Fibulin-5 Suppresses Lung Cancer Invasion by Inhibiting Matrix Metalloproteinase-7 Expression

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Introduction

The 5-year survival rate of lung cancer is below 20%, with most patients dying from distant metastasis (1). However, the mechanism of lung cancer metastasis is poorly understood. Tumor metastasis is characterized by cell detachment from primary tumors and invasion of recipient tissues (2). A critical step in this process is degradation of the basement membrane, which contains extracellular matrix (ECM) proteins and functions as a barrier to surrounding tissues. The degradation of the basement membrane is catalyzed by proteolytic enzymes including matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinases (TIMP; ref. 3). The activities of these enzymes are regulated by cell-surface receptor molecules known as integrins (4). Integrins not only mediate cell adhesion to the ECM, but also regulate intracellular signaling through kinases such as extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK; ref. 5). Accumulating evidence suggests that the activation of MMPs and TIMPs through integrin signaling plays an important role in cancer invasion and metastasis (5, 6). Nevertheless, the genetic and epigenetic alterations driving MMP/TIMP activation and tumor invasion remain to be identified (7).

The fibulin family, including fibulin-1-6, is a group of widely expressed ECM proteins localized to the basement membrane, stroma, and ECM fibers (8). Fibulins are characterized by repeated epidermal growth factor–like domains and a unique carboxyl-terminal structure (9). They mediate cell-to-cell and cell-to-matrix communication, and provide organization and stabilization to ECM structures during organogenesis and vasculogenesis (9). It has been shown that fibulin family members are aberrantly expressed in tumors, and can either suppress or promote cancer cell growth depending on cell types and cellular contexts (10–13).

Fibulin-5 is a recently identified fibulin family member (14). Distinguished from other fibulins, it contains a conserved RGD motif that binds to integrins and mediates endothelial cell adhesion (15, 16). Fibulin-5 can suppress angiogenesis in an RGD-dependent manner (17). The expression of fibulin-5 is induced in response to pathologic conditions such as lung injury and pulmonary hypertension (18, 19), and is regulated by transforming growth factor-β (TGF-β; ref. 11). Fibulin-5–deficient mice exhibited emphysematous changes (16, 20), suggesting an important physiologic function in pulmonary tissues. Although fibulin-5 is down-regulated in several types of tumors (12, 14), its functional role in lung cancer has not been characterized.

In this study, we identified fibulin-5 as a frequently silenced gene in lung cancer. Our results indicate that fibulin-5 functions as a metastasis suppressor of lung cancer, and down-regulation of fibulin-5 drives ERK-mediated MMP-7 induction, and therefore lung cancer cell invasion.

Materials and Methods

Bioinformatics analysis. The expression of fibulin family members was analyzed using the National Center for Biotechnology Information (NCBI) SAGE databases.4 CpG islands were identified using the CpG Island Searcher5 program.

Tissue samples. The acquisition of the tissues was approved by the Institutional Review Board at the University of Pittsburgh. Tissue microarray slides (US Biomax) are described in Supplementary Table S1. Frozen specimens from the University of Pittsburgh Cancer Institute lung cancer program are described in Supplementary Table S2.

Immunohistochemistry. Immunohistochemistry was performed with the mouse antibodies against fibulin-5 (R&D system) and MMP-7 (EMD BioSciences) as previously described (21).

Bisulfite sequencing and methylation-specific PCR. Isolation of genomic DNA and bisulfite modification were performed as previously described (21). The primers for fibulin-5 bisulfite sequencing

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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were 5'-AGGGAGAATTGGGAATGAGG-3' and 5'-CCCACCTTTTATTCC-TAAAC-3'. For methylation-specific PCR (MSP), methylated fibulin-5 promoter was amplified using the primer pair 5'-TGTAGTGGTGGAGGATTTCCGCG-3'/5'-TTCTCAACATATCCAAAACCGCCTT-3', whereas unmethylated fibulin-5 promoter was amplified using the primer pair 5'-TGTAGTGGTGGAGGATTTTCGGC-3'/5'-TTCTCAACATATCCAAA-CACACA-3'. MSP products were analyzed by electrophoresis on 2% agarose gels.

**Stable cell clones.** A549, H1299, and H460 cells were transfected with fibulin-5 or control empty vector, and were selected by G418 (400 ng/mL for A549 and H1299; 600 ng/mL for H460). Stable clones expressing fibulin-5 were identified by Western blotting.

**Antibodies and Western blotting.** Western blotting was performed as previously described (22). The antibodies included monoclonal antibodies against V5 (Invitrogen), fibulin-5 (R&D system), MMP-7, α-tubulin (EMD Biosciences), and FAK (BD Biosciences), as well as rabbit antibodies against ERK, phospho-ERK (Thr202/Tyr204), e-Jun-NH₂-kinase (JNK), phospho-JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180/Tyr204; Cell Signaling Technology), and phospho-FAK (Tyr-397; Biosource International). Monoclonal anti-ILK and rabbit anti-PINCH antibodies were previously described (23).

**Matrigel invasion assay.** Invasion assays were performed in triplicate in six-well trans-well units with 8-μm filters coated with Matrigel at 1:5 dilution (BD Biosciences). Each well was loaded with ~2 × 10⁵ cells. After incubation for 36 h, cells passing through the filters into bottom wells were fixed in formalin and stained with Crystal Violet (Sigma-Aldrich). Cell numbers in 10 randomly selected fields (∼200) from each well were counted.

**Analysis of secreted MMP-7.** Concentrations of secreted MMP-7 were determined in triplicate by ELISA using Human Total MMP-7 Quantikine ELISA kit (R&D Systems) according to the manufacturer's protocol.

**RNA interference.** Small interfering RNA (siRNA) duplexes were from Dharamco. Fibulin-5 was knocked down using ON-TARGETplus siRNA J-017621-05 and -06. MMP-7 was knocked down by MMP-7 757 (GGCAUU-CAGAAACCUAUAG) and MMP-7 877 (GCACUGUUCCUCACUCCA).

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism IV software. P values of <0.05 were considered to be statistically significant. The means ± one SD were displayed in the figures.

**Results**

**Down-regulation of fibulin-5 in lung cancer.** In light of our previous finding of fibulin-3 (EFEMP-1) inactivation in lung cancer (21), we analyzed the expression of all six fibulin family genes using the NCBI SAGE databases containing 159,059 transcripts from lung cancer, and 159,917 transcripts from normal lung tissues (24). In addition to fibulin-3, the expression of fibulin-1, fibulin-2, and fibulin-5 was also lower in lung cancer (Fig. 1A). Fibulin-5, whose expression was decreased by almost 10-fold, was the most significantly down-regulated fibulin family member in lung cancer (Fig. 1A). Down-regulation of fibulin-5 was confirmed by reverse transcription-PCR (RT-PCR) in lung tumors and lung cancer cell lines (Fig. 1B).

Fibulin-5 expression was further analyzed by immunohistochemistry in a tissue microarray containing 95 non–small cell lung cancer (NSCLC) and 46 normal lung specimens, including 32 matched tumor/normal pairs (Supplementary Table S1). A highly significant (P < 0.001, Fisher's exact test) difference between the

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**Figure 1.** Reduced expression of fibulin-5 in lung cancer. **A**, expression of fibulin family members in SAGE databases containing 159,128 transcripts from three lung adenocarcinomas and 159,917 transcripts from three normal lung specimens. The expression (copies/cell) was normalized as done by Yue and colleagues (41). **B**, RT-PCR was used to analyze fibulin-5 expression in five matched pairs of normal (N)/tumor (T) lung tissues and seven lung cancer cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene, was used as an internal control. **C**, summary of fibulin-5 expression determined by immunohistochemistry in 95 lung tumors and 46 normal lung samples. Correlations between loss of fibulin-5 expression and tumor status and grade were analyzed by Fisher’s exact test.
normal and tumor tissues was observed as follows: 73.9% (34 of 46) of normal lung specimens expressed fibulin-5, whereas only 22.1% (21 of 95) of NSCLC samples were positive for fibulin-5 (Supplementary Fig. S1A and B; Fig. 1C). Among the 32 pairs, 17 (53.1%) tumors completely lost fibulin-5 expression compared with the matched normal tissues (Supplementary Table S1). Fibulin-5 expression was detected in the cytoplasm of normal bronchial epithelial cells and fibulin-5–positive tumor cells (Supplementary Fig. S1A and B). Importantly, loss of fibulin-5 expression correlated with tumor grade ($P < 0.05$, Fisher’s exact test), with fibulin-5 staining detected in 50.0% (6 of 12) of grade 1 tumors, but in only 26.5% (9 of 34) of grade-2 tumors and 16.3% (8 of 49) of grade-3/4 tumors (Supplementary Table S1; Fig. 1C).

**Silencing of fibulin-5 by promoter hypermethylation.** To determine whether down-regulation of fibulin-5 is due to epigenetic silencing, eight lung cancer cell lines were treated with 5-aza-2′-dC, a pharmacologic inhibitor of DNA methyltransferase. Fibulin-5 expression was significantly elevated in four cell lines following 5-aza-2′-dC treatment by real-time RT-PCR (Fig. 2A). Bioinformatics analysis identified a CpG island in the 5′ promoter region of fibulin-5 (Fig. 2B). Bisulfite sequencing revealed that this CpG island was completely methylated in the four cell lines that were responsive to 5-aza-2′-dC treatment, but not in the other four that were insensitive to 5-aza-2′-dC (Supplementary Fig. S2A; Fig. 2A and B), suggesting that promoter hypermethylation underlies down-regulation of fibulin-5. Using a MSP assay, we found fibulin-5 promoter methylation in 11 of 22 (50.0%) lung cancer cell lines (Fig. 2C).

The relationship between fibulin-5 down-regulation and promoter methylation was further analyzed using 30 matched sets of frozen tissue samples. Each set included a tumor and histologically normal lung tissues adjacent and distal to the tumor (Supplementary Table S2). Although promoter methylation was found in 13 (43.3%)
tumors, it was detected in only 3 (10%) adjacent normal, and in no (0%) distal normal samples (Supplementary Fig. S2B; Fig. 2D). Immunohistochemistry confirmed the loss of fibulin-5 expression in all of the 13 tumors with promoter methylation (Supplementary S2C; Fig. 2D). Among the 19 cases where tumors lost fibulin-5 expression compared with distal normal, promoter methylation was observed in 10 (52.6%) tumors, but in none of the corresponding normal specimens (Fig. 2D). In contrast, promoter methylation was not detected in any of the 6 cases where tumors expressed fibulin-5 (Fig. 2D). The correlation between loss of fibulin-5 expression and promoter methylation was statistically significant ($P < 0.05$, Fisher’s exact test). Together, these results indicate that promoter hypermethylation is the major, but not the only, mechanism leading to fibulin-5 silencing in lung cancer.

Suppression of lung cancer cell invasion and MMP-7 expression by fibulin-5. Based on the biochemical properties and the RGD-containing feature of fibulin-5 (14), we hypothesized that it is involved in regulating cancer cell invasion. To test this hypothesis, A549, H460, and H1299 lung cancer cells, which contain promoter methylation and lack fibulin-5 expression (Fig. 2A–C), were used to establish stable fibulin-5–expressing cell lines by transfection (Supplementary Fig. S3; Fig. 3A). Analysis of cell invasion by Matrigel assays revealed that fibulin-5 expression significantly suppressed invasion of all three cell lines ($P < 0.01$, Fisher’s exact test; Supplementary Fig. S3; Fig. 3A). However, fibulin-5 expression did not significantly affect cell growth determined by soft agar and 5-bromo-2-deoxyuridine incorporation assays (Supplementary Fig. S4A and B), or spontaneous and drug-induced apoptosis (Supplementary Fig. S4C). Transfection of fibulin-5 into H1299 cells altered the expression of fibulin-2 and fibulin-6, but not other fibulins (Supplementary Fig. S4D).

To determine how fibulin-5 inhibits lung cancer cell invasion, genes encoding several proteolytic enzymes involved in degrading the basement membrane, including three TIMPs and five MMPs,
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were analyzed by RT-PCR in A549 and H1299 cells following fibulin-5 transfection. Only the expression of MMP-7 was consistently down-regulated by 60% to 80% following fibulin-5 transfection (Fig. 3B). Concordantly, fibulin-5 expression suppressed MMP-7 protein expression (Fig. 3C), the level of secreted MMP-7 (Fig. 3D), and the activity of an MMP-7 luciferase reporter (Supplementary Fig. S5A).

Fibulin-5 inhibits lung cancer cell invasion by down-regulating MMP-7. The role of MMP-7 in lung cancer cell invasion was then investigated. Transfection of MMP-7 into fibulin-5-expressing A549 cells restored their invasiveness (Fig. 4A). Conversely, knockdown of MMP-7 by two independent siRNA significantly decreased the invasiveness of both A549 and H1299 cells (Fig. 4B). To determine if fibulin-5 silencing alone is sufficient to drive MMP-7 expression and cell invasion, siRNA was used to knock down fibulin-5 in H1752 cells (Fig. 4C), which expressed a relatively high level of fibulin-5 with no detectable fibulin-5 promoter methylation (Fig. 2A and B). Transfection with two independent fibulin-5 siRNA, but not the control siRNA, led to increased H1752 cell invasion as well as elevated MMP-7 expression (Fig. 4C), suggesting that fibulin-5 down-regulation alone is sufficient for stimulating lung cancer cell invasion through up-regulation of MMP-7.

MMP-7 expression was then analyzed by immunohistochemistry using the aforementioned tissue microarray (Supplementary Table S1), in comparison with fibulin-5 expression (Supplementary Fig. S1C; Fig. 4D). No MMP-7 immunoreactivity was detected in the 46 normal lung specimens (data not shown), consistent with the previous finding that MMP-7 is not expressed in normal lung (25). In contrast, 39 of 95 (41.1%) NSCLC samples were positive for MMP-7 (Supplementary Table S1). Remarkably, a statistically significant ($P < 0.05$, two-tailed $\chi^2$ test) inverse correlation between fibulin-5 and MMP-7 expression was found, with 54.7% (52 of 95) of tumors expressing either MMP-7 or fibulin-5, but only 5.5% (5 of 95) expressing both proteins (Fig. 4D). Together, these results suggest that fibulin-5 suppresses lung cancer cell invasion by inhibiting the expression of MMP-7.

RGD-dependent inhibition of MMP-7 by fibulin-5 through the ERK pathway. To investigate the mechanism of MMP-7 regulation by fibulin-5, we determined the functional role of its RGD motif. Several deletion mutants of fibulin-5 were constructed and expressed in A549 cells (Fig. 5A). A deletion fragment containing the amino-terminal 1/3 of fibulin-5 (N136) including the RGD motif retained most of the activities in suppressing MMP-7 protein expression and its reporter activities (Supplementary Fig. S5B; Fig. 5B). In contrast, all deletion mutants without the RGD motif, including a microdeletion of the RGD residues only, lost over 80% of the activities in suppressing MMP-7 expression and the level of secreted MMP-7 (Fig. 5B), suggesting that the inhibition of MMP-7 by fibulin-5 is mediated by the RGD motif through integrin signaling.

To further delineate the mechanism by which fibulin-5 inhibits MMP-7 expression, several kinases downstream of integrin signaling, including ERK, p38, and JNK, were analyzed. Phosphorylation of ERK, but not that of p38 and JNK, was markedly reduced following fibulin-5 transfection (Fig. 5C). The inhibition of ERK phosphorylation by fibulin-5 was RGD dependent (Fig. 5C). ERK inhibitor PD98059 blunted MMP-7 expression in A549 and H1299 cells (Fig. 5D). Furthermore, fibulin-5 also suppressed serum- or epidermal growth factor–stimulated ERK and FAK phosphorylation, as well as the expression of the integrin downstream effectors ILK and PINCH (Supplementary Fig. S5C and D). These observations suggest that fibulin-5 functions as an inhibitor of
ERK signaling via its RGD motif to inhibit MMP-7 expression and lung cancer cell invasion.

**Potent inhibition of H460 tumor metastasis by fibulin-5.** The previously described NCI-H460 metastasis model was then used to determine whether fibulin-5 can suppress tumor metastasis (26). Parental and fibulin-5–expressing H460 cells, which had similar growth rate (Supplementary Fig. S6A), were injected i.v. into nude mice. After 5 to 7 weeks, the mice receiving the parental H460 cells had a number of lung metastasis nodules (Supplementary Fig. S6B; Fig. 6A) and lymph node metastasis (Supplementary Table S3). In contrast, mice injected with fibulin-5–expressing H460 cells had much fewer lung metastasis nodules (Fig. 6A and B), and no lymph node metastasis (Supplementary Table S3). The mice receiving the parental H460 cells also had significantly lower body weights compared with those injected with fibulin-5–expressing H460 cells (Supplementary Table S3). Furthermore, the parental H460 lung tumors expressed much higher levels of MMP-7 and phosphorylated ERK compared with those expressing fibulin-5 (Fig. 6C). These results suggest that fibulin-5 functions as a suppressor of lung cancer metastasis by inhibiting MMP-7 expression and ERK signaling.

**Discussion**

Similar to tumor initiation, invasion and metastasis are also driven by genetic and epigenetic alterations (27). Our results show that fibulin-5 functions as a suppressor of lung cancer invasion, and epigenetic inactivation of fibulin-5 contributes to lung cancer progression. However, deregulation in fibulin-5 does not seem to be a major factor in tumor initiation, as fibulin-5 silencing was mostly found in high-grade lung tumors. Previous studies also described down-regulation of fibulin-5 mostly in advanced malignancies (11). Knockdown of fibulin-5 in mice is not sufficient to affect tumor incidence (20). Our data revealed down-regulation of multiple fibulin family members in lung cancer. This consistent pattern seems to be distinguished from the context-specific expression of fibulin family members in other tumor types (10, 11).

Fibulin-5 is down-regulated in lung cancer largely by promoter hypermethylation. Interestingly, fibulin-5 hypermethylation was found in several histologically normal tissues adjacent to the tumors (Fig. 2D). This might be due to an epigenetic field effect and/or infiltrating tumor cells described in other studies (28). Additional mechanisms, such as expression of the oncoproteins c-Myc and vascular endothelial growth factor, may also lead to fibulin-5 silencing (29, 30).

Fibulin-5 is localized on 14q32.1, a tumor suppressor-containing region (31). Mutations or homozygous deletions of fibulin-5 have yet to be found. The effects of fibulin-5 on lung cancer are mediated by the inhibition of MMP-7, which is up-regulated in lung cancer (32, 33), and plays an important role in cancer invasion (4, 6). MMP-7 overexpression is associated with poor prognosis of NSCLC (34). The frequent change of fibulin-5, along with the inverse correlation between fibulin-5 and MMP-7 expression, suggests that epigenetic
silencing of fibulin-5 might be a major mechanism underlying MMP-7 overexpression in NSCLC. Fibulin-5 can also modulate the expression of MMP-2, MMP-3, TIMP-1, and TIMP-3 in suppressing the angiogenesis of fibrosarcoma cells (17), indicating a broad relevance in regulating the activities of the MMP/TIMP families. The antiangiogenic activity may also be a contributing factor for its inhibitory effect on lung cancer invasion. Our results suggest a model in which fibulin-5 inhibits ERK activity through RGD-mediated integrin signaling, leading to transcriptional repression of MMP-7 and suppression of cell invasion. Integrin signaling is not only critical for cell migration and invasion, but is also involved in the regulation of MMP and TIMP activities (5). The RGD motif of fibulin-5 binds to integrins αvβ3, αvβ5, and α9β1 (16). It remains to be determined which integrin subunit(s) mediates the inhibitory effect of fibulin-5 on MMP-7 in the lung. It is also unclear how MMP-7 expression is down-regulated through the ERK pathway. Several binding sites of the ETS transcription factors, which have been implicated in regulating MMP expression (35), were found in the promoter region of MMP-7 (data not shown). The roles of ETS and other transcription factors in the inhibition of MMP-7 by fibulin-5 need to be further investigated.

Tumor metastasis is stimulated upon inactivation of a group of metastasis suppressors, such as NM23, KAI1, and MKK4 (36). These genes are down-regulated in metastatic tumors and possess the activity of reducing the metastatic propensity of cancer cells in animal tumor models. Identifying additional metastasis suppressors is critical for understanding the mechanisms of tumor metastasis. Our data strongly suggest that fibulin-5 functions as a metastasis suppressor. Several additional lines of evidence support this notion. During mouse development, fibulin-5 is widely expressed in the so-called “epithelial-mesenchymal transition” (EMT) region (14). EMT is a critical process in cancer metastasis, during which epithelial cells acquire phenotypes of motile fibroblasts. Emerging evidence indicates that ERK/FAK-mediated integrin signaling and tumor-associated MMPs stimulate EMT (37, 38). Fibulin-5 expression is regulated by TGF-β (11), which plays an important role in cancer metastasis and EMT (39). Therefore, epigenetic silencing of fibulin-5 and subsequent activation of MMP-7 may be events involved in EMT. However, this speculation seems to contradict a recent finding that fibulin-5 can promote EMT in breast tumors (40), reinforcing the context specificity involved in the regulation of fibulin family proteins.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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