Early Loss of Fhit in the Respiratory Tract of Rodents Exposed to Environmental Cigarette Smoke

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Abstract

The Fhit gene, encompassing the most active common human chromosomal fragile region, FRA3B, has been shown to act as a tumor suppressor. Several studies have shown significant Fhit alterations or Fhit protein loss in lung cancers from smokers compared with lung cancers from nonsmokers. To evaluate the role of Fhit under controlled experimental conditions, we exposed rodents to environmental cigarette smoke (ECS) and evaluated Fhit expression or Fhit protein in the respiratory tract. After 14 days of exposure to ECS, loss of Fhit protein in the bronchial/bronchiolar epithelium affected half of the tested B6-129(F1) mice, either wild type or Fhit+/−. After 28 days, it affected the vast majority of the tested SKH-1 hairless mice and of A/J mice and all (UL53-3 × A/J)F1 mice, either wild type or F53+/−. In Sprague-Dawley rats, exposure to ECS for up to 30 days caused a time-dependent loss of Fhit in pulmonary alveolar macrophages. Moreover, ECS down-regulated Fhit expression and significantly decreased Fhit protein in the rat bronchial epithelium. The oral administration of N-acetylcysteine attenuated the ECS-related loss of Fhit, whereas oltipraz, 5,6-benzoflavone, phenethyl isothiocyanate, and indole 3-carbinol, and their combinations had no significant effect. Parallel studies evaluated a variety of molecular, biochemical, and cytogenetic alterations in the respiratory tract of the same animals. In conclusion, there is unequivocal evidence that Fhit is an early, critical target in smoke-related lung carcinogenesis in rodents, and that certain chemopreventive agents can attenuate the occurrence of this gene alteration. (Cancer Res 2006; 66(7): 3936-41)

Introduction

Ten years ago, the Fhit gene was discovered at the common fragile site FRA3B (1). Fhit encompasses the carcinogen-sensitive and viral integration–susceptible fragile region. The first coding exon 5 was found susceptible to deletion in many tumors, resulting in inactivation of Fhit protein. Thus far, almost 550 reports of Fhit studies have appeared, and Fhit was shown altered in a large proportion of tumors, including lung cancer (reviewed in ref. 2).

Several studies, using either immunohistochemical or molecular methods, showed a significant loss of Fhit in lung cancers from smokers compared with lung cancers from nonsmokers (3–9). Interestingly, a dose-dependent decrease of Fhit methylation was observed in bronchoalveolar lavage cells from cancer-free patients undergoing fiber optic bronchoscopy, as related to the number of cigarettes smoked in a lifetime (10). A small proportion of histologically normal bronchial epithelium from smokers displayed either Fhit loss, as detected by immunohistochemistry (11), or an increased Fhit methylation (12). On the other hand, negative results were reported in other studies evaluating the association of Fhit loss with smoking history (13–17).

Thus, on the whole, the assumption that the Fhit gene is a target for cigarette smoke is mostly supported by studies evaluating Fhit alterations or Fhit loss in lung tumors. Moreover, the conclusions of the studies on this subject are not univocal. As a further consideration, it is not clear whether the Fhit loss is an early consequence of exposure to cigarette smoke or the result of the multiple alterations occurring in preneoplastic or neoplastic cells. These premises prompted us to evaluate changes in Fhit gene expression and loss of Fhit protein in the respiratory tract of rodents exposed to environmental cigarette smoke (ECS) for short periods of time, up to a maximum of 30 days, before appearance of any ECS-related histopathologic alteration. In these studies, we used Sprague-Dawley rats and mice belonging to various strains and genotypes, including B6-129(F1) mice, either wild type or Fhit+/−; SKH-1 hairless mice, A/J mice, and (UL53-3 × A/J)F1 mice, either wild type or F53+/−. Part of these animals have been used in parallel studies evaluating the occurrence of ECS-related induction of lung tumors and modulation of intermediate biomarkers in cells of the respiratory tract (18–23). In addition, in one of the herein reported studies, we exposed hairless mice not only to ECS but also to ECS plus light or light alone, which induced skin tumors in mice (24, 25) as well as molecular and biochemical alterations not only in skin but, surprisingly, even in the respiratory tract and bone marrow cells (19, 21).

A further goal of the present study was to evaluate whether the oral administration of putative cancer chemopreventive agents may influence the ECS-related Fhit loss in rodent bronchial/bronchiolar epithelial cells. The investigated agents included sulindac, a nonsteroidal antiinflammatory drug; 5,6-benzoflavone, a synthetic flavonoid; the thiol N-acetylcysteine; the dithiolthione oltipraz; the natural compound phenethyl isothiocyanate, contained in watercress; and the indole glucosinate indole 3-carbinol (I3C), a decomposition product of cruciferous vegetables.

The results obtained show that exposure of rats and mice, belonging to various strains and genotypes, to ECS, for up to 30 days, consistently causes a significant and time-related decrease of Fhit gene expression, as detected both by reverse transcriptase-PCR (RT-PCR) and quantitative real-time PCR (QPCR), and a loss of Fhit protein, as detected both by immunohistochemistry and Western blot. Of the chemopreventive agents tested, only...
N-acetylcysteine was successful to attenuate the ECS-induced Fhit down-regulation and Fhit loss.

Materials and Methods

Animals. A total of 97 adult male Sprague-Dawley rats, weighing 315 to 320 g at the start of the experiments, were purchased from Harlan Italy (Correzzana, Milan, Italy). A total of 34 adult female B6-129(F1) mice, 1/4 of which were wild type and 20 heterozygous for Fhit (Fhit<sup>+/−</sup>) weighing on average 35 g, were produced at the Kimmel Cancer Center (Thomas Jefferson University, Philadelphia, PA) and shipped to the University of Genoa. Sixteen adult female SKH-1 hairless mice, weighing 21 to 22 g, and 10 adult female A/J mice, weighing 20 to 21 g, were purchased from Charles River (Calco, Lecco, Italy). Twenty adult female (UL53-3 x A/J) F<sub>1</sub> mice, 10 of which were wild type and 10 were F53 transgenic, weighing 17 to 18 g, were produced at the Ohio State University (Columbus, OH) and kindly supplied by Dr. Ming You (University of Columbus, OH) to the University of Genoa. All animals were housed in Makrolon cages on sawdust bedding and maintained on standard rodent chows for mice (MII, Morini, S. Polo d’Enza, Italy) and rats (Teklad TRM, Harlan) and tap water ad libitum. The temperature of the animal room was 23 ± 2°C, with a relative humidity of 55% and a 12-hour day/night cycle. The housing and treatments of animals were in accordance with our national and institutional guidelines.

Exposure of animals. A whole-body exposure of rats and mice, for the times indicated in Results, was achieved by burning Kentucky reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) in a smoking machine (model TE-10, Teague Enterprises, Davis, CA). 2R1 cigarettes, having a declared content of 44.6 mg tar and 2.5 mg nicotine each, were used in the study with SKH-1 mice. 1R3 cigarettes, having a declared content of 22.8 mg tar and 1.5 mg nicotine each, were used in all other studies. Before use, the cigarettes were kept for 48 hours in a standardized atmosphere humidified with a mixture of 70% glycerol and 30% water. Each smoldering cigarette was puffed for 2 seconds, once every minute, for a total of eight puffs, at a flow rate of 1.05 L/min to provide a standard puff of 35 cm<sup>3</sup>. The smoking machine was adjusted to burn five cigarettes at one time and to produce a mixture of sidestream smoke (89%) and mainstream smoke (11%), mimicking exposure to high-dose ECS. Exposure to ECS was 6 h/d, divided into two rounds with a 3-hour interval, for the number of days indicated in Results.

Exposure of SKH-1 mice to light was obtained by using halogen quartz bulbs (12 V, 50 W), supplied by Leuci (File, Lecco, Italy) and equipped with UV-C filters (WG 290, Schott Optics Division, Mainz, Germany), at an illuminance level of 10,000 lx. Eight mice were exposed to the light for 9 h/d. Additional 16 mice were exposed to daily cycles of both light and ECS, and half of them received sulindac (19).

Treatment with chemopreventive agents. Six chemopreventive agents were used, either individually or in combination, for assessing their influence on Fhit protein loss in ECS-exposed rats and mice. Sulindac and N-acetylcysteine were purchased from Sigma Chemical Co. (St. Louis, MO); N-acetylcysteine was purchased from Zambon Italia (Vicenza, Italy); and oltipraz, phenethyl isothiocyanate, and I3C were supplied by the Division of Cancer Prevention Repository of the National Cancer Institute (Rockville, MD). Sulindac and N-acetylcysteine were given with the drinking water, at a calculated intake of 45 and 1,000 mg/kg body weight, respectively. The other agents were incorporated in diets, prepared once per week, containing the concentrations of 400 mg/kg diet (oltipraz), 500 mg/kg diet (N-acetylcysteine and phenethyl isothiocyanate), or 2,500 mg/kg diet (I3C). Treatment with the chemopreventive agents started 3 days before the first exposure of rodents to ECS and/or light.

Collection and preparation of pulmonary alveolar macrophages and lung samples. At the times indicated in Results, the animals were deeply anesthetized with diethyl ether and killed by cervical dislocation. Rat pulmonary alveolar macrophages (PAM) were collected by bronchoalveolar lavage (19) and spun onto slides by means of a cytocentrifuge. The slides were air-dried and fixed in absolute methanol. The right lung from variously treated rats was immediately stored at −80°C for molecular analyses. The left lung of each animal was fixed in formalin and routinely processed for Fhit immunohistochemistry.

RNA extraction and Fhit gene expression. RNA was extracted from the pooled lung samples of rats, either sham exposed or ECS exposed or treated with N-acetylcysteine, alone or in combination with exposure to ECS, by sequential proteinase K-DNase I and phenol/chloroform treatments and isopropanol precipitation, as previously reported (20). Fhit mRNA expression was evaluated both by semiquantitative RT-PCR, and by QPCR. Specific primers for Fhit were designed by using a commercially available software (Primer Premier 4, Premier Biosoft International, Palo Alto, CA), as follows: 5’-TGGCTTGTCACCTGCCTGTC-3’ (P<sub>1</sub>) and 3’- CCTT-TTGGGGAAACATGGACC-5’ (P<sub>2</sub>). Complementary DNA was amplified by plateau-PCR amplification.

For RT-PCR analyses, the reaction product of 184 bp was separated by agarose gel electrophoresis, staining with ethidium bromide, and identified at 231 bp. For QPCR analyses, a Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) reaction was done in a rotating thermocycler (Rotor-Gene 3000, Corbett Research, Mortlake, Australia), using SYBR GREEN fluorescence (Invitrogen) as a tracer. Ubiquitin mRNA, as detected by 5’-CCTTGCTCCTCCGCTTAG-3’ (P<sub>1</sub>) and 3’-GTACGCCTGGTATGCCTCTTG-5’ (P<sub>2</sub>) primers, was tested as an internal positive standard used for data normalization. Negative controls (DNA-free samples) were included in each analysis.

Fhit protein. Fhit protein was detected by immunohistochemistry in the bronchial/bronchiolar epithelium of rats and mice and in rat PAM. A commercially available kit (Histomouse-SP kit, Zymed Laboratories, San Francisco, CA) was used following the manufacturer’s instructions. A rabbit anti-Fhit polyclonal antibody (ZB4, Zymed Laboratories) was used for rat bronchial/bronchiolar epithelium and PAM, at a final concentration of 3 pg/μL. A rabbit anti-Fhit polyclonal antibody, kindly supplied by Dr. Kay Huebner (Ohio State University Comprehensive Cancer Center, Columbus, OH), was used for mouse bronchial/bronchiolar epithelium, at final dilution of 1:2,000. Blind-coded slides were evaluated by two readers, each one scoring 1,000 cells per slide.

In addition, the Fhit protein was detected in the pooled lung samples from either sham-exposed or ECS-exposed rats by Western blot. SI2 fractions (100 μg protein per sample) were transferred to acrylamide gel and subjected to electrophoresis. The gel was blotted to an Immun-blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and labeled first with a primary rabbit anti-Fhit polyclonal antibody (ZB44, Zymed Laboratories) and then with a fluorescent secondary antibody labeled with Cy3 (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The signals were detected by fluorescence laser scanning (Scanarray, Packard Bioscience, Billerica, MA), and the Fhit protein band (17 kDa) was identified by marking reference to a DNA ladder (pUC18Dpnl Digest, Sigma Chemical). The cDNA amounts were quantified by densitometric analysis using a digital acquisition equipment (DC 120 Zoom Digital Camera, Eastman Kodak, Rochester, NY) and a specifically designed software (1D Image Analysis Software, Eastman Kodak). The results were standardized by making reference to the expression of the ubiquitin housekeeping gene, detected at 231 bp.

Results

Fhit loss in the bronchial/bronchiolar epithelium of smoke-exposed mice. Table 1 summarizes the results obtained in four
strains of mice exposed to ECS for either 14 days [B16-129(F1) strain] or 28 days (all other strains). Figure 1 shows examples of immunohistochemical analyses of two sections of bronchiolar epithelium from A/J mice, either sham exposed (A) or exposed to ECS for 28 days (B). Due to the difficulty of calculating the percentage of cells positive for Fhit protein, which is localized in the cytoplasm, especially in bronchioli, the results shown in Table 1 are expressed in terms of mice showing an evident loss of Fhit out of those examined. There was a sharp difference between sham-exposed mice and ECS-exposed mice. In fact, all samples from the 40 sham-exposed mice were diffusely positive for Fhit in all cells of the bronchial/bronchiolar epithelium, similar to that shown in Fig. 1A. In contrast, the majority of ECS-exposed mice (29 of 40; i.e., the 72.5%) displayed a situation similar to that depicted in Fig. 1B. The Fhit loss affected half of B6-129(F1) mice after just 14 days of exposure to ECS and was only marginally and not significantly higher in Fhit+/− mice. After 28 days of exposure, a Fhit loss was observed in the 87.5% of SKH-1 hairless mice, in the 80.0% of A/J mice, and in the 100% of (UL53-3 × A/J)F1 mice, irrespective of the p53 status.

In SKH-1 hairless mice, no loss of Fhit was observed in the bronchial/bronchiolar epithelium after exposure of the mice to the light emitted by UV-C-filtered halogen lamps. On the other hand, all eight mice exposed to daily cycles of light and ECS exhibited Fhit loss, and this result was unchanged in mice that additionally received sulindac in drinking water (data not shown).

**Fhit protein loss and Fhit gene down-regulation in the respiratory tract of smoke-exposed rats: effect of chemopreventive agents.** Only a tiny proportion of bronchial epithelial cells from sham-exposed Sprague-Dawley rats, kept in filtered air for 28 days, did not show the presence of Fhit protein detectable by immunohistochemistry (Fig. 2). The proportion of Fhit-negative cells increased 3.5-fold after exposure to ECS for 28 days. Of the seven chemopreventive treatments tested in ECS-exposed rats, only N-acetylcycteine, given with the drinking water, was successful to significantly attenuate the ECS-related loss of Fhit. In contrast, administration with the diet of oltipraz, phenethyl isothiocyanate, 5,6-benzoflavone, I3C, and the combination of phenethyl isothiocyanate with I3C did not significantly affect the ECS-induced Fhit loss. The proportion of Fhit-negative cells was lower in rats receiving the combination of N-acetylcycteine with oltipraz compared with rats exposed to ECS in the absence of any chemopreventive agent, but this difference was not statistically significant (Fig. 2).

In addition to immunohistochemical analyses, the levels of Fhit protein were evaluated by Western blot in lung S12 fractions from rats, either sham exposed or exposed to ECS. As assessed by testing two replicates of each sample in two separate experiments, the Fhit protein underwent a 45% decrease (P < 0.01) following exposure to ECS for 28 days (Fig. 3A).

The level of Fhit transcription was evaluated by both RT-PCR and QPCR analyses in the lung of rats, either sham exposed (SHAM), or smoke exposed (ECS), or receiving oral N-acetylcycteine alone, or smoke exposed and treated with N-acetylcycteine (ECS + N-acetylcycteine). RT-PCR (Fig. 3B) resulted in the production of an amplified sequence of 184 bp corresponding to Fhit mRNA. As assessed by testing three replicates of each sample in three separate experiments, N-acetylcycteine alone did not appreciably decrease Fhit expression, which was 17% lower than in SHAM. ECS halved Fhit expression (−51%, P < 0.05), an effect which was attenuated in rats cotreated with N-acetylcycteine (−33% compared with SHAM, P = 0.09 compared with ECS). QPCR resulted in the amplification of fluorescent products (Fig. 3C). As
assessed by testing three replicates of each sample, N-acetylcysteine alone did not appreciably decrease Fhit transcription, which was 13% lower than in SHAM. ECS significantly attenuated Fhit transcription (−46%, P < 0.001 compared with SHAM), an effect that was partially but significantly reversed in rats cotreated with N-acetylcysteine (−33% compared with SHAM, P = 0.05 compared with ECS).

Time course loss of Fhit in PAMs from smoke-exposed rats.
Exposure of Sprague-Dawley rats to ECS for up to 30 days resulted in a time-dependent loss of Fhit. The proportion of Fhit-positive PAM was 61.6 ± 3.6% at time 0 and 50.3 ± 1.0% after 30 days. This decrease was statistically significant (Fig. 4). Figure 4 also shows the appearance of two binucleated PAM, one positive (Fhit+) and one negative (Fhit−) for this protein in immunohistochemistry. The correlation between Fhit levels in PAM and time of exposure to ECS (r = −0.994) was statistically significant (P < 0.001). The regression line is \( y = 62.05 - 0.38x \), where \( y \) is the percentage of Fhit-positive PAM, and \( x \) is the exposure time in days. Based on this equation, and provided that the loss of Fhit continues to be linear after 30 days of exposure, the half-life of Fhit loss in PAM of ECS-exposed rats, under our experimental conditions, would be expected to be 81.8 days.

Discussion
The results of the present study provide evidence that a relatively short-term exposure of rodents to ECS causes a significant down-regulation of Fhit gene expression, as assessed by both RT-PCR and QPCR, as well as a loss of Fhit protein, as assessed by both immunohistochemistry and Western blot. These effects were detected in apparently healthy cells of the respiratory tract. In particular, a time-dependent loss of Fhit was recorded in PAM after exposure of rats to ECS for varying periods of time. Evaluation of Fhit by immunohistochemistry in PAM is particularly convenient because, due to the localization of Fhit in the cytoplasm, it is more reliable to score individual cells rather than cells within tissues. PAM are sweeping, sentinel cells that can easily be recovered by bronchoalveolar lavage, which are particularly useful for studying molecular, cytogenetic, and biochemical alterations in both humans and animal models (18, 19, 26–28). As many as 1 to 5 × 10⁶ PAM are removed every hour from human terminal airways via the mucociliary escalator (29). The only study in the literature reporting a cigarette smoke–related effect of Fhit in bronchoalveolar lavage cells from cancer-free smokers is the one by Kim et al. (10), who showed an increase of Fhit methylation that was dose-dependently related to the number of pack-years. In general, the de novo methylation of CpG islands within the promoters of tumor suppressor genes is one of the frequent mechanisms of gene inactivation in lung carcinogenesis (30). Based on the above findings and taking into account that the half-life of Fhit in PAM of ECS-exposed rats was <3 months, the possibility of evaluating...
Fhit gene or Fhit protein changes in PAM as a marker of exposure to cigarette smoke or other inhalant carcinogens and, perhaps, as a tool for distinguishing susceptible smokers requires further studies. In addition, loss of Fhit protein in the bronchial/bronchiolar epithelium affected about half of the B6-129(F1) mice exposed to ECS for 14 days only, irrespective of the Fhit status. This result may be in accordance with a previous experiment in which wild-type and Fhit+/− mice treated with the pulmonary carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone did not exhibit significantly different frequencies of lung lesions (31). The vast majority of the tested SKH-1 hairless mice and of A/J mice and all (UL53-3 × A/J)F1 mice, either wild type or with a germ line inactivation of one allele of P53, which is a hallmark of Li-Fraumeni syndrome (32), displayed a loss of Fhit after 28 days of exposure to ECS. It is noteworthy that Fhit and P53 work synergistically as tumor suppressors and Fhit-mediated inactivation of mdm2 leads to P53 stabilization (33). Our findings document, under well controlled experimental conditions, that Fhit is indeed a critical target for cigarette smoke, and that this molecular change occurs early in the carcinogenesis process and not as a consequence of the development of the neoplastic mass. This is accordingly to a number of previous studies (reviewed in ref. 34). The decreased transcription of Fhit in ECS-exposed rats suggests that Fhit loss is the result of genomic changes, such as deletion or methylation, and not of postgenomic alterations, such as production of mutated protein or posttranscriptional regulation.

On this ground, prevention strategies can be aimed either at inhibiting Fhit alterations or at restoring the damaged gene. The latter approach has already been pursued by oral gene transfer, using adenoviral or adeno-associated viral vectors expressing the latter approach has already been pursued by oral gene transfer, inhibiting Fhit of postgenomic alterations, such as production of mutated proteins in the lung of the same animals, and had various effects when we investigated, by cDNA microarrays, their ability to modulate the expression of 4,858 genes in lung and liver of both unexposed and ECS-exposed rats (22). In addition, when studying by antibody microarray the levels of 518 proteins in the lung of the same animals, N-acetylcysteine did not change per se the level of any tested protein but decreased the number of ECS-induced proteins (38).

N-acetylcysteine is an analogue and precursor of l-cysteine and reduced glutathione, which has been shown to exert a variety of protective effects and mechanisms in mutagenesis and carcinogenesis (39, 40). Interestingly, in the same rats used in the present study, N-acetylcysteine and its combination with olitpraz were the only treatments capable of significantly decreasing the frequency of apoptotic cells in the bronchial/bronchiolar epithelium. In contrast, phenethyl isothiocyanate showed an opposite trend, and all other treatments had no significant effect (23). These data are in line with the results of other studies showing a decrease by N-acetylcysteine and an increase by phenethyl isothiocyanate of apoptotic PAM in rats exposed to ECS, and a decrease by N-acetylcysteine of apoptotic cells in the bronchial/bronchiolar epithelium of rats exposed to mainstream cigarette smoke (23).

Enhancement of apoptosis is conceptually a double-edged sword, because it provides a protective mechanism in carcinogenesis but may contribute to the pathogenesis of other degenerative diseases. On the other hand, inhibition of apoptosis by chemopreventive agents, as observed with N-acetylcysteine, is expected to reflect their ability to counteract certain upstream signals, such as genotoxic damage, redox imbalances, and other forms of cellular stress that trigger apoptosis (23). The circumstance that N-acetylcysteine was the only agent able to attenuate both apoptosis and Fhit protein loss in the respiratory tract of ECS-exposed rats is meaningful, because it suggests that Fhit alterations and the apoptotic process are triggered by the same or parallel mechanisms. In this regard, it is noteworthy that Fhit has been involved in the regulation of apoptosis (41). In terms of mechanisms, previous studies done on lung tumors from mice treated with the carcinogen vinyl carbamate provided evidence that loss of Fhit expression and promoter/exon 1/intron 1 methylation status were correlated (42).

In conclusion, the herein reported data support and elucidate the role of Fhit as an early target in cigarette smoke–related lung carcinogenesis and indicate that certain chemopreventive agents can attenuate the induction of this gene alteration.

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