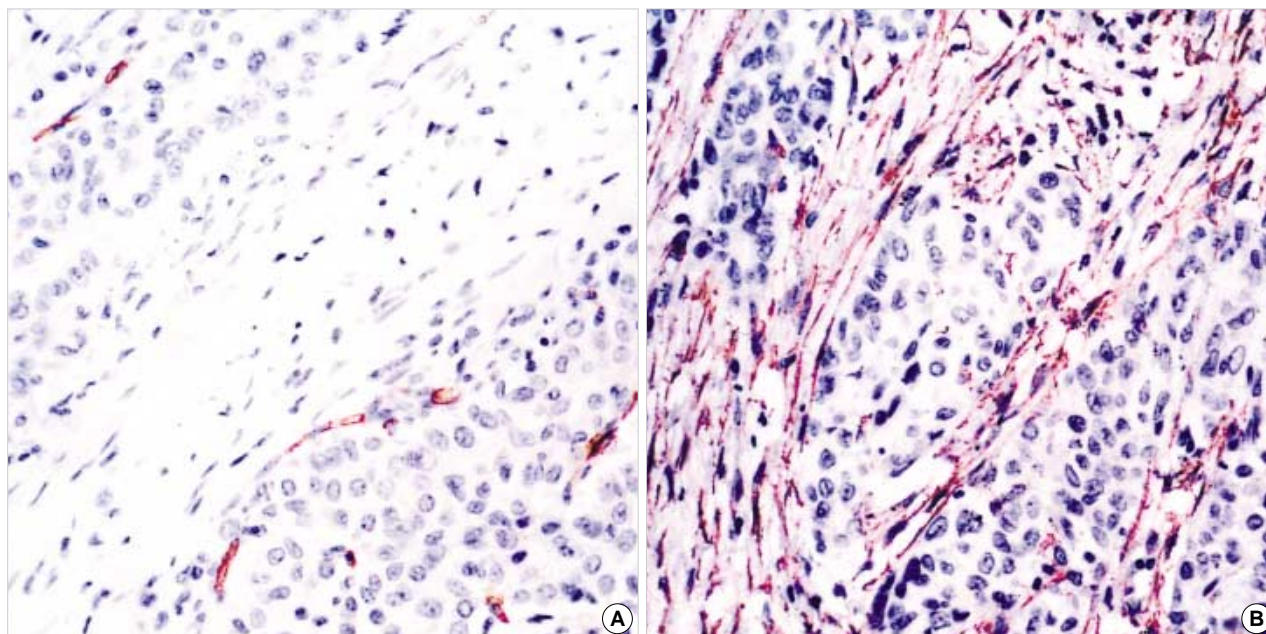

Fig. 3. In a case of the invasive group, peritumoral fibroblasts are uniformly negative for CD34, whereas small caliber vessels are positive for CD34 (A) and diffusely positive for smooth muscle actin (B).

normal alveolar framework. In 13 (81.2%) of 16 cases, CD34+ fibroblasts were scattered uniformly throughout the thickened stroma associated with the BAC components. However, in two of the remaining three cases, the number of CD34+ fibroblasts were reduced, while the other showed complete loss of the CD34+ fibroblasts. SMA-reactive myofibroblasts were noted in three cases,

one of which showed complete loss of the CD34+ fibroblasts.

In contrast to the normal alveolar framework or stroma of the BAC components, the number of CD34+ fibroblasts was reduced in the stroma of the invasive components in the mixed group, although capillaries with CD34 reactive endothelia were still detectable. In most cases, loss of CD34 expression was accom-

panied by the acquisition of SMA; however, this relation was not absolute, with a gain of SMA (87.5%) being more frequent than a loss of CD34 (75.0%). In one case, coexistence of SMA-reactive myofibroblasts and CD34+ fibroblasts was observed in the stroma of invasive component.

Expression of CD34 and SMA in the invasive group
(Table 1, Fig. 3)

In 19 (90.5%) of 21 cases in the invasive group, the stroma showed complete loss of the CD34+ fibroblasts. In these cases, the transition from tumor-free stroma to an invasive carcinoma was clear-cut. In the other two cases, a focal residual population of CD34+ fibroblasts was detected at the periphery of the infiltrating carcinoma. The endothelium of the vessels located in the stroma of an invasive carcinoma and of tumor-free lung tissue showed similar degrees of CD34 immunostaining.

SMA-reactive myofibroblasts were detected in the stroma of 20 (95.3%) cases of invasive carcinoma. The myofibroblasts were haphazardly arranged, spindle-shaped and showed plump cigar-shaped nuclei.

DISCUSSION

The present study is the first to correlate the presence and distribution of CD34+ fibroblasts as well as SMA-reactive myofibroblasts with the invasion of primary lung adenocarcinomas. The data reported herein indicate that the loss of CD34+ fibroblasts is a sensitive and specific marker of the stromal changes associated with invasive lung adenocarcinomas. In contrast, the accumulation of SMA-reactive myofibroblasts was seen more frequently than the loss of CD34+ fibroblasts, and could be observed in both noninvasive BAC components and invasive components of the lung. Similar findings have been reported in the studies of cervical[11] and breast cancers,[12] so the presence of SMA-reactive myofibroblasts in the stroma is considered a less specific marker for the evaluation of stromal invasion.

The loss of CD34+ fibroblasts in an invasive carcinoma is not site specific to the lung, as similar findings have been reported for stromal alterations occurring in ductal carcinomas of the breast,[10,12] colon,[13] skin[16] and cervix.[11] Barth *et al.*[11] reported that cervical intraepithelial neoplasia III disclosed a dense network of CD34+ fibroblasts, whereas Chauban *et al.*[12] reported that the loss of CD34 was significantly more frequent in high grade tumors than in those of low or intermediate grades for in situ

ductal carcinomas of the breast. In our study, the noninvasive BAC components mostly showed preserved CD34+ fibroblasts in the stroma. Interestingly, one case with a complete loss of CD34+ fibroblasts showed SMA-reactive myofibroblasts in the BAC components of the mixed group. Although this case was small in size (1.8 cm in diameter), it showed regional lymph node metastasis and frequent lymphovascular invasion. This may indicate different functional states of the epithelial-mesenchymal interactions, which are important in determining their invasive potential.

We found that the stromal fibroblasts from invasive adenocarcinoma tissue and the fibroblasts from normal alveolar framework had different characteristic phenotypes. The mechanisms mediating this process are far from understood. Barth *et al.*[11] suggested two alternative mechanisms: the tumor might induce apoptosis of the CD34+ fibroblasts, which were subsequently replaced by SMA-reactive myofibroblasts, or the immunophenotypic change may occur in a cell population that downregulates the CD34 and increases SMA expression. Nakamura *et al.*[17] also proposed two explanations for these results. First, invasive carcinoma cells may require specific environmental conditions, and invasive tumor cells may select only the most suitable fibroblasts for their stroma. Alternatively, the tumor cell environment may affect the fibroblasts, altering their phenotype in such a way that they become stable in culture. If tumor cells require specific stromal fibroblasts to exhibit invasive growth, therapeutic interventions targeted at eliminating stromal fibroblasts may be effective for reducing tumor invasion.

To elucidate the mechanism of tumor invasion, proliferating fibroblasts in tumor stroma must be analyzed more extensively. Experimental studies have shown that blood-borne CD34+ fibroblasts invade sites of tissue damage and are capable of connective tissue matrix synthesis.[18] Besides their function as a matrix-producing cell, CD34+ fibroblasts are a potent antigen-producing cell capable of priming naive T cells in situ.[19] Therefore, it has been claimed that CD34+ fibroblasts may play an important role in the host response to tissue damage, irrespective of the cause. Bearing in mind that CD34+ fibroblasts may mediate specific immunologic reactions related to antigen-presentation and T-cell priming, the loss of this cell population might play an important role in that part of the host response directed against invasive cancer cells; that is, mediated by infiltrating T-cells. Nakamura *et al.*[17] immunohistochemically examined the expression profile of proliferating fibroblasts in tumor stroma and normal bronchus tissues and found up-regulation of MLH1 and down-regulation of Cox1 in cancerous tissue in vivo. These

results indicate that proliferating fibroblasts in pulmonary adenocarcinomas were phenotypically different from the fibroblasts in normal bronchus tissues.

The pathologic features of invasion, such as stromal disruption have been shown to be of prognostic value in adenocarcinomas. Although a diagnosis cannot be based exclusively on immunohistochemical results, the distribution of stromal CD34+ fibroblasts and SMA-reactive myofibroblasts might be used as a possible diagnostic marker in the classification of invasive components in lung adenocarcinomas. These findings may present a helpful tool in characterizing the stromal remodeling associated with invasive cancer.

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