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Advances in Brief

Frequent Abnormalities of *FHIT*, a Candidate Tumor Suppressor Gene, in Head and Neck Cancer Cell Lines[1]

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Abstract

Loss of heterozygosity at the short arm of chromosome 3 occurs frequently in head and neck squamous cell carcinoma (HNSCC). *FHIT*, a candidate tumor suppressor gene, was recently identified at 3p14.2, and abnormalities of the gene were found in several types of human cancers. To investigate a potential role of the *FHIT* gene in HNSCC, we examined 16 HNSCC cell lines from 11 patients for abnormalities of the gene by using microsatellite analysis, reverse transcription-PCR, sequencing, and Southern blot analysis. We found that 13 of 16 (81%) cell lines exhibit loss of heterozygosity at 3p14.2. Seven cell lines from six individuals exhibited abnormal transcription patterns, including lack of a *FHIT* transcript in three lines and shortened transcripts in four lines. A further examination of coding sequences of *FHIT* in all lines with *FHIT* transcripts revealed a deletion of exon 4 in one line, a deletion of exons 5 to 7 in one line, and a deletion of exons 5 to 7 plus multiple small insertions between exons 4 and 8 in two lines derived from a primary tumor and a metastasis in the same individual. These results indicate that *FHIT* may have been inactivated in six cell lines from five (45%) individuals. We also observed two common polymorphism sites at codons 88 and 98 of the gene. These data indicate that abnormal transcription of the *FHIT* gene is common in HNSCC cell lines; however, other tumor suppressor gene(s) may reside at the same chromosomal region.

Introduction

HNSCC[3] is one of the common human cancers, with an incidence of 500,000 cases per year worldwide (1). Recent studies have demonstrated that multiple genetic alterations are involved in the tumorigenic process of this disease (2–6). Frequent LOH at many chromosome loci, the most striking alteration, indicates that multiple tumor suppressor genes are important in HNSCC tumorigenesis (3, 4).

Recently, Ohta *et al.* (7) identified a candidate tumor suppressor gene, *FHIT*, at 3p14.2 by positional cloning. Abnormal transcripts that contain deletions of one or more coding exons, homozygous deletion of a region containing the gene, and genomic DNA rearrangement have been found frequently in lung cancer, gastrointestinal tumors, and Merkel cell carcinoma (7–9). However, after a study of 31 colon cancer cell lines and xenografts, a more recent report (10) contradicted the Ohta group's report (7) by suggesting that abnormal *FHIT* is rare in colon cancers. Previous studies had shown that LOH at 3p, including 3p14, is one of the most frequent alterations in HNSCC (11). Recently, we reported that LOH at 3p14 is a frequent event in oral premalignant lesions and is associated with oral cancer development. This finding suggested that tumor suppressor gene(s) at this region may play an important role in early tumorigenesis of this tumor type (12). To investigate whether *FHIT* plays a role in the tumorigenesis of HNSCC, we examined 16 HNSCC cell lines for LOH status at 3p14.2, transcription pattern, potential mutation in coding region, and genomic DNA rearrangement of the gene.

Materials and Methods

Cell Lines. HNSCC cell lines used in this study included UMSCC10A, UMSCC10B, UMSCC11A, UMSCC11B, UMSCC14A, UMSCC14B, UMSCC17A, UMSCC17B, UMSCC22A, UMSCC22B, and UMSCC38 (gifts from Dr. Thomas E. Carey, University of Michigan, Ann Arbor, MI); 183A, MAD886Ln, and 1483 (gifts from Dr. Peter G. Sacks, Memorial Sloan Kettering Institute, New York, NY); TR146 (a gift from Dr. Alfonse Balm, The Netherland Cancer Institute, Amsterdam, the Netherlands); and SqCC/Y1 (a gift from Dr. Michael Reiss, Yale University, New Haven, CT); these lines were derived from 11 patients. Cell line pairs, designated A and B with the same UMSCC number, were derived from a primary tumor and a metastasis in the same patient, respectively (13).

DNA, RNA Extraction, and RT-PCR. Cultured cells were collected and digested in 2 ml of 50 mM Tris-HCl (pH 8.0) containing 1% SDS-proteinase K and incubated at 60°C for 12 h. DNA was extracted following the method described previously (14). Total RNA was extracted from cell lines using TRI REAGENT (MRC, Inc., Cincinnati, OH) according to the manufacturer's protocol. For RT-PCR, 2 μg of total RNA was subjected to reverse transcription with random hexamer, deoxynucleotide triphosphates, and 200 units of Superscript II RNase H$^-$ reverse transcriptase (Life technologies, Inc., Gaithersburg, MD) in a 20-μl reaction volume. PCR amplification was performed by using primers FHIT1S (5′-TCATTCCCAGCTGTCAAC-3′) and FHIT1AS (5′-GCGGTCTTCAAACTGGTTG-3′) for the *FHIT* cDNA fragment. The product was run on 1% agarose gel and visualized by ethidium bromide staining. The same cDNA was also used for amplifying a 577-bp *DPC4* cDNA fragment as a control (15).

LOH Analysis. D3s1234, D3s1285, D3s1312, D3s1313, D3s1480, and D3s1481 (Research Genetics, Huntsville, AL) at 3p14 were used for LOH analysis. For PCR amplification, one of two primers from each marker was end-labeled with [γ-^{32}P]ATP (4500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) and T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA). PCR reactions were carried out in a 12.5-μl volume as described previously (14). PCR products were separated on 6% polyacrylamide-urea-formamide gel, which was then autoradiographed. LOH was determined only when a single allele was displayed in all six highly polymorphic microsatellite markers (heterozygosity rates of 66–83%).

Sequence Analysis. RT-PCR products derived from primers FHIT1S and FHIT1AS were separated on 1% agarose gel. Normal-sized and shortened bands were cut out separately and eluted from the gel slices by using a QIAquick column (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's protocol. After ethanol precipitation and recovery in an appropriate amount of distilled water, an aliquot of DNA was used for each direct sequencing reaction. Briefly, purified DNA and sequencing primers labeled with [γ-^{32}P]ATP or [γ-^{33}P]ATP as described were subjected to PCR amplification for 30 cycles using the AmpliCycle sequencing kit (Perkin-Elmer, Branchburg, NJ). Each amplified product (3 μl) was run on 6% Long-Range gel (FMC BioProducts, Rockland, ME) and exposed to film for 12–48 h. Each identified alteration was confirmed by repeat sequence analysis. The primers

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; FHIT, fragile histidine triad; LOH, loss of heterozygosity; RT-PCR, reverse transcription-PCR.

Table 1 *FHIT status in head and neck cancer cell lines*

Cell line	LOH at 3p14	*FHIT* (RT-PCR)	cDNA sequence	Southern blot
UMSCC 10A	Yes	Normal transcription	Wild-type, codons 88 and 98 polymorphisms	Rearranged
UMSCC 10B	Yes	No transcription	No cDNA	Rearranged
UMSCC 11A	Yes	Normal transcription	Wild-type	Rearranged
UMSCC 11B	Yes	Normal transcription	Wild-type	Rearranged
UMSCC 14A	Yes	Normal transcription	Wild-type, codon 98 polymorphism	Rearranged
UMSCC 14B	Yes	Normal transcription	Wild-type, codon 98 polymorphism	Rearranged
UMSCC 17A	No	200–300 bp shortened	Exons 5–7 deletion and insertion	Rearranged
UMSCC 17B	No	200–300 bp shortened	Exons 5–7 deletion and insertion	Rearranged
UMSCC 22A	Yes	Normal transcription	Wild-type, codon 88 polymorphism	Normal
UMSCC 22B	Yes	Normal transcription	Wild-type, codon 88 polymorphism	Rearranged
UMSCC 38	Yes	Normal transcription	Wild-type	Rearranged
183A	No	Normal transcription	Wild-type, codons 88 and 98 polymorphisms	Rearranged
MAD 886Ln	Yes	300 bp shortened	Exons 5–7 deletion	Normal
1483	Yes	100 bp shortened	Exon 4 deletion	Rearranged
TR146	Yes	No transcription	No cDNA	Rearranged
SQCC/Y1	Yes	No transcription	No cDNA	Normal

used for sequencing included FHIT1S, FHIT1AS, FHIT2S (5′-ATCCTGCCACTGAGGACT-3′), FHIT2AS (5′-CTCCTCATAGATGCTGTC-3′), and FHIT3S (5′-CTCCAGAAACATGACAAGG-3′).

Southern Hybridization. For Southern blots, 5 μg of genomic DNA were digested with *Bam*HI (10 units/μg) for 15 h as specified by the manufacturer (Boehringer-Mannheim, Indianapolis, IN), run on 0.8% agarose gel, transferred to a Zata-Probe GT nylon membrane (Bio-Rad, Hercules, CA), and probed with 25 ng of the 748-bp PCR product of *FHIT* cDNA from −240 to 508 by using primers FHIT1S and FHIT1AS and a 594-bp cDNA fragment from −240 to 354, after random primer labeling (16). The blots were then exposed to film for 24 h to 5 days for visualization.

Results and Discussion

Frequent LOH at 3p14. We examined all 16 cell lines for LOH status at 3p14 by using six highly polymorphic microsatellite markers. The 75% average heterozygous frequency of the six markers was estimated according to the information obtained form Research Genetics. For each marker, individual DNA samples have a 75% chance to display a heterozygous pattern. Statistically, the chance not to display a heterozygous pattern in at least one of the six markers approaches zero if the DNA contains two alleles at this chromosomal region (17). Therefore, the homozygous pattern displayed in all six markers for a particular sample strongly indicated the probability of LOH at this chromosomal region. We found that only three cell lines from two individuals displayed heterozygous patterns in at least one marker (Table 1), which suggested frequent LOH at 3p14 in HNSCC cell lines (13 of 16 lines or 81%) and is in concordance with previous observations (4, 11, 17). However, the allelic status for any particular cell line is not known because the method used here is entirely statistical. No homozygous deletion was identified in any of these cell lines, although it is possible that small homozygous deletions reside in regions between the markers tested.

Abnormal Transcripts of the *FHIT* Gene. To investigate the potential abnormality of *FHIT* gene transcription in HNSCC cell lines, we used RT-PCR to examine the transcription patterns of the *FHIT* gene in all 16 HNSCC cell lines (Fig. 1). We found that three cell lines, UMSCC10B, SqCC/Y1, and TR146, from three different individuals did not have an *FHIT* transcript; four cell lines, UMSCC17A, UMSCC17B, MAD-886Ln, and 1483, from three other individuals, displayed clear, shortened *FHIT* transcripts. Interestingly, UMSCC17A and UMSCC17B displayed a fuzzy band about 200–300 bp shorter than the normal transcript, suggesting the possibility of variant transcripts at the band (Fig. 1). Indeed, exons 5–7 were found to be deleted, and variant insertions were identified between exons 4 and 8 by sequencing analysis of these two lines (Fig. 2*A*). Because the DNA was purified from the specific bands that were migrated between 200 and 300 bp shorter than the normal transcript and the size from exon 5 to exon 7 is 296 bp, these abnormal inserts should be about 100 bp shorter. Since the predicted translation starting site of the *FHIT* gene is at exon 5, observed alterations in these two cell lines are likely to have inactivated the gene. The shortened transcript of MAD-886Ln was found to lack exons 5–7 (Fig. 2*B*), which may have abolished the gene. The shortened transcript of 1483 was found to have only exon 4 deletion (Fig. 2*C*), which did not interfere with the coding sequence of the gene. Sequencing analysis of normal-sized transcripts from other lines did not reveal any mutation (Table 1). Taken together, these data suggest that the *FHIT* gene may have been inactivated in six HNSCC cell lines from five patients (45% of 11 patients) and indicate that abnormal transcription of the *FHIT* gene is common in HNSCC cell lines, as has been reported for other tumor types (7–9). Furthermore, the similar pattern of alterations observed in UMSCC17A and UMSCC17B derived from the same patient indicates that these alterations may occur *in vivo*. Because these two cell lines retained two alleles at marker D3s1234 (located between exon 8 and exon 9 of the *FHIT* gene), the abnormal transcripts displayed may be derived from different alleles. UMSCC10B cell line derived from a lymph node metastasis did not express the *FHIT* transcript, however, whereas UMSCC10A derived from a primary tumor of the same patient expressed the wild-type *FHIT* gene. These results suggest that

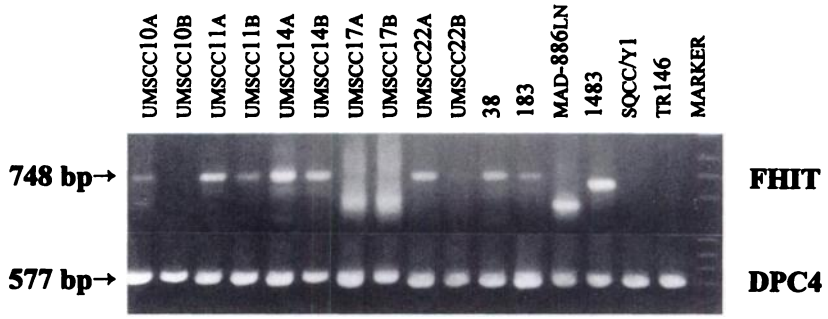


Fig. 1. Expression of the *FHIT* gene in HNSCC cell lines detected by RT-PCR. A 748-bp normal fragment could be observed by using the *FHIT* primers FHIT1S and FHIT1AS. A 577-bp fragment from *DPC4* cDNA was used as a control for cDNA quality. No product was observed in UMSCC10B, SQCC/Y1, and TR146. Shortened bands were seen in UMSCC17A, 17B, MAD-886LN, and 1483. All cell lines exhibited comparable RT-PCR products in the control panel. DNA marker and sizes are indicated at the sides of the panels.

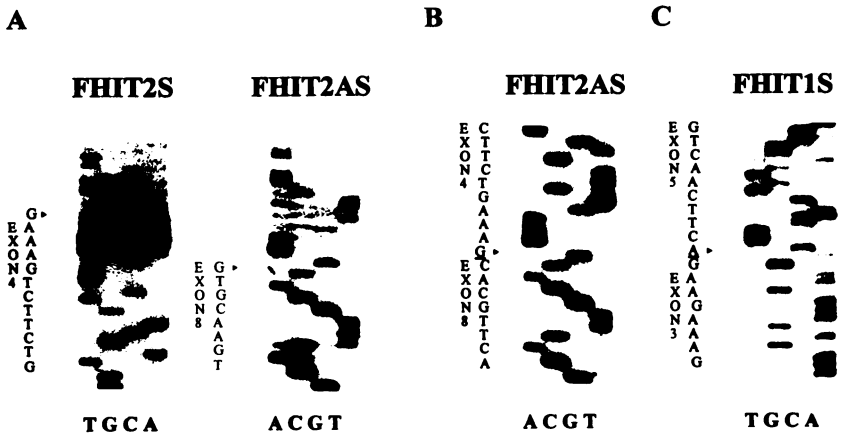


Fig. 2. Sequencing analysis of shortened *FHIT* cDNA products. *A*, exons 5–7 were deleted, and multiple small DNA fragments were inserted between exons 4 and 8 in the cell line UMSCC17A. *B*, exons 5–7 were deleted, and exon 4 was connected directly to exon 8 in the cell line MAD-886Ln. *C*, exon 4 was deleted, and exon 3 was connected directly to exon 5 in the cell line 1483. Names of sequencing primers are indicated at the *top* of each panel. *Underlines* and *arrows*, boundaries between exons.

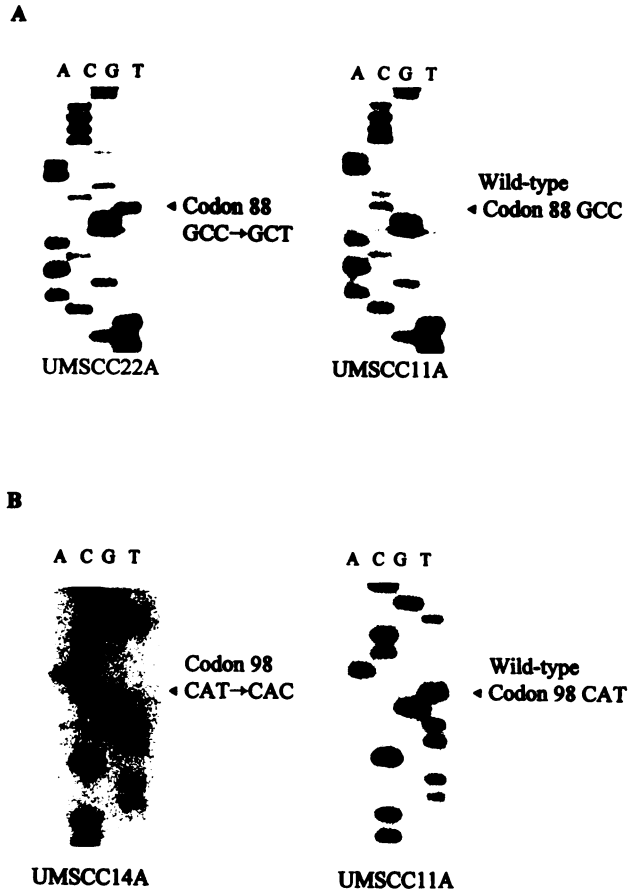
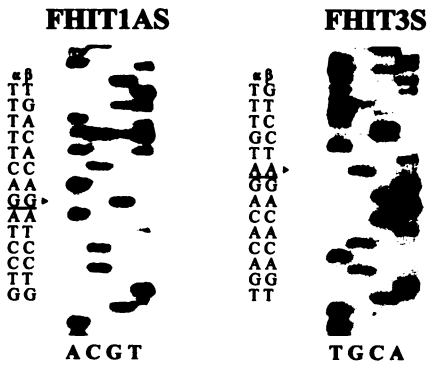


Fig. 3. Polymorphism and alternative splicing of *FHIT*. *A*, polymorphism at codon 88 (GCC→GCT) was shown in the cell line UMSCC10A. *B*, a polymorphism at codon 98 (CAT→CAC) was exhibited in the cell line UMSCC14A. *Arrows*, alternative nucleotides.

the alteration in UMSCC10B may occur after malignant transformation or during *in vitro* culture.

Polymorphisms and Alternative Splicing. During the sequencing analysis of the *FHIT* cDNA, we identified two codons, 88 and 98, with frequent polymorphisms that did not change amino acids. Three of seven individuals exhibited polymorphism at codon 88 (GCC→GCT; Table 1; Fig. 3*A*), and three of seven individuals showed polymorphism at codon 98 (CAT→CAC; Table 1; Fig. 3*B*). Although these two polymorphisms occurred frequently, we, in fact, did not observe a heterozygous pattern at these two sites in each individual sequence, which further indicated that most of these cell lines actually contain only one copy of the *FHIT* gene, as identified by the LOH analysis. We also found a common *FHIT* transcript with an alternative splicing, which contained an 11-bp deletion at the beginning of exon 10 of the gene (termed *FHITβ*), which was observed previously in some lung tumors (8), in all cell lines sequenced (Fig. 4). According to the density of bands on sequencing gels, the abundance of *FHITβ* is at least as great as that of previously reported *FHIT* (we term it *FHITα*; Ref. 7; Fig. 4). *FHITβ* was also observed in all cDNAs produced from normal tissues (data not shown). Although *FHITβ* does not change coding sequences, the question of a functional difference between these two transcriptional forms needs to be investigated further.

DNA Rearrangement. To learn whether genomic DNA rearrangement contributes to the abnormal transcription observed in these cancer cell lines, we first examined DNA extracted from lymphocytes of four normal individuals to determine the normal patterns. By cleaving the genomic DNA with *Bam*HI and probing with an 748-bp *FHIT* cDNA probe, seven major restriction fragments ranging from 1.2 to 18 kb were visualized (Fig. 5). A 5.5-kb fragment was observed in two cases, but the fragment was missing in the other two (Fig. 5), which we considered to be a common polymorphism. Because the Southern blot patterns were different from those reported previously



Exon 9 Exon 10

FHITα 5'-CAGTGACACAG**ATGTTTTTCAG**ATCCTGAA-3'
FHITβ 5'-CAGTGACACAG·····ATCCTGAA-3'

Fig. 4. The alternative splicing (*FHITβ*), which contains an 11-bp deletion at the 5′ end of exon 10, was confirmed by sequencing analysis from both sides of the deletion. *Underlines* and *arrows*, the boundaries of exons. Sequences of *FHITα* and *FHITβ* are shown at the *bottom*.

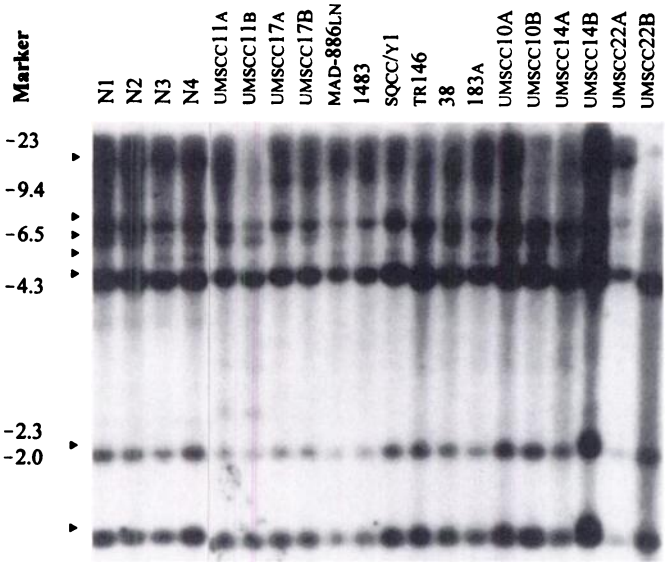


Fig. 5. Southern hybridization DNA was digested by *Bam*HI, separated on 0.8% agarose gel, and probed with 748-bp *FHIT* cDNA. DNA from four normal individuals (*N1–N4*) showed seven major restriction fragments ranging from 1.2–18 kb (*arrows*). A 5.5-kb fragment was missing in *Lanes 3* and *4*. DNA rearrangement or missing major restriction fragments were observed in 13 HNSCC cell lines.

with use of the same restriction enzyme (8), we validated the accuracy of the enzyme used for DNA digestion by cleaving pC53-SN3, a vector containing *Bam*HI sites at each side of a wild-type *p53* gene insert (18). The release of an expected 1.8-kb p53 fragment confirmed the specificity of the enzyme (data not shown). We also used a shorter *FHIT* cDNA probe, excluding exons 9 and 10, to hybridize with the same blot, and we observed the same patterns except for weaker 18- and 2.1-kb fragments (data not shown). After a further examination of Southern blot patterns for all 16 HNSCC cell lines using the same restriction enzyme, we found DNA rearrangements in 13 cell lines from nine patients (Table 1 and Fig. 5). Five of these 13 cell lines exhibited abnormal transcripts, whereas the others had a normal transcription pattern and a wild-type sequence of the *FHIT* gene (Table 1). Three cell lines exhibited normal Southern blot patterns (Table 1). However, only one line displayed a normal transcript. These data suggested that other mechanisms, such as a mutation in the promoter region or hypermethylation, may play a role in controlling the gene expression.

The putative tumor suppressor gene or genes at 3p14 is considered to be important in HNSCC because LOH in this chromosomal region occurs frequently and early in tumorigenesis. The *FHIT* gene was identified at 3p14, a common fragile site in the human genome (7, 19). It is a human homologue of a *Schizosaccharomyces pombe* enzyme, diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) asymmetrical hydrolase (20), which cleaves the Ap_4A substrate asymmetrically into ATP and AMP. As an early study (21) showed, Ap_4A stimulates the initiation of DNA synthesis in baby hamster kidney cells that were arrested in G_1 by serum deprivation. These data suggest that *FHIT* may have potential tumor suppressor function by regulating Ap_4A concentration in the cell cycle. Alterations observed in many primary tumors and tumor cell lines may inactivate *FHIT* and stimulate cell proliferation. However, all alterations (except small numbers of homozygous deletions) observed thus far are altered transcripts, mainly deletions of one or more exons. Although rearranged genomic DNA has been observed in many tumors, it is not associated with the abnormal transcriptions observed, suggesting that other mechanisms may contribute to control of gene expression. In this study, moreover, eight cell lines from five individuals, which exhibited LOH at 3p14, showed a normal *FHIT* transcript and wild-type *FHIT* sequences, suggesting that one or more other tumor suppressor gene(s) in the same region may also be important in HNSCC. Further studies of primary HNSCC are needed to determine whether these alterations are frequent events *in vivo*.

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