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Advances in Brief

Surfactant Protein Gene Expression in Metastatic and Micrometastatic Pulmonary Adenocarcinomas and Other Non-Small Cell Lung Carcinomas: Detection by Reverse Transcriptase-Polymerase Chain Reaction[1]

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Abstract

Reverse transcriptase (RT)-PCR with primers specific for surfactant protein A (*SP-A*), B (*SP-B*), C (*SP-C*), and D (*SP-D*) genes was applied to detect metastatic non-small cell lung carcinomas. Forty-one paratracheal and subcarinal lymph nodes obtained from 41 patients with non-small cell lung carcinomas, 11 lymph nodes from 11 patients with extrapulmonary adenocarcinomas, and eight control lymph nodes from patients without cancer were analyzed using RT-PCR. PCR products corresponding to *SP-B* gene products were found in all eight control lymph nodes, offering evidence of *SP-B* gene expression in cells of lymphatic tissue. SP-A, SP-C, and/or SP-D transcripts were detected in 11 (84.6%) of 13 lymph nodes with histologically identifiable metastases of pulmonary adenocarcinomas and in 10 (55.5%) of 18 lymph nodes that were tumor free on routine histological examination. These findings provide evidence of micrometastatic nodal involvement which remains undetectable by conventional light microsopy but can be evaluated by surfactant RT-PCR. Gene expression of SP-A and SP-C was restricted to metastatic pulmonary adenocarcinomas but *SP-D* gene activity has been also detected in two of four metastases of pulmonary large cell carcinomas, one adenosquamous carcinoma, and nine extrapulmonary adenocarcinomas as well.

Introduction

NSCLCs[3] represent about 75–80% of all lung cancers. Lobectomy or pneumonectomy combined with lymphadenectomy of regional lymph nodes is the treatment of choice. Assessment of the lymph node status is critical since spread in the mediastinal lymph nodes is associated with a highly unfavorable prognosis. Immunohistochemical investigations by Chen *et al.* (1) have previously demonstrated that occult lymph node micrometastases occur in about two thirds of the patients whose nodes did not contain metastases on routine histological examination. According to these investigators, median survival of this group of patients is shorter than that of patients with no detectable lymphatic spread but longer than that of patients with metastases detected on routine microscopy.

Recently an increasing number of studies have used RT-PCR to detect micrometastases or minimal residual diseases of nonhematological malignancies which escape detection by conventional light microscopy (2–5). Detection of specific gene sequences with the use of RT-PCR has been shown to be a more sensitive technique than serial sectioning or immunohistochemistry and has further increased the detection rate of micrometastases (4). SP-A, SP-B, SP-C, and SP-D are synthesized and secreted by type II alveolar epithelia. Gene expression of SP-A, SP-B, and SP-D has also been detected in Clara cells. Both cell types are assumed to be progenitor cells of PACs, and indeed subpopulations of NSCLC have been reported to express SP-A, SP-B, and SP-C (6–8). It was the purpose of this study to determine whether metastases and micrometastases of non-small cell lung cancer are detectable using RT-PCR targeted at these surfactant protein genes. RT-PCR was further applied on lymph node metastases of nonpulmonary adenocarcinomas to evaluate whether detection of surfactant proteins by RT-PCR can be used to discriminate between metastases of pulmonary and extrapulmonary adenocarcinomas.

Materials and Methods

Tissue. Thirty-one paratracheal and subcarinal lymph node specimens were selected following lymphadenectomy of 31 patients undergoing surgery for primary PACs. Intrapulmonary and hilar lymph nodes which are adjacent or within the lung tissue were excluded from this investigation to prevent false positive RT-PCR data. Special care was taken in the preparation of the lymph nodes evaluated. Each lymph node was stripped carefully of adipose tissue and blood, rinsed with saline, and bisected with a clean surgical knife. One half was snap frozen in liquid nitrogen and stored at −70°C before RNA extraction. The rest was formalin fixed and paraffin embedded for routine histology. Hematoxylin and eosin staining of three 5-μm sections revealed histologically detectable metastases in 13 lymph nodes while the remaining 18 lymph nodes were free of metastases as determined by conventional light microscopy.

An additional 10 lymph nodes from the same location with histologically detectable metastases were obtained from 10 patients with pulmonary squamous carcinomas ($n = 4$), large cell carcinomas ($n = 4$), adenosquamous carcinoma ($n = 1$), and carcinoid tumor ($n = 1$).

Eight mesenterial lymph nodes and one tonsil from patients who demonstrated no evidence of malignancy, and 11 lymph nodes from patients with histologically detectable metastases from mammary ($n = 4$), prostatic ($n = 2$), gastric ($n = 2$), colonic ($n = 1$), pancreatic ($n = 1$), and testicular ($n = 1$) carcinomas were analyzed as control specimens.

Cells. Type II alveolar cells were isolated from tumor-free lung tissue as previously described (9). For the preparation of lymphocyte suspensions, tonsil tissue was minced using a metal sieve. Lymphocytes were subsequently isolated by Ficoll-Hypaque density centifugation.

RNA Isolation. Total cellular RNA was extracted using the acid guanidinium-phenol-chloroform method (10) and diluted to a final concentration of 1 μg/μl diethylpyrocarbonate-treated water. The integrity of RNA was checked electrophoretically.

Oligonucleotide Primers and Probes. Oligonucleotide primers and probes for β-actin, SP-A, SP-B, SP-C, and SP-D were designed from previously published sequences (11–15). To avoid spurious amplification of genomic DNA, each primer set was selected from two different exons with at least one intervening intron.

Human β-actin primers (11) were as follows: primer I: 5′-GGGTGGGGCGCCCCAGGCACCA-3′ and primer II: 3′-CATCGCTCCTTAATGTCACGCACGATTTC-5′. The β-actin Pr-1 probe was obtained from Oncogene Science (Hamburg, FRG). Surfactant protein primers and probe sequences were as follows: SP-A (12) primer I: 5′-TTTCTTGGAGCCTGAAAAGA-3′; primer II: 3′-GGAGGCCGAAGGCCAGAGAGCGT-5′; and

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; PAC, pulmonary adenocarcinoma; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; SP-D, surfactant protein D; RT, reverse transcriptase; FRG, Federal Republic of Germany.

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probe: 5′-GTCGTGGAGTGTGGCTTGGAGCTCCTCATCTAGATGAGC-3′; SP-B (13) primer I: 5′-CATCGACTACTTCCAGAACCAGAC-3′; primer II: 3′-GCAGATTGCCGCCCGCCACCAGAGG-5′; and probe: 5′-CAGAG-GTACCACGCGGCACACCTGGGCCACTGCCACAGCTAGCGCACCCT-TGGGAATCAT-3′; SP-C (14) primer I: 5′-ATGGATGTGGGCAGCAA-AGAGGT-3′; primer II: 3′-AGATGTAGTAGAGCGGCACCTC-5′; and probe: 5′-GATAAGAAGGCGTTTCAGGTGCACTGGGCAGCAGGGAAT-3′; and SP-D (15) primer I: 5′-TTTGCCAGGAGCTGCAGGGCAA-3′; primer II: 3′-AAGTGCTCGCAGACCACAAGACG-5′; and probe: 5′-AAAGCAGCCTGGAGGTGCTGTACTTGTC-CCTGTAAGGCCTCAACCTGCTGC-3′.

RT-PCR. First-strand cDNA was synthesized by using Moloney mouse leukemia virus RT (GIBCO, Eggenstein, FRG). Five μg total cellular RNA were added to 1 μl enzyme (200 units), 2 μg dT15 primer (TIB-Biomol, Berlin, FRG), 1 μM random hexamers (Promega, Heidelberg, FRG), 50 units RNasin (Promega, Heidelberg, FRG), 1× transcription buffer (GIBCO), 10 mM DTT (GIBCO), and 4 mM deoxynucleotide triphosphate (Promega) to form a total reaction volume of 25 μl. The reaction was allowed to proceed at 37°C for 1 h.

The 50-μl total PCR volume consisted of 1–6 μl cDNA, 1 unit *Taq* polymerase (Amersham, Braunschweig, FRG), 1× amplification buffer (United States Biochemical, Braunschweig, FRG), 1.75 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, and 0.5 μM of each primer (TIB-Biomol).

The PCR reaction was then performed using a Cyclone thermocycler (Integra, Fernwald, FRG) under the following conditions: (*a*) β-actin: 25 amplification cycles, denatured at 94°C for 50 s, annealed at 65°C for 1 min, extended at 72°C for 1.5 min; (*b*) SP-A: 35 amplification cycles, annealing temperature was 58°C; (*c*) SP-B: 30 amplification cycles, annealing temperature was 66°C; (*d*) SP-C: 35 amplification cycles, annealing temperature was 60°C; and (*e*) SP-D: 35 amplification cycles, annealing temperature was 60°C.

Control RT-PCR reactions were always performed using all of the reagents as for the experimental samples, but without added RNA.

Gel Electrophoresis and Southern Blotting. After completion of the amplification cycles, 10 μl of each PCR product was run in a 1.5% agarose gel containing ethidium bromide. All specimens were analyzed et least twice to confirm a positive or negative outcome. For hybridization probes were labeled with with [^{32}P]dATP (DuPont, Bad Homburg, FRG) using terminal deoxynucleotidyl transferase (GIBCO). PCR products were transferred onto a positively charged nylon membrane (Hybond N^+; Amersham) by overnight alkali capillary blotting with the use of 0.25 M NaOH/1.5 M NaCl. Prehybridization was performed in a solution containing 50% (v/v) formamid, 4× SSC, 5× Denhardt's solution, 10 mM Tris, 10 mM EDTA, 0.1% SDS, 1 mg/ml denaturated salmon sperm DNA at 42°C for 3 h. After this time the labeled probe was added to the solution, and hybridization was allowed to proceed for 16 h. Filters were subsequently washed twice in 2× SSC at 60°C for 15 min and then in 0.2× SSC for 15 min. Exposure of the film lasted for 16 h at −70°C.

Sequencing. To identify PCR products obtained by SP-A, SP-B, SP-C, and SP-D primers, samples were purified from excess PCR primers by using a preparative 1.5% low-melting point agarose gel. Following digestion of the gel with gelase (Biozym, Heidelberg, FRG), purified cDNA was extracted and subsequently cloned into the pCR-script SK(+) phagemid by using the reagents of the corresponding cloning kit (Stratagene, Heidelberg, FRG), as described by the manufacturer. Cloned PCR products were sequenced by using a T7 sequencing kit (Pharmacia, Freiburg, FRG) and [^{35}S]dATP according to the instruction manual of the company.

Results

Specificity of RT-PCR in Detecting Surfactant Protein mRNA Expression. RNA integrity was confirmed in all samples by the detection of a 550-bp β-actin product in ethidium bromide-stained gels. Ethidium bromide staining of the amplified *SP-A*, *SP-B*, *SP-C*, and *SP-D* gene products from total RNA of type II alveolar cells (Fig. 1*a*) revealed bands of the expected molecular size (857 bp for SP-A, 367 bp for SP-B, 598 bp for SP-C, and 943 bp for SP-D). Subsequent sequencing of the PCR products and comparison to the published sequences (12–15) confirmed the identity of the fragments as cDNA

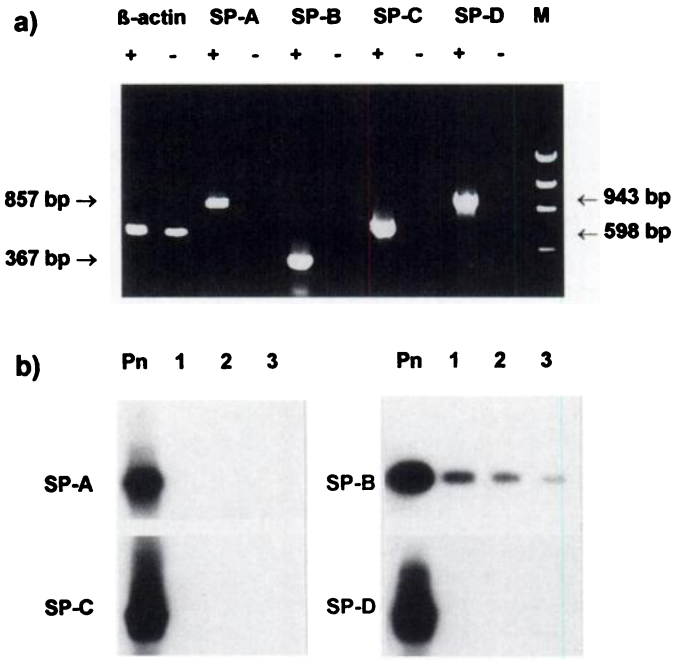

Fig. 1. *a*, ethidium bromide-stained gel showing electrophoresed PCR products obtained from RT-PCR amplification of RNA extracted from freshly isolated human type II alveolar epithelia (+) and A549 lung epithelial cells (−). RT-PCR was performed using primers specific for β-actin, SP-A, SP-B, SP-C, and SP-D. *Lane M*, DNA ladder markers. *b*, autoradiograph of Southern blot showing hybridization of ^{32}P-labeled SP-A, SP-B, SP-C, and SP-D probes to RT-PCR products derived from freshly isolated human alveolar type II cells (*Pn*) and three representative uninvolved lymph nodes (*Lanes 1–3*). A positive SP-B signal is seen in all three samples from uninvolved lymph nodes.

of surfactant proteins. SP-A, SP-B, SP-C, and SP-D transcripts were not detected in A549 lung epithelial cells (Fig. 1*a*), which is in accordance with previous data obtained using Northern blot analysis (7).

Normal lymphatic tissue including eight lymph nodes from patients without cancer and one tonsil were examined using RT-PCR. They did not contain detectable SP-A, SP-C, and SP-D transcripts. In contrast, a 367-bp DNA fragment of SP-B was amplified in all eight lymph nodes and the tonsil (Fig. 1*b*). According to these data the presence of *SP-B* gene activity in lymphatic tissue precludes the use of metastatic lung cancer detection by RT-PCR for this particular gene.

Sensitivity of RT-PCR in Detecting Surfactant Protein mRNA Expression. Sensitivity was determined by performing serial dilutions of type II alveolar cells and preparing mixes with tonsil lymphocytes to represent 10^3, 10^2, 10, and 1 type II alveolar cell(s)/10^7 lymphocytes (Fig. 2). Three sample mixes were prepared at each dilution and tested by RT-PCR. mRNA of SP-A and SP-C was detected by RT-PCR at concentrations as low as a single alveolar type II cell mixed in 10^7 tonsil lymphocytes. In contrast SP-D mRNA was detected at concentrations as low as 10 alveolar type II cells mixed in 10^7 tonsil lymphocytes.

Detection of Surfactant Protein mRNA Expression in Metastases and Micrometastases of NSCLC. In accordance with the data obtained in non-neoplastic lymphatic tissue, *SP-B* gene activity was found in all 41 paratracheal and subcarinal lymph nodes (Table 1). These data indicate that no sufficient discrimination between normal and involved lymph nodes is possible on the basis of the expression of the *SP-B* gene.

In contrast to *SP-B*, selective patterns of expression were found with respect to the *SP-A*, *SP-C*, and *SP-D* genes. The data are summarized in Table 1. Of 13 histologically positive lymph nodes

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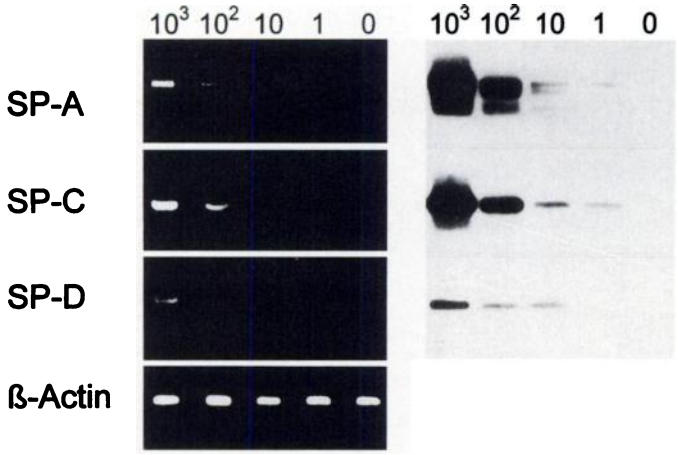

Fig. 2. Sensitivity of RT-PCR in detecting SP-A, SP-C, and SP-D mRNA from freshly isolated human type II alveolar cells mixed in 10^7 lymphocytes. Cells (10^3, 10^2, 10, and 1 human alveolar type II) were mixed with 10^7 lymphocytes, and mRNA was prepared from each of the mixtures to be used for RT-PCR. Positive control reactions (β-actin) for each mRNA preparation was performed using primers specific for β-actin. *Left*, PCR products stained with ethidium bromide; *right*, PCR products detected using Southern blot hybridization.

Table 1 *Surfactant protein mRNA in metastases and micrometastases of PACs*

No.	SP-A	SP-C	SP-D	SP-B[a]
1[b]	+[c]	++	++	++
2	–	++	–	++
3	++	+	++	++
4	++	–	–	++
5	–	–	++	++
6	+	–	+	++
7	–	–	–	++
8	–	–	++	+
9	–	–	+	++
10	–	–	+	++
11	–	–	–	++
12	+	+	+	++
13	–	–	+	+
14[d]	++	++	++	++
15	++	++	++	+
16	++	++	+	++
17	+	–	+	++
18	++	–	++	++
19	–	++	+	++
20	+	–	–	+
21	++	–	–	++
22	–	–	+	+
23	–	–	+	+
24	–	–	–	++
25	–	–	–	++
26	–	–	–	+
27	–	–	–	+
28	–	–	–	+
29	–	–	–	+
30	–	–	–	+
31	–	–	–	+

[a] *SP-B* gene activity was also detected in eight control lymph nodes from patients without cancer.
[b] Nos. 1–13, 13 histologically positive lymph nodes from patients with PACs.
[c] +, gene expression as detected using RT-PCR and subsequent Southern blot hybridization; ++, gene expression as detected using RT-PCR and ethidium bromide staining.
[d] Nos. 14–31, 18 histologically negative lymph nodes from patients with PACs.

from 13 patients with PACs (Fig. 3), all but two gave positive signals for at least one of the surfactant protein mRNAs A, C, and D. In addition, two of four lymph node metastases from large cell carcinomas and a lymph node metastasis from an adenosquamous carcinoma showed gene activity for SP-D but not for SP-A or SP-C. Two lymph node metastases of large cell carcinomas, all four metastases of squamous carcinomas, and one lymph node metastasis of a pulmonary carcinoid tumor did not contain detectable SP-A, SP-C, and SP-D transcripts.

Of 18 lymph nodes from patients with PACs that were diagnosed to be devoid of metastases by conventional light microscopy, 10 gave positive signals for at least one of the SP-A, SP-C, or SP-D mRNAs. Since normal lymphatic tissue does not show any gene activity of SP-A, SP-C, and SP-D, these findings indicate that occult tumor spread within the lymphatic tissue which is not detectable by conventional light microscopy might be the site of biosynthesis of the surfactant protein transcripts.

Taken together, 6 of 23 cases with lymph node metastases or occult tumor spread showed gene activity of all three surfactant proteins, 4 cases showed gene activity of two surfactant proteins, and 11 cases contained transcripts of only one surfactant protein. The percentage of SP-D-positive lymph nodes (73.9%) with metastases or occult tumor spread of PACs was higher than the percentage of SP-A-positive cases (52.2%). SP-C transcripts were selectively found in only 8 (34.8%) of 23 cases.

Detection of Surfactant Protein mRNA Expression in Lymph Node Metastases from Nonpulmonary Adenocarcinomas. Eleven lymph nodes with histological evidence of metastatic nonpulmonary adenocarcinomas were examined using RT-PCR and subsequent Southern blot hybridization to detect SP-A, SP-C, and SP-D transcripts. Metastases of four mammary carcinomas, two prostatic carcinomas, two gastric carcinomas, one colonic carcinoma, one pancreatic carcinoma, and one embryonal carcinoma of the testis did not contain detectable SP-A and SP-C transcripts. In contrast, a 943-bp long SP-D fragment was amplified from the metastases of all four mammary carcinomas, two prostatic carcinomas, one of two gastric carcinomas, one colonic carcinoma, and one pancreatic carcinoma. Only the embryonal carcinoma of the testis and one gastric carcinoma did not contain detectable SP-D transcripts.

Discussion

The use of RT-PCR for the detection of occult tumor spread in nodal tissue relies on the amplification of genes in tumor cells which are not expressed in non-neoplastic lymphatic tissue. The high level of sensitivity of the RT-PCR technique also requires high levels of specificity with respect to the cell markers used. Cytokeratins 8, 18, and 19, which have successfully been used to detect carcinoma cells in lymph nodes and bone marrow by immunohistochemistry, are only of limited value for RT-PCR analysis since low levels of cytokeratin mRNA are also detected in nonepithelial cells and especially in tumor-free lymphatic tissue (3, 16). Other tissue-specific markers like tyrosinase (2), prostata-specific antigen (5), and the epithelial mucin-associated protein MUC1 (4) have not been detected in normal lymphatic tissue or bone marrow and were succesfully applied for the

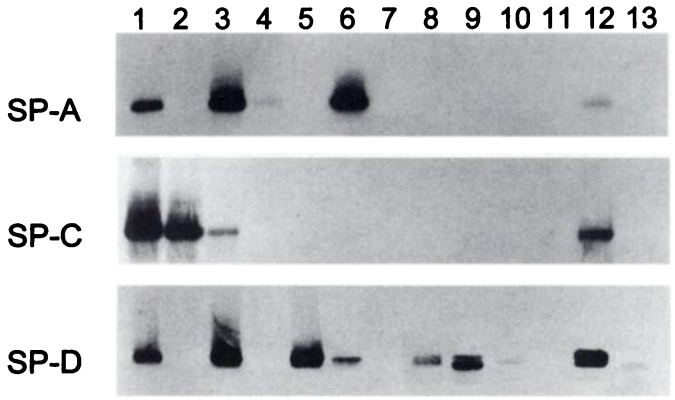

Fig. 3. Expression of SP-A, SP-C, and SP-D in 13 lymph node metastases of 13 PACs. PCR products were detected using Southern blot hybridization.

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detection of disseminated malignant melanomas, prostatic cancer, and breast cancer cells, respectively.

The intention of this study was to evaluate the possible use of surfactant protein RT-PCR for the detection of lung cancer metastases and micrometastases. The presence of *SP-B* gene activity in nonneoplastic lymphatic tissue precludes RT-PCR analysis of this particular gene as a detector of metastatic pulmonary carcinomas. Further investigations are needed to identify the cell type within the nodal tissue that demonstrates *SP-B* gene activity.

The *SP-A* gene, which is normally expressed by type II alveolar cells, Clara cells, some tracheal epithelia and tracheal glands (17), was detected in nearly one half (12/23 cases) of the metastatic and micrometastatic PACs. This value is in agreement with previous results that were obtained on primary or metastatic PACs and on lung cancer cell lines using immunohistochemistry (6), Northern blot analysis (7), and *in situ* hybridization (8). None of the extrapulmonary adenocarcinomas tested showed detectable amounts of *SP-A* gene activity.

SP-C, which in normal human lung tissue is only synthesized by type II alveolar cells, also has a restricted expression pattern in PACs. Only 8 of 23 lymph node metastases and micrometastases were SP-C positive. The incidence of SP-C-positive tumors observed in our study is higher than previously obtained values in lung cancer cell lines using Northern blot analysis (7). The differences might by due to the higher sensitivity of the RT-PCR technique. They also might indicate that lung cancer cells may often lose their potential to synthesize SP-C when they are kept in culture.

Previous investigations have demonstrated that SP-D is expressed in extrapulmonary tissues as well. Immunochemical investigations have demonstrated SP-D and SP-B in rat intestinal epithelium (18), and a recent study with combined immunohistochemical studies and RT-PCR analysis reports on the expression of SP-D in the rat gastric mucosa (19). As far as we know, no data are available on the expression of SP-D in human tumors. The present data demonstrate a variable expression of the *SP-D* gene in PACs, large cell carcinomas, and one pulmonary adenosquamous carcinoma. SP-D is also detectable in mammary, prostatic, gastric, colonic, and pancreatic carcinomas.

We show in this pilot study that RT-PCR using SP-A, SP-C, or SP-D as tumor markers might be a valuable tool to detect micrometastases of PACs, large cell carcinomas, and adenosquamous pulmonary carcinomas. The detection rate of micrometastases by this approach is limited by the fact that each of these cell markers is expressed only by a subset of tumors. SP-D was the most commonly detected surfactant protein in 17 of 23 metastases or micrometastases. The detection rate of metastatic PACs increased up to 84.6% (11/13 cases) when SP-A, SP-C, and SP-D were evaluated together, but even then about 15% of the metastatic PACs escaped detection because they did not synthesize surfactant proteins. A combination of surfactant proteins with other tissue-specific markers such as mucin-associated proteins (4) might possibly further increase the sensitivity of this approach. With respect to the tissue specificity, RT-PCR of SP-A and SP-C might be useful in diagnostic practice to identify the potential lung origin for metastatic lesions with unknown primary tumors. This is obviously not the case for RT-PCR of SP-D, which has also been detected in 9 of 11 nonpulmonary adenocarcinomas. Finally, additional studies will be needed to evaluate the prognostic significance and therapeutic implications of occult micrometastatic tumor spread that has been detected by surfactant protein RT-PCR.

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