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# ADVANCES IN ONCOLOGY®

JUNE • 1999 VOl 15 • NO 2

**Scientific Research**

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**Clinical Update: Non-Hodgkin's Lymphoma**

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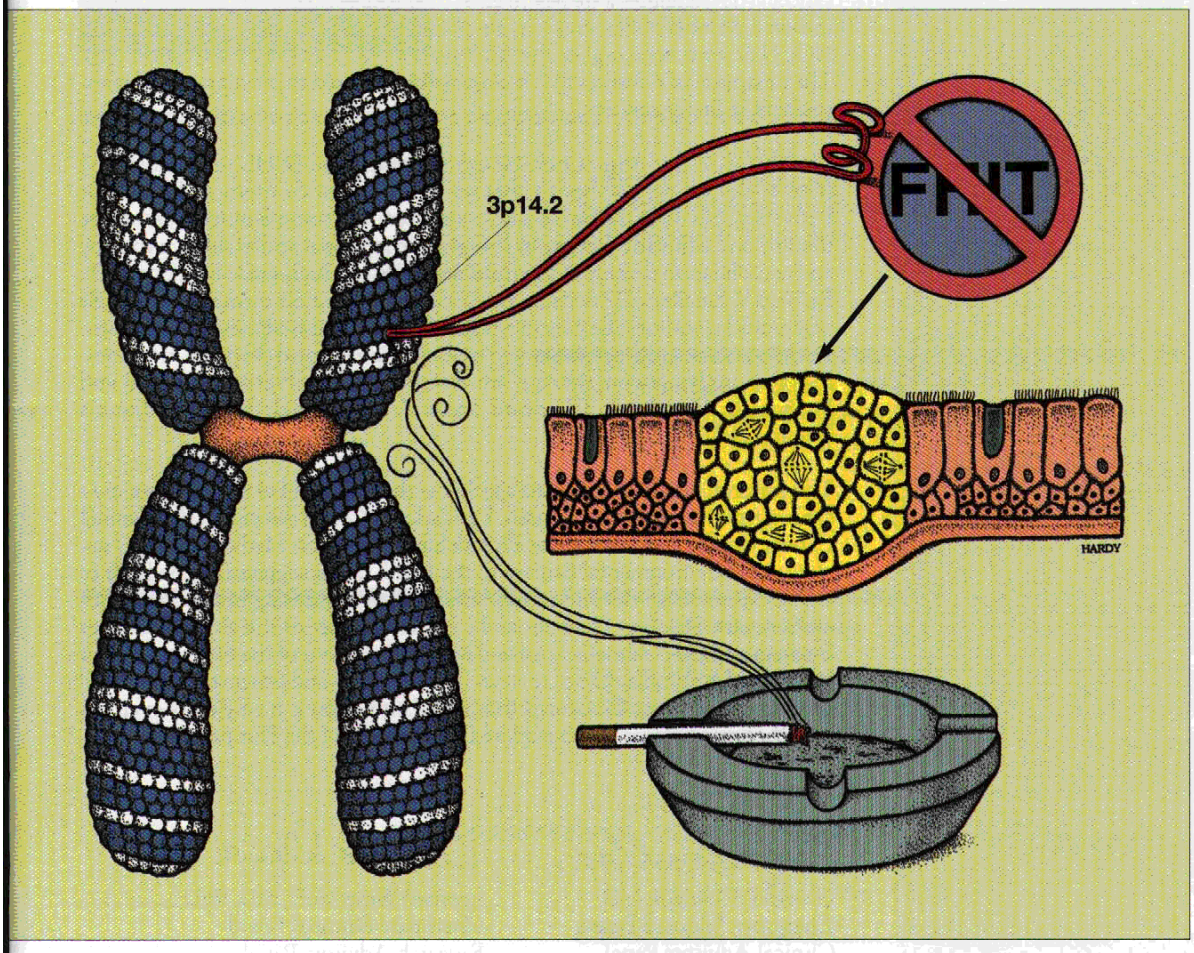


Tobacco carcinogens inactivate *FHIT* gene in lung cancer epithelial cells

CLIGGOTT PUBLISHING CO.
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ADVANCES IN ONCOLOGY®
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Dear Doctor,

The National Comprehensive Cancer Network (NCCN) and the American Cancer Society (ACS) recently made public the first version of the NCCN breast cancer treatment guidelines for patients. The guidelines, which were originally developed for oncologists by the NCCN, were adapted by the ACS to satisfy the informational needs of patients with breast cancer and their families. Among the topics presented, in layperson's language, are types of breast cancer, stages of disease, treatment options, important questions for patients to discuss with their physicians, information about clinical trials, and a glossary of terms commonly used in breast cancer therapy. Also included in the patient guidelines are visual aids and flowchart algorithms of therapy for different stages of breast cancer. The guidelines were described at the Fourth Annual NCCN Conference in Fort Lauderdale, Fla, and may be obtained from the NCCN (at 888-909-NCCN or www.nccn.org) or the ACS (at 800-ACS-2345 or www.cancer.org). The next issue of *Advances In Oncology* will be devoted to prevention and management of breast cancer.

The theme of this issue is non-Hodgkin's lymphoma (NHL)—specifically 3 state-of-the-art approaches to therapy. Dr. Richard S. Stein of Vanderbilt University discusses the use of autologous stem cell transplantation as salvage therapy and adjuvant therapy in low-grade lymphoma and as salvage therapy and consolidation therapy in intermediate-grade and high-grade lymphoma. Dr. Richard V. Smalley of Synertron, Inc, a small and privately owned clinical trials organization, outlines the controversial role of interferon-alfa in NHL management by reviewing 9 key randomized trials. Antibody-based therapies, now coming of age in lymphoma therapy, are described by Drs. Pratik S. Multani and Michael L. Grossbard of Harvard Medical School and the Massachusetts General Hospital.

The research article in this issue spotlights the other end of the continuum—cancer genetics and carcinogenesis. Dr. Carlo M. Croce, director of the Kimmel Cancer Center and Institute at Philadelphia's Jefferson Medical College, explains how environmental damage to the *FHIT* tumor suppressor gene leads to loss of expression of the Fhit protein and, eventually, to lung cancer. In collaboration with Drs. Kay Huebner and Charles Brenner of the Kimmel Cancer Institute and Drs. Gabriella Sozzi and Marco A. Pierotti of the National Tumor Institute of Milan, Dr. Croce reviews how his group and others tied the *FHIT* gene to lung cancer, discovered that *FHIT* alteration is a very early event in bronchogenic neoplasia, and explored the function of the Fhit protein.

Sincerely,

George P. Canellos, MD
Chairman, Editorial Board
Clinical Advisory Panel

Leonard Weiss, ScD, MD, PhD
Chairman, Editorial Board
Research Advisory Panel

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V. Craig Jordan, PhD, DSc

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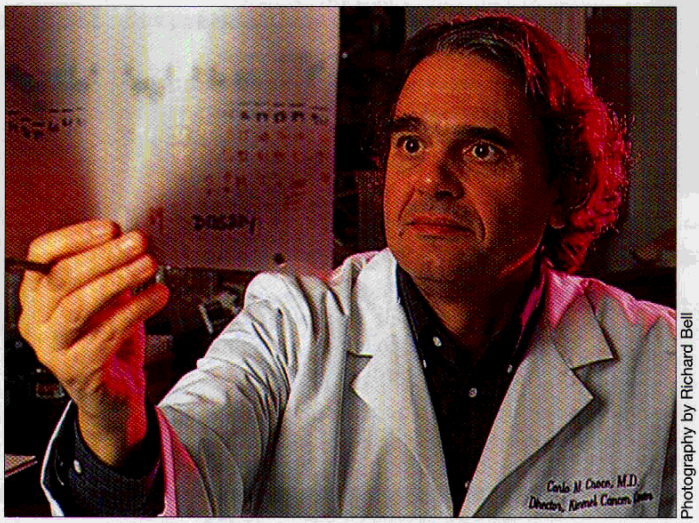
# Carlo M. Croce, MD

Kimmel Cancer Institute, Thomas Jefferson University

The "Century of Physics," winding down as the millennium approaches, is about to pass the baton of preeminence to the "Century of Genetics." The stage was set for the dominance of genetics in the 21st century by the discovery of the structure of DNA almost 50 years ago. To the extent that cancer is a disease of genes gone bad, the key to its control and cure will lie in the understanding of human genetics—how and why genes become defective, what turns them on and off, and how they can be repaired. Leading the way in the increasingly complex field of human cancer genetics is Carlo M. Croce, MD.



Photography by Richard Bell

For many years, Dr. Croce's work has focused on the initiating events responsible for the pathogenesis of hematopoietic malignancies and on the early events in the pathogenesis of human cancers, including leukemias and lymphomas, head and neck cancers, and gastrointestinal cancers. More recently, in collaboration with Kay Huebner, PhD, he has turned his attention to lung cancer. In the work he and his colleagues describe here, the intimate relationship between bronchogenic neoplasia and the *FHIT* tumor suppressor gene, which contains the most common fragile site of the human genome, *FRA3B*, is at center stage. In particular, they have shown that *FRA3B* is prone to damage by common environmental carcinogens, including tobacco smoke and polyaromatic hydrocarbons, and that the damage results in lung cancer.

Very recent studies have shown that loss of heterozygosity, particularly at band 3p14.2 on the short arm of chromosome 3, occurs in the nonneoplastic airway cells of smokers and persists in the lungs of former smokers. Working with scientists at the Kimmel Cancer Institute in Philadelphia and the National Tumor Institute in Milan, Italy, Dr. Croce has shown that more than 80% of squamous cell lung carcinomas have completely lost expression of the Fhit protein at that site. He is now in pursuit of a means to deliver the *FHIT* gene by a suitable vector so that genetically damaged cells in high-risk patients may be eliminated—possibly even before the onset of cancer.

Dr. Croce, director of the Kimmel Cancer Center and the Kimmel Cancer Institute at Jefferson Medical College of Thomas Jefferson University in Philadelphia and professor and chairman of the department of microbiology and immunology at Jefferson Medical College, was born in Milan. He earned his medical degree (summa cum laude) at the University of Rome. His career as a cancer geneticist began immediately thereafter, almost 3 decades ago at the Wistar Institute of Anatomy and Biology in Philadelphia.

Working with Hilary Koprowski at Wistar, Dr. Croce published a series of papers on fusion of gametic and somatic cells, the genetics of cell transformation associated with SV40, regulation of hybrid cells, genetic linkage, tumorigenicity, and the genetics of human cancer. In 1980, he became the Wistar Professor of Human Genetics at the University of Pennsylvania School of Medicine and the Institute Professor and associate director at the Wistar Institute.

From 1988 to 1991, Dr. Croce was director of the Fels Institute for Cancer Research and Molecular Biology at Temple University in Philadelphia, where he was also professor of medicine and professor of pathology. He received Outstanding Investigator Awards from the NCI and the NIH in 1985 and in 1992 for his work on the molecular genetics of hematopoietic malignancies.

Dr. Croce has been a pioneer in understanding the genetic causes of cancer. Among many accomplishments, he has elucidated the roles of chromosome alterations in human leukemias and lymphomas and has identified several genes involved in blood cancers. A genetic profile of these genes is already being used in clinical settings to detect remaining disease in treated patients.

More specifically, Dr. Croce and his team have isolated and named the *ALL1* gene, which is critical to the development of acute leukemia. *ALL1* is involved in acute leukemias including acute lymphoblastic leukemia, acute myelogenous leukemia, acute myelomonocytic leukemia, and acute monocytic leukemia. In addition to recent work on the *FHIT* gene and lung cancer, Dr. Croce's group at the Kimmel Cancer Institute has shown that *ALL1* can fuse with itself (in a mechanism called "self-fusion"), leading to acute myelogenous leukemia, and that expression of the gene named *ARP*, one of the targets of *ALL1*, is completely lost in all acute leukemias carrying chromosome translocations involving the *ALL1* locus at 11q23. *TCL-1*, which is involved in chronic T-cell leukemia and adult T-cell leukemia, is another gene target identified by the Croce group.

Among the group's most recent work is an ongoing investigation of the role of the gene *TAL1/SCL/TCL5* in the pathogenesis of human leukemias and hematopoietic differentiation. Dr. Croce has developed a *TAL1* transgenic mouse model in which overexpression of the gene leads to acute leukemia or high-grade lymphoma. He has also described the molecular genetic events that result in Burkitt's lymphoma, mantle cell lymphoma, follicular lymphoma, and lymphomas in patients with HIV infection. His achievements in identifying and cloning the *FHIT* gene and in creating knockout and transgenic mouse models to investigate it continue to yield vital information on carcinogenesis. ■

# Fhit Loss in Lung Cancer: Diagnostic and Therapeutic Implications

Kay Huebner, PhD; Gabriella Sozzi, PhD; Charles Brenner, PhD; Marco A. Pierotti, PhD; and Carlo M. Croce, MD

Lung cancer is the major cause of cancer deaths worldwide, and the predominant cause of lung cancer is cigarette smoking. After smoking cessation, the risk of lung cancer diminishes only gradually over decades, and available therapies have lead to cure in only 10% of cases. Novel methods for early diagnosis, prevention, and treatment are urgently needed.

**Lung cancer and the *FHIT* gene**

Non–small cell lung carcinoma (NSCLC) accounts for about 80% of all lung tumors and includes 3 main histologic subtypes: adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma.[1] SCC has been the most common type for years, but adenocarcinoma is increasing, especially in women, and is now the more prevalent subtype in some countries.

Normal lung epithelium does not show squamous differentiation, but squamous metaplasia occurs after smoking injury and progresses through dysplasia, carcinoma in situ, and invasive tumor. About 60% of SCCs are treated by surgery, with a resulting 5-year survival rate of less than 40%. For untreated SCC, survival is frequently less than 1 year. Lung ADC occurs mostly in the upper lobes, grows rapidly, and often invades the pleura. Fewer than 40% of ADCs can be surgically treated, and only 25% of patients with resected tumors survive 5 years.

Small-cell lung carcinoma (SCLC) is a highly malignant tumor arising from the basal cells of the bronchial epithelium. Most SCLC occurs in male cigarette smokers and has metastasized by the time of diagnosis. Recent advances in chemotherapy for metastatic SCLCs have led to improvement in prognosis. Tumors limited to the lung are also treated by radiation.

• *The short arm of chromosome 3 in lung cancer genetics*. The short arm of human chromosome 3 (3p) contains multiple suppressor genes for various tumors, including breast, lung, kidney, cervical, ovarian, and head and neck cancers.[2] Regions on 3p (3p25-26, 21-22, 14, 12-13) probably harbor suppressor genes involved in the pathogenesis of lung, breast, and kidney cancers. Lung cancer suppressor loci have been mapped by several groups to 3p25, 3p21.3 and 3p12-13.

A suppressor gene at 3p25, the von Hippel-Lindau gene (*VHL*), responsible for some familial kidney carcinomas, was isolated several years ago and is being characterized for function in normal tissue and kidney tumors. The *VHL* gene does not appear to be involved in lung cancer.[3] Another potential kidney cancer suppressor gene, the *FHIT* gene, at 3p14.2 was also isolated several years ago.[4]

A gene in the 3p14.2 region had been pursued by a number of groups because this chromosome band was the site of a balanced chromosome translocation (t[3;8][p14.2;q24]) associated with familial clear cell renal carcinomas. The translocation was known to be close to another interesting genetic landmark, the locus known as *FRA3B*, the most inducible of the common human chromosomal fragile sites.[2] The *FHIT* gene, greater than 1 megabase in size, encompasses both of these genomic landmarks, as well as an unexpected number of homozygous deletions in cancer cell lines.

Fragile sites are chromosome regions that reveal cytogenetically detectable gaps after exposure of cells to specific reagents. They have been shown to be involved in numerous types of recombinant events, including sister chromatid exchange, interchromosomal and intrachromosomal rearrangements, deletions and translocations in hybrid cells, and plasmid and viral DNA integration.[2] Expression of fragile sites can trigger intrachromosomal gene amplification, lending support to the hypothesis that they play a key role in the amplification of some oncogenes during tumor progression.[5] Based on coincident chromosomal positions of some fragile sites and cancer specific chromosomal alterations, it was suggested that fragile sites could be involved in generation of cancer specific chromosome breaks.[6]

Initially, no lung tumor suppressor gene was believed to be at band 3p14.2. Many had given up the idea of a kidney cancer tumor suppressor there because of the notion that the kidney cancer-associated familial break in 3p14.2 was just a way of losing the *VHL* gene.[7] Clear cell kidney cancers, however, almost always involve loss of a combination of different loci on chromosome 3; so, loss of *VHL* does not preclude involvement of *FHIT*.[8] The definitive experiment to determine whether the remaining *FHIT* allele in the familial tumors is altered or the Fhit protein inactivated has not been done because the tissue has not been available.

The *FRA3B* fragile site researchers remained interested in 3p14.2 and contributed a number of genomic maps, markers and insights that facilitated characterization of the *FHIT* gene and locus. Because fragile sites coincide with chromosome bands nonrandomly altered in specific types of cancer, there may be oncogenes or suppressor genes at fragile sites that are targets of alteration and clonal selection in carcinogenesis.[6] Thus the *FHIT* gene, encompassing the t(3;8) translocation, a human papillomavirus (HPV) integration site from a cervical cancer, numerous 3p breaks in somatic cell hybrids, plasmid integration sites, the entire *FRA3B*, and numerous hemizygous and homozygous deletions in cancer cells, was a candidate tumor suppressor gene at a fragile site that was frequently altered in cancers.[2,4] In fact, until markers within the *FHIT* gene were tested, many deletions within 3p14.2 would have been undetectable, because both the hemizygous and homozygous deletions can be much smaller than other common regions of loss of heterozygosity (LOH) and the chromosome can appear unaltered cytogenetically (Figure 1).

• FHIT *alteration is a very early event in lung*

**KAY HUEBNER, PHD; GABRIELLA SOZZI, PHD; CHARLES BRENNER, PHD; MARCO A. PIEROTTI, PHD**

Dr. Huebner is professor and laboratory head, and Dr. Brenner is assistant professor and laboratory head at the Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia. Dr. Sozzi is professor and laboratory head, and Dr. Pierotti is professor and director of the division of experimental oncology A at the National Tumor Institute in Milan, Italy.

*cancer*. More than 80% of SCLCs are negative for Fhit protein expression, as are most preneoplastic lesions.[9,10] Fhit inactivation is significantly more frequent in lung cancers of smokers than nonsmokers and is a more frequent alteration than *p53* mutation.

Sozzi and colleagues,[9,11] who previously linked cigarette smoking with lung cancer, demonstrated that loss of Fhit protein occurs for molecular markers in lung tumors at the highest frequency reported thus far, reaching nearly 90% in SCC and preneoplastic lesions. Loss of Fhit occurs at the earliest clinically detectable stage of neoplasia. Thus, analysis of Fhit expression in biopsies or cytologic bronchial specimens could be useful for early detection. Tomizawa and associates[10] concluded Fhit loss correlated with squamous histology, smoking, and poor survival in early-stage NSCLC and Fhit expression would be a useful marker for stage I NSCLC that might respond to more aggressive treatment.

Although differing in many important respects, the 2 studies agree on important points. First, there is a significantly higher proportion of Fhit-negative SCC among smokers. Second, Fhit loss correlates with undifferentiated morphology, particularly in ADC. Both studies see a role for assessment of Fhit expression in lung cancer screening programs, and both show that the *FHIT* gene is a sensor of smoke-related carcino-

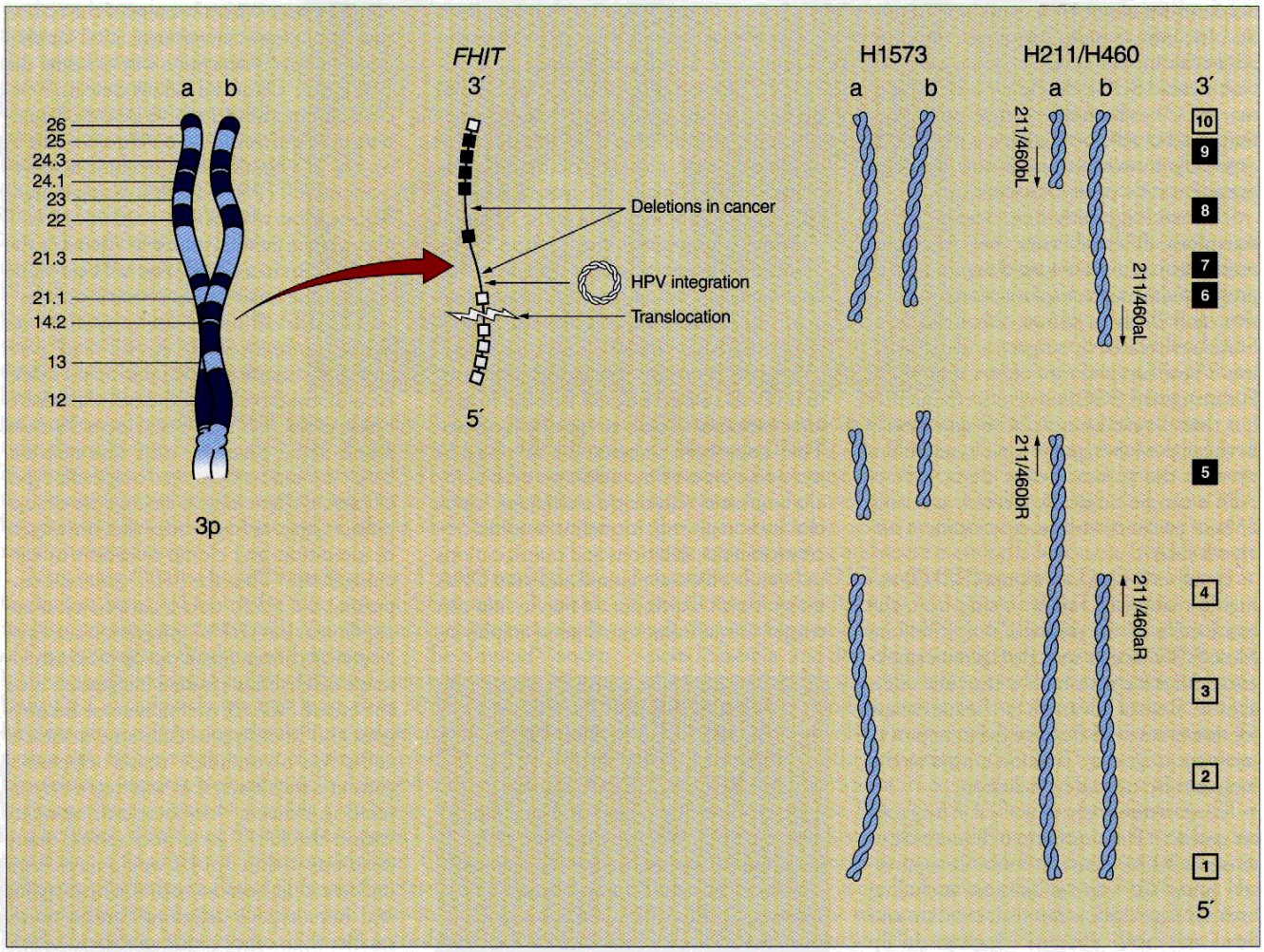



**Figure 1.** *The* FHIT *gene at chromosome region 3p14.2 encompasses* FRA3B, *which is the most fragile region of the human genome, and numerous chromosomal alterations observed in cancer cells. The 3p14.2 band of chromosome 3 contains the* FHIT *gene, which is about 1 megabase. This region is highly susceptible to carcinogen-induced breakage in all individuals and when damaged can lead to deletions, insertions, and translocations. The chromosome alterations frequently lead to loss of portions of the Fhit protein encoding sequence and consequent loss of expression of the Fhit protein. (HPV, human papillomavirus.)*

genic damage leading to Fhit loss. Compensation for Fhit loss or elimination of cells missing Fhit could be important goals in prevention and therapy trials.

Loss of heterozygosity of 3p14.2 alleles occurs in the nonneoplastic airway cells of smokers and persists in the lungs of former smokers. Mao and colleagues[12] tested 54 current and former smokers without cancer for LOH at 3 loci within or near candidate lung tumor suppressor genes (3p14, 9p21, 17p13) in DNA microdissected from bronchial biopsies. Current smokers tended to have a higher frequency of overall LOH than former smokers (82% *v* 62%), but the difference was not significant, meaning that nearly two thirds of former smokers harbored clonal genetic alterations in their bronchial epithelia. Current smokers, however, had a significantly higher frequency of LOH at 3p14 than former smokers ($P$=.01). In addition, a lower frequency of LOH at 3p14 in former smokers was linked to reversal of squamous metaplasia following smoking cessation.

Wistuba and associates[13] obtained biopsy specimens from a similar group of smokers, former smokers, and nonsmokers without cancer. Histologically normal and abnormal specimens were assessed for LOH on 5 chromosomal arms with 15 microsatellite markers. Among smokers and former smokers, 86% showed LOH in at least 1 biopsy specimen, while none of the nonsmokers' specimens did. About half of the normal specimens from smokers showed LOH, which was more frequent at 3p and 9p than at the other loci. This study found no significant differences in the frequencies or patterns of allelic loss between current and former smokers, even those who had ceased smoking for more than 10 years.

Both of these studies found that *FHIT* loss occurs in the lungs of smokers without cancer and that 3p LOH is more frequent than LOH at other involved loci. It is clear that the *FHIT/FRA3B* locus is an early and frequent target of loss in lung cancer and that the genetic damage leads to loss of the Fhit protein (See "Inactivation of *FHIT* in Lung Cancer."). Nelson and colleagues[14] went one step further and showed an association of *FHIT* exon loss with smoking duration and asbestos exposure.

• *Other genes and loci in lung cancer genetics*. The progression from normal lung epithelia to invasive neoplasia proceeds through the occurrence of morphologic lesions of various grades. Many studies have been directed toward detection of molecular changes in early and progressive lesions in order to describe the molecular history of disease progression and provide markers for early diagnosis and risk assessment. These studies have culminated in preliminary multistep models of lung carcinogenesis.[15-18]

Most studies agree that 3p LOH is the earliest change detected in hyperplasia, followed closely by 9p LOH and the onset of aneuploidy (chromosomal instability) in the dysplastic lesions. Frequently, 17p13 LOH, *p53* mutation, and *ras* mutation occur in carcinoma in situ (Figure 2). Some studies find that *HER2/neu* overexpression also occurs before squamous metaplasia or hyperplasia.[17] *myc* Overexpression, *Rb1* inactivation, telomerase activation, and *Bcl2* overexpression are also observed at the carcinoma in situ stage. At least 1 target of the 3p LOH in up to 100% of lung cancers is the *FHIT* gene; the *CDKN2* locus is the likely target of the 9p losses. 3p LOH and aneuploidy are the 2 most characteristic events in lung cancer, occurring in nearly 100% of cases.

**Assessment of *FHIT* gene alterations**

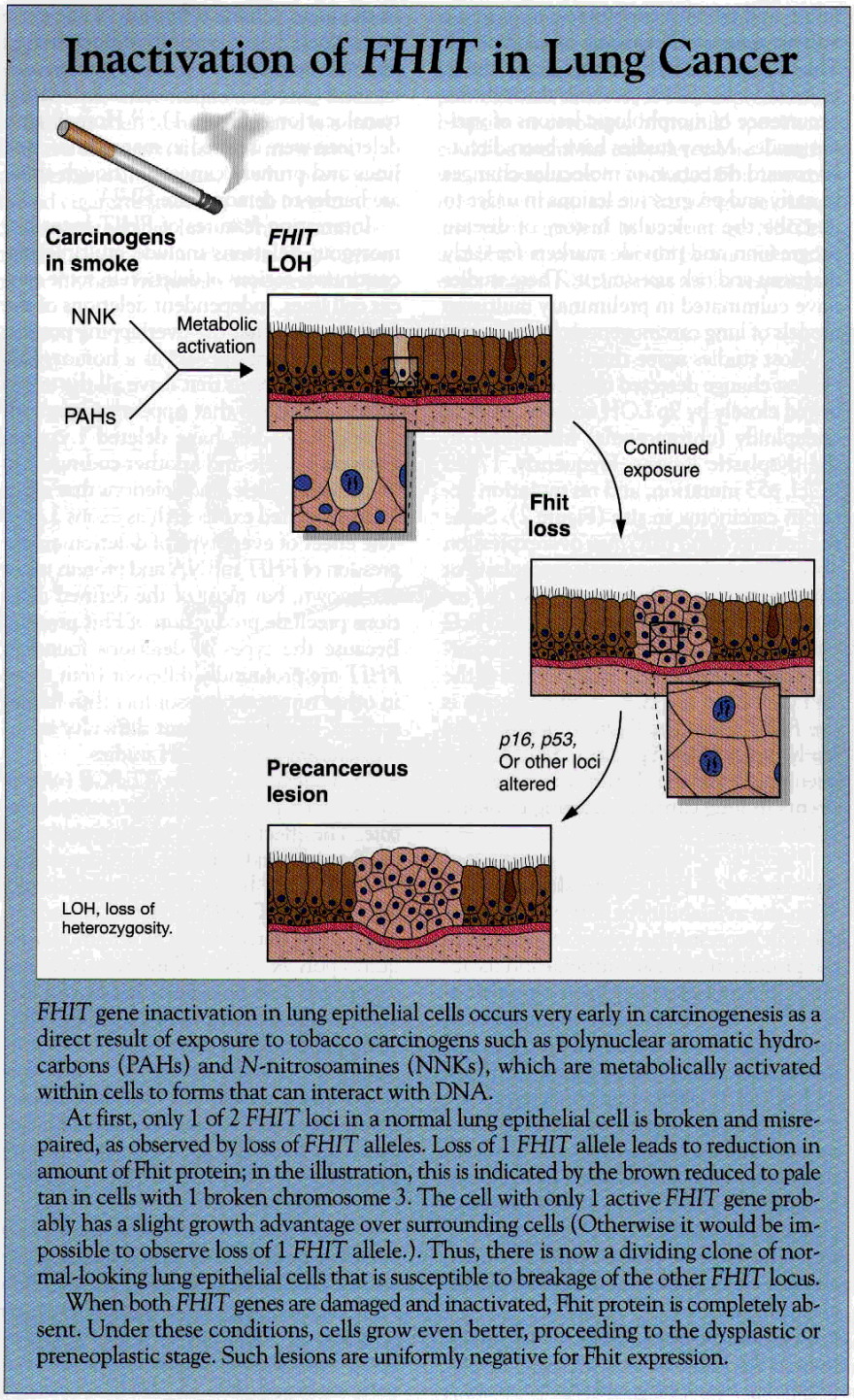
With the availability of antiserum against the Fhit protein, the robust expression of the protein in normal epithelia and its absence in neoplasias have been demonstrated.[19-25] Absence of a protein in cancer cells, however, does not necessarily prove a role in development of the cancer. To prove such a link, it is important to demonstrate the mechanism of inactivation of the gene product and show that the cells in which the inactivation has occurred have clonally expanded in the preneoplastic and neoplastic cells. Ideally, it should be possible to show the biologic process through which absence of the protein contributes to clonal expansion.

• *DNA alterations*. Chromosomal alterations in the *FHIT* locus in cancer cells contributed to the cloning and characterization of the gene. Characterization of the gene and sequencing of portions of it facilitated discovery and definition of many more cancers and cancer cell lines with deletions and translocations (Figure 1).[2,26] Homozygous deletions were defined in many cancer cell lines and primary cancers although these are harder to demonstrate.[4,14,27-33]

Interesting features of *FHIT* locus homozygous deletions include multiple discontinuous regions of deletion in some cancer cell lines, independent deletions of the 2 alleles such that the overlapping portion of the deletions appears as a homozygous deletion, deletions that leave all the exons intact, deletions that appear to spare all coding exons but have deleted 1 coding exon on 1 allele and another coding exon on the other allele, and deletions that affect 5′ untranslated exons such as exons 3 or 4. The effect of every type of deletion on expression of *FHIT* mRNA and protein is not yet known, but most of the defined deletions preclude production of Fhit protein. Because the types of deletions found in *FHIT* are profoundly different from those in other tumor suppressor loci thus far described, they may present difficulty in interpretation even in LOH studies.

• *RNA alterations and the RT-PCR (reverse transcription-polymerase chain reaction) debate*. The effect on level of transcription of the frequent and complex deletions within the *FHIT* gene has not been systematically assessed. *FHIT* mRNA is not abundant, and routine detection by Northern blot requires poly $A^+$ RNA. Thus, relatively few cancer cell lines have been assessed for level of *FHIT* transcripts by Northern blotting, and the levels have been almost uniformly low or undetectable.[4,26,34,35] It is likely that many cancer cell lines and cancers barely express *FHIT* mRNA, probably because the locus has been damaged in ways that affect level of transcription, maturation, or stability of mRNA.

Because poly $A^+$ mRNA is usually not available from primary cancers, most studies of *FHIT* expression have been by RT-PCR methodology (that is, reverse transcription of small amounts of RNA followed by PCR amplification of the cDNA). Early studies of *FHIT* cDNA in cancer cell




*FHIT* gene inactivation in lung epithelial cells occurs very early in carcinogenesis as a direct result of exposure to tobacco carcinogens such as polynuclear aromatic hydrocarbons (PAHs) and *N*-nitrosoamines (NNKs), which are metabolically activated within cells to forms that can interact with DNA.

At first, only 1 of 2 *FHIT* loci in a normal lung epithelial cell is broken and misrepaired, as observed by loss of *FHIT* alleles. Loss of 1 *FHIT* allele leads to reduction in amount of Fhit protein; in the illustration, this is indicated by the brown reduced to pale tan in cells with 1 broken chromosome 3. The cell with only 1 active *FHIT* gene probably has a slight growth advantage over surrounding cells (Otherwise it would be impossible to observe loss of 1 *FHIT* allele.). Thus, there is now a dividing clone of normal-looking lung epithelial cells that is susceptible to breakage of the other *FHIT* locus.

When both *FHIT* genes are damaged and inactivated, Fhit protein is completely absent. Under these conditions, cells grow even better, proceeding to the dysplastic or preneoplastic stage. Such lesions are uniformly negative for Fhit expression.

lines were designed to look for point mutations in primary cancers; what they showed was a significant frequency of primary cancers that exhibited short *FHIT* RT-PCR products relative to products from matched normal tissues.[4] Because similar short RT-PCR *FHIT* products were found in many cancer cell lines with defined homozygous deletions, this was taken as evidence that *FHIT* alleles in the primary cancers had suffered deletions.

Impressive evidence has accumulated that *FHIT* alterations in cancer are important, but a number of laboratories have reported that RT-PCR products in specific tumor types look completely normal and therefore *FHIT* is not involved (in spite of high frequency *FHIT* LOH) and short RT-PCR products are widely observed in normal and tumor cells and thus are not cancer specific or relevant to cancer.[2,26,35] There are several possible reasons. First, some laboratories have used isotopically labeled RT-PCR products and autoradiographic exposures to detect the products. These methods lead to detection of minor products.[30,36] Second, because the *FRA3B* is the most inducible of the common fragile regions, it is likely that many normal tissues include a few cells that have sustained damage to the *FHIT* gene, possibly producing a low level of aberrant products. Thus, while the RT-PCR assay has been extremely useful in some laboratories, in suggesting which tumor types are likely to exhibit altered Fhit expression, it has pitfalls, and results must be interpreted with caution.

• *Point mutations*. Very few point mutations in the *FHIT* open reading frame have been observed.[14,37-39] Polymorphisms have been observed, but most of those in the protein coding region cause no amino acid change, suggesting the Fhit protein may not tolerate amino acid sequence divergence. Polymorphisms in untranslated exons and in introns have been observed but not assessed for an effect or expression on splicing.

The major route for inactivation of the *FHIT* gene is probably through carcinogen-induced double-strand breaks and misrepair. It will be of great interest to examine types of damage to the *FHIT* locus and other fragile regions in the repair deficient can-

cers associated with mutations in mismatch and double-strand break repair genes.[25]

**Suppression of tumorigenicity by Fhit**

Several studies have explored the ability of the Fhit gene product to suppress growth of cells and tumors. Siprashvili and colleagues[19] transfected 4 cancer cell lines lacking endogenous Fhit with a *FHIT* expression vector and isolated Fhit-expressing clones, most of which grew as well as the parental control cell in 1% and 10% fetal bovine serum. Each of the Fhit-expressing clones was suppressed for growth of tumors in nude mice. While it may be concluded that Fhit does not affect growth of cells in tissue culture, this is not necessarily valid because the investigators were selecting for constitutive Fhit expression compatible with cell growth.

The conclusion can only be tested using a conditional expression vector with Fhit turned off during selection of colonies or by efficient transient transfection with a *FHIT* vector. It was difficult to isolate Fhit-expressing clones from each of the transfected cancer cell lines. Very few of the colonies picked (as few as 1 in 40 for some transfections) expressed detectable Fhit, suggesting that in fact overexpression of Fhit may be selected against in tissue culture.

In ongoing studies of the stably transfected lung cancer clones, H460/*FHIT*, expressing exogenous Fhit protein, a significant fraction of the cells have been observed to undergo apoptosis at each subdivision. This suggests that in these lung cancer clones the overexpression of Fhit may affect a proapoptotic pathway.

Otterson and associates[40] transfected a HeLa cell line that lacked Fhit expression and tested tumorigenicity of a clone expressing Fhit. Tumors were observed in the nude mice, but the immunohistochemistry findings suggested that Fhit was not expressed in most of the tumor (that is, apparently Fhit expression was being lost with growth of the tumor). We have also isolated HeLa cell clones expressing exogenous Fhit and have tested their tumorigenicity. Tumors that appeared after injection of parental cells or transfected clones were excised, fixed, and tested for expression of

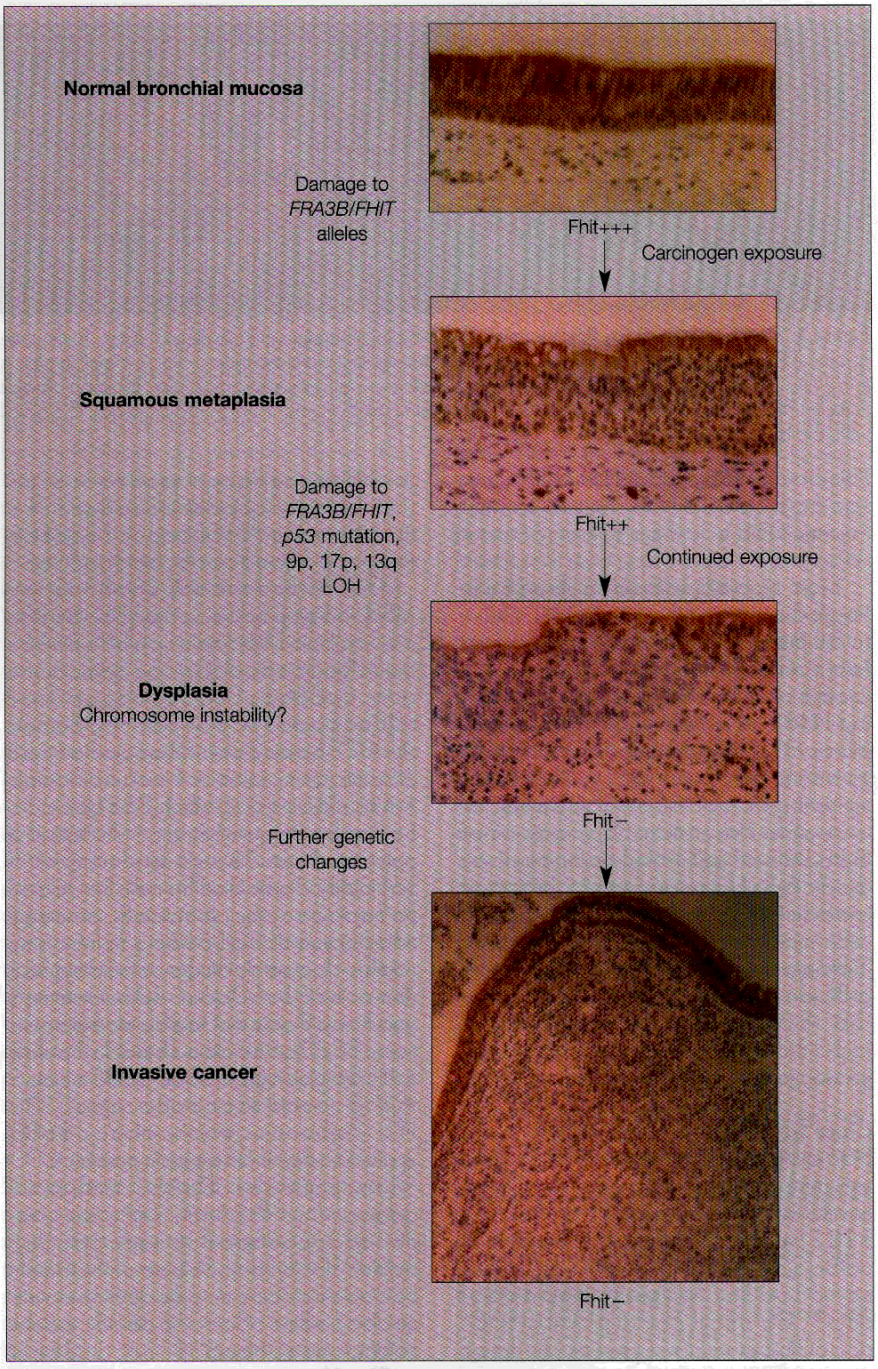



**Figure 2.** *Early morphologic lesions on the path to lung cancer. Fhit expression was assessed in progressive bronchial lesions and correlated with our interpretation of molecular events that occur during the morphologic progression. (LOH, loss of heterozygosity.) (Photographs reprinted with permission from Sozzi et al.[9])*

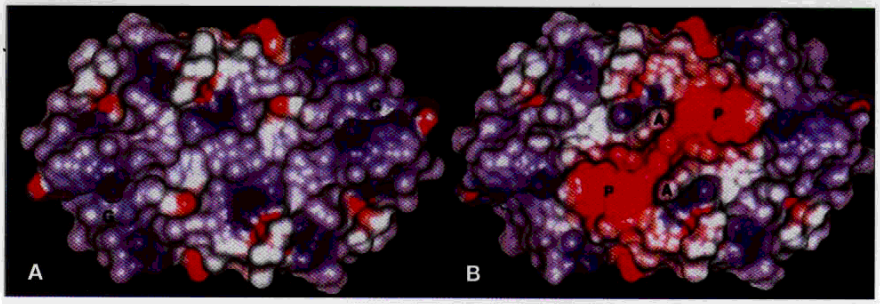



**Figure 3.** *The Fhit protein dimer. Binding dinucleoside polyphosphate changes its shape and charge. In the empty form of Fhit, the negatively charged surfaces of a computer-generated electrostatic surface potential are shown in red* (**A**). *When the groove is filled by binding of 2 diadenosine polyphosphate molecules, the dimer changes to become the active signaling form of Fhit* (**B**). *The bright red blobs marked P are phosphate groups. (Reprinted with permission from Pace et al.*[44])

Fhit protein. Three tumors lost Fhit expression in the majority of cells, and 1 tumor expressed Fhit in perhaps 40% of the tumor cells.

Most recently, a study by Ji and colleagues[41] used an adenovirus *FHIT* vector to study the effect of Fhit expression on lung cancer cells. Some of the same lung cancer cell lines used in our studies, including H460, were infected with the adenovirus *FHIT* vector and analyzed for cell growth in vivo and in vitro, with appropriate testing for expression and enzymatic activity of the Fhit protein. Overexpression of Fhit inhibited cell growth in human lung cancer cells but not in normal bronchial epithelial cells, with fewer than 20% of cells surviving at 7 days after infection. Tumorigenicity of the infected lung cancer cells was eliminated. The conclusion was that the *FHIT* gene, when delivered at high efficiency, functions as a tumor suppressor gene in vitro and in vivo.

### Clues to biologic function of Fhit

Fhit is a member of a recently discovered superfamily of nucleotide-binding proteins termed Histidine Triad (HIT).

• *HIT proteins*. Proteins in this superfamily utilize a distinct protein fold and 3 conserved histidine residues to bind nucleotides.[42] The initial clue to potential function of Fhit protein was provided by detection of 69% similarity to the enzyme diadenosine tetraphosphate ($Ap^4A$) hydrolase from the yeast *Schizosaccharomyces pombe*.[4] Several lines of evidence indicate that the tumor-suppressing function of Fhit may depend on Fhit acting as the cellular receptor for dinucleoside polyphosphates.

One of the most well-known nucleotide-binding proteins is Ras p21, which is activated in a high proportion of human tumors. Ras is activated by oncogenic mutations that lead to diminished guanosine triphosphatase (GTPase) activity. Because the GTP-bound form of Ras stimulates cell division, these mutations are transforming.

• *Nucleotides and signaling*. Fhit appears to be regulated by binding to diadenosine polyphosphates. Though these compounds are readily cleaved by Fhit and Fhit-related proteins into the commonly occurring adenosine mononucleotides, a mutant form of Fhit that binds diadenosine polyphosphates well but cleaves them extremely poorly was functional in a tumor suppressor assay.[19,43] This indicates that it is binding with, not cleavage of, a nucleotide substrate that generates a molecular signal. The Fhit–diadenosine polyphosphate complex appears to signal for tumor suppression.

• *Structural studies*. The structural consequences when Fhit forms a substrate complex were investigated by x-ray crystallography.[44] To stabilize what would normally be a transient enzyme-substrate intermediate, key features of both the enzyme and the substrate were altered.[45,46]

The Fhit dimer in its empty form contains a positively charged cleft that is filled by 2 diadenosine polyphosphate substrates, converting the concave electropositive surface to a bulging electronegative surface (Figure 3).[44] GTP-binding by Ras causes that protein to undergo a conformational change that mediates binding of Ras-GTP to mitogenic effectors. In the Fhit system, because presentation of the dinucleoside polyphosphate substrates is the most striking change in protein appearance, we hypothesized that the tumor-suppressing effectors of Fhit recognize the nucleotide-bound surface and that an effector binding to Fhit-substrate complexes may stabilize these complexes, sustaining the active (signaling) form of Fhit.

• *Fhit expression in tissues*. Assessment of the tissue specificity by Northern blot and RT-PCR analysis suggested that *FHIT* RNA was expressed in most, if not all, tissues at a low level.[4] During immunohistochemical analysis of expression of Fhit in human cancers, we have also observed the pattern of expression in the normal cells within lung, stomach, pancreas, breast, kidney, colon, and oral cavity.[9,23,24,33,34] Others have studied uterine cervix, kidney, and lung.[10,20,47]

In each of these organs, Fhit is strongly detected in the epithelial cells lining the tubular or ductal structures, as if the protein has an important function in epithelial cell shape, structure, polarity, or secretion—functions that involve cell-cell communication and interaction. Stromal cells within those organs either do not express or express much less Fhit. The cells of lung squamous metaplasia and dysplasia, which show reduced Fhit expression, show alterations in cell shape and cell interactions.

• *The murine* Fhit *gene*. From our very early studies of the murine *Ptprg* gene, now known to be near the 5′ end of the *Fhit* gene, we knew the locus was on mouse chromosome 14 near the centromere, in a region already known to be altered by deletions and translocations in murine tumors.[48,49] We also observed a homozygous deletion in the 5′ end of *Ptprg* in the commonly used murine L cell line established in the 1940s by a protocol involving carcinogen treatment of mouse cells. These observations raised the questions: Is the mouse *Fhit* locus fragile? And will murine

cancer cell lines also show deletions within *Fhit*? If so, mice could be used as a model system to understand the interactions among carcinogens, *Fhit* fragility, Fhit protein loss, and murine cancers.

We have isolated and characterized the murine *Fhit* gene, determined its chromosomal location, and studied its expression in normal and tumor-derived cell lines.[50] In parallel, Glover and associates[51] have also isolated a murine *Fhit* cDNA and genomic clones and have assessed the fragility of the locus. The cDNA and structure are similar to those of the human gene, with exons 5 through 9 encoding the protein. The gene is fragile and murine cancer cell lines show homozygous deletions within *Fhit*.

We have now tested primary carcinogen-induced murine lung and mammary carcinomas for expression of Fhit by immunohistochemistry and have found a large fraction of both tumor types negative for Fhit expression. The results were similar to those we have found for human lung and breast cancers. It will be interesting to analyze the effects of known carcinogens on induction of alterations at the *Fhit* gene in murine lung cancers. Such studies will complement ongoing analyses of alteration of the human *FHIT* gene in lung and other cancers.

• *NitFhit, the invertebrate Fhit, is a fusion protein*. Clues to protein function can also be turned up by studying the gene and protein in invertebrate model organisms, especially now that some of their genomes have been entirely sequenced. Recently, we cloned and characterized *Fhit* genes from *Drosophila melanogaster* and *Caenorhabditis elegans* and found that both genes encode fusion proteins in which the Fhit domain is fused with a novel domain showing homology to bacterial and plant nitrilases.[52]

In human and mouse, the nitrilase homologues and Fhit are encoded by 2 different genes, *FHIT* and *NIT1*. The tissue specificity of expression of murine *Fhit* and *NIT1* genes is nearly identical. Because fusion proteins with dual or triple enzymatic activities have been found to carry out specific steps in a given biochemical or biosynthetic pathway, we postulate that Fhit and Nit1 proteins collaborate in a biochemical or cellular pathway.[52] Structural, biochemical and cellular experiments are in progress to define the interactions between Nit and Fhit and to elucidate their consequences in tumor suppression.

## Diagnostic and therapeutic implications

• FRA3B *sequence alterations*. We have been especially interested in the instability and recombinogenicity of the *FRA3B* in cancer cells and have used the complete sequence of the central part of the *FHIT* gene to define exact end points of homozygous deletions in cancer cells and to isolate and sequence those end points.[29] Sequence analysis of more than thirty cancer cell-associated deletion end points has shown that this locus is a frequent target of homologous recombination between LINE repetitive elements, resulting in *FHIT* gene internal deletions secondary to carcinogen-induced damage at the *FRA3B* fragile region. These studies lend support to the long-standing hypothesis that chromosome fragile sites may be "weak links" in the human genome, contributing to cancer susceptibility.

Because *FRA3B* is constitutively fragile, it might be supposed that this locus would be equally vulnerable to carcinogen breakage in all individuals. It is possible, however, that there are polymorphisms within *FRA3B*, perhaps at repeat clusters or regions of high flexibility or other identifiable sequences, that could cause variations in susceptibility to carcinogen damage, variations in ability to repair fragile gaps, or variations in likelihood of HPV integration and that could contribute to the individual risk of cancer. Identification of such polymorphisms will be possible with the availability of the complete sequence of *FHIT/FRA3B*.

• *Fhit protein*. Assessment of the level of Fhit protein in biopsy or cytologic specimens from smokers and former smokers might help to reinforce smoking cessation programs but could also be part of a strategy to assess high risk patients for prevention regimens. If the correlation of Fhit positivity with survival holds up in larger studies, especially for nonsmokers with adenocarcinoma, then detection of Fhit might be a useful prognostic factor in this subpopulation. Lung cancer cell death due to Fhit overexpression after infection with an adenovirus *FHIT* vector suggests therapeutic approaches through delivery of the *FHIT* gene into preneoplastic lesions or tumors.[41]

## Conclusion

There is no doubt that early skepticism of the *FHIT* gene as a tumor suppressor affected progress in understanding the function of Fhit.[53-55] This negative response was partly because Fhit wasn't p16; that is, it was not obviously involved in cell cycle or growth regulation. Many characteristics of normal cells, however, are altered in cancer cells, especially cancers arising from epithelium. These include cell shape, cytoskeleton, cell movement, polarity, cell-cell interactions, and cell-environment interactions. Alterations in some of these characteristics are among the earliest changes observed in squamous metaplasia.

Loss of heterozygosity, particularly at band 3p14.2, occurs in the nonneoplastic airway cells of smokers and persists in the lungs of former smokers. More than 80% of squamous cell lung carcinomas have completely lost expression of the Fhit protein at band 3p14.2, as have most preneoplastic lesions. Because Fhit is lost very early in lung carcinogenesis, before the onset of chromosomal and genetic instability, it is likely that treatment strategies aimed at eliminating Fhit-negative cells may be useful. Moreover, delivery of the *FHIT* gene, in a suitable vector, may eliminate the genetically damaged cells from high-risk individuals at the preneoplastic stage or even before. ■

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