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Original Paper

FHIT Gene Abnormalities in Both Benign and Malignant Thyroid Tumours

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***FHIT*, a candidate tumour suppressor gene, has recently been identified at chromosomal region 3p14.2, and deletions of the gene have been reported in many types of human cancers. Loss of heterozygosity (LOH) at this region has also been found frequently in follicular thyroid carcinoma (FTC). To investigate the potential role of *FHIT* in thyroid tumorigenesis, we examined 57 thyroid tumour specimens (eight benign adenomas, 40 papillary, four follicular and five anaplastic carcinomas), and two thyroid carcinoma cell lines (NPA, SW579) for genetic alterations by using reverse transcription–polymerase chain reaction (RT–PCR), PCR product sequencing, single-strand conformation polymorphism (SSCP) and Southern blot analysis. Two cervical carcinoma cell lines (C-33A, HeLa) were included as positive controls. We detected truncated *FHIT* transcripts in three of eight (38%) benign adenomas, nine of 40 (23%) papillary, and two of five (40%) anaplastic carcinomas, and in three cell lines (SW579, C-33A, HeLa). Most of the truncated transcripts lacked exons 4 or 5 to 7 or 8 of the gene and were presumably non-functional as the translation start site is located in exon 5. SSCP analysis of the coding exons failed to detect any point mutations among the samples without abnormal *FHIT* transcripts. Southern blot analysis demonstrated either loss or reduced intensity of major Bam HI restriction fragments in the three cell lines found to have abnormal *FHIT* transcripts, indicating, respectively, either intragenic homozygous or heterozygous deletions of the *FHIT* gene. Intragenic homozygous deletions were also found in two papillary thyroid carcinoma specimens: one was missing a 13 kb Bam HI fragment which contains exon 4, the other had deletions of 15.5, 13 and 4.2 kb fragments which contain exons 2 and 9, 4, and 5, respectively. The absence of a defective *FHIT* gene in FTC indicates that an additional tumour suppressor gene may reside in this region and be involved in the development of FTC. Given that defective *FHIT* genes were found in both benign and malignant thyroid tumours, the inactivation of this putative tumour suppressor gene is likely to be an early event in the pathogenesis of some forms of thyroid neoplasms.**

Key words: tumour suppressor gene, *FHIT*, fragile histidine triad, thyroid neoplasm
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INTRODUCTION

THYROID EPITHELIAL tumours represent a unique model to study the molecular genetics of tumorigenesis, as these tumours exhibit a broad spectrum of neoplastic pathology, ranging from benign colloid adenomas to uniformly fatal anaplastic carcinomas [1]. Genetic alterations of several oncogenes (*ras*, *ret*, *trk*) [2–5] and tumour suppressor genes (*p53*, *Rb*, *p16/CDKN2*, *p21/Waf1*) [6–10] have been studied to elucidate the molecular lesions which could determine thyroid tumour development and progression. *ras* Oncogene activation is an early event and appears to be specifically involved in the genesis of follicular adenoma and carcinoma. The rearrangements of *ret* and *trk*, and overexpression of *met*

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proto-oncogenes are specific for papillary carcinoma [11]. Loss of heterozygosity (LOH) involving chromosomal regions 11q13 and 3p has been implicated in thyroid tumours [12, 13]. LOH at 3p was reported to occur frequently in follicular thyroid carcinoma (FTC) [13] as well as in many other human cancers [14], suggesting the involvement of one or more potentially important tumour suppressor genes in this region.

A candidate tumour suppressor gene, *FHIT* (fragile histidine triad), was recently identified at chromosomal region 3p14.2 [14]. It comprises 10 exons which encode a 1.1 kb transcript, encompassing approximately 1 Mb of genomic DNA. The *FHIT* gene contains the most common fragile site, FRA3B, and the t(3;8) chromosomal translocation breakpoint associated with hereditary renal cell carcinoma. *FHIT* is a member of the histidine triad gene family [15]. The cognate protein is 69% homologous to the *Schizosaccharomyces pombe* enzyme, diadenosine 5′,5″-p^1, p^4-tetraphosphate (Ap_4A) asymmetrical hydrolase, which cleaves the Ap_4A substrate into ATP and AMP [16]. The human FHIT protein has recently been characterised as a dinucleoside 5′,5‴-p^1, p^3-triphosphate (Ap_3A) hydrolase [17]. Although its substrate, Ap_4A may be involved in DNA replication and cell cycle control [18, 19], the exact role of *FHIT* in tumorigenesis remains unknown.

Defects of the *FHIT* gene, such as abnormal transcripts containing deletions of one or more coding exons, intragenic homozygous deletions and genomic DNA rearrangement, have been found frequently in many cancer cell lines, as well as in primary tumours, including those of lung, breast, oesophagus, stomach, Merkel cell, and head and neck [14, 20–23]. To investigate whether *FHIT* plays a role in the tumorigenesis of thyroid carcinoma, we examined 57 thyroid tumour specimens, and two thyroid carcinoma cell lines (NPA, SW579) for abnormal transcript patterns, potential point mutations in the coding region, and genomic deletions or rearrangements. Two cervical carcinoma cell lines (C-33A, HeLa) were included as positive controls.

MATERIALS AND METHODS

All thyroid tumour tissues were obtained at surgery, and were immediately frozen in liquid nitrogen and stored at − 70°C until processed. The clinical staging of thyroid cancer was based on the TNM classification introduced in 1987 by the International Union Against Cancer [24]. Fifty-seven thyroid tumours were studied: eight benign adenomas, 40 papillary, four follicular and five anaplastic carcinomas. The thyroid squamous carcinoma cell line SW579 and cervical carcinoma cell lines HeLa and C-33A were obtained from ATCC (Rockville, Maryland, U.S.A.). The thyroid papillary carcinoma cell line NPA was kindly provided by James A. Fagin (University of Cincinnati Medical Center, Cincinnati, Ohio, U.S.A.).

The human *FHIT* cDNA probe was obtained by reverse transcription–polymerase chain reaction (RT–PCR) using primers derived from exons 3 and 10 of the gene (see below). The resulting PCR product was verified by DNA sequencing following subcloning into a TA cloning vector (Invitrogen Co., San Diego, California, U.S.A).

RT–PCR and single-strand conformation polymorphism (SSCP) procedure

Total RNA from the tumour samples and cell lines was extracted as described previously [25]. Five micrograms of total RNA was reverse transcribed into cDNA in 15 μl volume, using a first-strand cDNA synthesis kit (Pharmacia, Vienna, Austria). The cDNA was then amplified by PCR in 35 cycles using the following two primers: 5′-TCCGTAGTGCTATCTACATC-3′ (sense) and 5′-CATGCTGATTCAGTTCCTCTTGG-3′ (antisense). The resulting PCR products contained the entire open reading frame of the *FHIT* gene. Samples were first denatured at 94°C for 2 min and then submitted to 35 cycles of amplification as follows: 40 sec denaturation at 94°C, 40 sec annealing at 48°C, and 40 sec extension at 72°C.

To facilitate SSCP analysis, the aforementioned PCR products were reamplified in 25 μl volume using two internal primers together with the flanking primers to generate two overlapping fragments, each approximately 300 bp long. The two internal primer sequences were as follows: 5′-TTGTTTCAGACGACCCAG-3′ (sense), 5′-GAGAGGTCCCATGGAAAT-3′ (antisense). The SSCP analysis was performed as described previously [26].

Sequence analysis

DNA sequencing was performed by the dideoxy chain termination method after cloning the PCR products into a TA cloning vector.

DNA extraction and Southern blot hybridisation

Genomic DNA extraction from the tumour samples and cell lines, and Southern blot hybridisation were performed as described previously [27]. Briefly, 10 μg DNA was digested with Bam HI, fractionated on a 0.8% agarose gel and blotted on to a Nylon membrane (Hybond-N, Amersham, Arlington Heights, Illinois, U.S.A.) by capillary transfer. The 707 bp *FHIT* cDNA probe (spanning exons 3–10) was labelled with [$\alpha - ^{32}$P]dCTP to a specific activity of 10^9 cpm/μg, using a random primer labelling kit (Pharmacia). Hybridisation was performed at 42°C for 18 h in 6×SSPE, 10 mM ethylene diamine tetraacetic acid (EDTA), 5×Denhardt's solution,

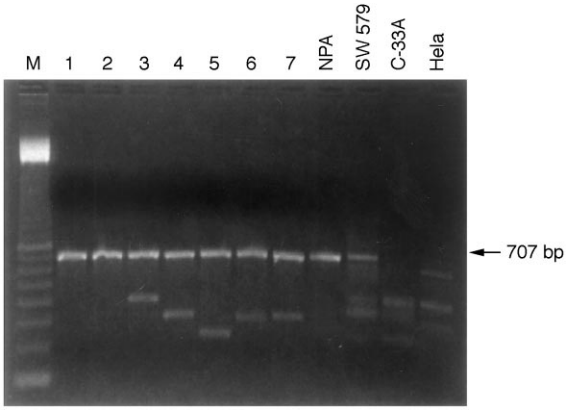

Figure 1. Reverse transcription–polymerase chain reaction (RT–PCR) analysis of *FHIT* transcripts from thyroid tumour specimens and cell lines. cDNA fragments were generated by RT–PCR using the primers covering the coding region of the *FHIT* gene. The PCR products were size fractionated on 1.6% agarose gels. Lane M contains a 1kb DNA ladder (Pharmacia). The wild-type product (707 bp), while present in most samples, was missing in the HeLa and C-33A cell lines. The abnormal fragments in lanes 3, 4, 5, 6, 7 were produced by the joining of exons 4–8, 4–9, 3–9, 3–8, and 4–9, respectively. Lanes 1 and 2 represent normal controls from normal thyroid tissues. Multiple abnormal transcripts were present in SW579, HeLa and C-33A cell lines.

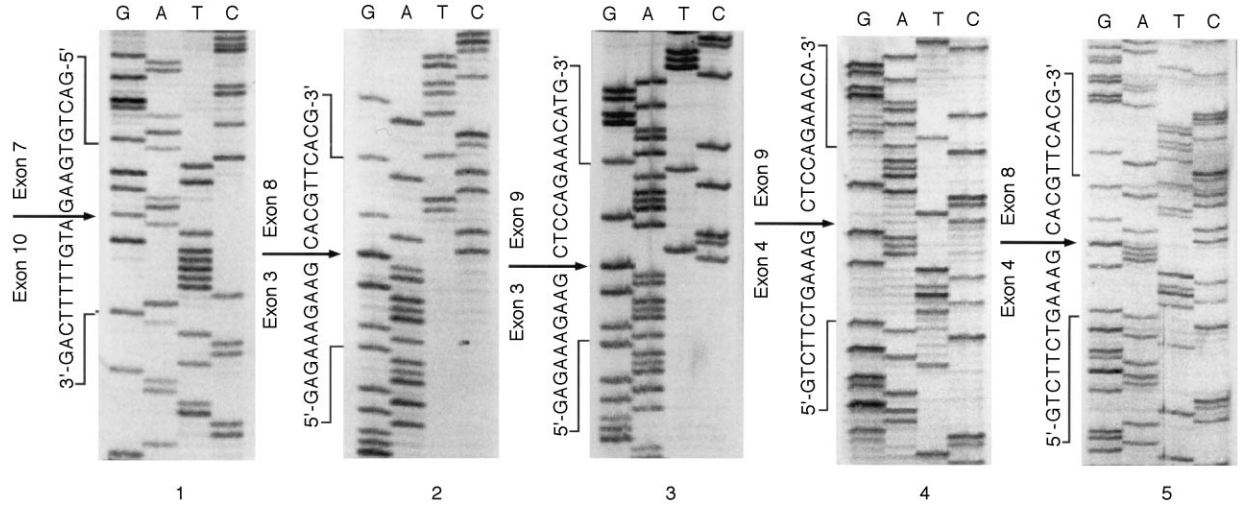

Figure 2. DNA sequence autoradiographs showing multiple exon deletions of the *FHIT* gene in thyroid tumour specimens. Five different patterns of deletion are illustrated as examples. The joining point is indicated by an arrow. Sequencing reactions were performed using single cDNA clones as templates.

0.5% sodium dodecyl sulphate (SDS), 100 µg/ml denatured salmon testis DNA and 50% formamide. The membranes were then washed twice in 2×SSPE at 65°C and exposed to Kodak X-OMAT AR-5 film at −70°C with intensifying screens.

RESULTS

Fifty-seven thyroid tumour specimens (eight benign adenomas, 49 carcinomas) and two thyroid (NPA, SW579) and two cervical (HeLa, C-33A) carcinoma cell lines were analysed by RT–PCR for the presence of aberrant *FHIT* gene transcripts. The RT–PCR products were analysed on 1.6% agarose gels following ethidium bromide staining (Figure 1). Altered transcripts were observed in three benign adenomas, eight papillary and two anaplastic carcinomas, as well as in three of four cell lines: SW579, HeLa and C-33A. Figure 1 shows RT–PCR results from a representative panel of tumour specimens and cell lines. All thyroid tumour specimens and cell lines contained transcripts of both normal and abnormal sizes, although the abundance of normal sized transcripts was reduced significantly in SW579. The wild-type *FHIT* transcripts were not found in HeLa and C-33A cell lines.

Sequence analysis of these abnormal transcripts revealed the presence of multiple exon deletions, resulting in fusion of exons 3 and 8, 3 and 9, 4 and 9, 4 and 8, or 7 and 10 (Figures 2 and 3, Table 1). Absence of exon 4 or 5 to exon 7 or 8 was the most frequent abnormality, accounting for 19 of the 22 truncated products sequenced. Two types of deletion were found in one adenoma specimen: one missing exons 4–7 and the other with an insertion of an 87 bp DNA sequence replacing exons 5–8 (Figure 3). The insertion created a chimeric polypeptide of 39 amino acids with eight amino acids at the N-terminus derived from the insert and the remaining 31 amino acids from exon 9 of the *FHIT* gene. It remains to be seen whether the chimeric polypeptide plays a role in tumorigenesis. In another case, insertion of a 72 bp DNA sequence between exons 4 and 5 was found in a papillary carcinoma sample. Multiple abnormal *FHIT* transcripts were found in SW579, C-33A and HeLa cell lines (Figure 1, Table 1). The information regarding tumour histology, patient history and pattern of abnormal *FHIT* transcripts confirmed by DNA sequencing is summarised in Table 1.

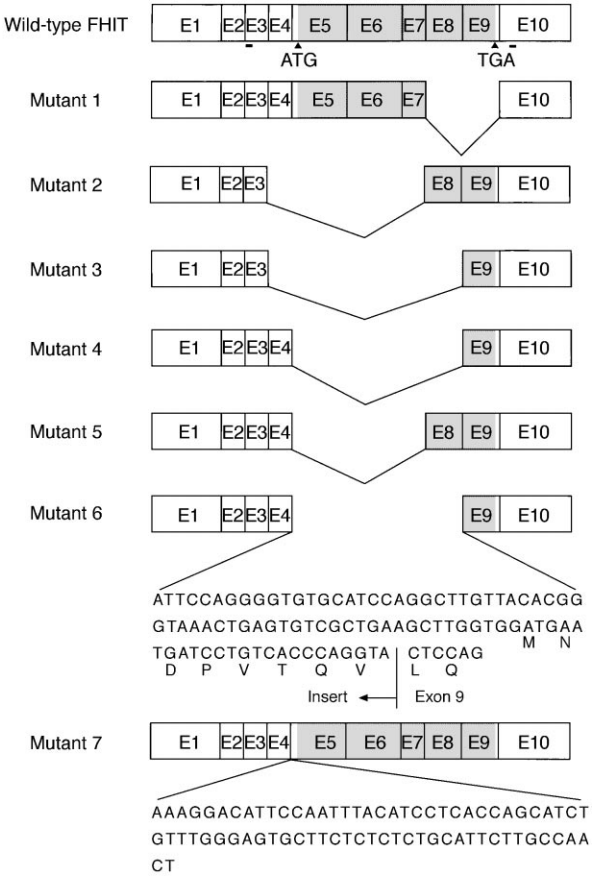

Figure 3. Schematic representation of different *FHIT* mutants present in thyroid tumour samples. The wild-type *FHIT* gene is shown at the top and seven different mutants are shown below. The dark bars under exons 3 and 10 for wild-type *FHIT* represent the primer set used to amplify the *FHIT* cDNA. Open and shaded boxes indicate untranslated and translated regions, respectively. Mutant 6 is missing exons 5–8 with insertion of an 87 bp DNA sequence, whereas in mutant 7, a 72 bp DNA fragment is inserted between exons 4 and 5.

Table 1. *Abnormal* FHIT *gene transcripts in thyroid tumours*

Tumour or cell line	Pathology	Stage	Patient		Deleted exons/insertions
			Age	Sex	
1	Adenoma		30	F	5–8
2	Adenoma		40	F	4–7 and 5–8 plus 87 bp insertion
3	Adenoma		50	M	5–7
4	Papillary Ca	I	52	F	4–7
5	Papillary Ca	II	26	F	4–7
6	Papillary Ca	II	24	F	5–8
7	Papillary Ca	III	60	M	5–8
8	Papillary Ca	I	47	F	4–7
9	Papillary Ca	III	41	F	72–bp insertion between exons 4 and 5
10	Papillary Ca	I	52	F	5–7
11	Papillary Ca	II	25	F	4–8
12	Anaplastic Ca	IV	81	M	4–7
13	Anaplastic Ca	IV	43	F	4–8
SW579	Thyroid Ca				4–7, 5–7 and 8–9
C-33A	Cervical Ca				5–7, 4–8 and 5–8
HeLa	Cervical Ca				4, 4–7 and 5–7

Ca, Carcinoma.

SSCP analysis was performed on cDNA from those tumour samples without abnormal *FHIT* transcripts to identify possible subtle point mutations. Different types of mobility shifts were demonstrated in four tumours (three papillary carcinomas, one adenoma). Sequence analysis revealed an 11bp deletion at the 5′ end of exon 10 in two papillary carcinomas and a silent nucleotide substitution at codon 98 (CAT to CAC) in one papillary carcinoma and one adenoma. The nucleotide substitution and the deletion have been reported previously to be a polymorphism and a variant of *FHIT* (*FHITβ*), respectively [22] (data not shown). The *FHITβ* variant arises by alternative splicing through the use of a cryptic acceptor site in exon 10 [22].

To investigate the molecular basis of the aberrant *FHIT* transcripts, we examined genomic DNAs from primary tumours and cell lines for the presence of alterations in the

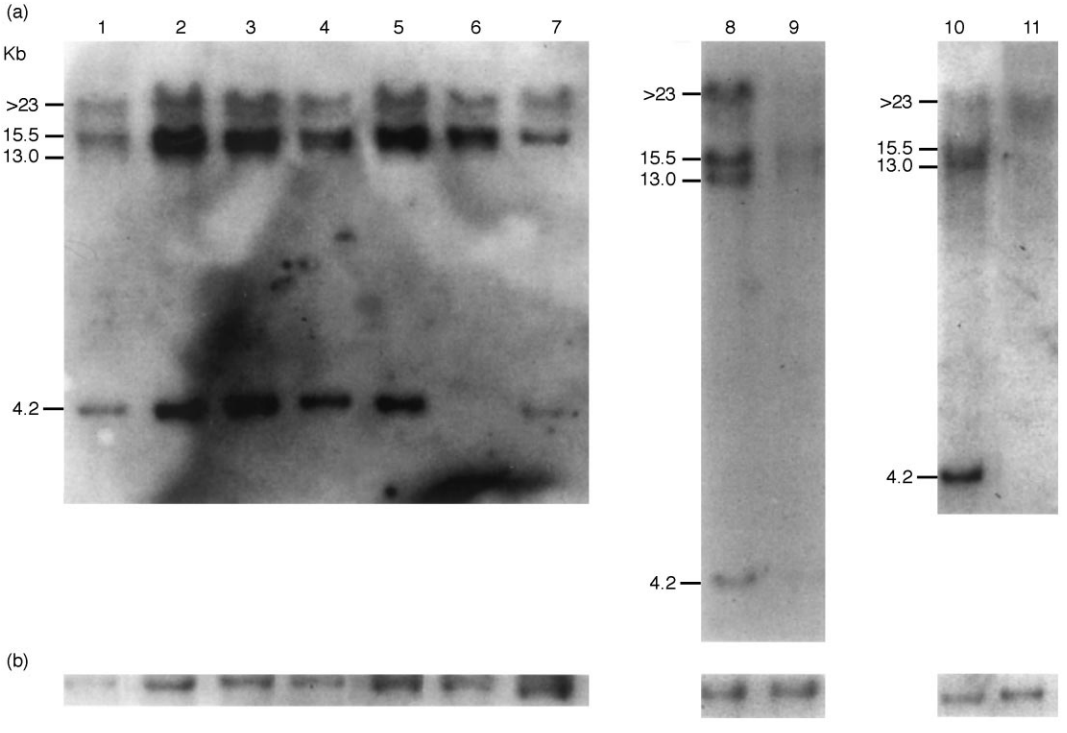

Figure 4. Southern blot analysis of the *FHIT* gene in thyroid tumour specimens and human tumour cell lines. Ten micrograms of genomic DNA digested with Bam HI was fractionated on a 0.8% agarose gel and blotted on to a Nylon membrane. The membranes were hybridised with a 707 bp *FHIT* cDNA probe containing exons 3–10 (a). The same blots were rehybridised with a human β-actin probe to monitor the sample loading (b). Lanes 1–3, papillary carcinomas corresponding to tumour nos 4, 7 and 5, respectively in Table 1; lanes 4, 8 and 10, wild-type; lanes 5–7 and 9, NPA, C-33A, HeLa and SW579, respectively; lane 11, papillary carcinoma. The 13 kb fragment was missing in lanes 1, 7 and 11, and its intensity was reduced in lane 9; the 4.2 kb fragment was lost in lanes 6 and 11, and its intensity was reduced in lanes 7 and 9; the 15.5 kb fragment was lost in lane 11, and its intensity was reduced in lane 9.

FHIT gene. Southern blot analysis showed that loss or reduced band intensity among the Bam HI restriction fragments was found in two papillary carcinoma specimens and three cell lines in which abnormal transcripts were detected, indicating homozygous and heterozygous deletions of the *FHIT* gene, respectively (Figure 4). One papillary carcinoma specimen was found to be missing 15.5 kb, 13 kb, and 4.2 kb Bam HI fragments which contain exons 2 and 9, 4, and 5, respectively [28]. We also tried to PCR amplify both exons 4 and 5 from tumour genomic DNA to confirm the large deletion shown on the Southern blot. No amplification was observed. RT–PCR analysis of this specimen showed no abnormal transcripts and only a weak wild-type band, most likely resulting from contaminating normal cells (data not shown). The other papillary carcinoma specimen was missing a 13 kb fragment and contained abnormal transcripts lacking exons 4–7, inclusive. In SW579 cells, the intensity of the major Bam HI restriction fragments is reduced at least by half as compared with the wild-type, indicating allelic deletion of the *FHIT* gene. In HeLa cells, the 13 kb fragment was lost and the intensity of the 4.2 kb fragment was reduced, probably resulting from a heterozygous deletion, whereas in C-33A cells the 4.2 kb band was missing.

Our results show that abnormalities in the *FHIT* gene were present in 38% (3/8) of benign thyroid adenomas, 23% (9/40) of papillary, 40% (2/5) of anaplastic, carcinomas and none (0/4) of the FTCs examined. Both normal and abnormal *FHIT* transcripts were present in the thyroid tumour samples and the SW579 cell line. However, only abnormal transcripts were observed in HeLa and C-33A cell lines, indicating that both alleles of the *FHIT* gene were altered.

DISCUSSION

The pattern of abnormal *FHIT* transcripts seen in the present study is similar to those reported in cancers of lung, breast, digestive tract, Merkel cell and head and neck [14, 20–23]. The majority of aberrant *FHIT* transcripts lack exons 4–7, 5–7, 5–8 or 4–8. In addition, a 72 bp insertion between exons 4 and 5 and an 87 bp insertion replacing exons 5–8, were also noticed. Two causative mechanisms may be involved in the formation of the abnormal *FHIT* transcripts: (i) genomic DNA deletion, as demonstrated by Southern blot analysis; or (ii) aberrant RNA splicing. The precise exonic deletions, insertions precisely spliced between or replacing wild-type exons and multiple transcripts seen in SW579, C-33A and HeLa cells, respectively, suggest abnormal RNA splicing as the more likely mechanism [29].

The presence of more abundant wild-type than aberrant *FHIT* transcripts in primary thyroid tumours may not be explained by wild-type contamination from normal cells such as stroma and inflammatory cells within the tumours. It is likely that both wild-type and mutant transcripts arise from tumour cells, which would argue against the proposed role of *FHIT* as a classic tumour suppressor gene. It is possible that the 'wild-type' transcripts may not actually be translated into protein because of mutations in the regulatory region upstream from the structural area examined. It has been demonstrated that even when DNA and RNA alterations of the *FHIT* gene are not detected, the FHIT protein may be absent or reduced [28, 30]. Another possibility is that thyroid tumours consist of heterogeneous cell populations, such that both alleles of the *FHIT* gene may be mutated in a subpopulation of thyroid tumour cells which coexist with other abundant tumour cell populations not harbouring a *FHIT* defect. It is known that tumour cell populations are not generally genetically homogeneous even in cell lines. Using fluorescence *in situ* hybridisation analysis of allelic losses of the *FHIT* gene, Virgilio and colleagues have demonstrated multiple cell populations with diverse regions of deletion of portions of the *FHIT* gene in 65% of head and neck squamous cell carcinoma cell lines [31]. The presence of only mutated *FHIT* products in some cell lines such as C-33A and HeLa supports the candidacy of *FHIT* as a tumour suppressor gene. Given the fact that the abundance of wild-type transcripts is significantly reduced in SW579 as compared with that in primary tumours (Figure 1), and is missing in C33-A and HeLa, the tumour cells with the *FHIT* defect likely gain more growth advantage than do those without.

We have not detected any *FHIT* abnormalities in four FTCs. Similar results were also reported by McIver and associates [32], even though they found a high rate of LOH at 3p14.2: 44% LOH with the microsatellite marker *D3S1234* located between exons 8 and 9, 70% LOH with the *D3S1300* marker positioned in the very large intron (more than 200 kb) between exons 5 and 6. These results indicate that an additional tumour suppressor gene(s) may exist in this region which may be involved in the development of FTC. The simultaneous presence of both wild-type and aberrant *FHIT* genes in primary thyroid tumours may account for the relatively low detection rate of *FHIT* abnormalities by Southern blot analysis.

Several groups have investigated the prevalence of LOH in thyroid neoplasms [33–35]. The highest prevalence of LOH was found in follicular carcinomas, with chromosomes 2p and 3p the most frequently affected sites. Fine mapping of 3p showed LOH clustered in the telomeric region (3p21-ter) as well as at *D3S1300* (3p14.2) where *FHIT* was located [33]. High rates of LOH at multiple chromosomal regions were also present in follicular carcinomas, indicating a greater degree of chromosomal instability. In contrast, papillary carcinomas had the lowest rate of LOH (primarily located at chromosomes 10q and 11p) without multiple chromosomal involvement. The significantly different rate of LOH between follicular and papillary carcinomas suggests a fundamental difference in the mechanisms leading to the pathogenesis of these two forms of cancer.

The significance of this study was the discovery of frequent *FHIT* abnormalities in both benign thyroid adenomas and malignant papillary and anaplastic carcinomas. These *FHIT* abnormalities have also been observed in benign lesions of breast and lung [29, 36]. It is likely that *FHIT* mutation is an early event in thyroid tumorigenesis and does not appear to be relevant to FTC. The issue of whether *FHIT* plays a causative role in tumorigenesis or is simply a molecular marker reflecting genomic instability at 3p14.2 cannot be resolved until the biological function of the cognate protein is fully characterised.

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