Frequent Loss of Gelsolin Expression in Non-Small Cell Lung Cancers of Heavy Smokers

Hirotoshi Dosaka-Akita, Fumihiro Hommura, Hisakazu Fujita, Ichiro Kinoshita, Motoi Nishi, Toshiaki Morikawa, Hiroyuki Katoh, Yoshikazu Kawakami, and Noboru Kuzumaki

First Department of Medicine [H. D.-A., F. H., I. K., Y. K.]; Division of Gene Regulation, Cancer Institute [H. F., N. K.]; Second Department of Surgery [T. M., H. K.], Hokkaido University School of Medicine; and Department of Public Health, Sapporo Medical University [M. N.], Sapporo, 060, Japan

ABSTRACT

Most lung and bladder cancers have been shown to be associated with smoking. We have previously demonstrated the frequent loss of gelsolin expression and its tumor suppressor activity in bladder cancer (M. Tanaka et al., Cancer Res., 55: 3228—3232, 1995). Here, we examined gelsolin expression in 12 cultured non-small cell lung cancer (NSCLC) cell lines. Furthermore, we analyzed gelsolin expression in relation to patients’ smoking habits in 88 surgically resected NSCLCs to investigate whether gelsolin could be a molecular target for tobacco-induced carcinogenesis of lung cancer. All 12 NSCLC cell lines showed low-to-undetectable expression of the gelsolin gene, compared to that in normal lung tissue, by Northern blot analysis. On the other hand, Southern blot analysis of genomic DNA did not show any gross rearrangements or deletions of the gene in the NSCLC cell lines. Western blot analysis of gelsolin expression showed low-to-undetectable gelsolin expression in all 12 NSCLC cell lines, compared to normal lung tissue. Immunocytochemical analysis of gelsolin expression in NSCLC cell lines showed results that were consistent with those obtained by Western blot analysis, using normal bronchial epithelial cells as a positive control: two cell lines with lower gelsolin expression by Western blot analysis had reduced but positive cytoplasmic immunostaining of gelsolin, compared with primary normal bronchial epithelial cells, whereas no such immunostaining was observed in two cell lines with much lower or undetectable gelsolin expression by Western blot analysis. Therefore, gelsolin expression was analyzed in surgically resected NSCLCs by immunohistochemistry. Reduced or undetectable gelsolin expression was observed in 48 of 88 (55%) resected NSCLCs. Such altered gelsolin expression significantly correlated with heavy smoking of patients (Pearson’s χ² test and P = 0.03 by multivariate logistic regression analysis), whereas there was no significant correlation between gelsolin expression and histological type, pathological tumor-node-metastasis (pTNM) stage, or survival. These findings suggest that the frequent loss of gelsolin expression may be involved in the development of NSCLCs as a potential molecular target of tobacco-induced carcinogenesis.

INTRODUCTION

The progressive accumulation of genetic changes plays a major role in the development of lung cancer (1). Central among these genetic changes is a limited set of genetic alterations that can disrupt strictly controlled cell functions, such as growth and differentiation. The pivotal genes involved are tumor suppressor genes and oncogenes, including the Rb, p53, CDKN2, and K-ras genes (2–6). Alterations of these genes have been shown to occur in association with exposure to carcinogens contained in tobacco smoke, experimentally and/or clinically (6–9), and are thought to be molecular targets for tobacco-induced carcinogenesis of lung cancer (7). The role of tobacco smoke is critical in the development of lung cancer (10, 11), which requires genetic alterations of more than several genes (1). Therefore, there may be other molecular targets of tobacco-induced carcinogenesis than these four genes in lung cancer. We have previously demonstrated frequent loss of gelsolin, an actin-binding protein, and its tumor suppressor activity in bladder cancer (12), which is also known to be a type of cancer caused by tobacco smoke (10, 11). Gelsolin is a key regulator of the actin cytoskeleton, which is essential for cell morphology and motility (13–15). Alterations of these phenotypes are frequently observed in lung cancer. In addition, it has been reported that chromosome 9q, on which the gelsolin gene is located (16), is frequently deleted in NSCLCs (17, 18). Moreover, gelsolin may be linked to signal transduction pathways involving phosphatidylinositol 4,5-bisphosphate and phospholipase C γ (17, 18), which is frequently activated in cancer (19). Nevertheless, gelsolin expression has not been previously examined in human lung cancer.

Here, to address the question of whether gelsolin might be a molecule that is inactivated and involved in the development of NSCLCs, in association with cigarette smoking, we examined gelsolin expression in cultured cell lines and surgically resected tumors of NSCLC and analyzed its correlation with smoking habits of NSCLC patients.

PATIENTS AND METHODS

NSCLC Cell Lines, Tumor Specimens, and Survival Data. Twelve NSCLC cell lines were cultured in RPMI 1640, supplemented with 10% FCS and 0.03% glutamine at 37°C in an atmosphere of 5% CO₂. They included six adenocarcinomas (A549, PC-3, RERF-LC-OK, VMRC-LCD, RERF-LC-MS, and ABC-1) and six squamous cell carcinomas (EBC-1, LC-1 sq, PC-10, VMRC-LCP, NCI-H226, and NK-2), all of which were obtained from the Health Science Research Resources Bank of Japan (Osaka, Japan), except for NCI-H226, which was kindly provided by Dr. H. Oie of the NCI-Navy Medical Oncology Branch, National Cancer Institute (Bethesda, MD).

Tumor specimens from 88 patients with NSCLC were obtained by surgery at Hokkaido University Medical Hospital, Sapporo Minami-Ichijo Hospital, and Keiyukai Sapporo Hospital (all in Sapporo, Japan) during 1990 and 1995. Normal lung tissue, including bronchi and alveoli, was obtained from eight patients by surgery. Normal bronchial tissue was obtained from one patient by biopsy under a fiberoptic bronchoscope. Tumor specimens and normal tissues were snap-frozen in liquid nitrogen and stored at −80°C until use. Normal bronchial epithelial cells were also obtained from three patients under a fiberoptic bronchoscope, directly adhered to glass slides without cell culture, and stored at −80°C until use. Sections of tumor specimens and normal tissues and cells were reviewed by a pathologist. According to the 1981 WHO classification (20), tumor specimens were histopathologically diagnosed as adenocarcinoma (n = 56), squamous cell carcinoma (n = 27), or large cell carcinoma (n = 5). The postsurgical pTNM stage was determined according to the guidelines of the American Joint Committee on Cancer (21). The tumor specimens represented 49 stage I, 7 stage II, 25 stage III, and 7 stage IV tumors.

Smokers who were analyzed here were current smokers, including individuals who stopped smoking 3 months before surgery. Ex-smokers who stopped smoking more than 3 months before surgery were not observed here. Non-smokers were those who had never smoked. Survival was analyzed for the 50
GELSON EXPRESSION IN NON-SMALL CELL LUNG CANCERS

patients who had undergone potentially curative resection for their stage I and II NSCLCs and who met the following criteria: survived for more than 3 months after surgery; did not die of causes other than lung cancer within 5 years after surgery; and were followed for more than 2 years after surgery (for patients who remained alive).

Southern and Northern Blot Analyses. Genomic DNA was extracted from NSCLC cells and normal lung tissue and digested with endonucleases for Southern blot analysis, as described previously (8). Total cellular RNA was prepared from NSCLC cells and normal lung tissue by guanidine isothiocyanate lysis and subsequent CsCl gradient ultracentrifugation for Northern blot analysis, as described previously (21). Southern and Northern blots were probed with a 2.4-kb BamHI-HindIII fragment of human gelsolin cDNA (kindly provided by Dr. D. J. Kwiatkowski, Harvard Medical School, Boston, MA) or with an 8.0-kb BamHI-EcoRI fragment of pHR83-3BR containing a human cardiac actin gene (provided by Dr. F. Gunning, Stanford University and Veterans Affairs Medical Center, Palo Alto, CA) as an internal control, as described previously (12).

Western Blot Analysis and Immunohistochemistry. Preparation of protein extracts from NSCLC cells and normal lung tissues was done for Western blot analysis, as described previously (22). Western blots that were probed with an antihuman gelsolin MAb (clone GS-2C4; BioMakor, Rehovot, Israel) or with an antihuman gelsolin MAb (clone GS-2C4; BioMakor, Rehovot, Israel) were visualized by chemiluminescence (Amersham International, Buckinghamshire, United Kingdom). An antiaction MAb (Boehringer Mannheim Biochemicals, Mannheim, Germany) was used as an internal control (12).

Immunohistochemistry for gelsolin expression was carried out as described previously (8, 12). Briefly, cultured NSCLC cells and primary bronchial epithelial cells, adhered to glass slides, as well as 5-μm frozen sections of tumor specimens and normal lung and bronchial tissues, were fixed in acetone at 4 for 10 min and reacted with a mouse antihuman gelsolin MAb (clone GS-2C4) or with control mouse isotype-specific immunoglobulin (IgGl) at 4°C overnight. Antigen retrieval procedure was not performed in this study. Immunostaining was performed by the biotin-streptavidin immunoperoxidase method with 3,3′-diaminobenzidine as a chromogen (SAB-PO kit; Nichirei, Tokyo, Japan). Methyl green was used for counterstaining.

Immunohistochemical staining was separately and independently evaluated by two investigators (H. D.-A. and I. K.). In the rare instance of a discrepancy in judgment, agreement was obtained by discussion at a microscope head. At the time of review, neither of these investigators were aware of the clinical and clinicopathological data of the NSCLCs because all of the slides had been coded; these data were disclosed only after both investigators were in agreement on the gelsolin status for a given specimen. Here, immunohistochemically stained sections were judged positive for gelsolin expression only when cancer cells in all areas of the specimen showed cytoplasmic staining. Tumors were regarded as negative for gelsolin expression when cancer cells in any areas of the specimen exhibited loss of cytoplasmic staining; surrounding normal stroma cells and the stroma itself showed adequate staining as positive internal controls within the same sections.

Densitometric Quantification. Gelsolin expression determined by Northern and Western blot analyses was quantitatively analyzed by a densitometer (Personal Densitometer and ImageQuant; Molecular Dynamics, Sunnyvale, CA). The levels of gelsolin mRNA and protein were normalized against the levels of cardiac actin mRNA and actin protein, respectively. The normalized levels of gelsolin mRNA and protein in the NSCLC cell lines were further normalized relative to those in the normal lung tissue control (1.000).

Statistical Analysis. The associations between loss of gelsolin expression and clinical and clinicopathological characteristics were analyzed by the χ² test or Fisher's exact test, as appropriate (23). The association between loss of gelsolin and age was analyzed by Student's t test. To examine the effect of more than one factor simultaneously on the loss of gelsolin, multivariate logistic regression analysis was used (24). Survival curves were estimated using the Kaplan-Meier method (25), and differences in survival distributions were evaluated by the generalized Wilcoxon test (26). The significance level chosen was P < 0.05, and all tests were two-sided.

RESULTS

We performed a Northern blot analysis to explore gelsolin gene expression in 12 NSCLC cell lines. As compared with that in normal lung tissue, the intensity of an approximately 2.4-kb single band of gelsolin transcripts was low to undetectable in all 12 NSCLC cell lines: the intensity was lower in PC-3, RERF-LC-OK, RERF-LC-MS, EBC-1, LC-1 sq, PC-10, and LC-2 (relative gelsolin levels of mRNA = 0.173–0.384); much lower in A549, ABC-1, VMRC-LCP, and NCI-H226 (relative gelsolin levels of mRNA = 0.024–0.098); and undetectable in VMRC-LCD (relative gelsolin level of mRNA = 1.000; Fig. 1 and Table 1).

Western blot analysis using an antigelsolin MAb also exhibited low-to-undetectable levels of gelsolin expression in all 12 NSCLC cell lines, compared to that of normal lung tissue: the level of expression was lower in RERF-LC-MS and EBC-1 (relative gelsolin levels of protein = 0.268 and 0.210, respectively); much lower in A549, PC-3, RERF-LC-OK, ABC-1, LC-1 sq, PC-10, VMRC-LCP, NCI-H226, and BK-2 (relative gelsolin levels of protein = 0.014–0.069); and undetectable in VMRC-LCD (relative gelsolin levels of protein = 1.000; Fig. 2 and Table 1).

To investigate whether immunocytochemistry was a suitable and reliable method to evaluate gelsolin expression, we further analyzed gelsolin expression by immunocytochemistry in NSCLC cell lines. Gelsolin expression by this method showed results that were consistent with those obtained by Western blot analysis. Cultured cells of NSCLC cell lines RERF-LC-MS and EBC-1 showed moderately reduced but positive cytoplasmic immunostaining for gelsolin, whereas VMRC-LCD and NCI-H226 did not show such immunostaining, as compared with primary normal bronchial epithelial cells. Normal bronchial epithelial cells obtained from all three patients

| Table 1 Densitometric quantification of mRNA and protein levels of gelsolin in 12 NSCLC cell lines |
|-----------------|-----------------|-----------------|-----------------|
| Cell line       | mRNA            | Protein         |
| Normal lung tissue | 1.000           | 1.000           |
| A549            | 0.098           | 0.014           |
| PC-3            | 0.173           | 0.068           |
| RERF-LC-OK      | 0.291           | 0.030           |
| VMRC-LCD        | 0.000           | 0.000           |
| RERF-LC-MS      | 0.366           | 0.268           |
| ABC-1           | 0.092           | 0.061           |
| EBC-1           | 0.280           | 0.210           |
| LC-1 sq         | 0.264           | 0.044           |
| PC-10           | 0.384           | 0.029           |
| VMRC-LCP        | 0.079           | 0.069           |
| NCI-H226        | 0.024           | 0.027           |
| BK-2            | 0.222           | 0.067           |

* Levels of gelsolin mRNA and protein in the NSCLC cell lines relative to those in normal lung tissue control (1.000) after normalization against the levels of cardiac actin mRNA and actin protein, respectively.
frozen tumor tissues from 88 resected NSCLCs to immunohistochemistry for gelsolin (Fig. 5). NSCLCs displayed a range of reactivities, including no or very little staining, heterogeneous staining, and homogeneous staining. Tumors were regarded as negative for gelsolin expression when cancer cells in any areas of the specimen exhibited loss of cytoplasmic expression. Forty-eight of 88 (55%) resected NSCLCs were scored as negative for gelsolin expression. Notably, in one normal bronchial and eight normal lung tissues, gelsolin expression was observed in bronchial epithelial cells, bronchial gland cells, capillary and lymphatic endothelial cells, fibroblasts, and bronchial and vascular smooth muscles, as well as in alveolar epithelial cells (Fig. 5 and data not shown).

exhibited sufficiently strong cytoplasmic immunostaining for gelsolin (Fig. 3).

To explore the mechanisms of the loss of gelsolin expression, as determined by Northern and Western blot analyses, Southern blot analysis of the gelsolin gene was performed in the NSCLC cell lines. Southern blot analysis of genomic DNA from the cultured cells after restriction with BamHI, EcoRI, or HindIII did not show any gross rearrangements or deletions of the gelsolin gene in the 12 NSCLC cell lines, compared to that of normal lung tissue (Fig. 4 and data not shown).

On the basis of the observation that gelsolin expression was frequently low or undetectable in NSCLC cell lines, we next examined gelsolin expression in surgically resected NSCLCs. For this analysis, we used immunohistochemistry with an antigelsolin MAb, because: immunocytochemistry suitably detected gelsolin expression, as paralleled with Western blot analysis in NSCLC cell lines; and immunohistochemistry could detect gelsolin expression in cancer cells, separate from that in admixed normal cells. To determine gelsolin expression in cancer cells with the surrounding normal stroma cells and stroma itself as positive internal controls, we subjected fresh-frozen tumor tissues from 88 resected NSCLCs to immunohistochemistry for gelsolin (Fig. 5). NSCLCs displayed a range of reactivities, including no or very little staining, heterogeneous staining, and homogeneous staining. Tumors were regarded as negative for gelsolin expression when cancer cells in any areas of the specimen exhibited loss of cytoplasmic expression. Forty-eight of 88 (55%) resected NSCLCs were scored as negative for gelsolin expression. Notably, in one normal bronchial and eight normal lung tissues, gelsolin expression was observed in bronchial epithelial cells, bronchial gland cells, capillary and lymphatic endothelial cells, fibroblasts, and bronchial and vascular smooth muscles, as well as in alveolar epithelial cells (Fig. 5 and data not shown).
Table 3 Multivariate logistic regression analysis for the correlation between gelsolin expression and clinical and clinicopathological characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Odds ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>1.04</td>
<td>0.17</td>
</tr>
<tr>
<td>Smoking*</td>
<td>2.40</td>
<td>0.03</td>
</tr>
<tr>
<td>Histology†</td>
<td>1.23</td>
<td>0.69</td>
</tr>
<tr>
<td>pStage</td>
<td>0.96</td>
<td>0.81</td>
</tr>
</tbody>
</table>

* For an increase of 1 yr in age.
† Squamous cell carcinoma vs. other histological types.
suggest the physiological importance of gelsolin for normal epithelial cell functions. On the other hand, an extremely low or undetectable level of gelsolin expression has been frequently observed in naturally occurring human cancers, including stomach, colon, bladder, and breast cancers, as well as lung cancer reported here (12, 29–32). Altered gelsolin expression was also demonstrated in murine fibroblasts transformed by the c-H-ras oncogene (33, 34) and in human fibroblasts and epithelial cells transformed with SV40 (35). These studies suggest that the alteration of gelsolin function may be one of the characteristics of malignant conversion in various types of cancers and transformed cells. Moreover, introduction of an exogenous murine mutant (His 321) or human wild-type cDNA of the gelsolin gene into murine and human cancer cell lines, respectively, causes morphological reversion and greatly reduces colony-forming ability in vitro, as well as tumorigenicity in vivo (12, 36). Taken together, the above information suggests that gelsolin may be a tumor suppressor for various types of cancers, although the exact role of the loss of gelsolin in the development of cancer remains to be determined. The absence of the gelsolin gene per se does not induce lung cancer because an increased incidence of lung cancer has not been noted in mice lacking gelsolin (37). However, such mice might be more susceptible to carcinogens, as well as to alterations of tumor suppressor genes and oncogenes.

It is well established that carcinogens in tobacco smoke are the principal cause of several kinds of cancer, including lung and bladder cancers (10, 11), both of which show down-regulated gelsolin expression (this study and Ref. 12). The significant association of low or undetectable gelsolin expression with heavy smoking in those NSCLC patients observed here suggests that gelsolin is a candidate molecular target of carcinogens contained in tobacco smoke. However, the sizes of both the gelsolin transcripts and proteins in NSCLC cell lines determined by Northern and Western blot analyses, respectively, were the same as those in normal lung tissues, suggesting that there were no major mutations in the coding sequence of the gelsolin gene. Likewise, gross rearrangements or deletions of the gene were not observed after restriction with endonucleases by Southern blot analysis. However, the cell line PC-10 showed abundant expression of gelsolin mRNA, with no or little protein product detected by Western blot analysis. These data might indicate that a point mutation within the coding portion of the gene resulted in protein truncation, causing protein degradation, supporting the hypothesis of gelsolin as a tumor suppressor gene. For other cell lines, however, the down-regulation is probably caused by some other mechanism, such as methylation, as with the CDKX2 gene for p16INK4A protein (38), and is potentially induced by cigarette smoking. Tobacco smoke contains a mixture of highly mutagenic compounds, such as 4-methyl nitrosamino-1-(3-pyridyl)-1-butanone and benzo(a)pyrene, that are responsible for the development of lung cancer. 4-Methyl nitrosamino-1-(3-pyridyl)-1-butanone exposure of mouse strains that are sensitive to tumor induction results in an early and significant increase in cytosine DNA-methyltransferase gene expression, which is responsible for CpG island methylation in type II pneumocytes, the precursor cells for lung adenocarcinomas in these animals (39). Benzo(a)pyrene is known to induce point mutations with a characteristic G-to-T transversion in vivo (40), which are frequently observed in the p53 and K-ras genes from smoking-associated lung cancers (7, 41), and is shown in vitro to form DNA adducts at the major mutational hot spots of the p53 gene in lung cancers (42). Taken together, these reports (7, 39–42) imply a direct link between specific genetic alterations and exposure to carcinogens in tobacco smoke.

There was no distinct correlation between gelsolin expression and the pTMM stage of resected NSCLCs, as has been reported in bladder cancer (12). This could be caused by small number of patients in each pTMM stage. Alternatively, this might be because loss of gelsolin expression is an early event during the carcinogenesis of lung and bladder cancers. Negative or positive gelsolin expression was not correlated with survival in the present cohort of NSCLC patients. This observation might have resulted from the smaller number of patients analyzed and the shorter periods of survival observations in each group. Larger studies that include patients with homogeneous stages of disease in longer periods of observations are required to confirm whether the loss of gelsolin expression can predict clinical outcome in NSCLC.

In conclusion, loss of gelsolin expression is frequently observed both in cell lines and resected tumors of NSCLC and may be involved in the development of NSCLCs as one of the events during carcinogenesis caused by tobacco smoke. The molecular basis of loss of gelsolin expression remains to be determined.

ACKNOWLEDGMENTS

We thank Drs. D. J. Kwiatkowski (Harvard Medical School) for providing human gelsolin cDNA, F. Gunning (Stanford University and Veterans Affairs Medical Center) for providing pHLR83-BR plasmid containing a human cardiac actin gene, and H. Oie (NCI-Navy Medical Oncology Branch, National Cancer Institute) for providing the NCI-H226 cell line. We also thank Dr. K. Inoue (Hokkaido University Medical Hospital) for pathological review of tissue sections.

REFERENCES

Gelsolin Expression in Non-Small Cell Lung Cancers


Frequent Loss of Gelsolin Expression in Non-Small Cell Lung Cancers of Heavy Smokers

Hirotoshi Dosaka-Akita, Fumihiro Hommura, Hisakazu Fujita, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/2/322

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.