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Human semaphorins A(V) and IV reside in the 3p21.3 small cell lung cancer deletion region and demonstrate distinct expression patterns

(growth cone guidance/collapsin/recessive oncogene/G protein)

YOSHITAKA SEKIDO*, SCOTT BADER*†, FARIDA LATIF‡, JEOU-YUAN CHEN*§, FUH-MEI DUH¶, MING-HUI WEI¶, JOSEPH P. ALBANESI‖, CHENG-CHI LEE**, MICHAEL I. LERMAN‡, AND JOHN D. MINNA*‖††

*Hamon Center for Therapeutic Oncology Research, ‖Department of Pharmacology, and ††Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235; ‡Laboratory of Immunobiology, and ¶Biological Carcinogenesis and Development Program, Science Applications International Corporation Frederick, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD 21702; and **Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030

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ABSTRACT **Semaphorins and collapsins make up a family of conserved genes that encode nerve growth cone guidance signals. We have identified two additional members of the human semaphorin family [human semaphorin A(V) and human semaphorin IV] in chromosome region 3p21.3, where several small cell lung cancer (SCLC) cell lines exhibit homozygous deletions indicative of a tumor suppressor gene. Human semaphorin A(V) has 86% amino acid homology with murine semaphorin A, whereas semaphorin IV is most closely related to murine semaphorin E, with 50% homology. These semaphorin genes are ≈70 kb apart flanking two GTP-binding protein genes, *GNAI-2* and *GNAT-1*. In contrast, other human semaphorin gene sequences (human semaphorin III and homologues of murine semaphorins B and C) are not located on chromosome 3. Human semaphorin A(V) is translated *in vitro* into a 90-kDa protein, which accumulates at the endoplasmic reticulum. The human semaphorin A(V) (3.4-kb mRNA) and IV (3.9- and 2.9-kb mRNAs) genes are expressed abundantly but differentially in a variety of human neural and nonneural tissues. Human semaphorin A(V) was expressed in only 1 out of 23 SCLCs and 7 out of 16 non-SCLCs, whereas semaphorin IV was expressed in 19 out of 23 SCLCs and 13 out of 16 non-SCLCs. Mutational analysis in semaphorin A(V) revealed mutations (germ line in one case) in 3 of 40 lung cancers. Our data suggest the need to determine the function of human semaphorins A(V) and IV in nonneural tissues and their role in the pathogenesis of lung cancer.**

The semaphorin/collapsin family of molecules plays a critical role in the guidance of growth cones during neuronal development (1). Using grasshopper semaphorin I sequences, Kolodkin *et al.* (2, 3) cloned two semaphorin genes in *Drosophila* and one in human (human semaphorin III; H-Sema III), providing evidence that semaphorins are a family of genes sharing amino acid homology across species and possessing characteristics of transmembrane and secreted proteins. Meanwhile, based on its *in vitro* ability to collapse growth cones from dorsal root ganglion neurons, collapsin was purified from chicken brain and was found to be highly homologous in sequence with semaphorins (4). The semaphorin/collapsin protein family are all about 750 aa long with a shared, highly conserved ≈500-aa semaphorin domain with or without a single immunoglobulin (Ig) domain. The members come in multiple forms: secreted with an Ig domain, transmembrane with an Ig domain, and transmembrane without an Ig domain. Five murine semaphorin genes (semaphorin A, B, C, D, and E) and four chicken collapsin genes (collapsin 2, 3, 4, and 5) have been isolated (5, 6). Recent genetic analysis of *Drosophila* embryos and *in vitro* cellular studies of vertebrate neural tissue have demonstrated that semaphorins regulate the guidance of axons during embryogenesis by repelling growth cones from regions of high semaphorin expression (7, 8).

Lung cancer arises because of mutations in dominant and recessive oncogenes (9). Some of the important putative lung cancer recessive oncogene(s) (tumor suppressor genes) reside on chromosome 3p (10–15). Chromosomal abnormalities and allelotyping studies show 3p loss of heterozygosity in >90% of small cell lung cancers (SCLCs) and >50% of non-small cell lung cancers (NSCLCs) and indicate the presence of approximately four different 3p recessive oncogenes located at 3p12–13, 3p21.3, and 3p25 (13, 16). The 3p21.3 site is particularly interesting because of its frequent involvement and because we and others (refs. 15 and 17; S.B., F.L., Y.S., M.-H.W., F.-M.D., J.-Y.C., K. Tartoff, C.-C.L., V. Kashuba, R. Gizatullin, E. Zabarovsky, G. Klein, B. Zbar, A. F. Gazdar, M.I.L., and J.D.M., unpublished results) have recently identified a series of overlapping homozygous deletions in SCLC cell lines narrowing the localization of one of the genes to <350 kb. We have constructed a complete cosmid/P1 contig covering this region, which was used to isolate candidate recessive oncogenes (18). In this process of positional cloning, we have found two previously uncloned human semaphorin genes lying within ≈70 kb of each other. We find that these genes have widespread but distinct patterns of expression in nonneural tissues and different patterns of expression in lung cancer.

MATERIALS AND METHODS

cDNA Library Screening. A contig of 22 cosmids covering 600 kb localized to the 3p21.3 SCLC homozygous deletions was isolated from a human placental cosmid library (Stratagene) or a chromosome 3-specific cosmid library by cosmid walking with insert-end probes starting from internal markers such as

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Abbreviations: H-Sema A(V), human semaphorin A(V); H-Sema IV, human semaphorin IV; H-Sema III, human semaphorin III; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription–PCR; SSCP, single-strand conformation polymorphism; EST, expressed sequence tag; Ig, immunoglobulin.

†Present address: Department of Pathology, University of Edinburgh Medical School, Edinburgh, United Kingdom.
§Present address: Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan.

‡‡The sequences reported in this paper have been deposited in the GenBank data base [human semaphorin A(V), U28369; human semaphorin IV clone H1.47, U32172, and human semaphorin IV clone H2.6, U32171].

GNAI-2 and *GNAT-1*. The integrity of each cosmid was verified by establishing collinearity with chromosome 3 DNA and its absence in the DNA of SCLC lines H740 and H1450. *Eco*RI-digested fragments from the cosmid c12 (see Fig. 1) were used to screen a human placenta cDNA library (Clontech), and clone 8.20 was isolated. The contig of 22 cosmids was used to screen a gridded placenta cDNA library (19), and clone 155C6 was isolated. Screening of a pancreatic islet cDNA library with the 155C6 insert yielded two clones, B6 and B8. The inserts of 8.20, 155C6, B6, and B8 were sequenced using the Sequenase version 2.0 kit and found to encode a semaphorin-related sequence of 2919 bp, which we named human semaphorin A(V) [H-Sema A(V); GenBank accession no. U28369].

Eighteen additional cDNA clones were isolated from an OLIGO-1-ligated placenta cDNA library using the direct selection method (20) and found to have semaphorin sequence homology. These clones (two of which were deposited as semaphorin IV sequences U32172 and U32171 in GenBank) as well as expressed sequence tag (EST) clones T98884 and Z21993 were subsequently found to represent different parts of another human semaphorin cDNA sequence deposited by J. Roche, F. Boldog, M. Robinson, L. Robinson, L. Varella-Garcia, M. Swanton, B. Waggoner, R. Fishel, W. Franklin, R. Gemmill, and H. Drabkin (unpublished results) as GenBank accession no. U33920 and by Xiang *et al.* (21) as GenBank accession no. U38276. We refer to this gene as human semaphorin IV (H-Sema IV). A 1.4-kb partial cDNA fragment of H-Sema IV was synthesized by reverse transcription–PCR (RT-PCR; sense primer, 5′-TGAAGGGGCGGATCGTCT; antisense primer, 5′-TTCTGGTCCTGGGGCTCA) using random-primed cDNA prepared from Epstein–Barr virus-transformed B-lymphoblastoid cell lines (22). Sequence editing, cDNA contig building, and alignment were performed by Lasergene software (DNASTAR, Madison, WI). GenBank searches were performed using the BLAST and ENTREZ search network services at the National Center for Biotechnology Information.

Cell Lines and Southern and Northern Blot Analyses. The tumor cell lines have been described and were deposited at the American Type Culture Collection (23, 24). DNA and RNA were prepared from cell lines by standard techniques (22), and membrane preparation, hybridization, and washing were performed as described (25). Human multiple tissue Northern blots I and II and human brain blot III were purchased from Clontech. The DNA probes used were the 2.7-kb cDNA insert of B6 as a probe for H-Sema A(V) and the 1.4- kb cDNA fragment as a probe for H-Sema IV.

***In Vitro* and *in Vivo* Translation of H-Sema A(V).** H-Sema A(V) expression constructs (sense, pCB11; and antisense, pCB14) were synthesized by ligation of the 2.7-kb cDNA insert of clone B6 into the *Eco*RI site of pcDNA3 (Invitrogen). *In vitro* translation was performed by TNT coupled reticulocyte systems (Promega). Products labeled by [^{35}S]methionine were separated by SDS/PAGE. Another H-Sema A(V) expression construct (pCB11-Flag) was prepared by insertion of synthetic oligonucleotides into *Cel* II and *Xba* II sites of pCB11 and tagged with the FLAG marker octapeptide of DYKDDDDK followed by a spacer of GGGSGG at the carboxyl terminus of the H-Sema A(V). Transfection assays in Cos-7 cells were performed by Lipofectamine (GIBCO/BRL). Anti-FLAG M2 antibody (Kodak) was used as the first antibody.

Mutational Analysis. Fourteen overlapping regions (150–250 bp in length) covering the entire coding frame of H-Sema A(V) were tested by single-strand conformation polymorphism (SSCP) analysis using genomic DNA or cDNA, and aberrant bands were sequenced as reported (25). Restriction endonuclease fingerprinting analysis using primers in the 1.4-kb cDNA region of H-Sema IV was performed as described (26).

RESULTS

Cloning, Location, and Orientation of Human Semaphorin Genes H-Sema A(V) and H-Sema IV. In our 3p21.3 recessive oncogene positional cloning effort, we prepared a long-range physical map, identified overlapping homozygous deletions, prepared a cosmid/P1 contig, and isolated cDNAs (S.B. *et al.*, unpublished results; ref. 18). The cDNAs were sequenced and localized to the 3p21.3 homozygous deletion, and GenBank searches revealed that several encoded semaphorin-related sequences for two different genes, which we named H-Sema A(V) and H-Sema IV (Fig. 1). Sequence analysis of the H-Sema A(V) cDNAs demonstrated a single long open reading frame predicting a 749-aa protein, which contains a putative signal sequence at the amino terminus, the semaphorin domain, and an Ig domain (Fig. 2*A*). A GenBank search revealed this to be distinct from H-Sema III (53% aa homology) but highly homologous to murine semaphorin A (86% aa homology), indicating that this gene is the human homologue of murine semaphorin A (Fig. 2*B*). *In vitro* transcription from the T7 promoter and translation of H-Sema A(V) expression constructs produced a protein of the expected size of 90 kDa (Fig. 2*C*). Cos-7 cells transfected with a FLAG-tagged H-Sema A(V) expression construct followed by immunofluorescence staining gave signals at the endoplasmic reticulum but not in other areas (data not shown), supporting the prediction that H-Sema A(V) is a secreted protein.

While screening cDNA libraries, we isolated 18 additional cDNA clones as well as finding identical matches with GenBank EST clones Z21993 and T98884, all of which mapped to cosmids GT1 and GT1–1 in the 3p21.3 region (Fig. 1). Since these clones exhibited homology with the semaphorins, a 1.4-kb cDNA fragment was amplified by RT-PCR by using separate primers from sequences U32172 and U32171, and mRNAs of 3.9 and 2.9 kb were detected by Northern blot (see below); we named this gene H-Sema IV. All of our cDNA clones were subsequently found to be nearly identical to GenBank sequence U33920 submitted by Roche *et al.* and sequence U38276 submitted by Xiang *et al.*, which they also found encodes an additional human semaphorin. The amino acid homology of H-Sema A(V) and H-Sema IV and the presumed phylogenetic relationships of H-Sema A(V), IV, and III and the known murine semaphorins A through E are shown (Fig. 2 *A* and *B*).

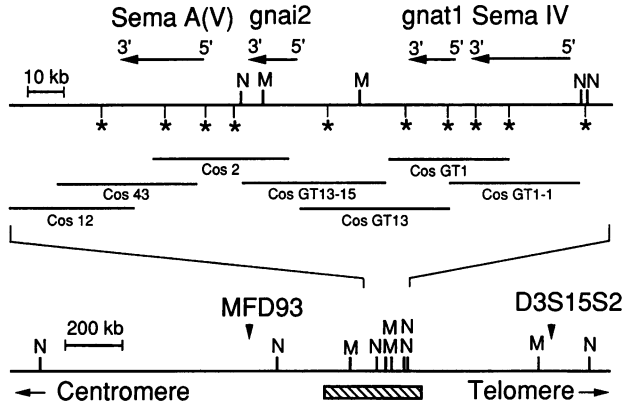

FIG. 1. Location and orientation of the H-Sema A(V), H-Sema IV, *gnai-2*, and *gnat-1* genes in a 160-kb region (upper map with 10-kb scale). Arrows indicate the relative locations of the genes and their orientations; putative CpG islands are indicated by asterisks. Seven clones of the 22-cosmid contig are shown. The lower map covering an ≈1800-kb region, with the 200-kb scale, indicates the position of the common region of overlap (hatched bar) in the SCLC homozygous deletions. N, *Not* I; M, *Mlu* I.

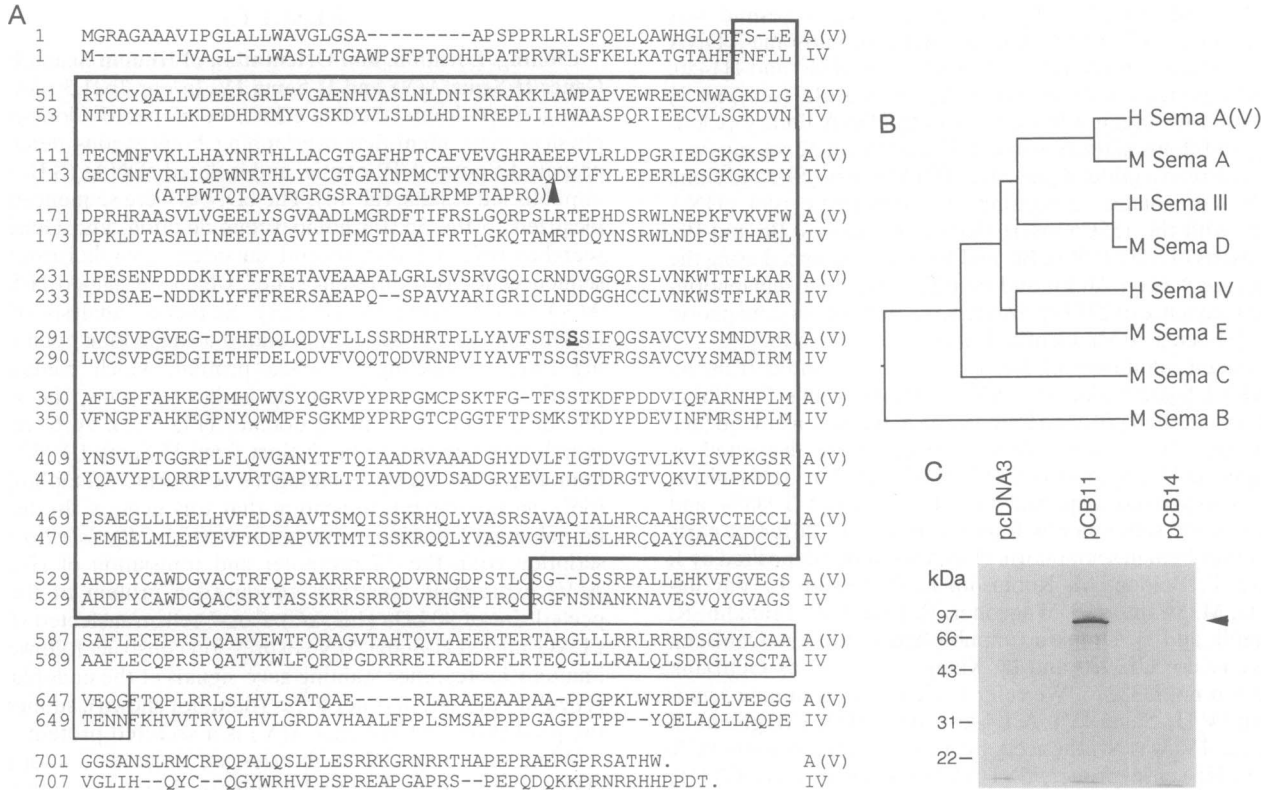

FIG. 2. (*A*) Predicted amino acid sequences of H-Sema A(V) and H-Sema IV. The nucleotide sequence used for H-Sema A(V) is U28369, and that used for H-Sema IV is U38276 (Xiang *et al.*). The H-Sema IV U33920 (Roche *et al.*) open reading frame sequence differs from that of U38276 by a 31-aa insertion between codons 152 and 153 (shown beneath the line with arrowhead), an insertion of R between codons 238 and 239, and amino acids AG instead of SA at codons 441 and 442. The semaphorin domain is given in the thick line box, and the Ig domain is given in the thin line box. H-Sema A(V) cDNA clone B8 compared to clone B6 had a 3-bp deletion at nt 1226, leading to a loss of S at codon 331 (shown as S). Alternative splicing seems the most likely explanation for the 3-bp difference because both forms were found expressed in many lung cancer samples with only one chromosome 3p allele, and two 3′ splice consensus sequences were found in tandem (CAG-CAG) in cosmid DNA. (*B*) Phylogenetic tree of human and mouse semaphorins. Alignments for *A* and *B* were obtained using the clustal method of the MEGALIGN program (Lasergene, DNASTAR). (*C*) *In vitro* transcription and translation of H-Sema A(V). An arrowhead indicates the expected 90-kDa product in a sense construct (pCB11) but not in the vector (pCDNA3) or an antisense construct (pCB14).

Southern blot analysis using the 600-kb cosmid contig showed that H-Sema A(V) and IV are located ≈70 kb apart with the *GNAI-2* and *GNAT-1* genes situated between these semaphorins (Fig. 1) (27, 28). These four genes were encompassed between two markers previously assigned to this region [D3S1235 (MFD 93) and D3S15S2] (17, 29). The 5′ to 3′ orientation of the four genes was determined by Southern blot analysis using different parts of the genes as probes. Our studies (18) detected the presence of CpG islands within the cosmid contig, and we found CpG islands in the 5′ regions of both semaphorin genes (Fig. 1).

The presence of two human semaphorins within ≈70 kb of each other raised the question whether other human semaphorin family members were also located in the 3p21.3 region. PCR-based screening using the 22 cosmid contig as a template was performed using conserved sequences in the semaphorin domain as primers, but no other semaphorin-related sequences were found. GenBank searches using H-Sema A(V), IV, and III sequences yielded a large number of human ESTs. Four human EST clones (R54387, H10623, T08621, and Z28925), homologous but not identical to H-Sema A(V), IV, or III, as well as H-Sema III, were prepared as probes by RT-PCR synthesis and used in Southern blot analysis to test for their presence in the 22 cosmid contig and in genomic DNAs from lung cancer cell lines. However, none of these hybridized to the 600-kb cosmid contig or were deleted in the ≈1500-kb H740 homozygous deletion. Furthermore, these four EST probes and the H-Sema III probe did not hybridize to monochromosome 3 hybrid cell (MCH906.15) DNA (30). Probes for mouse semaphorins B and C were also prepared by RT-PCR. While these cross-hybridized in Southern blots to human genomic DNA, they were not deleted in H740 DNA and were not found in human DNA from the monochromosome 3 hybrid cells. Thus, we excluded the other currently known human and human homologous of mouse semaphorin genes from 3p21.3 and human chromosome 3 regions.

Expression of H-Sema A(V) and IV. Expression of H-Sema A(V) (3.4-kb mRNA) and H-Sema IV (3.9- and 2.9-kb mRNAs) was detected in a variety of human adult tissues by Northern blot analysis (Fig. 3). The expression pattern of the two semaphorin genes differed (e.g., higher H-Sema IV levels in lung), with substantially elevated levels of both genes found in certain tissues. Using total RNAs from lung cancer cell lines, expression of H-Sema A(V) was only detected in 4% (1 out of 23) of SCLCs and 44% (7 out of 16) of NSCLCs, whereas H-Sema IV expression was found in 83% (19 out of 23) of SCLCs and 81% (13 out of 16) of NSCLCs (representative examples are shown in Fig. 4 and summarized in Table 1). Thus, expression of H-Sema A(V) differs between SCLCs and NSCLCs, with H-Sema A(V) and H-Sema IV apparently independently regulated in lung cancer cell lines. In addition, there were dramatic differences in the levels of expression between the tumors expression H-Sema A(V) or H-Sema IV.

Searching for Mutations of H-Sema A(V) and IV. Since H-Sema A(V) and IV are localized within the 350 kb shortest region of overlap of the SCLC homozygous deletions (Fig. 1), they are candidate tumor suppressor genes and were thus tested for mutations in lung cancer cell lines. Southern blot analysis was

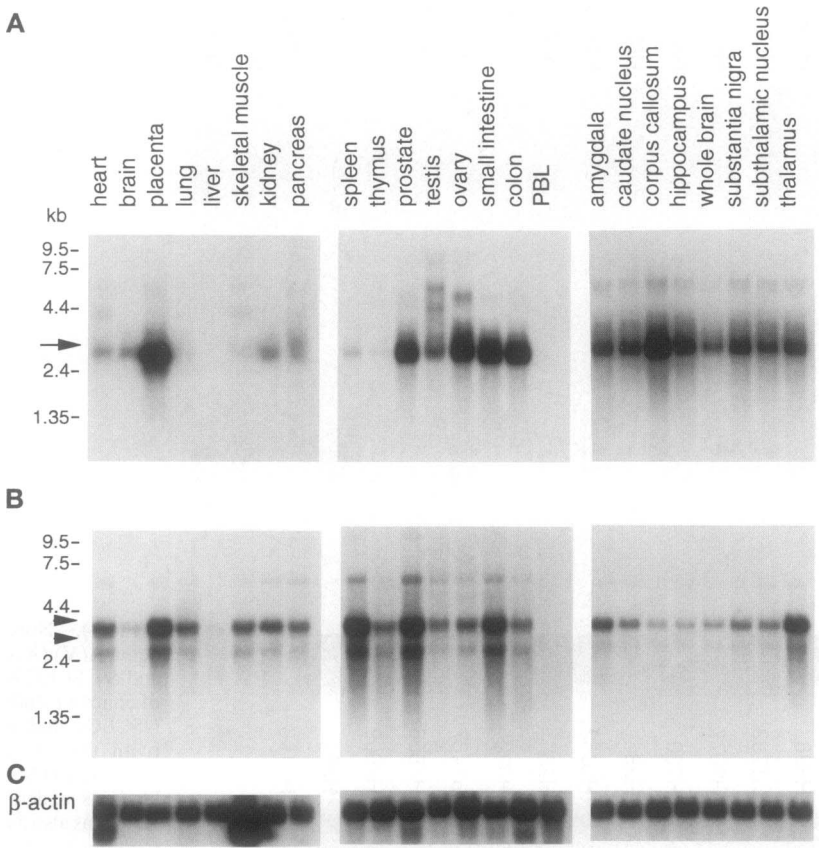

FIG. 3. Expression of H-Sema A(V) (*A*) and H-Sema IV (*B*) in normal human tissues. Northern blots purchased from Clontech had 1 μg of poly(A)$^+$ RNA loaded per lane. The films were exposed between 48 and 72 hr using an intensifying screen. An arrow indicates the 3.4-kb mRNA of H-Sema A(V) and arrowheads indicate the 3.9-kb and 2.9-kb mRNAs of H-Sema IV. A β-actin control for mRNA integrity (*C*) detects a 2.0-kb band in all lanes and a 1.8-kb band in some tissues. PBL, peripheral blood leukocytes.

performed using DNA from 120 lung cancer cell lines using a 2.6-kb H-Sema A(V) cDNA probe and a 1.4-kb H-Sema A(V) cDNA probe, but no other deletions or rearrangements were detected (data not shown). Next, we employed SSCP analysis for the H-Sema A(V) gene in 40 lung cancer samples and found three NSCLCs with abnormal SSCP migration changes (data not shown), which were sequenced. NCI-H358 had a G → C substitution at nt 1424, leading to an Asp → His substitution at codon 397. NCI-H1155 had a C → T substitution at nt 1277, leading to Arg → Cys at codon 348. The lack of normal DNA from these patients did not allow us to determine whether these were constitutional changes. NCI-H1648 had a G → A change at nt 1360, with no amino acid change, and a C → T change at nt 1479, leading to Thr → Ile at codon 415. Study of constitutional DNA from this patient (Epstein–Barr virus transformed B-cell line, BL10) revealed that BL10 was heterozygous at both nt 1360 and nt 1479, whereas the wild-type allele was deleted in NCI-H1648 (data not shown), indicating that this cell line has rare nucleotide polymorphisms, at least one of which results in an amino acid substitution in the H-Sema A(V) gene. We performed a partial mutation screen of H-Sema IV using the restriction endonuclease fingerprinting technique covering 456 aa in our 1.4-kb cDNA region (nt 1031 to nt 2399 of the U33920 sequence) but found no abnormalities.

DISCUSSION

We report the isolation of two additional members of the human semaphorin family, H-Sema A(V) and H-Sema IV, which are located ≈70 kb apart at 3p21.3. The presence of two GTP-binding protein genes (*GNAI-2* and *GNAT-1*) between these semaphorins, coupled with the 5′ to 3′ orientation of the four genes, provides information on the evolution of this region. Recently, others have deposited the entire cDNA sequence of H-Sema IV in GenBank (Roche *et al.* accession no. 33920; and Xiang *et al.*, accession no. U38276). Homology searches indicated that H-Sema A(V) is the human homologue of murine semaphorin A (86% aa homology), H-Sema IV does not yet appear to have an isolated murine semaphorin homologue although it shares 50% aa homology with murine semaphorin E, and H-Sema III is the homologue of murine semaphorin D (94% aa homology).

H-Sema A(V) and H-Sema IV are candidate tumor suppressor genes located in the overlapping SCLC homozygous deletion region where we are isolating other genes. H-Sema A(V) shows amino acid substitutions in NSCLCs and greatly decreased or absent expression in both SCLCs and many NSCLCs. However, H-Sema A(V) mutation frequency (3 out of 40) was much lower than predicted compared to the frequency of mutation of the *p53* or *rb* genes (both mutated in >90% of SCLCs and a substantial fraction of NSCLCs) (9). In addition, the changes in NCI-H1648 are constitutional, and further studies are needed to determine if this is a rare polymorphism or an inherited change predisposing to cancer. In contrast to H-Sema A(V), H-Sema IV is frequently expressed in both SCLCs and NSCLCs, although wide variations in the level of expression are seen among lung cancers. So far ≈60% of the open reading frame of H-Sema IV has been screened for mutations, but none were found. Differences in semaphorin expression levels may be due to nonstructural changes such as methylation, and we have located CpG islands in the 5′ regions of both H-Sema A(V) and IV.

Another striking feature of our data was the expression of H-Sema A(V) and H-Sema IV in a variety of adult nonneural

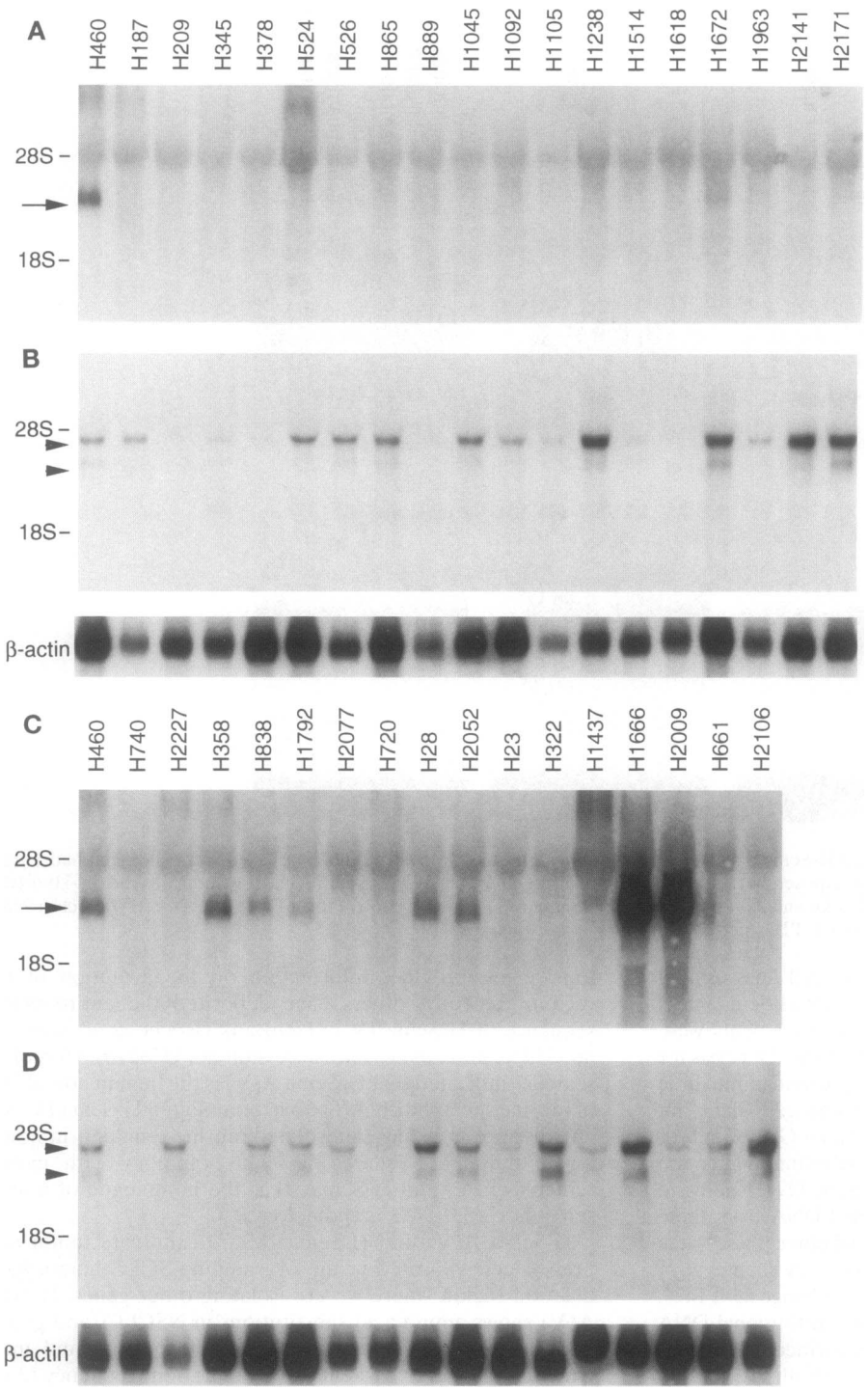

FIG. 4. Northern blot analysis of H-Sema A(V) (*A* and *C*) and H-Sema IV (*B* and *D*). SCLC RNAs (with the exception of control H460) are in *A* and *B*, whereas NSCLCs and mesothelioma cell lines (with the exception of SCLC controls H740 and H2227) are in *C* and *D*. Each lane was loaded with 10 μg of total RNA. H460 was also used as a positive, and H740 (deletion) was used as a negative control. An arrow indicates a 3.4-kb mRNA of H-Sema A(V), and arrowheads indicate 3.9-kb and 2.9-kb mRNAs of H-Sema IV. A β-actin control for RNA integrity is shown at the bottom. The histologic types and scored semaphorin expression patterns (H-Sema A(V)/H-Sema IV) were SCLC: H187 (−/+), H209 (−/−), H345 (−/trace), H378 (−/trace), H524 (−/2+), H526 (−/2+), H865 (−/2+), H889 (−/−), H1045 (−/2+), H1092 (−/+), H1105 (−/+), H1238 (−/3+), H1514 (−/trace), H1618 (−/−), H1672 (trace/3+), H1963 (−/+), H2141 (−/3+), H2171 (−/3+), H740 (−/−), and H2227 (−/+); NSCLC: H460 (2+/+), H358 (2+/−), H838 (+/+), H1792 (+/+), H2077 (−/+), H720 (−/−), H23 (−/+), H322 (−/3+), H1437 (trace/+), H1666 (4+/3+), H2009 (4+/+), H661 (−/+), and H2106 (−/3+); and mesothelioma: H28 (2+/2+) and H2052 (2+/2+). Only some of the semaphorin expression data summarized in Table 1 are shown.

tissues as well as in multiple sites in the nervous system and the frequent expression of H-Sema IV in lung cancer cells of all histologic types. Püschel *et al.* (5) reported on the expression of murine semaphorins including semaphorin A in the thoracic region of developing mouse embryos, where semaphorin A was expressed in muscle and vertebrate, and speculated on the role of semaphorins in the differentiation of the organ systems. If semaphorins play a role in selectively repelling neuronal growth cones, it is a reasonable hypothesis that they are involved in determining local cell type interactions in nonneuronal tissues such as contact inhibition. Thus, loss of contact inhibition seen in cancer could occur because of alteration in semaphorin expression or function. It is possible that mutation or downregulation of H-Sema A(V) and/or H-Sema IV may provide some growth or survival advantage to tumor cells. One possibility is that both H-Sema A(V) and H-Sema IV need to be functionally expressed to suppress malignancy, a situation found in only 24% (10 out of 42) of the lung cancers tested. Thus, mutation screening continues as well as introduction of wild-type copies of these genes into lung cancer cells to test for the ability of these genes to regulate the *in vitro* and/or *in vivo* growth of lung cancers. Finally, Kolodkin *et al.* (3) noted that semaphorins were encoded by vaccinia and variola virus genes and postulated a potential function in immune suppression of the secreted viral products. In this regard, it is interesting to speculate, as Kolokdin did, that semaphorins (such as H-Sema IV produced by lung cancer cells) may function as natural immunosuppressants and thus could participate in the host response to cancer.

Table 1. Expression of H-Sema A(V) and H-Sema IV in lung cancer cell lines

Expression pattern*		Number of cancers with pattern			
A(V)	IV	SCLC	NSCLC	Mesothelioma	Total
+	+	1	6	3	10
+	−	0	1	0	1
−	+	18	7	0	25
−	−	4	2	0	6
Total		23	16	3	42

*+ = detectable expression including trace to 4+, and − = undetectable on Northern blot analysis of total RNA.

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