Published in final edited form as:

Cancer Lett. 2010 May 28; 291(2): 230–236. doi:10.1016/j.canlet.2009.10.017.

Fhit loss in lung preneoplasia: relation to DNA damage response checkpoint activation

Roberto Cirombella^a, Giuseppe Montrone^b, Antonella Stoppacciaro^a, Simona Giglio^a, Stefano Volinia^c, Paolo Graziano^b, Kay Huebner^{c,*}, and Andrea Vecchione^{a,c,*}

^a Division of Pathology, II University of Rome “La Sapienza”, Ospedale Santo Andrea, Rome, Italy

^b Division of Pathology, Ospedale San Camillo Forlanini, Rome, Italy

^c Department of Molecular Virology, Immunology and Medical Genetics and Comprehensive Cancer Center, Ohio State University, OH, USA

Abstract

Loss of heterozygosity at the FHIT locus is coincident with activation of DNA damage response checkpoint proteins; thus damage at fragile loci may trigger checkpoint activation. We examined preneoplastic lesions adjacent to non-small cell lung carcinomas for alterations to expression of Fhit and activated checkpoint proteins. Expression scores were analyzed for pair-wise associations and correlations among proteins and type of lesion. Hyperplastic and dysplastic lesions were positive for nuclear H2AX expression; 12/20 dysplastic lesions were negative for Fhit expression. Fhit positive lesions showed expression of most checkpoint proteins examined, while Fhit negative lesions showed absence of expression of Chk1 and phosphoChk1. The results show that loss of expression of Fhit is significantly directly correlated with absence of activated Chk1 in dysplasia, and suggest a connection between loss of Fhit and modulation of checkpoint activity.

Indexing terms

Fhit; Preneoplastic lesions of lung; DNA damage response checkpoint; chromosome fragile sites; squamous dysplasia

1. Introduction

Common chromosomal fragile sites are preferential targets of replication stress in preneoplastic lesions, resulting in deletions involving fragile genes encoded at these conserved chromosome regions [1]. It has been suggested that damage to fragile sites, with lost function of genes located at these sites, may play a significant role in carcinogenesis [2]. Expression of the tumor

*Correspondence should be addressed to: Kay Huebner, Ohio State University Comprehensive Cancer Center, Biomedical Research Tower, Rm 916, 460 W. 12th Ave., Columbus, OH 43210, ph 614-292-4850; Fax: 614-688-8675; kay.huebner@osumc.edu or Andrea Vecchione, Division of Pathology, II University of Rome “La Sapienza”, Ospedale Santo Andrea, Rome, Italy; andrea.vecchione@osumc.edu.

Conflict of interest statement

The authors have no relevant affiliation or financial involvement with any organization or entity with a financial interest in or conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, or patents received or pending, or royalties.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

suppressor proteins, Fhit and Wwox, encoded at the most active fragile loci FRA3B (3p14.2) and FRA16D (16q23.3), is lost coordinately in breast cancers [3] and Fhit expression is frequently lost in preneoplastic lesions of the lung [4–6], esophagus, stomach, cervix, and other organs [7–10], suggesting that loss of Fhit expression, due to the susceptibility of FHIT/FRA3B to carcinogen damage, plays a role in initial stages of multi-step carcinogenesis.

Precancerous cells undergo selective pressure to escape from the cell cycle block induced by checkpoint responses to DNA damage. Studies of hyperplasias and dysplasias [11–13] of skin and lung showed that from early dysplastic stages, an ataxia telangiectasia mutated (ATM)-regulated DNA damage response network is activated, delaying or preventing cancer; the ataxia telangiectasia and Rad3-related (ATR)-regulated damage response was not examined in these earlier studies. Allelic loss within the FHIT gene is coincident with these events in early lesions [11,12]. Mutations compromising this checkpoint would allow cell proliferation and tumor progression, leading to more DNA replication stress and increased genomic instability. Recently we reported that the FHIT tumor suppressor gene encodes a protein that is necessary for protecting cells from accumulation of DNA damage through its role in modulation of checkpoint proteins [14–16], suggesting the paradox that the FHIT locus is a target of DNA damage due to replication stress and encodes a protein that itself is necessary for the integrity of the DNA damage response. Introduction of exogenous Fhit into cells *in vitro* led to up-modulation of expression of checkpoint proteins Hus1 and Chk1 at mid-S checkpoint, a modulation that led to induction of apoptosis in esophageal cancer cells but not in normal cell cultures. The results suggested that the DNA damage-susceptible FRA3B/FHIT-encoded Fhit protein is necessary for protecting cells from accumulation of DNA damage through its role in modulation of checkpoint proteins [14]. Yutori et al [16] assessed association between phosphorylated Chk2 (pChk2) and Fhit expression in oral squamous cell carcinoma (OSCC). Loss of Fhit expression was an early event during oral carcinogenesis and a decrease in the number of pChk2-positive cells was associated with tumor progression. When OSCC cells were exposed to ionizing radiation, pChk2 levels were upregulated and knockdown of Fhit diminished the ionizing radiation-induced Chk2 phosphorylation. Furthermore, Fhit-deficient mice demonstrated a decrease in the number of pChk2-positive cells in some dysplastic lesions and in N-nitrosobenzylamine-induced papilloma of the forestomach. The findings suggested that Fhit plays a role in regulating the ATM–Chk2 DNA damage response during oral carcinogenesis.

To better understand this phenomenon, we studied the expression of the Fhit protein and its genomic status in preneoplastic lesions of the lung in relation to the status of proteins involved in the ATM and ATR checkpoints. Exposure to internally or externally generated stresses, such as hypoxia, UV, ionizing radiation, or carcinogens in cigarette smoke, cause cellular DNA damage. In normal cells, double-strand and single-strand DNA breaks lead to the activation of checkpoint responses and cell cycle arrest in the G1/S and G2/M phases, or to apoptosis. The phosphatidylinositol 3-kinase-related serine/threonine protein kinases ATM and ATR, are key proteins controlling DNA-damage checkpoint responses [17,18]. ATM responds to ionizing radiation-induced DNA double-strand breaks (DSBs), leading to activation by autophosphorylation [17] and ATR to DNA adducts and stalled replication forks [18]. Downstream targets of ATM and ATR, Chk2 and Chk1 respectively, play pivotal roles in regulation of the G1/S and G2/M checkpoint responses [19–21]. Interestingly, expression of ATR and Chk1 is essential for protection of the integrity of the common fragile site loci and thus absence of expression of one of these proteins leads to increased activation of fragile sites and the concomitant accumulation of deletions and rearrangements at these unstable genomic loci [22,23].

Lung cancer is the leading cause of cancer deaths worldwide and global case numbers are set to rise [24]. Despite advances in therapy, the frequency of survival for lung cancer patients

remains only 15% [25], likely due to the relatively advanced stage of disease at diagnosis. In fact, screening trials have not had an impact on survival; screening has been able to detect small (2–3 mm in size), asymptomatic nodules, but even these nodules may be malignant, and therefore late in the course of disease [26]. If preneoplastic lesions can be better defined, early detection methods combined with methods to prevent progression might significantly improve survival. To define preneoplastic lesions, we need to understand the molecular events that occur during bronchogenic carcinogenesis. Carcinogenesis is thought to be a multistep process consisting of the accumulation of gene mutations. Preneoplastic lesions may be morphological phenotypes of the different steps in this progression from normal to malignant tissue. We are just beginning to identify the presumed preneoplastic lesions in the bronchial epithelium [27]. The World Health Organization recently published a tumor classification system that defines preneoplastic lesions of the bronchial epithelium: squamous dysplasia and carcinoma *in situ*, referred to here as low grade and high grade squamous dysplasia (DL and DH), which may be precursors to squamous cell carcinoma; atypical adenomatous hyperplasia (AAH), which may be the progenitor lesion for adenocarcinoma. Additional possible preneoplastic lesions and conditions include squamous metaplasia (progressing to DL and DH) and adenomatous hyperplasia (progressing to AAH).

Gorgoulis et al [11] and Barkova et al [12] have performed systematic analyses of precancerous and cancer lesions of lung and other cancers to determine when during cancer development a DNA damage response checkpoint is detectable. Activation of the DNA damage response was detected by monitoring expression of phosphorylated histone H2AX (H2AX), phosphorylated Chk2 (pChk2) and other proteins, by immunohistochemical staining of fixed tissues. In these studies, the hyperplasias showed signs that a DNA damage checkpoint pathway had been activated. In the dysplasias and cancers there were also signs of a DNA damage response, shown by 53BP1 localization and H2AX and pChk2 expression. The results suggested that a DNA damage checkpoint is activated in preneoplastic lesions. The conclusions of these studies were that in precancerous lesions, replication stress, leading to formation of DNA DSBs, activates a DNA damage checkpoint, which in turn induces cell cycle arrest or apoptosis. Authors proposed that tumor suppressor loci would eventually be targeted, releasing the cells from the suppressive effects of the DNA damage checkpoint and leading to tumor progression [11–13]. To illustrate that the cells within the lesions had undergone replication stress that could have led to DNA DSBs, investigators showed that there was allelic loss specifically at the fragile FHIT/FRA3B locus in the preneoplastic lesions [11,12]; chromosome fragile regions are known to be highly susceptible to formation of small deletions due to replication stress [1,28].

Since the FHIT/FRA3B locus was damaged in the lesions examined, damage to this fragile locus may itself cause activation of the checkpoint [15,22] and subsequent loss or reduction of expression of the haploinsufficient Fhit tumor suppressor, may contribute to alteration or inactivation of checkpoint responses [14,15], allowing lesion progression. To examine the relation between Fhit expression and checkpoint activation, we have focused on metaplasias, hyperplasias and dysplasias found in lung tissue sections near NSCLCs.

2. Materials and Methods

2.1. Tissues

After Institutional Review Board approval of this study, we examined fixed lung cancer sections from numerous archived blocks, in order to find cases with concomitant preneoplastic lesions. We then examined 15 hyperplastic lesions (9 subcolumnar hyperplasia and 6 micropapillary hyperplasia), 15 metaplastic lesions and 20 dysplastic lesions of the lung (squamous dysplasias: 4 DL, 5 DH and 11 AAH. All lesions were adjacent to NSCLCs and contained normal epithelium as an internal control. Among the hyperplastic lesions, the

micropapillary are benign, reactive lesions, while for subcolumnar hyperplasias, there is some evidence that they could be preneoplastic. Normal adjacent cells were also examined for expression of the selected proteins. In order of abnormality then, the lesions might be ranked as follows: normal <metaplasia <hyperplasia (micropapillary <subcolumnar) <dysplasia (AAH and squamous dysplasia, with DL < DH).

2.2. Immunohistochemistry and Immunofluorescence

Routine deparaffinization of all sections (2 µm thick) mounted on positive charge slides was carried out according to standard procedures [29], followed by rehydration through an ethanol series. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase in methanol for 30 min. The sections were then incubated with the appropriate antiserum at the indicated dilutions: anti-H2AX (Ser 139; 1:100; Upstate); anti-53BP1 and anti-Chk2 (hybridoma supernatants, 1:20); anti-pChk2 (Thr 68; 1:100; Cell Signaling Technology); anti-pATM (1:200, Rockland), anti-Chk1 (1:50 Abcam, Carboxyterminal end), anti-pChk1 (1:100, Bethyl), anti-Fhit (1:1000, Zymed Laboratories) overnight at room temperature. Primary antiserum was omitted and replaced by PBS for negative controls. After this incubation, sections were treated with biotinylated rabbit or mouse antiserum and streptavidin-biotin-peroxidase (Histostain-SP Kit; Zymed Laboratories). Antibody localization was detected with diaminobenzidine as a chromogen substrate. Finally, sections were washed in distilled water and weakly counterstained with Harry's modified Hematoxylin. Immunostaining was classified in the following two groups: negative, 0–10% of cells stained, or positive, staining was detected in >10% of the cells.

Immunofluorescence staining for detection of 53BP1 was performed as described for immunohistochemistry. Anti-FITC secondary antibody was diluted 1:1000 and placed on slides for 1 hr at rm temp. Nuclei were colored with Hoechst 33258. Immunofluorescence-labeled cells were examined using a confocal laser-scanning microscope (Leka TSC2) or a Leica epifluorescent microscope equipped with filters that distinguish between FITC and Hoechst fluorescence. Images were acquired using a digital camera and the Leica IM1000 software.

2.3. FISH analysis

For fluorescence *in situ* hybridization (FISH) analysis at the FHIT locus, we used the ZytoLight SPEC FHIT/CEN 3 Dual color Probe following the manufacturer's instructions (Zyto Light, Bremerhaven, Germany). Briefly 10 µl of prewarmed dual color probe was applied on each sample covered with a coverslip and denatured at 77 C on a hot plate. Slides were then transferred to a hybridization oven and hybridized overnight at 37 C followed by various washing steps. FISH analysis was also performed on formalin fixed, paraffin embedded 293T, which retain and express FHIT loci, as control for detection of concurrent presence of 3cen and 3p14.2 signals.

2.4. Statistical analysis

Fisher's Exact test was used to analyze the association between two biomarkers. Then Holm's step-down procedure was used to adjust the Fisher's Exact P-values, due to the fact that multiple pairwise associations were tested simultaneously [30]. P<0.05 was considered to be statistically significant. Binary logistics regressions and Ordinal regressions were also used to assess correlations among pairs of protein markers.

3. Results

3.1. Expression of Fhit in specific lesions

Table I lists the frequency of Fhit reactivity in the various types of lesions, as well as the frequency of detection of H2AX in the same lesions. Fhit immunostaining was observed in normal lung tissue (Fig. 1, for examples) and in the 15 squamous metaplastic lesions examined (Table I, for summary). In hyperplastic lesions we observed loss of Fhit protein expression in 22% of subcolumnar hyperplasias (2 of 9), while in the micropapillary hyperplasias the protein was consistently expressed (Table I). The modulation of Fhit expression changed dramatically in dysplastic lesions; results showed that Fhit protein was lost in 25% of DL, in 80 % of DH and in 64% of AAH examined (Fig. 1, for examples, and Table I). We also examined expression of the other frequently altered fragile tumor suppressor, Wwox, in these lesions but did not observe loss of Wwox expression in preneoplastic lesions of the lung.

3.2. Expression of DNA damage response proteins in preneoplastic lesions

Occurrence of double-strand DNA breaks or DNA replication fork-stalling in cells can be monitored by assessment of expression of phosphorylated histone H2AX (H2AX) [31]. In the normal lung epithelium and in the metaplastic lesions, lack of nuclear H2AX expression was consistent with the absence of DNA damage. However, three cases of subcolumnar hyperplasia showed evidence of DNA damage documented by nuclear expression of H2AX (Table II). All dysplastic lesions, precursors of adenocarcinoma or squamous cell carcinoma, showed expression of H2AX, a sign that DNA DSBs or replication fork-stalling had occurred in each of these lesions, in accord with earlier studies [11,12]

The DNA damage response network, signal transduction pathways consisting of sensors, transducers and effectors, is composed of phosphoinositide kinase-related proteins, including the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia related (ATR) genes that, in response to DNA damage, phosphorylate key proteins, checkpoint kinases Chk2 or Chk1; the phosphorylated forms of ATM (pATM), Chk2 (pChk2), ATR (pATR) and Chk1 (pChk1) are the activated forms.

Investigation of the unphosphorylated forms of ATM and Chk2 showed expression of the proteins in all cases examined. Next we examined the expression of the activated forms of these checkpoint proteins. Results, summarized in Tables II and III, showed no activation of ATM or Chk2 in squamous metaplasia or papillary hyperplasia (not shown), whereas three of nine cases of subcolumnar hyperplasia showed expression of activated ATM and Chk2 (pATM and pChk2) (see Table II). We also examined the ATR pathway phosphorylation target, Chk1 and its activated form, pChk1, though we were unable to examine expression of ATR itself due to lack of appropriate antisera for immunohistochemical staining. We did not detect expression of Chk1 or pChk1 in any metaplastic or hyperplastic lesions, including subcolumnar lesions (Table II).

3.3. Checkpoint protein expression in dysplasias

Eleven precursor lesions for adenocarcinoma of the lung, AAH lesions 1–11, and nine squamous cell carcinoma precursor lesions, four low grade (DL 1–4) and five high grade (DH 1–5) dysplasias, were also stained for expression of H2AX and the ATM and ATR pathway proteins. Nuclear H2AX was detected in all twenty dysplasias, as was Chk2, while pChk2 was detected in 8/11 AAHs and all but 1 squamous dysplasia. Activated ATM (pATM) was expressed in 4 of 5 DHs, all 4 DLs and 4 of 11 AAH lesions. Chk1 and activated Chk1 (pChk1) expression showed identical patterns of expression in the dysplastic lesions with 4 of 11 AAH, all DL and 1 DH dysplasias showing expression. The expression pattern for Fhit was nearly

identical to the Chk1/pChk1 expression profile (Table III for summary, Fig. 1 for examples of staining).

Pair-wise comparisons of expression patterns of these proteins, as described in methods, showed a statistically significant direct correlation of Fhit and Chk1/pChk1 ($p=0.0001$) expression. There was also a significant direct association between Fhit and pATM expression ($p=0.04$) and significant association between H2AX and Chk2/pChk2, which are all expressed in most of the dysplasias, and between H2AX and pATM.

In the normal epithelium 53BP1 did not show discrete nuclear foci, while from hyperplastic to dysplastic lesions the nuclear distribution changed, as shown in Fig. 1, reflecting its recruitment to nuclear foci [11,12].

To assess possible molecular evolution between dysplastic lesions and invasive carcinoma of the lung, we compared expression of the DNA damage response proteins in the two types of lesions. Results of immunohistochemical analysis of the cancers concurrent with the dysplasias showed very similar patterns of expression.

3.4. Loss of heterozygosity (LOH) at the FHIT locus

Dual-color FISH was performed using DNA probes for bands 3p14.2 (FHIT locus; the FHIT genomic locus covers 3p14.2 almost completely, without presence of other genes) and chromosome 3 centromere. In all specimens, FHIT deletions were not found in normal epithelium, 15/15 metaplastic lesions or 6/6 micropapillary hyperplasia samples. However, FHIT hemizygous deletions were observed in 2/9 (22%) subcolumnar hyperplasias, 6/11 (64%) AAHs, 1/4 (25%) DL, and 4/5 (80%) DH dysplasias (data not shown). Just one case of AAH showed a homozygous deletion. Concordance was observed between FHIT deletion status and the overall cellular Fhit protein expression levels, as assessed by immunohistochemistry; ie, each lesion that showed evidence of deletion of at least one FHIT allele, also showed absent or very reduced Fhit expression.

4. Discussion

Loss of Fhit expression in NSCLCs, normal bronchial mucosa and preinvasive lesions was previously found to be a frequent alteration in precancerous lesions, was significantly higher in tumors of smokers than of nonsmokers, and was an independent and more frequent event than p53 over-expression [4]. Also, chromosome 3p allele loss is one of the most frequent and earliest genetic events in lung cancer pathogenesis [6]; FHIT allele losses reportedly occurred in 96% of the cancers and 78% of the preinvasive lesions, were often multiple and discontinuous, with areas of LOH interspersed with areas of retention of heterozygosity, as might be expected for a genomic region affected by DNA damage due to replicative stress. It has also been shown that absence of Fhit expression does not always correlate with loss of FHIT alleles but can be due to silencing through epigenetic mechanisms or combinations of LOH and promoter methylation [32]. In this study we have observed loss of FHIT alleles in most dysplastic lesions, in concordance with reduced or lost Fhit expression. But since most of the lesions showed hemizygous loss of a FHIT allele, we do not know the mechanism of complete loss of Fhit protein expression. It is possible that small deletions within the FHIT locus would not be detected with the commercial probe used.

In this study we were most interested in the relation between loss of Fhit protein expression and activation of the DNA damage response checkpoint. Checkpoint proteins are activated in preneoplastic and neoplastic lesions of skin, lung and breast and activation of checkpoints may be barriers to cancer progression until mutations in checkpoint genes allow growth of cells through the checkpoint in spite of DNA damage, leading to genome instability and cancer

progression. LOH at the FHIT locus was concomitant with activation of the ATM checkpoint [11,12]. We hypothesized that DNA breakage at the fragile gene loci may be involved in triggering DNA damage responses; paradoxically, loss of expression of the FHIT gene due to this breakage alters the DNA damage response checkpoint, since Fhit is involved in normal responses to DNA damage [14–16]. Since lung preneoplastic lesions show loss or reduction of Fhit protein, cells within the lesions are likely to have defective DNA damage response checkpoints, increasing the likelihood that the Fhit negative dysplastic lesions will progress along a path to malignancy.

Loss of Fhit expression concomitantly with absence of Chk1/pChk1 in 11 of 12 dysplasias, but not with pATM, suggests that the ATR/Chk signal pathway is already altered in dysplasia, though this pathway was activated in the earlier lesions. This is in accord with their more advanced stage in progression and suggests that dysplasias have already broken through at least some checkpoint barriers to progression and are likely to progress further. This is also consistent with the pathological designation of DL and DH dysplasias as high and low grade since the high grade squamous dysplasias each showed loss of pChk1, while the low grade dysplasias did not. The schema shown in Fig. 2 shows our interpretation of the molecular evolution of the dysplastic lesions. Clearly, other interpretations are possible. We looked for a biological effect of loss of pChk1 expression by assessing the lesions for differences in frequency of apoptotic cells in different types of lesions by TUNEL assay (not shown), but did not find differences, possibly because of the small sample size. It would be useful to design a larger study of lung lesion biopsies, in order to seek insights into biological differences between the Fhit/pChk1 positive and the Fhit/pChk1 negative lesions. It is possible that by treatment with specific checkpoint response inhibitors, such as PARP1 inhibitors, it might be possible to inhibit progression of preneoplastic lesions to the dysplastic stage, since such inhibitors might preferentially cause apoptosis in the lesions with activated checkpoints, as shown for Brca1/2 deficient cells [33].

Acknowledgments

Supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC) and by grants, CA132453 and CA115965, from the National Cancer Institute of the USPHS. We acknowledge the expert assistance of the OSUCCC Biostatistics Shared Resource for statistical analyses.

The work in the laboratory of KH is partially supported by a grant from the National Cancer Institute of the US Public Health Service for the study of Fhit function.

Abbreviations

Fhit	fragile histidine triad protein
Wwox	WW-domain and oxidoreductase containing protein
H2AX	gamma histone 2AX
ATM	ataxia telangiectasia gene, ATR, ataxia telangiectasia related gene
Chk1/2	checkpoint kinase proteins 1 and 2
53BP1	p53 binding protein 1
NSCLC	non-small cell lung cancer
AAH	atypical adenomatous hyperplasia
DSBs	double-strand breaks
CT	computer tomography scanning

FISH	fluorescent <i>in situ</i> hybridization
LOH	loss of heterozygosity

References

1. Tsantoulis PK, Kotsinas A, Sfrikakis PP, Evangelou K, Sideridou M, Levy B, Mo L, Kittas C, Wu XR, Papavassiliou AG, Gorgoulis VG. Oncogene-induced replication stress preferentially targets common fragile sites in preneoplastic lesions. A genome-wide study. *Oncogene* 2008;27:3256–3264. [PubMed: 18084328]
2. Thavathiru E, Ludes-Meyers JH, MacLeod MC, Aldaz CM. Expression of common chromosomal fragile site genes, WWOX/FRA16D and FHIT/FRA3B is downregulated by exposure to environmental carcinogens, UV, and BPDE but not by IR. *Mol Carcinog* 2005;44:174–182. [PubMed: 16187332]
3. Guler G, Uner A, Guler N, Han SY, Iliopoulos D, Hauck WW, McCue P, Huebner K. The fragile genes, FHIT and WWOX, are inactivated coordinately in invasive breast carcinoma. *Cancer* 2004;100:1605–1614. [PubMed: 15073846]
4. Sozzi G, Pastorino U, Moiraghi L, Tagliabue E, Pezzella F, Ghirelli C, Tornielli S, Sard L, Huebner K, Pierotti MA, Croce CM, Pilotti S. Loss of FHIT function in lung cancer and preinvasive bronchial lesions. *Cancer Res* 1998;58:5032–5037. [PubMed: 9823304]
5. Tseng JE, Kemp BL, Khuri FR, Kurie JM, Lee JS, Zhou X, Liu D, Hong WK, Mao L. Loss of Fhit is frequent in stage I non-small cell lung cancer and in the lungs of chronic smokers. *Cancer Res* 1999;59:4798–4803. [PubMed: 10519387]
6. Wistuba II, Behrens C, Virmani AK, Mele G, Milchgrub S, Girard L, Fondon JW 3rd, Garner HR, McKay B, Latif F, Lerman MI, Lam S, Gazdar AF, Minna JD. High resolution chromosome 3p allelotyping of human lung cancer and preneoplastic/preinvasive bronchial epithelium reveals multiple, discontinuous sites of 3p allele loss and three regions of frequent breakpoints. *Cancer Res* 2000;60:1949–1960. [PubMed: 10766185]
7. Kitamura A, Yashima K, Okamoto E, Andachi H, Hosoda A, Kishimoto Y, Shiota G, Ito H, Kaibara N, Kawasaki H. Reduced Fhit expression occurs in the early stage of esophageal tumorigenesis: no correlation with p53 expression and apoptosis. *Oncology* 2001;61:205–211. [PubMed: 11574776]
8. Zhao P, Liu W, Lu YL. Clinicopathological significance of FHIT protein expression in gastric adenocarcinoma patients. *World J Gastroenterol* 2005;11:5735–5738. [PubMed: 16237777]
9. Butler D, Collins C, Mabruk M, Leader MB, Kay EW. Loss of Fhit expression as a potential marker of malignant progression in preinvasive squamous cervical cancer. *Gynecol Oncol* 2002;86:144–149. [PubMed: 12144820]
10. Connolly DC, Greenspan DL, Wu R, Ren X, Dunn RL, Shah KV, Jones RW, Bosch FX, Muñoz N, Cho KR. Loss of fhit expression in invasive cervical carcinomas and intraepithelial lesions associated with invasive disease. *Clin Cancer Res* 2000;6:3505–3510. [PubMed: 10999736]
11. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 2005;434:907–913. [PubMed: 15829965]
12. Bartkova J, Horejsi Z, Koed K, Krämer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Ørntoft T, Lukas J, Bartek J. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 2005;434:864–870. [PubMed: 15829956]
13. Bartek J, Bartkova J, Lukas J. DNA damage signaling guards against activated oncogenes and tumour progression. *Oncogene* 2007;26:7773–7779. [PubMed: 18066090]
14. Ishii H, Mimori K, Inoue H, Inageta T, Ishikawa K, Semba S, Druck T, Trapasso F, Tani K, Vecchione A, Croce CM, Mori M, Huebner K. Fhit Modulates the DNA Damage Checkpoint Response. *Cancer Res* 2006;66:11287–11292. [PubMed: 17145874]
15. Pichiorri F, Ishii H, Okumura H, Trapasso F, Wang Y, Huebner K. Molecular parameters of genome instability: roles of fragile genes at common fragile sites. *J Cell Biochem* 2008;104:1525–1533. [PubMed: 18393361]

16. Yutori H, Semba S, Kumori T, Yokozaki H. Restoration of fragile histidine triad expression restores Chk2 activity in response to ionizing radiation in oral squamous cell carcinoma cells. *Cancer Sci* 2008;99:524–530. [PubMed: 18167129]
17. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J Biol Chem* 2003;278:12207–12213. [PubMed: 12519769]
18. Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL, Friend SH. Overexpression of a kinaseinactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. *EMBO J* 1998;17:159–169. [PubMed: 9427750]
19. Ahn JY, Schwarz JK, Piwnica-Worms H, Canman CE. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Res* 2000;60:5934–5936. [PubMed: 11085506]
20. Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* 1998;282:1893–1897. [PubMed: 9836640]
21. Helt CE, Cliby WA, Keng PC, Bambara RA, O'Reilly MA. Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. *J Biol Chem* 2005;280:1186–1192. [PubMed: 15533933]
22. Durkin SG, Arlt MF, Howlett NG, Glover TW. Depletion of CHK1, but not CHK2, induces chromosomal instability and breaks at common fragile sites. *Oncogene* 2006;25:4381–4388. [PubMed: 16732333]
23. Arlt MF, Durkin SG, Ragland RL, Glover TW. Common fragile sites as targets for chromosome rearrangements. *DNA Repair (Amst)* 2006;5:1126–1135. Review. [PubMed: 16807141]
24. Rom WN, Hay JG, Lee TC, Jiang Y, Tchou-Wong KM. Molecular and genetic aspects of lung cancer. *Am J Respir Crit Care Med* 2000;161:1355–1367. [PubMed: 10764334]
25. Alberts WM. Introduction: Diagnosis and Management of Lung Cancer: ACCP Evidence-Based Clinical Practice Guidelines (2nd Edition). *Chest* 2007;132:20S–22S. [PubMed: 17873157]
26. Patz EF Jr, Goodman PC, Bepler G. Screening for lung cancer. *N Engl J Med* 2000;343:1627–1633. [PubMed: 11096172]
27. Wistuba II, Gazdar AF. Lung cancer preneoplasia. *Annu Rev Pathol* 2006;1:331–348. [PubMed: 18039118]
28. Durkin SG, Ragland RL, Arlt MF, Mulle JG, Warren ST, Glover TW. Replication stress induces tumor-like microdeletions in FHIT/FRA3B. *Proc Natl Acad Sci U S A* 2008;105:246–251. [PubMed: 18162546]
29. Indinnimeo M, Giarnieri E, Stazi A, Cicchini C, Brozzetti S, Valli C, Carreca I, Vecchione A. Early stage human colorectal cancer: prognostic value of nm23-H1 protein overexpression. *Cancer Lett* 1997;111:1–5. [PubMed: 9022121]
30. Holm S. A simple sequentially rejective multiple test procedure. *Scand J Statist* 1979;6:65–70.
31. Stucki M, Jackson SP. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst)* 2006;5:534–543. [PubMed: 16531125]
32. Verri C, Roz L, Conte D, Liloglou T, Livio A, Vesin A, Fabbri A, Andriani F, Brambilla C, Tavecchio L, Calarco G, Calabrò E, Mancini A, Tosi D, Bossi P, Field JK, Brambilla E, Sozzi G. EUELC Consortium, Fragile histidine triad gene inactivation in lung cancer: the European Early Lung Cancer project. *Am J Respir Crit Care Med* 2009;179:396–401. [PubMed: 19096006]
33. Lord CJ, Ashworth A. Targeted therapy for cancer using PARP inhibitors. *Curr Opin Pharmacol* 2008;8:363–369. Review. [PubMed: 18644251]

Fig. 1.

Representative micrographs illustrating expression of the DNA damage response related proteins in preneoplastic lesions adjacent to NSCLCs. Top row: normal bronchial epithelium showing strong Fhit expression, absence of DSBs or replication fork damage indicated by lack of H2AX expression and lack of expression of DNA damage response associated proteins. Second row: in a Fhit negative AAH, nuclear H2AX is expressed, demonstrating recognition of DNA damage; pChk2 is also expressed, indicating activation of the ATM/Chk2 checkpoint pathway, and 53BP1 is expressed in nuclear foci. Third row: A Fhit positive AAH showing activation of the ATM/Chk2 and the Chk1 pathways, as well as nuclear foci of 53BP1. Fourth row: A Fhit negative DH with H2AX expression and abortive activation of the ATM pathway, lacking evidence of Chk2 or Chk1 activation and showing only an occasional cell with 53BP1 foci. Fifth row: A Fhit negative DL showing evidence of activation of the ATM/Chk2 pathway but not the Chk1 checkpoint, and some cells with large 53BP1 foci.

Fig. 2.
Hypothetical sequence of molecular events in progression of high grade lung dysplasia.

Table I

Fhit and H2AX expression in all lesions

	Fhit			H2AX			
	Positive	Negative	Total	%Fhit-	P ^a	Positive	Negative
Metaplasia	15	0	15	0	0.006 *	0	15 *
Microapillary	6	0	6	0	0.18	0	6
Subcolumnar	7	2	9	22	0.3	3	6
AAH	4	7	11	64	0.66	11	0 *
DL	3	1	4	25	0.99	4	0
DH	1	4	5	80	0.52	5	0

P values were calculated by the Fisher Exact Probability Test (two-tailed); p< or = to 0.05 was considered statistically significant *.

Table II

Fhit and checkpoint proteins in hyperplastic lesions

Subcolumnar	Fhit	H2AX	Chk2	pChk2	pATM	Chk1	pChk1
A	+	+	+	+	+	-	-
B	+	+	+	+	+	-	-
C	+	+	+	+	+	-	-
D	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-
F	+	-	-	-	-	-	-
G	+	-	-	-	-	-	-
H	+	-	-	-	-	-	-
I	+	-	-	-	-	-	-

For subcolumnar hyperplasia, there is some evidence that these lesions are preneoplastic. Indeed, several of the subcolumnar lesions were negative for Fhit expression (D, E), several others showed an activated Atr/Chk1 checkpoint (A–C), while lesions F–I exhibited the expression pattern observed for normal and metaplastic tissues.

Table III

Fhit and checkpoint proteins in dysplasias

Diagnosis	Fhit	H2AX	Chk2	pChk2	pATM	Chk1	pChk1
AAH-7	+	+	+	+	+	+	+
AAH-8	+	+	+	+	+	+	+
AAH-3	+	+	+	+	+	+	+
AAH-4	+	+	+	-	-	+	+
AAH-1	-	+	+	+	+	-	-
AAH-2	-	+	+	+	-	-	-
AAH-9	-	+	+	+	-	-	-
AAH-10	-	+	+	+	-	-	-
AAH-11	-	+	+	+	-	-	-
AAH-5	-	+	+	-	-	-	-
AAH-6	-	+	+	-	-	-	-

DL-4	+	+	+	+	+	+	+
DL-2	+	+	+	+	+	+/-	+/-
DL-3	+	+	+	-	+	+	+
DL-1	-	+	+	+	+	+	+

DH-1	+	+	+	+	+	+/-	+/-
DH-2	-	+	+	+	+	-	-
DH-3	-	+	+	+	+	-	-
DH-5	-	+	+	+	+	-	-
DH-4	-	+	+	+	-	-	-