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ORIGINAL ARTICLE

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Reduced Fhit protein expression in human malignant mesothelioma

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Abstract Human malignant mesothelioma (MM) is an aggressive neoplasm related to occupational exposure to asbestos and characterised by a long latency time. Multiple chromosomal deletions and DNA losses have been revealed in MM by studies performed with karyotypic, comparative genomic hybridisation and loss of heterozygosity (LOH) analyses. Among frequently deleted chromosomal sites, LOH at chromosome 3p has been detected in MM, suggesting the presence of one or several tumour suppressor genes that have an important role in development of the disease. The *FHIT* (fragile histidine triad) tumour suppressor gene, located at 3p14.2, has been proposed to be a target to major human lung carcinogens, such as tobacco smoke and asbestos. Although many studies have indicated decreased Fhit protein expression in a variety of malignancies, there is no report of *FHIT* gene aberrations or Fhit protein abnormalities in MM. We examined expression of the Fhit protein and LOH at the *FHIT* gene in malignant mesothelioma. Altogether, 13 paraffin embedded MM tumours were analysed for Fhit protein expression, and 21 fresh tumours and 10 cell cultures for LOH at the *FHIT* gene with two intragenic microsatellite markers. All tumours showed less intense immunostaining than normal bronchial epithelium or mesothelium. Fhit expression was absent or reduced in 54% (7 of 13) of the tumours, with the weakest staining observed in poorly differentiated areas. Allele loss was seen in 3 of 10 (30%) of the MM cell lines, but only in 1 of the 21 fresh tumours studied, suggesting concealment of LOH by normal cells present in MM tumours. In conclusion, our present data indicate a frequent decrease of Fhit protein expression, thus supporting the significance of *FHIT* inactivation in development of MM.

Keywords Malignant mesothelioma · *FHIT* gene · Fhit protein expression · Loss of heterozygosity · Asbestos exposure

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Introduction

Human malignant mesotheliomas (MM) are aggressive tumours originating from mesothelial cells lining pleural, peritoneal and pericardial cavities [1]. Although MM is a relatively rare neoplasm, its incidence has been rising during the past decades, mainly due to increased occupational exposure to asbestos, which is the primary causal factor in the aetiology of MM [8]. A long latency between the exposure and onset of the disease is characteristic of MM, suggesting that multiple somatic genetic events are required for malignant transformation of mesothelial cells [19].

Studies performed with cytogenetic and comparative genomic hybridisation as well as loss of heterozygosity (LOH) analyses demonstrate occurrence of several genetic alterations, most commonly losses of chromosomal regions in human MM [11]. Most frequently deleted chromosomal regions are 22q and 9p [18]. Both these regions harbour a tumour suppressor gene, *NF2* (neurofibromatosis type 2) and *p16/CDKN2A*, respectively, which have been shown to be altered in many MMs [3].

Among losses at different chromosomal sites, frequent deletions of the short arm of chromosome 3 in MM have been reported [21, 32, 38]. The *FHIT* (fragile histidine triad) tumour suppressor gene [23], located at 3p14.2, has been suggested to be a target of major human lung carcinogens, such as tobacco smoke and asbestos [9]. The Fhit protein has been reported to be expressed in most non-neoplastic human tissues, and the highest levels of expression have been detected in epithelial cells. The protein is also consistently expressed in benign mesothelium [39]. Although *FHIT* abnormalities, either allele deletions or loss of protein expression, have been investigated extensively in many types of human cancer, including lung cancer [13, 22, 26, 31], there is no report, to our knowledge, of alterations in the *FHIT* gene aberration or Fhit protein expression in MM.

We examined Fhit protein expression and *FHIT* allele loss in MM tumours and cell lines. Most patients had been exposed to asbestos at work. Protein expression was decreased in all tumour samples examined relative to normal bronchus epithelium and mesothelium. Markedly reduced expression was seen in 54% of the tumours, and tumour cells with the poorest differentiation showed the weakest immunostaining. LOH of the *FHIT* gene was detected in three of ten cell lines (30%) and in one (1/21, 5%) fresh tumour sample. Frequent Fhit protein decrease suggests its involvement in the development of MM.

Materials and methods

Study population

Altogether, 26 MM cases were studied. Data for demographic, clinico-pathological and exposure characteristics of the cases are given in Table 1. The diagnosis of malignant mesothelioma was confirmed for each case by the Finnish National Mesothelioma Panel. According to occupational history and/or fibre analysis [34], 19 of 25 (76%) patients had been exposed to asbestos at work (concentration $\geq 1\times10^6$ fibres per gram dry tumour tissue), and 6 cases were non-exposed ($<1\times10^6$ fibres/g); this data was missing from 1 case. Most of the tumours represented a mixed histological type; however, all the tumours analysed for protein expression were of epithelial origin. Cultured MM cells or cell lines (10 cases) from surgically resected tumours [24] and fresh frozen tumour specimens from 21 MM cases were analysed (Table 1). Four of the cell lines had been established previously and were long term (M9 K, M10 K, M33 K and M38 K); whereas six were newly established cell cultures and examined in passages 1–4 (M138 K, M143 K, M144 K, M146 K, M150 K and M153 K). Tumour samples were stored at −70°C prior to DNA extraction. For each specimen, we had access to the corresponding normal tissue (blood or peripheral pleura) of the patient. Immunohistochemical staining for Fhit protein expression was studied in 13 paraffin-embedded tumour samples.

PCR and LOH analysis

DNA was extracted from blood, frozen fresh tumours or cell cultures using proteinase-K digestion and phenol/chloroform purification followed by ethanol precipitation. Special care was taken by the pathologist when dissecting the tumour specimen to avoid contamination by normal tissue. DNA was amplified by polymerase chain reaction (PCR) with two highly polymorphic

Table 1 Malignant mesothelioma cases and samples studies. Fresh tumour samples and cell lines were analysed for loss of heterozygosity (LOH), and paraffin-embedded samples for immunohistochemical staining. + sample analysed, − sample not analysed

Tumor no.	Age (years)	Sex	Asbestos exposure	Histology	Sample analysed for LOH or protein expression		
					Fresh tumour	Cell line	Paraffin-embedded tumour
M13	72	Male	Yes	Mixed	+	−	−
M28	54	Male	Yes	Epithelial	+	−	+
M30	46	Male	Yes	Mixed	+	−	+
M65	63	Male	Yes	Epithelial	+	−	+
M79	61	Male	Yes	Mixed	+	−	+
M91	72	Male	Yes	Epithelial	+	−	−
M92	44	Male	Yes	Mixed	+	−	−
MO3	57	Male	Yes	Mixed	−	−	+
M119	49	Male	Yes	Mixed	+	−	−
M122	51	Male	Yes	Mixed	+	−	−
M127	55	Male	Yes	Not specified	+	−	−
M129	65	Female	Yes	Not specified	+	−	−
M9	52	Male	Yes	Mixed	+	+	−
M10	52	Male	Yes	Epithelial	+	+	+
M33	42	Male	Yes	Mixed	+	+	−
M38	55	Male	Yes	Mixed	+	+	−
M138	57	Male	Yes	Mixed	−	+	−
M144	75	Male	Yes	Mixed	−	+	+
M150	59	Male	Yes	Mixed	+	+	−
M100	39	Male	No	Epithelial	+	−	+
M101	79	Male	No	Epithelial	+	−	−
M143	49	Female	No	Epithelial	+	+	+
M146	58	Male	No	Mixed	−	+	+
M153	60	Male	No	Mixed	+	+	+
M42	57	Female	No	Mixed	+	−	+
M83	No data	No data	No data	Epithelial	−	−	+

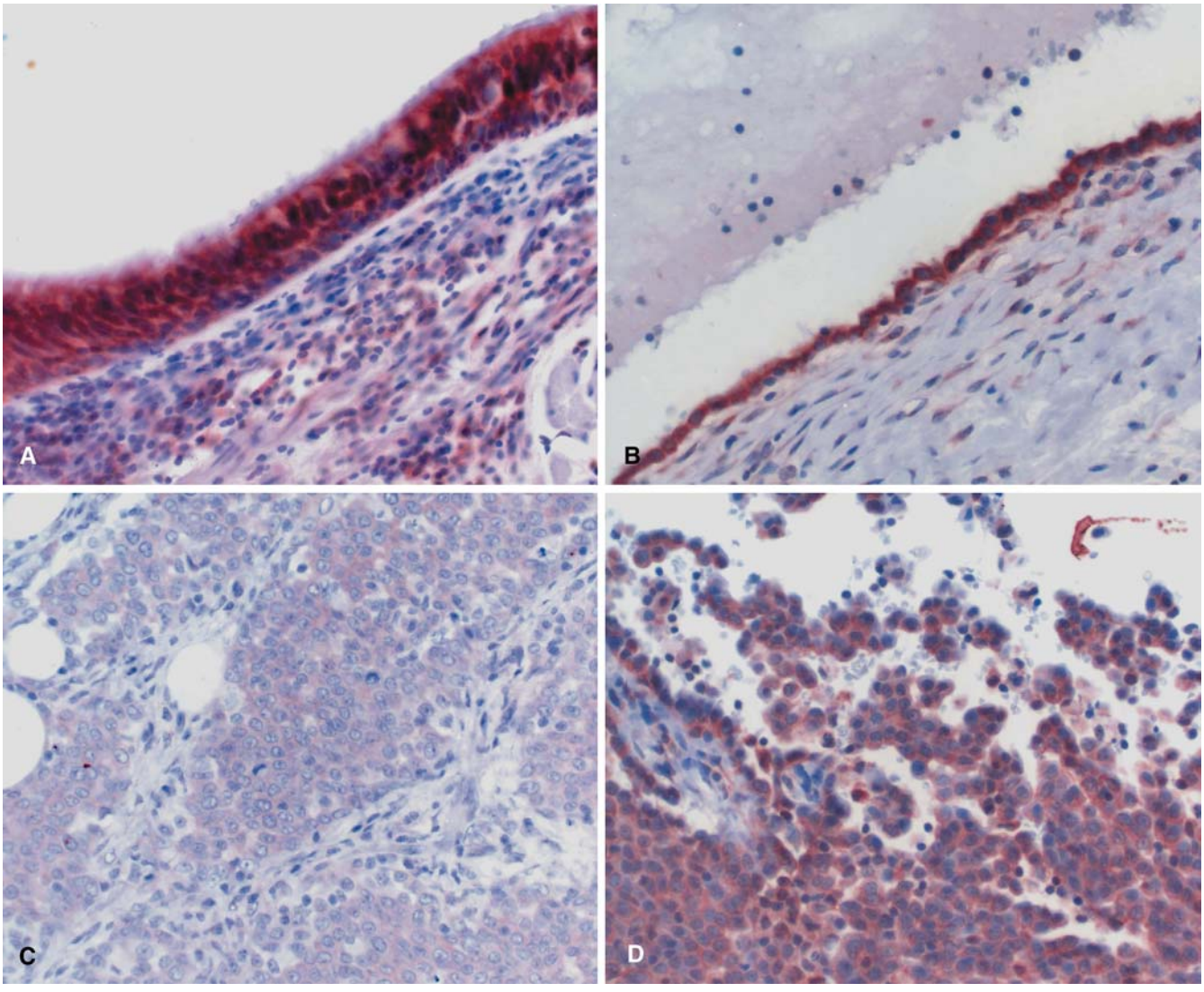

Fig. 1 Positive fragile histidine triad immunostaining in normal bronchial epithelium (**A**) and in mesothelium (**B**). Poorly differentiated papillary mesothelioma (**C**) versus well-differentiated area (**D**) of the same tumour; the former representing approximately 5% of the tumour

intragenic microsatellite markers, D3S1313 and D3S1234. The markers were obtained from Research Genetics (Huntsville, AL).

Genomic DNA (100 ng) was amplified (30 cycles) according to standard protocols in a volume of 10 μl. PCR products were radiolabelled during the amplification reaction and separated by electrophoresis in 6% polyacrylamide gels (7.7 M urea). After electrophoresis, the gels were dried and exposed to X-ray film. Absence or significant decrease of one allele in the tumour compared with the normal reference tissue was considered LOH.

Immunohistochemical studies

Expression of the Fhit protein was examined in a set of paraffin-embedded tumour (PET) specimens (n=13). A rabbit polyclonal antibody raised against glutathione S-transferase-Fhit protein, a generous gift from Dr. Kay Huebner, Kimmel Cancer Center, Philadelphia, Pennsylvania, was used in the immunohistochemical analysis. Sections of 4–5 μm were cut from the PET samples, deparaffinised and microwaved for 4×5 min in 0.01 M Na-citrate buffer (pH 6.0). Immunostaining was performed with the help of a TechMate Horizon (Dako A/S, Glsotrup, Denmark) autostainer (room temperature, 30 min). An automated protocol named MSIP and recommended by the manufacturer was used. All reagents are made by Dako (A/S) for routine use with the stainer. The dilution of the antibody was 1:500. As a control, non-specific rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a concentration corresponding to that of the Fhit antibody was used to stain all sections using an identical protocol.

Evaluation of the Fhit positivity (H.W.) was based on the intensity of staining using scoring from 1 to 5 (1=marginal, close to background; 2=weak; 3=moderate; 4=high; 5=overstaining) and the percentage of positively staining cells. Normal columnar epithelium of the bronchus and the mesothelium indicated positive immunostaining with the intensity score 4. The combination of staining intensity and percentage of positive stained cells was classified in the following three groups:

a. Absent or markedly reduced expression: staining intensity $\leq$1 or staining intensity 2 in <30% of cells;
b. Reduced expression: staining intensity 2 or 3 in 30–50% of the cells;

Table 2 Immunostaining of the fragile histidine triad (Fhit), and loss of heterozygosity (LOH) for the *FHIT* gene in malignant mesothelioma tumors and cell lines. The results are compared with those of lung cancers from cases with occupational exposure

Tumour	Fhit staining			LOH at *FHIT* [2]		
	Total number studied	Negative or reduced		Total number studied	LOH detected	
		n	(%)		*n*	(%)
Malignant mesothelioma						
Tumours	13	7	(54)	20	1	(5)
Cell lines	No data	No data	No data	10	3	(30)
Non-small cell lung cancer[1]	19	13	(68)	24	7	(29)
Squamous cell carcinoma	9	8	(89)	11	4	(36)
Adenocarcinoma	9	4	(44)	10	1	(10)
Large-cell carcinoma	1	1	(100)	2	1	(50)
Adeno-squamous cell carcinoma	0	0	(0)	1	1	(100)

[1] Pylkkänen et al. 2002 [26]
[2] The intragenic markers studied were D3S1234 and D3S1313

c. Positive expression: staining intensity 3 or 4 in >50% of the cells.

The scorer was blinded to results obtained from the LOH analysis.

Results

Protein expression

Intensive immunohistochemical staining for the Fhit protein was observed in the normal columnar epithelium of the bronchus and mesothelium. Compared with this positive staining, the protein expression was decreased in all tumours examined. Four cases showed markedly reduced expression (staining intensity 1 in 1–100%, or 2–3 in <30% of the cells), three cases showed considerably reduced expression (2–3 in 30–50% of the cells), and six cases showed a positive expression (intensity 3 in >50% of the cells). Heterogeneous staining with more strongly positive Fhit expression was seen in areas of well-differentiated tumour, and low staining intensity was noted in areas of poor differentiation. Examples of Fhit expression in normal bronchial epithelium and mesothelium as well as in a MM tumour with well-differentiated papillary cells and poorly differentiated area are shown in Fig. 1.

Allele loss

Altogether, 21 MM tumours and ten cell cultures were investigated for the presence of allele deletion at the *FHIT* gene with two intragenic microsatellite markers (D3S1234 and D3S1313). Allele loss was detected in three of ten cell cultures (30%). One of the corresponding fresh tumour samples also showed LOH. The low LOH frequency in fresh tumour samples was most likely caused by an unavoidable presence of normal cells in the tumour specimens, subsequently covering the existence of LOH. Table 2 summarises the results of immunostaining of the Fhit protein and LOH analyses in malignant mesothelioma tumours and cell lines, and in lung cancer cases associated with asbestos exposure.

Discussion

Several recurrent genomic alterations are characteristic to MMs, but no single specific gene has been yet indicated. Deletions or mutations of two known tumour suppressor genes (*NF2* at 22q.12 and *p16/CDKN2A* at 9p21) have been observed in MM [3, 10, 25, 27]. However, MM appears to involve other genes as well, since inactivation of these genes often occurs with low frequency. In a recent study, we detected recurrent allelic deletion in several chromosomal regions in MM cell cultures [26]. In addition to frequent loss at *NF2* and *p16/CDKN2A* gene regions, we also detected frequent deletions at 3p14.2, where the *FHIT* gene resides. The inactivation of the *FHIT* gene is involved in lung cancer [9, 17, 30, 35] and many other cancer types [5, 14, 29, 36, 37]. The *FHIT* gene suppresses growth of cancer cells [28], and, in carcinogen-treated *FHIT*-deficient mice, development of multiple tumours was inhibited by transfection of an intact human *FHIT* gene [12].

The Fhit protein is expressed in most non-neoplastic human tissues, and the highest levels of expression is detected in epithelial cells. Also, mesothelial cells from benign body cavity effusion consistently express strong immunoreactivity for the Fhit protein [39]. In the present study, all MM tumours showed a decrease in the expression of the Fhit protein, compared with the normal mesothelium. Immunohistochemical analysis indicated absent or reduced expression in 7 of 13 (54%) of the cases studied. Interestingly, high intensity of Fhit staining was seen in the areas of well-differentiated tumours, and low intensity of staining was noted in those of poor differentiation. A similar expression pattern was reported in tongue carcinoma, where Fhit expression was higher in the well-differentiated areas than in poorly differentiated areas [20]. A correlation between the absence or reduction of Fhit expression and advanced tumour stage has been observed in bladder and lung cancers [2]. In a recent study, reduction or absence of Fhit protein was shown to

be associated with high proliferation and large tumour size of breast carcinomas [4]. Such observations may suggest that *FHIT* inactivation is a relatively late event in the neoplastic progression in MM as well.

LOH was detected in three of ten (30%) MM cell cultures studied with two highly polymorphic loci intragenic to the *FHIT* gene. The low rate of LOH detected in fresh tumour samples is probably due to the presence of normal cells in tumour samples typical to MM. Another point is that Fhit staining in many tumour types is often heterogeneous, displaying a mixed pattern of both low and high staining intensity [5, 7, 16, 33]. In addition, the *FHIT* gene, as with many other tumour suppressor genes, may be inactivated by alternative mechanisms. Tumour-acquired methylation of the CpG islands of the promoter regions of *FHIT* gene resulting in silencing of transcription has been reported to occur in lung cancer. In a recent study, *FHIT* methylation was detected in 37% of primary non-small cell lung cancers and 65% of lung cancer cell lines, and methylation significantly correlated with loss of Fhit expression [40]. We detected LOH in MM cells in very early passages (1–3) in culture, which may suggest that a relatively large proportion of malignant cells in the original tumours, in fact, carry allele loss at 3p.

Association between asbestos exposure and reduced Fhit expression has been suggested in lung cancer [22, 26]. In this study, malignant mesothelioma cases associated with occupational asbestos exposure showed frequent reduction of Fhit protein expression. These observations further support previous suggestions that the *FHIT* gene alterations may be involved in the development of neoplasms associated with environmental carcinogen exposure [2, 6, 22, 26]. In conclusion, our present data show that absent or markedly reduced Fhit expression was frequent in MM. However, *FHIT* inactivation may, instead, be a late event, as suggested by the pattern of immunostaining of the tumour cells. As in nearly all MM patients, the first manifestation of malignancy is bloody effusion of the pleural cavity [15]. Immunostaining of Fhit protein could perhaps serve as a biomarker for detection of malignant cells in the effusions.

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