

# Semenogelins Are Ectopically Expressed in Small Cell Lung Carcinoma

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## ABSTRACT

Two proteins recovered from cell surface adhesion complexes in a small cell lung carcinoma (SCLC) cell line were identified as fragments of the seminal plasma proteins semenogelin I and semenogelin II. Association of both proteins with the adhesion complexes was induced by epidermal growth factor. Expression of semenogelins was previously thought to be highly specific to seminal vesicles, but Western blot analysis demonstrated that semenogelin II is widely expressed in SCLC cell lines and occasionally in other malignant cell lines. Although semenogelin expression is normally restricted to males, two SCLC cell lines from female patients were also positive for semenogelin II expression. Immunohistochemical analysis demonstrated diffuse expression of semenogelins in 12 of 13 SCLC tumors and focal expression in a minority of lung squamous and adenocarcinomas. Semenogelins were secreted into the medium by cultured SCLC cells, which suggested that these proteins may be useful markers for detecting residual tumor burden or recurrence of SCLC after treatment.

## INTRODUCTION

Circulating markers have proved to be valuable for assessing disease progression in prostate, ovarian, and colorectal cancers (1-5). After treatment, these markers often provide a non-invasive and sensitive assessment of residual tumor burden and regrowth after treatment of the primary malignancy. SCLC<sup>2</sup> accounts for approximately 25% of lung cancers but has the lowest 5-year survival of these cancers (reviewed in Refs. 6-8). Although SCLC initially responds well to chemotherapy, the cancer recurs with high frequency and is often refractory to further treatment. Therefore, to improve the treatment of this

disease, sensitive methods to detect residual SCLC and its regrowth are needed (see "Molecular targets for therapy of small cell lung cancer," National Cancer Institute, 1999).<sup>3</sup> Unfortunately, relatively few circulating markers for SCLC have been described, and their measurement has been of limited utility in monitoring this disseminated cancer<sup>3</sup> (9-11). Therefore, better markers are needed to detect residual disease and assess tumor burden in SCLC patients.

MHS-5 is a monoclonal antibody that recognizes two polypeptides in human semen (12-14). The predominant proteins constituting semen coagulum are semenogelin I, a  $M_r$  50,000 protein, and semenogelin II, which exists in a nonglycosylated and glycosylated form at  $M_r$  71,000 and  $M_r$  76,000, respectively (15-18). MHS-5 recognizes both of the intact forms of semenogelin I and semenogelin II, as well as several proteolytic fragments released during liquefaction of the gel. The genes for semenogelin I and semenogelin II are located 11.5 kb apart on the p arm of chromosome 20 (19), and each gene contains three exons that encode the secreted protein.

Histological analyses have failed to detect semenogelin expression in any normal human tissue other than seminal vesicle epithelium (14, 20, 21). This specificity has led to the use of semenogelin antibodies for forensic detection of seminal fluid in sexual assault. EST-expression profiling demonstrated that semenogelin I and semenogelin II were expressed almost exclusively in cDNA libraries from normal and malignant prostate (see Unigene Cluster Hs; 180016 and HS; 1968<sup>4</sup>) correlating with the path of seminal fluid passage during ejaculation. However, two isolated observations from the Cancer Genome Anatomy Project database<sup>4</sup> suggest that these genes may be sporadically expressed in malignancies derived from other tissues. Sequencing of a papillary renal cell carcinoma EST library (NCI CGAP Kid1, papillary renal cell carcinoma, Krizman protocol 1) detected semenogelin I in 1 of 1114 clones sequenced. Semenogelin II was detected in 1 of 4153 clones sequenced from a colon cancer EST library (NCI CGAP Co10).

The present study demonstrates that semenogelins are expressed ectopically by multiple SCLC lines derived from both male and female patients and in a minority of other lung cancer cell lines, but not in breast cancer or lymphoma cell lines. Furthermore, membrane association of the semenogelin proteins was enhanced in OH-1 SCLC cells by the addition of EGF when the cells attached to thrombospondin-1 peptides. Semenogelin immunoreactivity was also observed in most SCLC tumors, which suggests these proteins may be useful markers for SCLC.

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<sup>2</sup> The abbreviations used are: SCLC, small cell lung carcinoma; LC/MS, liquid chromatography/mass spectrometry; EST, expressed sequence tag; EGF, epidermal growth factor.

<sup>3</sup> Internet address: <http://www.conference-cast.com/webtie/sots/lung/default.htm>.

<sup>4</sup> Internet address: <http://gap.nci.nih.gov/Genes/GeneFinder>.



## MATERIALS AND METHODS

**Proteins and Peptides.** A synthetic peptide containing the thrombospondin-1 sequence that binds to the  $\alpha_3\beta_1$  integrin, FQGVNQVRFVF (peptide 678) was prepared as described previously (22). Semenogelin was purified from seminal plasma as described (15). Recombinant EGF was obtained from R&D Systems.

**Cell Lines and Reagents.** Human lung cancer (OH-1, NCI-N592, NCI-N417, NCI-H378, NCI-H570, NCI-H727, NCI-H157, NCI-H520, and A549), breast carcinoma (MDA-MB-231, MCF-7, and T47D), melanoma (C32, and A2058), and Jurkat T lymphoma cell lines were grown in RPMI 1640 containing 10% FCS (15% FCS for OH-1 cells).

**Adhesion Assays.** OH-1 SCLC cells (23) were dissociated by replacing the growth medium with 2.5 mM EDTA in PBS and incubating at 37°C for 10 min. Cells were then triturated and collected by centrifugation, suspended in M199 medium, and plated on the respective thrombospondin-1 peptide-coated plates (Falcon 1029). OH-1 cell adhesion to thrombospondin-1 peptide 678 (10  $\mu$ M) was assessed with and without EGF (5 ng/ml). Cells were incubated for 2 h at 37°C and then aspirated and washed three times with Dulbecco's PBS. Cytoskeleton-associated adhesion complexes were isolated by detergent extraction of the cells using CSK buffer [0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 20  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 10 mM PIPES (pH 6.8)] for 1 min followed by sonication for 30 s to remove cell bodies (24). The cell surface adhesion complexes, which remained attached to the peptide substrates, were then extracted using 0.5 ml of 1× immunoprecipitation buffer [50 mM Tris (pH 7.2), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride]. The plates were scraped, the recovered extracts were centrifuged, and the supernatant fractions were collected.

**Isoelectric Focusing/Two-Dimensional Gel Electrophoresis.** Proteins extracted from the thrombospondin-1 peptide-associated adhesion complexes were mixed with an equal volume of rehydration buffer (8 M urea, 1% immobilized pH gradient buffer, and 2% 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate in H<sub>2</sub>O) in a strip holder with a gel strip (pH 3–10; Amersham Pharmacia Biotech) and incubated overnight at 20°C. The strip holders and hydrated strips were placed in an IPGphor unit and subjected to isoelectric focusing for 4 h. The gels were equilibrated with 100 mg of DTT in 10 ml of H<sub>2</sub>O for 1 h followed by the addition of 100 mg of iodoacetamide and shaking for 15 min. The equilibrated gel strip was applied to a SDS/10% polyacrylamide gel and subjected to electrophoresis on a Hoefer TE Transphor for 15 min at 20 mA and then for 5 h at 40 mA. The gels were removed and stained with Coomassie Blue stain for 1 h and destained (10% acetic acid + 35% methanol in H<sub>2</sub>O) overnight.

**Cell Line Screening.** OH-1 cells were harvested and extracted as described in the adhesion assay protocol. Extracted proteins, after clarifying by centrifugation, were separated by SDS-PAGE in Mini Protean II system (Bio-Rad) for 55 min at 170 V. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane in trans-

fer buffer (20% methanol in 1× Tris/glycine; Bio-Rad) at 70 V for 2.5 h and washed three times with 1× Tris/glycine buffer. The membrane was blocked overnight in Dulbecco's PBS containing 1% BSA and 0.1% Tween 20. The blot was then incubated with biotinylated MHS-5 antibody (Humagen Fertility Diagnostics, Charlottesville, VA) at 1:1000 for 2 h at 37°C followed by four washes with Dulbecco's PBS containing 0.1% Tween (13). The membrane was then incubated with streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:20,000 for 1 h. Surface proteins were visualized with a chemiluminescent detection kit (Amersham Pharmacia Biotech).

**Immunoprecipitation.** Unlabeled MHS-5 antibody was prebound to Protein A agarose in Dulbecco's PBS containing 1% BSA and 0.1% Tween 20 for 2 h at 4°C. Beads containing the bound antibody were then incubated with 500  $\mu$ l of extracted proteins overnight at 4°C. The beads were washed three times with Tris-buffered saline and then were eluted by heating with sample buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, and bromophenol blue) at 95°C for 5 min, and separated, and transferred as described above. The blot was then incubated with biotinylated MHS-5 antibody and streptavidin-horseradish peroxidase and visualized by chemiluminescence.

**LC/MS Identification of Proteins.** Proteins of interest contained in one- or two-dimensional gels were analyzed by LC/MS to determine their identity. The protein spot detected in the stained gel was excised and placed in a microfuge tube. The gel piece was washed with methanol/ammonium bicarbonate buffer, dried *in vacuo*, and then treated with trypsin overnight. The resulting peptides were extracted, separated, and analyzed on a Finnigan LCQ LC/MS system. The resulting run files were first analyzed using Sequest database searching software. If no identification resulted, then further database searching and/or *de novo* sequencing was carried out.

**Immunohistochemistry and Slide Preparation.** Formalin-fixed, paraffin-embedded representative tissue specimens were immunostained with monoclonal antibody against MHS-5 (dilution 1:100). Staining was performed with the EnVision system (DAKO). Endogenous peroxidase activity was quenched by treatment with 5% hydrogen peroxide in methanol for 30 min at room temperature. Antigen retrieval using Target retrieval (pH 7.0; DAKO) and microwave treatment for 20 min in an 800-W microwave oven was performed. A blocking step with serum-free protein block (DAKO) was used. The primary antibody was then applied for 120 min at room temperature. The sections were rinsed with washing buffer (Dulbecco's PBS + 0.1% Tween) at room temperature and incubated with EnVision system reagents for 30 min. at room temperature. The slides were rinsed with washing buffer and treated with a solution containing 0.05% diaminobenzidine hydrochloride and 0.1% hydrogen peroxide in 0.05 M TRIS-buffered saline (pH 7.4), at room temperature for 5 min. After rinsing in distilled water for 3 min, the slides were counterstained with modified Harris hematoxylin, dehydrated, and mounted. Negative control sections were treated in an identical fashion except for lack of primary antibody. An appropriate positive control (human seminal vesicle) was run concurrently.



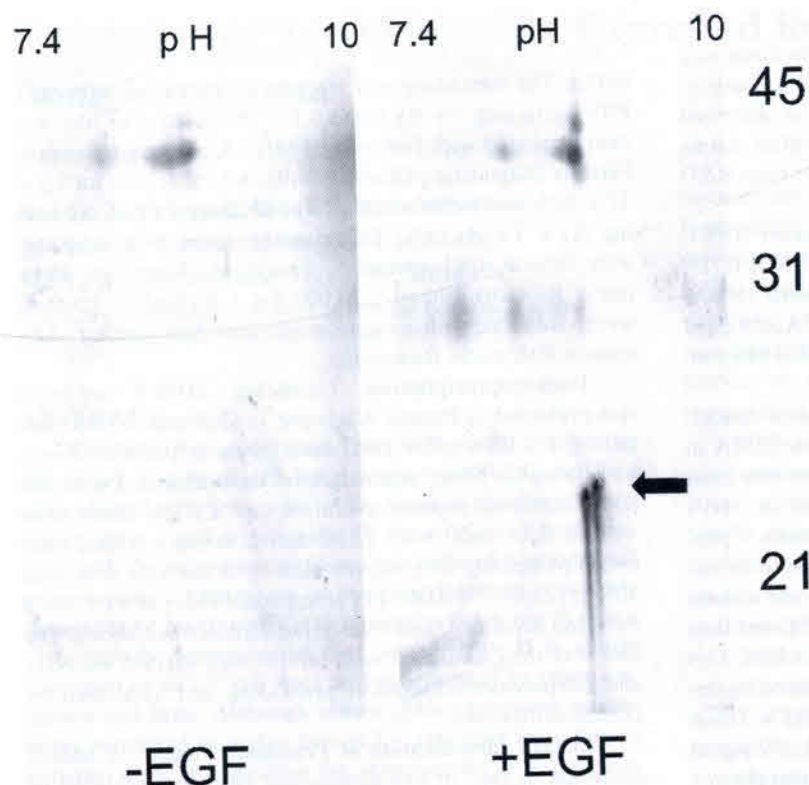


Fig. 1 Induction of semenogelin I and II association with adhesion complexes by EGF in OH-1 SCLC cells after adhesion on thrombospondin-1 peptide 678. Matched portions of the two-dimensional isoelectric focusing and SDS gels are presented, demonstrating the presence of two protein spots, with  $M_r$  25,000–28,000, isoelectric point = 9 (black arrow), that were specifically induced by adding EGF to the medium (right side).

Table 1 Semenogelin tryptic peptide fragments isolated from OH-1 SCLC cells that could be specifically assigned to semenogelin I or semenogelin II

Peptide sequence	SG <sup>a</sup>	Position <sup>b</sup>	MassA <sup>c</sup>	Xcorr <sup>d</sup>	DelCn <sup>e</sup>	Ions <sup>f</sup>
QHLGGSQQLLNK	SGII	98–110	1486.7	2.6062	0.399	14/24
HLGGSQQLLNK	SGI	99–110	1332.5	3.6396	0.508	18/22
GHYQNVVDVR	SGII	218–227	1187.3	2.6330	0.460	15/18
GHYQNVVEVR	SGI	218–227	1201.3	3.6777	0.497	14/18
QDLLSHEQK	SGII	535–543	1098.2	2.3372	0.334	13/16
EQDLLSHEQK	SGI	414–423	1227.3	2.9229	0.397	13/18

<sup>a</sup> SG, semenogelin protein to which the peptide sequence matched.

<sup>b</sup> Position in the amino acid sequence based on an Xcalibur database search. SEM1\_HUMAN covers 452 amino acids and SEM2\_HUMAN spans 582 amino acids.

<sup>c</sup> MassA is the mass of the  $M^{+1}$  molecular ion.

<sup>d</sup> Xcorr is raw cross-correlation score of the peptide; only statistically significant sequences with Xcorr > 2.2 are presented.

<sup>e</sup> DelCn is the  $\delta$  correlation score between the top two candidate peptide matches and is significant if the value is >0.2.

<sup>f</sup> The number of peptide fragment ions matched/the total number of expected fragment ions.

## RESULTS

**Isolation and Identification of Semenogelin Proteins in SCLC Cells.** Analysis of adhesion complexes isolated from OH-1 SCLC cells by two-dimensional gel electrophoresis showed that two proteins with  $M_r$  of 25,000–28,000 and an isoelectric point of 9, were induced to associate with the adhesion complexes by the addition of EGF (Fig. 1). The two protein spots were isolated and sequenced by mass spectrometry and found to contain multiple peptides homologous to portions of semenogelins I and II (Table 1).

Mass spectrometric analyses of tryptic peptides from these two fragments yielded peptides that covered 42.6% of the semenogelin I protein sequence and 38.5% of the semenogelin II

protein sequence. Several peptides detected were identical in both proteins, as expected from their sequence known homologies, but several of the ion peaks were assigned to peptides that could be assigned specifically to semenogelin I or II (Table 1). For example, a tryptic peptide from an  $NH_2$ -terminal sequence of semenogelin I (HLGGSQQLLNK) differed from the corresponding semenogelin II sequence (QHLGGSQQLLNK) by the insertion of a single glutamine in the semenogelin II peptide  $NH_2$  terminus and a two-amino acid substitution on the  $COOH$ -terminal end. This resulted in distinct  $M^{+1}$  ions for peptides derived from the two proteins. Both of these molecular ions were detected in analysis of the tryptic digest, and the identities of both peptides were confirmed by the corresponding



mass spectrometry/mass spectrometry spectra (Table 1). Similarly, a semenogelin I COOH-terminal sequence (EQDLLSHEQK) contained an extra NH<sub>2</sub>-terminal glutamic acid that is not in the semenogelin II protein sequence (QDLLSHEQK; Table 1), and both molecular ions and peptide sequences were detected in the LC/MS analysis. Therefore, the recovered protein spots contain NH<sub>2</sub>- and COOH-terminal fragments of both semenogelin I and II. These results suggest that both of the semenogelins were cleaved somewhere in the middle of the protein to generate the observed fragments and proves that both of the proteins are expressed in the OH-1 SCLC line (Fig. 1). In total, we identified three sets of peptides that could distinguish expression of the two semenogelin proteins and thereby verified that both of the proteins are expressed in this SCLC cell line.

**Biochemical Detection of Semenogelin Proteins in Human Cancer Cell Lines.** Nearly all of the SCLC lines screened by Western blotting and immunoprecipitation demonstrated the presence of a doublet between  $M_r$  70,000–80,000 corresponding to semenogelin II (Fig. 2A; Table 2). Only the classic SCLC line OH-1, which grows as tight aggregates *in vitro*, was positive for semenogelin I. Semenogelin II was detected in all of the SCLC and some of the non-SCLC cell lines, including some squamous cell carcinomas. Semenogelin II expression was also detected in two melanoma cell lines (A2058 and C32) but was absent in the other cancer cell lines examined, including H727 carcinoid cells; MDA-MB-231, MCF7, and T47D breast carcinoma cells; and Jurkat T-cell lymphoma cells (Fig. 2A; Table 2). However, the SCLC lines typically demonstrated stronger semenogelin II expression than squamous cell lung carcinoma and other types of cancers.

Interestingly, semenogelin II was found in cell lines derived from SCLC patients of either gender. Expression of semenogelin II in the female lines H378 and N417 further supports specific ectopic production of the semenogelins by the SCLC cells (Fig. 2A; Table 2). The previous rare observations of semenogelin ESTs outside the prostate were only in tumors from males.<sup>4</sup>

Semenogelin II was also secreted into the medium by OH-1 SCLC cells (Fig. 2B) but not by H570 squamous cell lung cancer cells (results not shown). Measurement of secreted semenogelin II, therefore, could potentially provide a quantitative method to assess SCLC tumor burden. In contrast, semenogelin I was not apparent in the supernatant of OH-1 cell medium (Fig. 2B), which suggests that, although membrane-associated, it is not actively secreted into the medium by these cells or remains bound to surface proteoglycans via its heparin-binding site (15).

**Immunohistochemical Detection of Semenogelin Expression in Lung Cancer.** To determine whether semenogelin proteins are also expressed in tumors from SCLC patients, we analyzed pathological slides of various lung cancer tissue specimens using the MHS-5 antibody (Fig. 3). Of 13 SCLC specimens analyzed, 12 were found to be diffusely positive with homogeneous, widespread antibody labeling throughout the cytoplasm of the tumor cells and surrounding matrix (Fig. 3, A and B; Table 3). In contrast, only 4 of 21 squamous lung cancer specimens showed positive MHS-5 staining, and 3 of those were only focally positive. All of the other squamous cell specimens were negative. (Fig. 3, C and D; Table 3). Lung adenocarcinoma



**Fig. 2** Specific expression of semenogelin (SG) proteins in lung cancer cell lines. **A**, semenogelin detection by Western blotting. Both semenogelin I and II were present on the surface of OH-1 SCLC cells. Only the semenogelin II doublet was detected in H378, H570, and A549 lung carcinoma cell lines, and no antigen was detected on MDA-MB-231 breast carcinoma cells. **B**, active secretion of semenogelin proteins by SCLC cells as detected by immunoprecipitation. OH-1 cells were incubated in solution, 500  $\mu$ l of clarified cell-free conditioned medium was immunoprecipitated using MHS-5, and semenogelins were detected by Western blotting using biotinylated MHS-5. Semenogelin II but not semenogelin I was secreted by OH1 SCLC cells. Medium alone was a negative control, and medium spiked with purified semenogelin was a positive control.



Table 2 Semenogelin (SG) I and II protein expression in SCLC and non SCLC cell lines

Cell Line	Type	Gender	SGI	SGII
OH-1	SCLC	Male	+	+
N592	SCLC	Male	—	+
N417	SCLC	Female	—	+
H378	SCLC	Female	—	+
H727	Lung carcinoid tumor	Female	—	—
H157	Lung squamous cell carcinoma	Female	—	+
H520	Lung squamous cell carcinoma	Male	—	+
H570	Lung squamous cell carcinoma	Male	—	+
A549	Lung carcinoma	Male	—	+
MDA-MB231	Breast carcinoma	—	—	—
MCF7	Breast carcinoma	—	—	—
T47D	Breast carcinoma	—	—	—
C32	Melanoma	—	—	+
A2058	Melanoma	—	—	+
Jurkat	T-cell lymphoma	—	—	—

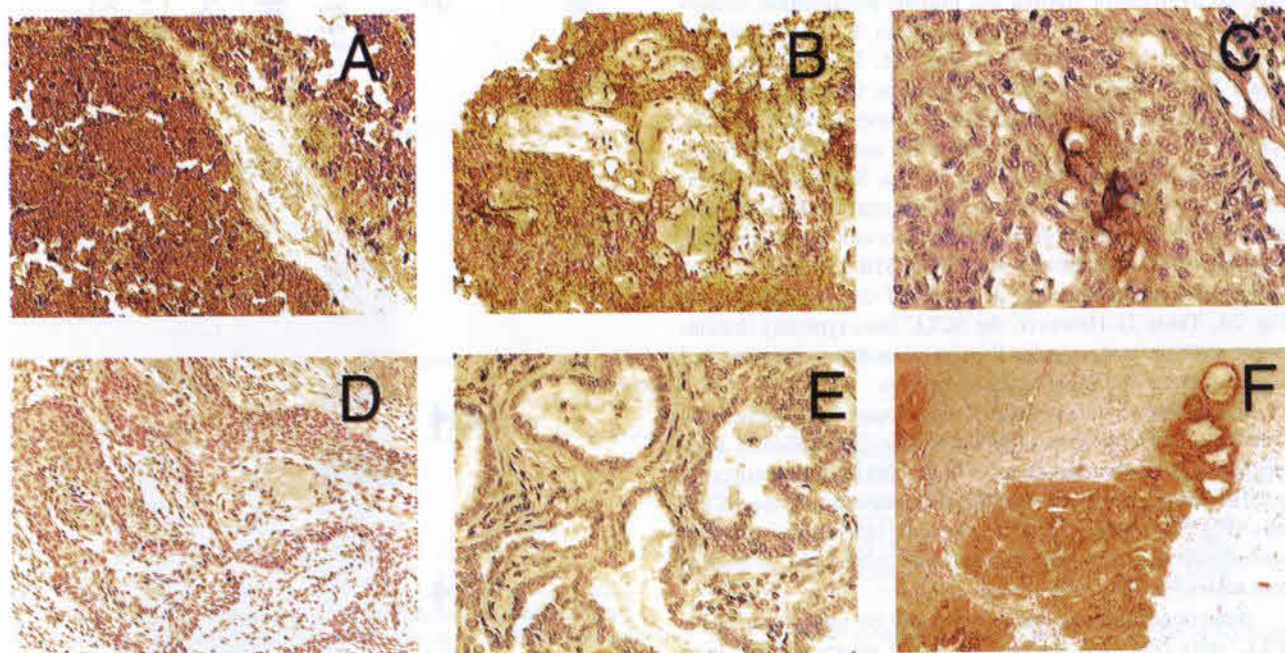


Fig. 3 Immunohistochemical analysis of surgical specimens of various lung cancers using antibody MHS-5 for detection of semenogelin proteins. A and B, representative SCLC specimens showing diffusely positive MHS-5 labeling throughout the tumor (brown); C, representative field of three squamous cell specimens showing only slight focally positive MHS-5 labeling (brown); D, representative field for 17 of 21 negative lung squamous carcinoma specimens; E, representative field for 5 of 7 lung adenocarcinomas that showed no labeling for semenogelin proteins; F, seminal vesicle tissue labeled with MHS-5 as a positive control for semenogelin expression.

specimens showed results similar to those with the squamous cell carcinomas, with two tissues showing a very slight focal positivity in the luminal epithelium cells, and all of the other specimens were negative except the seminal vesicle control (Fig. 3E; Table 3). All three of the lung cancers produce predominantly centrally arising tumors, which suggests that the semenogelin proteins are a relatively specific marker of SCLCs.

## DISCUSSION

Expression of semenogelin proteins was previously determined to be highly specific to seminal vesicles and seminal plasma (10, 13–16, 18–21, 25–28), but our results demonstrate

that this is not the case in patients with SCLC. By two-dimensional gel analysis of proteins recovered from cell surface adhesion complexes in OH-1 SCLC cells, we identified fragments of the seminal plasma proteins semenogelin I and semenogelin II. In particular, semenogelin II is widely expressed in SCLC cell lines and occasionally in certain other malignant cell lines. However, semenogelin I seems to be specifically expressed only in the OH-1 SCLC cells. Furthermore, semenogelin II expression is not restricted only to males, because SCLC cell lines from two female patients were also positive for semenogelin II expression. Association of both proteins with adhesion complexes stimulated by EGF on substrates coated with the throm-



Table 3 Incidence of MHS-5 immunohistochemical detection of semenogelin proteins in lung cancers

Diagnosis	No. of specimens	Positive <sup>a</sup>		Negative
		Diffuse	Focally	
Small cell carcinoma	13	12	0	1
Squamous cell carcinoma	21	1	3	17
Adenocarcinoma	7	0	2	5

<sup>a</sup> Archival surgical specimens were diagnosed on the basis of pathological characteristics and identified as focally positive or diffusely positive by immunohistochemical staining using MHS-5.

bospondin-1 peptide p678 suggested that the increased surface expression of semenogelin protein may be correlated with proliferation and differentiation of the OH-1 SCLC cell lines (29). However, semenogelins were also found to be secreted into the medium by cultured OH-1 SCLC cells, which suggests that these proteins may be useful circulating markers for detecting SCLC. Further study is required to test the utility of circulating semenogelin assays.

Immunohistochemical analysis of surgical specimens from various lung cancers demonstrated diffuse expression of semenogelins by MHS-5 antibody labeling in 92% of SCLC tumors, whereas lung squamous and adenocarcinomas showed only focal expression in a minority of specimens. These data confirm the biochemical findings that semenogelins are ectopically expressed by SCLC and can, thus, be used as a tumor marker for this particular lung cancer. On the basis of the previous observation of semenogelin ESTs in two isolated cancer libraries<sup>4</sup> and our detection of semenogelins in two melanoma cell lines, this protein may be sporadically expressed in a variety of malignancies. However, none that we have examined to date show the consistent expression that we observed in both SCLC cell lines and tumors.

Small-cell lung carcinomas are highly metastatic neuroectoderm tumors derived from neural crest cells of the developing fetal central nervous system. Neuroectoderm tumors frequently secrete ectopic proteins, such as reported for parafollicular carcinomas of the thyroid (30, 31) and pheochromocytomas of the adrenal medulla (32–34). On the basis of the high level of expression of semenogelins in the SCLC cell lines and strong staining of tumor specimens, semenogelins may be useful markers for detecting SCLC, although they probably cannot differentiate SCLC from non-SCLC tumors. Because semenogelins are secreted proteins (15) and SCLC cells in culture secrete the antigen, semenogelins could also be useful circulating markers for SCLC tumor burden. We are currently developing methods to detect semenogelins in peripheral blood and will examine their potential for assessing residual burden and early detection of regrowth after chemotherapy of SCLC. Early detection of this regrowth may allow early treatment to decrease the emergence of drug resistance and improve survival rates for SCLC.

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