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Fragile histidine triad protein expression in nonsmall cell lung cancer and correlation with Ki-67 and with p53

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ABSTRACT: Fragile histidine triad (FHIT) is a tumour suppressor gene, which is altered in a variety of epithelial tumours, including lung cancer. Biochemical and functional pathways of its tumourigenicity are not yet understood. Its role in tumour proliferation is particularly controversial. The purpose of this study was to correlate the expression of FHIT protein in nonsmall cell lung cancer (NSCLC) with tumour proliferation as estimated by Ki-67 antigen and with p53, a suppressor gene.

FHIT, Ki-67 and p53 expression were evaluated by immunohistochemistry in 119 resected NSCLC.

Altogether, 58 tumours were negative (expression <10%) for FHIT. The median expression in tumours was 15% positive cells, in comparison with 100% in normal matched lung tissue. The expression was as strong as in normal tissue in only 19 cases. FHIT expression was significantly lower in squamous cell carcinoma (SCC) (5%) than in adenocarcinoma (ADC) (64%). The median expression of Ki-67 was 20% and 69% of tumours were positives (expression >10%). Ki-67 expression was significantly higher in SCC (33.3%) than in ADC (10%). The loss of FHIT protein was not correlated with the expression of p53 (median: 7.5%, 58% of positive tumours for a cut-off of 10% of positive cells) or Ki-67. But percentage of labelled cells for p53 and Ki-67 were significantly correlated.

The results suggest that for fragile histidine triad, the pathway of tumourigenesis is independent of p53 and of tumoural proliferation, as reported previously *in vitro*.

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Fragile histidine triad (FHIT) is a nonclassical tumour suppressor gene encompassing the most fragile site of the human genome, FRA3B. FHIT, located on the short arm of chromosome 3, at locus 3p14.2, is homologous to a family of genes characterised by a histidine triad. Abnormalities in this gene are among the most common genetic changes occurring in human cancers. Alteration of FHIT transcript, loss of heterozygocity of the FHIT gene by deletion and loss of the FHIT protein occur in a variety of epithelial tumours including lung cancer [1], head and neck tumours [1, 2], oesophageal, stomach and colon cancers [3], pancreatic carcinoma [4], breast cancer [5], cervical [6] and endometrial [7] carcinoma, renal carcinoma [8], bladder tumours [9] and hepatocellular carcinoma [10].

In lung cancer, loss of FHIT has been documented in 80–100% of small cell lung cancer and in 40–80% of nonsmall cell lung cancer (NSCLC). Reduction of FHIT expression is more important in squamous cell carcinoma (SCC) than in adenocarcinoma (ADC) and in smokers than in nonsmokers [1]. Loss of FHIT does not seem to correlate with the prognosis of lung cancer and already occurs in preneoplastic lesions [1, 11, 12].

Biochemical and functional pathways of FHIT tumourigenicity are not yet understood. FHIT is a diadenosine triphosphate (Ap3A) hydrolase, but the role of this enzyme in suppressing tumours is still unclear. Some recent trials suggest that FHIT could play a role in apoptosis, but this is perhaps not its only biological function. The role of FHIT protein in intrinsic cellular proliferation remains particularly controversial. *In vitro*, FHIT expression does not seem to alter the growing cell [13, 14], but these trials were inconclusive, since the FHIT-expressing clones were selected for constitutive FHIT expression. In contrast, *in vivo* studies performed in mice [14] suggest that FHIT expression strongly affects the ability of tumoural cells to grow. Based on those considerations, the present study was undertaken to correlate the FHIT protein expression using immunohistochemistry, in a series of resected NSCLC with a tumoural proliferation index (estimated by Ki-67 expression). FHIT expression was also correlated with patient characteristics and another tumour suppressor gene, p53.

Material and methods

Tissue specimens

Invasive NSCLC (n=119) was resected by wedge resection, lobectomy or pneumectomy at the Dept of Surgery, St Peter Hospital, January 1993–July 2000. Normal matched lung tissues were also assessed.

Immunohistochemical studies

Antibodies. For FHIT [14, 15], a polyclonal rabbit antibody anti-GST-FHIT (dilution 1 in 25, final titration 40 $\mu g \cdot mL^{-1}$,

ZR44; Zymed, San Francisco, CA, USA), with cytoplasmic staining, was used. Tumour proliferation was studied with a murine monoclonal antibody (clone MIB-1) to Ki-67 antigen [16], a human recombinant peptide corresponding to a 1002 bp Ki-67 complementary deoxyribonucleic acid (cDNA) fragment (dilution 1 in 50, final titration 1 $\mu g \cdot mL^{-1}$, Immunotech, Marseille, France). p53 expression was assessed by a mouse monoclonal antibody, MS 186 R7 [17], to suppressor protein Ab-5 (clone DO-7) (dilution 1 in 50, final titration 1.9 $\mu g \cdot mL^{-1}$, Neomarkers, Union City, CA, USA). Ki-67 and p53 staining were unclear.

Staining. After surgical resection, all tissues were fixed in 10% neutral buffered formalin and routinely embedded with paraffin. Immunohistochemistry was performed according to a standard streptavidin-biotin-peroxidase complex [18]. Sections of 5 μm-thickness were cut from paraffin-embedded tissues, deparaffinised in xylene and rehydrated in ethanol. The slides were submitted to antigen retrieval in citric buffer, 0.01M pH 6.0, consisting in three periods of 5 min of microwave treatments at 650 W. After recooling at room temperature for 20 min, slides were rinsed twice in tris-hydroxymethyl-amino-methane HCl buffer (Tris-HCl 0.05 M, NaCl 0.09%, pH 7.6, Tris-HCl buffer) for 10 min. All the following steps were followed automatically at 37°C in the NexES system (Ventana Medical Systems, Tucson, AZ, USA). The endogenous peroxydases were quenched with hydrogen peroxide for 4 min. The primary antibodies were deposited and incubated for 30 min. The complexes between the FHIT, p53 or Ki-67 proteins and their respective antibodies were fixed with glutaraldehyde and 0.9% NaCl. The secondary biotinylated antibody was incubated for 8 min. The slides were then stained using a diaminobenzidine tetrahydrochloride (DAB) detection kit (Ventana Medical System), counterstained with haematoxylin and mounted with permount.

Negative controls for the three antibodies were carried out by omitting the primary antibody and substituting normal mouse (for p53 and Ki-67) and rabbit (for FHIT) immunoglobulin for the primary antibody. Positive controls used an internal control, the normal lung epithelial cells for FHIT and, for p53 and Ki-67, external controls from breast tumours well known to be positive for each antibody.

Evaluation of the staining. Observers (n=3) independently evaluated the results of the immunohistochemical staining without any knowledge of the clinical data. The level of positivity was expressed as percentage (0–100%) of tumour cells in the total field of a single section showing cytoplasmic (for FHIT) or nuclear (for Ki-67 and p53) staining.

For FHIT, the tumour expression was considered as negative when the expression was <10% and positive when >10% [1]. Positivity >90% was considered to be equal to normal lung tissue. For p53, a tumour was also considered as positive when expression was >10% [19, 20].

The tumour proliferation index, as estimated by the expression of Ki-67, was expressed as a percentage of stained cells in the total field of a single section and a tumour was considered as positive when >10% of cells were immunoreactive [21, 22].

Statistical analysis

Statistical analysis was performed with nonparametric tests, since the distribution of FHIT, Ki-67 and p53 expression was non-normal. The correlation between continuous and dichotomic variables was measured using the Mann-Whitney U-test. Comparisons between the dichotomic variables were performed using corrected Chi-squared tests. The correlation between biological variables, all considered as continuous in order to avoid the problem of a subjective choice of level of positivity, was assessed by Spearman ranks correlation coefficients. Its significance was assessed by testing a null hypothesis of equality to zero of this coefficient. The median values of the distributions of the biological variables were compared according to staging, sex, age, tobacco or histology by Mann-Whitney U-test for dichotomic variables, or using the Kruskal-Wallis test for categorical variables. Survival was measured from the date of surgery. Survival distribution was estimated by the Kaplan-Meier method. Survival comparison was performed by two-sided log-rank tests. The criterion for statistical significance was $p<0.05$.

Results

The patient population consisted of 97 males (82%) and 22 females (18%) with a median age of 63 yrs (range 27–86 yrs). Histology, according to the new World Health Organisation 1998 classification [23], was ADC in 52, SCC in 59, adenosquamous cell carcinoma in two and undifferentiated large cell carcinoma in six. Seventeen patients had stage IA disease (14%), 52 stage IB (44%), four stage IIA (3%), 28 stage IIB (24%), 10 stage IIIA (8%), six stage IIIB (5%) and two stage IV (2%, due to the presence of two tumours in the same lung), according to the 1997 International Staging System [24]. There were 40 active smokers (34%), 60 former smokers (50%) and five nonsmokers (4%). Information was missing in 14 (12%). Smoking ranged 13–140 packs-yr with a median of 48 packs-yr.

The median expression of FHIT protein was 15% (range 0–100%) in NSCLC in comparison with 100% in normal matched lung tissue (fig. 1, d). Among tumours, 58 (48.7%) were negative for the FHIT protein and 61 (51.3%) positive. Only 19 (16%) of samples showed an expression as strong as normal tissue. One hundred (84%) thus had a reduced or negative FHIT expression. The loss of FHIT protein was not significantly associated with age, sex, stage or tobacco (table 1). Expression (fig. 1, e, f, g, h) was significantly lower ($p<0.001$) in SCC (median: 5%, range 0–100%) than in ADC (median: 64%, range 0–100%). The median expression of Ki-67 in NSCLC (fig. 1 a, b) was 20% (range 0–92%) and was not significantly different according to age, sex, stage or tobacco, but was higher ($p<0.001$) in SCC (33.3%, range 0–90%) than in ADC (10%, range 0–92%). Using a cut-off of 10%, 69% of the tumours were positive for Ki-67. The median expression of p53 in those tumours (fig. 1c) was 7.5% (range 0–100%) and there was no association with any patient characteristic. For p53, tumours were positive in 48% of the cases.

If the variable is considered continuous, FHIT expression (table 2) was not correlated with either Ki-67 (globally and according to histology) or p53 expression. However, p53 expression was significantly correlated with the expression of Ki-67 ($r=0.32$, $p=0.0005$). If only p53 is considered as a dichotomic variable, the results were the same, showing no significant association between FHIT and p53 expressions ($p=0.12$, Mann-Whitney U-test) but a significant correlation between p53 and Ki-67 ($p<0.0001$). If both FHIT and p53 are considered as dichotomic variables, and if FHIT is evaluated according to positive *versus* negative (cut-off 10%), and according to strong *versus* reduced as compared to normal tissue (cut-off 90%), there was no significant correlation between FHIT and p53, with p-values of 0.07 and 0.47, respectively. If FHIT, p53 and Ki-67 were each considered as dichotomic variables, there was also no significant correlation between FHIT and Ki-67 expression ($p=0.39$ and $p=0.70$,

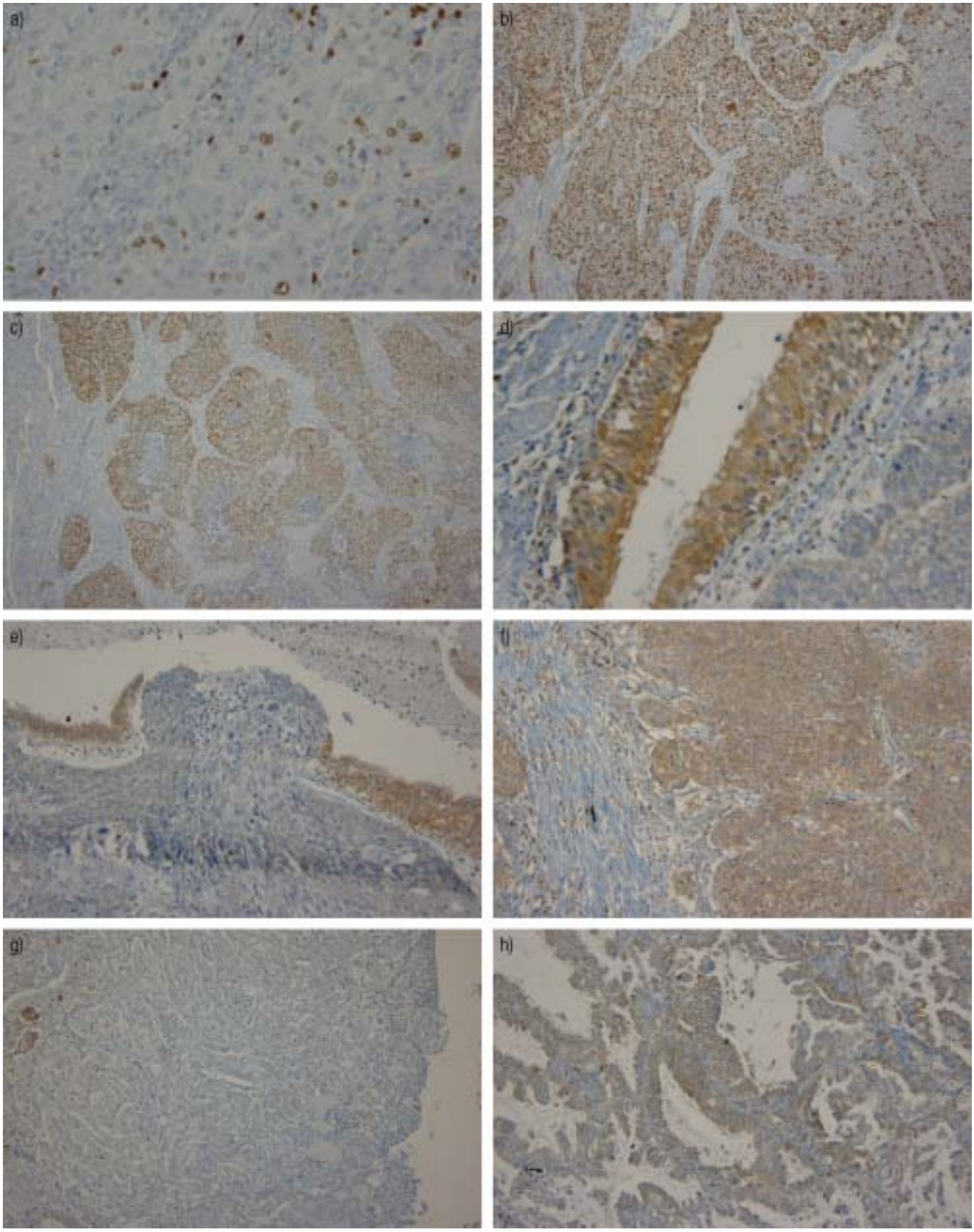

Fig. 1. – Samples with Ki-67, p53 and fragile histidine triad (FHIT) staining by immunohistochemistry. a) Adenocarcinoma (ADC) with a low nuclear staining of Ki-67. b) squamous cell carcinoma (SCC) with a strong nuclear staining of Ki-67. c) SCC with nuclear staining of p53. d) Strong cytoplasmic staining with FHIT in normal epidermal lung cell. e) Low cytoplasmic staining with FHIT in a SCC with internal control contrast. f) Strong cytoplasmic staining with FHIT in a SCC. g) Low cytoplasmic staining with FHIT in an ADC. h) Strong cytoplasmic staining with FHIT in an ADC.

Table 1. – Correlation between biological variables and patient characteristics

Patients' characteristics	Subjects n	FHIT		Ki-67		P53	
		Median	p-value	Median	p-value	Median	p-value
Sex M/F	97/22	10/25	0.18	23.33/15.83	0.45	3.75/35	0.21
Age yrs	63 (27–86)[#]		0.13[¶]		0.07[¶]		0.17[¶]
			0.18		0.42		0.08
Histology							
Squamous cell	59	5	<0.001	33.33	<0.001	25	0.24
Adenocarcinoma	52	64		10		1.9	
Stage							
I	69	20	0.77	26.67	0.71	3.75	0.92
II	32	17.5		17.5		60	
III	16	10		20		8.75	
IV	2	43.75		38.33		31.88	
I+II		20	0.61	20	0.33	5	0.79
III+IV		10		20		8.75	
Tobacco packs·yr^{-1}	48 (13–140)[#]		0.05[¶]		0.06[¶]		0.08[¶]
			0.60		0.57		0.47
Smokers	40	20	0.69	20	0.63	10	0.50
Nonsmokers	5	10		20		0	
Smokers		20	0.92	20	0.34	10	0.71
Former smokers	60	20		20		3.75	
Former smokers		20	0.68	20	0.34	3.75	0.49
Nonsmokers		10		20		0	

FHIT: fragile histidine triad. [#]: median (range); [¶]: r value.

respectively, with a cut-off of 10% and 90% for FHIT), but there was still a significant correlation between p53 and Ki-67 expressions ($p<0.001$).

The median follow-up was 28 months (range 0.3–109). Among the 119 patients, 51 were dead at the time of analysis. Survival rates at 1 and 3 yrs were 80% (95% confidence interval (CI) 72–88%) and 59% (95% CI 49–69%), respectively. The median survival was 53 months. No significant difference in terms of survival distribution according to FHIT expression (fig. 2) was observed. There were 24 and 27 dead patients with positive and negative tumours, respectively, for FHIT expression at the time of analysis.

Discussion

The results from the present study confirm the reduced expression of FHIT protein in lung cancer with a proportion similar to observations reported by others [1, 11, 12]. It also confirms a higher loss of FHIT in SCC as compared to adenocarcinoma [1, 11]. In addition, the expression of Ki-67 was significantly different according to histology, as reported previously [25, 26]. No correlation was observed between FHIT expression in NSCLC and tumour proliferation index (estimated by expression of Ki-67), nor with p53 expression, though p53 was significantly correlated with the Ki-67 expression. These results are the same irrespective of the method used to assess the expression of the different markers (as dichotomic variables or as continuous variables) showing that there was no bias due to the choice of the cut-off. This suggests a lack of interference of FHIT with cellular proliferation, as suggested previously by *in vitro* studies [13, 14], and a biological pathway of FHIT in tumourigenesis independent of tumour proliferation and of p53. One potential bias has nevertheless to be considered: Ki-67 estimates a measure of cellular proliferation index, but this index is not the only parameter determining tumour proliferation, because

Table 2. – Spearman correlation between Fragile Histidine Triad (FHIT), Ki-67 and p53 in nonsmall cell lung cancer

	FHIT	Ki-67	p53
Expression of markers median (range) %	15 (0–100)	20 (0–92)	7.5 (0–100)
FHIT			
r		-0.06	0.16
p-value		0.54	0.10
Ki-67			
r	-0.06		0.32
p-value	0.54		0.0005
p53			
r	0.16	0.32	
p-value	0.10	0.0005	

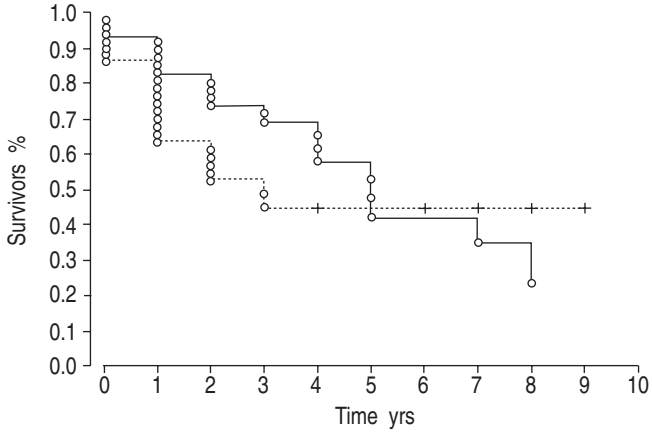

Fig. 2. – Survival according to fragile histidine triad expression. –: positive; ·····; negative.

duration of cellular cycle and cell death rate are other important factors for tumour growing [27]. Ki-67 only evaluates the intrinsic cellular proliferation, not the other two factors determining tumour proliferation. In addition, differences according to tobacco consumption were not demonstrated, though the sample of nonsmokers was too small (five patients) to be conclusive. In terms of survival, preliminary analysis did not detect any significant difference, but a longer duration of follow-up leading to a higher number of events is required to perform a definitive analysis or a larger sample size.

The choice of immunohistochemistry (IHC) to assess expression of FHIT was based on previous publications [15, 27, 28] suggesting that this method was the best way to assess the level of FHIT involvement in a clinical setting because of its high sensitivity and reproducibility as compared with Western-blot analysis or reverse transcriptase polymerase chain reaction. For Ki-67, the clone MIB-1 was chosen as an antibody to estimate the proliferation index, since, in opposition to other factors detecting Ki-67, it can be used with paraffin-embedded tissue [27].

If it is considered that FHIT does not interfere with tumoural proliferation, which other biological pathways could be implicated for this protein? An Ap3A hydrolase activity had never been previously described to suppress tumours. *In vivo*, the expression of FHIT suppresses tumourigenicity of cell lines transfected in nude mice [14]. The ability to cleave Ap3A is not required for tumour suppression [14], but the binding to Ap3A regulates the activity of FHIT and the FHIT-Ap3A complex could send the tumour-suppressor signal. Interestingly, Ap3A accumulates in cultured cells in response to cellular stress [29, 30]. The loss of FHIT is a very early event in various tumours, including bronchial preneoplastic lesions [1], breast hyperplasia [31], oral dysplastic lesions [32] and endometrial hyperplasia [7]. Conversley, FHIT does not seem to correlate with prognosis in several trials [1, 11, 12]. These two last points suggest an early role of FHIT in carcinogenesis, in the initiation of tumourigenic process rather than in the progression to the invasive and metastatic disease.

A proapoptotic function of FHIT has recently been reported by *in vitro* [33–35] and *in vivo* [33] experiments. Interestingly, apoptosis is associated with a decrease of free Ap3A level in human cultured cells [19, 36] suggesting a possible involvement of FHIT in induction of apoptosis through a bound form with Ap3A. Further investigations are required for a better understanding of these potential pathways.

In conclusion, the data from this study suggest that the pathway for fragile histidine triad to suppress tumours seems to be independent of cellular proliferation and different to the p53 pathway. These results need to be confirmed by further analysis, such as cell-cycle profile in fragile histidine triad re-expressing cells. The role of fragile histidine triad in the initiation of cancerogenesis, perhaps at the level of induction of apoptosis, should be investigated in further studies. Common implications in most epithelial tumours and alterations at the earlier stage of tumourigenesis suggest an important role of fragile histidine triad in cancer biology.

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