Characterization of MAST9/Hevin, a SPARC-like Protein, That is Down-Regulated in Non-Small Cell Lung Cancer

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Abstract

In the search for genes down-regulated in non-small cell lung cancer (NSCLC), we have identified a cDNA fragment, termed MAST9. Cloning, sequencing, and characterization of the full-length MAST9 cDNA revealed that the entire 2808 nucleotide sequence had an open reading frame of 1992 nucleotides encoding a Mr 75,000 protein. Sequence analysis disclosed a striking homology to SPARC, known to be involved in tumorigenesis. The recently identified “Hevin” cDNA isolated from high endothelial venules is identical to MAST9. Using Northern and Western blot analysis, we showed that MAST9 was down-regulated in the tumor samples of nine non-small cell lung carcinoma patients. Furthermore, we demonstrated that both the bacterially expressed and the endogenous MAST9 proteins form homodimers. The lack of expression in non-small cell lung carcinomas and its homology to SPARC suggest a putative role of MAST9 in lung tumor formation.

Introduction

The development of lung cancer is a multistep process that is accompanied by a number of genetic alterations. These genomic changes include the deregulation of growth-promoting oncogenes and the inactivation of growth-constraining tumor suppressor genes (1). To identify new, still unknown genes that are inactivated in NSCLCs, we applied the magnet-assisted subtraction technique (known as “MAST”) (2). Six different cDNA fragments were isolated that were down-regulated in histologically different primary tumors of four NSCLC patients. Five of the six cDNAs could be adjoined to known sequences. One clone, termed MAST9, was unknown (3).

To better understand the role of MAST9 in NSCLC development, we cloned the full-length MAST9 cDNA and defined its tissue distribution. Analysis of the purified NH2-terminal MAST9 domain demonstrated the formation of MAST9 homodimers. Extending the Northern and Western analysis to nine additional matched normal/tumor samples we confirmed the originally observed down-regulation, indicating that the inactivation of MAST9 could be a common event in lung tumorigenesis.

Materials and Methods

MAST9 cDNA Isolation, Sequencing, and Database Analysis. To identify the human full-length MAST9 clone, we screened a human heart cDNA library in Agt11 (Clontech) using the isolated MAST9 fragment (3) as a probe. Three positive clones were identified and sequenced. To obtain the missing 5’ end of the cDNA’s reading frame, a modified 5’-rapid amplification of cDNA ends method was applied (4). The resulting 2808-nt full-length cDNA was termed MAST9 (GenBank accession no. X86693). Sequence analysis was perform using the Wisconsin Package (version 9.1; Genetics Computer Group Madison, WI) and the National Center for Biotechnology Information GenBank database.

Normal/Tumor Tissues of the Lung. Normal and corresponding primary tumor tissues from nine NSCLC patients (five squamous cell carcinomas, three adenocarcinomas, and one large cell carcinoma) were placed into liquid nitrogen immediately after resection.

RNA Extraction and Northern Blots. Total RNA was extracted from pulverized normal/tumor tissue as described (5). Twenty µg of total RNA of matched normal/tumor samples were fractionated on a 1% agarose gel containing formaldehyde and transferred to Hybond-N nylon membranes (Amer sham Corp.). For tissue distribution analysis, the multiple tissue Northern (MTN) blot from Clontech was used. Northern blots were hybridized with 32P-labeled 2.8-kb MAST9 cDNA for 16 h at 65°C in 1% BSA, 1 mm EDTA, 0.5 M NaH2PO4 (pH 7.2), and 7% SDS. The filters were washed in 2X SSC, 1% SDS at 65°C for 10 min and in 0.1X SSC, 0.1% SDS at 65°C for 10 min and exposed to X-ray film for 3 days at —70°C.

MAST9 Protein Expression, Purification, and Antibody Production. The 902-bp BamHI/HindIII MAST9a fragment, encoding the first 1-289 aas of MAST9, was cloned into the pDS56/RBSII.6xHis(-1) expression vector (6). The 6xHis MAST9a protein was bacterially expressed as described (6). Protein chromatography using the nickel chelate procedure was performed using the His-Bind Resin from Novagen. The chicken polyclonal antisera against the purified 6xHis MAST9a protein was obtained from Davids Biotechnologie (Regensburg, Germany).

Western Blot Analysis. About 0.1 µg of each of the nine paired normal and tumor tissues was sonicated in 100 µl of Complete protein inhibitor mixture (Boehringer Mannheim) using a microsonicator apparatus. After 5 min of boiling, the samples were centrifuged (12,000 × g for 2 min). Twenty-five µl of the supernatant were mixed with 25 µl of 2X SDS-PAGE sample buffer and heated for 5 min at 90°C. Half of the denatured protein extract was immediately applied to nonreduced SDS-PAGE (10%), and, after gel electrophoresis, proteins were transferred electrophoretically to Immobilon P membranes for 1 h at 300 mA. Western blots were reacted with 1:100,000 diluted chicken anti-human MAST9a IgY (14 mg/ml). Immunoreactive bands were identified using horseradish peroxidase-conjugated donkey antichicken IgGs at a 1:10,000 dilution (La Roche, Milan, Switzerland), the SuperSignal reagents (Pierce), and Kodak X-OmatAR film.

Results

Full-Length MAST9 cDNA Screening and Sequence Analysis. The 128-bp MAST9 cDNA clone (3) was used to screen a human heart cDNA library. Three clones, of which the largest was 2211 bp in size, were isolated and sequenced on both strands. The missing 5’ end of the MAST9 reading frame was amplified using a combination of λ library screening and a modified 5’-rapid amplification of cDNA ends method (4). The entire 2808-nt MAST9 cDNA consists of a 5’-nontranslated sequence of 322 nts, an open reading frame of 1992 nts, and a 3’-noncoding sequence of 491 nts (Fig. 1). The predicted 664-aa MAST9 protein has a calculated molecular weight of Mr 75,200, with an isoelectric point of 4.54.

Sequence analysis revealed a hydrophobic domain at its NH2 terminus (3-13 aas) followed by an extensive acidic region that includes two repeated motifs (187-193 aas and 262-268 aas) in its center and...
a SPARC-like domain (431-664 aas) at the COOH terminus (Fig. 1).

The SPARC domain consists of a conserved cysteine-rich follistatin-like domain and an extracellular Ca²⁺-binding module (EC domain) with two calcium-binding EF-hands. The SPARC domain of MAST9 revealed significant homologies with SPARC-like proteins from Caenorhabditis elegans (7), Xenopus (8), chicken (9), mouse (10), bovine (11), and human (12); the quail retinal protein QR1 (13); and the mouse (14) and rat (15) brain-derived SC1 proteins. Highest homology was found to the mouse/rat matrix glycoprotein SC1 (accession no. U77330/U27562; 92%/91% identity), followed by the quail QR1 protein (accession no. M61908; 73% identity) and the human SPARC/osteonectin/BM40 protein (accession no. J03040; 62% identity). Preliminary fluorescence-activated cell sorting studies with different cell lines indicated that the hydrophobic domain of MAST9 serves as a transmembrane anchor rather than a signal sequence for secretion (data not shown). The presence of six potential Asn-linked glycosylation sites and the SPARC module define MAST9 as an additional member of the SPARC protein family of extracellular multidomain proteins.

TM-domain
acidic region
FS-domain
EC-domain

SPARC-domain

Fig. 1. Full-length cDNA and corresponding protein sequence of MAST9. Top, the 2808-nl MAST9 cDNA and the corresponding 664 aa reading frame. The numbers on both sides correspond to the cDNA positions, whereas the protein sequences appear on the right (in italics). The predicted transmembrane region, the two acidic motifs, the follistatin-like domain, and the extracellular calcium-binding domain (EC domain) are boxed. The putative N-linked glycosylation sites are boxed. Bottom, schematic summary of the different predicted MAST9 domains. The protein domains are numbered according to their aa positions. Y, glycosylation site. The long stretches of negatively charged aa (---) within the acidic region and the two acidic motifs (187-193 and 262-268) are shown. The follistatin-like domain (FS-domain) is indicated by the increased amount of cysteines (Q. Ca²⁺s represent the EF-hands within the extracellular calcium-binding domain (EC-domain).
glycoproteins. Comparison of the MAST9 cDNA sequence against the GenBank database revealed a striking homology to the most recently identified 2645-nt human Hevin cDNA isolated from high endothelial venules (accession no. X82157). The MAST9 and Hevin proteins were found to be identical except at positions 46 aa (W/R), 49 aa (D/A), 106 aa (H/D), and 116 aa (T/S).

MAST9 mRNA Is Widely Expressed in Normal Tissues But Is Down-Regulated in Lung and Colon Cancers. Previously, we could show that the 2.8-kb transcript of the MAST9 gene was absent in four primary NSCLCs (3). To confirm the inactivation of MAST9 as a common event in NSCLC tissues, we extended our analysis to nine additional NSCLC patients. Total RNA from matched normal/tumor pairs of NSCLC patients was hybridized using the full-length MAST9 cDNA as a probe. In the tumor samples, MAST9 was downregulated or decreased independently of the histological subtype (Fig. 24). Interestingly, differential expression of MAST9 was also observed in paired normal/tumor tissues in three of four colon carcinoma patients (data not shown). The single transcript of 2.8 kb is highly abundant in the human heart, placenta, skeletal muscle, and pancreas; even more abundant in heart and brain; and at lower levels in the kidney, but it is absent in the liver (Fig. 3).

MAST9 Protein Forms Homodimers and Is Down-Regulated in NSCLC. MAST9 expression was further studied on the translational level. For this purpose, we expressed a six histidine-tagged MAST9 deletion protein (6xHis MAST9Δ) in Escherichia coli and purified it using the nickel chelate procedure (Fig. 4). For antibody production, the divergent NH2-terminal part of MAST9 (position 1–289 aa) was used to exclude cross-reactivity between the different SPARC-like proteins. Western blot analysis with the chicken antiserum was performed with equal amounts of freshly prepared protein extracts derived from normal and tumor tissues of nine NSCLC patients. MAST9 expression was only detected in the normal lung tissue but not in the corresponding NSCLC tumors, confirming the Northern blot results (Fig. 2B). Two major bands, Mf 75,000 and 150,000, were obtained, suggesting that the wild-type MAST9 protein forms a Mf 150,000 homodimer. This stable dimer formation could be reproduced with the divergent NH2-terminal region of MAST9 (position 1–289 aa), indicating that the dimerization domain is located in the NH2-terminal region. The interaction was disrupted under reducing conditions (Fig. 4). Weak protein bands observed in tumor tissues may be explained by contamination of normal cells (Fig. 2B). Only freshly frozen tissues were used to detect the MAST9 protein in Western blots. MAST9 protein detection decreased when the tissues were thawed and refrozen, suggesting the fragile nature of MAST9. In addition to lung tissue, the chicken antiserum recognized the endogenous MAST9 protein in fresh human spleen, pancreas, and kidney tissues (data not shown).

Discussion

MAST9, a cDNA clone that is differentially expressed in matched normal/tumor tissues from NSCLC patients was cloned, sequenced, and characterized. The COOH-terminal region of the deduced aa sequence exhibited homology to the SPARC domain. On the basis of this finding, MAST9 belongs to the modular SPARC-like family of extracellular glycoproteins. MAST9 is identical to Hevin, which was recently isolated from human tonsils (16). We observed four aa changes between MAST9 and Hevin, which most likely represent polymorphisms. Hevin was proposed to play a role in specialized properties of high endothelial venules that are important for lymphocyte migration (17). However, additional functions of Hevin/MAST9 have to be postulated. The finding that MAST9 was expressed in a wide range of tissues, e.g., brain, heart, lung, kidney, pancreas, spleen, colon, skeletal muscle, and placenta (Fig. 3), support this hypothesis. Hevin was additionally detected in prostate, testis, ovary, small intestine, and lymph nodes (16). In contrast to our results, Girard and Springer (16) did not observe Hevin in the kidney. Conceivably, this high abundance in different tissues indicates a more general, not tissue-specific role.

The highly conserved SPARC domain could help to elucidate the function of MAST9. The secreted SPARC protein has widespread extracellular tissue localization and is spatially and temporally expressed during development (18, 19). In the adult organism, SPARC is produced in matrix-producing tissues by osteoblasts, odontoblasts, chondrocytes, and endothelial cells; in corticosteroid-secreting tissues by astrocytes and Leydig cells; and in megakaryocytes; in highly proliferative epithelium of the skin, gut, salivary, and lactating mammary glands; and in renal tubules of the kidney. SPARC is believed to have multiple roles in anti-adhesive cell-matrix interactions, e.g., regulating cytokine actions in cell migration and spreading, induction of cell rounding, and modulation of cell cycle progression (19). The fact that
SPARC and MAST9 have not only overlapping but also distinct tissue expression suggesting besides similar, and also additional dissimilar, physiological roles. Mok et al. (20) have recently observed SPARC to be expressed at high levels in normal human ovarian surface epithelial cells but to be down-regulated in ovarian cancer cells. Transfection of full-length SPARC cDNA into the SKOV3 ovarian cell line with impaired SPARC expression showed reduced growth rate and reduced the ability of the cells to form tumors in nude mice. It is particularly interesting that MAST9, a newly characterized member of the SPARC family, is down-regulated in NSCLCs and colorectal carcinomas, and thus, in cancers of epithelial origin. Therefore, similar actions of MAST9 and SPARC are proposed in these tissues. In contrast to ovarian cancer, Ledda et al. (21) showed that SPARC inhibition is associated with loss of tumorigenicity in melanoma cells. Antisense expression of SPARC abrogated the tumorigenicity of melanoma cells in nude mice. The authors presented evidence that suppression of SPARC expression reversed the invasive and metastatic phenotype in human melanoma cells. SPARC-like matricellular protein family, to be down-regulated in primary tumors of cancers of the lung and the colon. However, the mechanisms that cause the inactivation of MAST9 in NSCLC still remain to be clarified. Therefore, it will be of particular interest to perform studies exploring the molecular events of the down-regulation of MAST9. The initial step to answer this question will be the identification and characterization of the genomic locus of MAST9, allowing deletion and mutation studies by RFLP, single-strand conformation polymorphism, or cycle sequencing. Moreover, transfection studies with the wild-type MAST9 gene using malignant MAST9-lacking cell types will evaluate the tumor-suppressive potential of MAST9.

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References


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