Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9770–9775, September 1996
Medical Sciences

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected on April 30, 1996.

FHIT gene alterations in head and neck squamous cell carcinomas

(fragile site/Ap_3A hydrolase/chromosome 3p14.2)

LAURA VIRGILIO*, MICHELE SHUSTER†, SUSANNE M. GOLLIN†, MARIA LUISA VERONESE*, MASATAKA OHTA*, KAY HUEBNER*, AND CARLO M. CROCE*

*Kimmel Cancer Institute and Kimmel Cancer Center, Jefferson Medical College, Philadelphia, PA 19107; and †Department of Human Genetics, University of Pittsburgh Graduate School of Public Health and the University of Pittsburgh Cancer Institute, Pittsburgh, PA 15261

Contributed by Carlo M. Croce, July 18, 1996

ABSTRACT **To determine whether the *FHIT* gene at 3p14.2 is altered in head and neck squamous cell carcinomas (HNSCC), we examined 26 HNSCC cell lines for deletions within the *FHIT* locus by Southern analysis, for allelic losses of specific exons *FHIT* by fluorescence *in situ* hybridization (FISH) and for integrity of *FHIT* transcripts. Three cell lines exhibited homozygous deletions within the *FHIT* gene, 55% (15/25) showed the presence of aberrant transcripts, and 65% (13/20) showed the presence of multiple cell populations with losses of different portions of *FHIT* alleles by FISH of *FHIT* genomic clones to interphase nuclei. When the data obtained by FISH and by reverse transcriptase–PCR analyses are combined, 22 of 26 cell lines showed alterations of at least one allele of the *FHIT* gene. Our data indicate that the *FHIT* gene is disrupted in HNSCCs and hence, loss of *FHIT* function may be important in the development and/or progression of head and neck cancers.**

Head and neck cancers represent 3% of all cancer in Western countries (1), and in some geographical regions such as India, the incidence is as high as 45% (2); 90–95% of these tumors are head and neck squamous cell carcinomas (HNSCC). HNSCC has a high mortality rate with a 5 year survival of 40%. Tobacco and alcohol have been recognized as etiological factors of these carcinomas, and the reported increase in the incidence of HNSCC by epidemiological studies is probably due to changes in consumption of these agents (2).

Several regions of loss of heterozygosity (LOH) have been identified in HNSCC recently, including regions of 3p, 9p, 11q, 13q, and 17p (3, 4). Among these loci, the 9p region presents the highest rate of genetic alteration at 75%, followed by alterations of 3p ranging between 45% and 55% (4, 5). LOH of 9p has been linked to the tumor suppressor gene, *CDKN2* (6–8), whereas LOH on 3p has not yet been associated with specific genes.

Deletions of the short arm of chromosome 3 are common in many human cancers, including sporadic and hereditary renal carcinomas, small-cell lung carcinomas (SCLC) (9, 10) and non-small-cell lung carcinomas (NSCLC) (11, 12) and carcinomas of the breast and cervix (13, 14). In this regard, it is interesting to note that the most common fragile site in humans, FRA3B, is at 3p14.2 (for reviews, see refs. 15 and 16), and if, as has been suggested by Yunis and Soreng (17), fragile sites are targeted areas of genetic lesions in human tumors, then 3p14.2 may be the site of a gene involved in several types of cancers. LOH of alleles on the short arm of chromosome 3 appears to be an early event in oral carcinomas, since it has been reported in oral dysplastic lesions at the precancerous stage (18, 19). Three distinct regions of LOH on chromosome arm 3p have been identified in HNSCC, 3p13-14, 3p21.3, and 3p25. All three regions present a loss of heterozygosity in 45–50% of the cases both at the precancerous and cancerous stages, with the exception of 3p21.3, that shows a lower incidence (30%) in dysplastic lesions (18). Furthermore, patients with dysplastic lesions with LOH either of 9p or 3p are at higher risk of developing tumors.

Recently, we have cloned the *FHIT* gene, at 3p14.2, which encompasses the FRA3B fragile site, is disrupted by the t(3;8) chromosomal translocation observed in a family with renal cell carcinoma, and spans a region commonly deleted in cancer cell lines (20, 21). We have also shown that ≈80% of SCLC and 40% of NSCLC express aberrant *FHIT* transcripts (22), suggesting that the *FHIT* gene is a frequent target for alteration in lung tumors. SCLC and HNSCC present the same type of histology, share major etiological factors such as tobacco (23, 24), and exhibit similar regions of LOH of the short arm of chromosome 3. To determine whether the *FHIT* gene is also disrupted and aberrantly transcribed in HNSCCs, we studied 26 early passage HNSCC-derived cell lines.

MATERIALS AND METHODS

RNA Extraction and Reverse Transcription–PCR (RT–PCR). Total RNA was extracted from cell lines using RNAzol (Tel-Test, Friendswood, TX) and cDNA synthesized from 1 μg of total RNA. RT was performed in a 20 μl volume of 1× first strand buffer (GIBCO), 10 mM DTT, 500 mM of each dNTP, 0.3 mg/ml random primers (GIBCO), and 300 units of SuperScript II (GIBCO) reverse transcriptase. The samples were first incubated 5 min at 65°C and then at 37°C for 60 min. One microliter of cDNA was used for PCR amplification with primers 5U2 and 3D2, from *FHIT* exons 1 and 10, respectively (21), in a final reaction volume of 25 μl containing 0.8 μg of each primer, 100 mM of each dNTP, 1× reaction buffer (Boehringer Mannheim), and 1.25 units *Taq* polymerase (Boehringer Mannheim). The PCR consisted of an initial denaturation step at 95°C for 3 min, followed by 25 cycles of 30 s at 94°C, 30 s at 62°C, and 40 s at 72°C. One microliter of the first PCR amplification product was used for a second PCR amplification reaction with primers 5U1 and 3D1, in *FHIT* exons 3 and 10 (21). The nested PCR amplification was for 30 cycles under the same conditions as the first, and PCR products were resolved on a 1.5% agarose gel.

Abbreviations: HNSCC, head and neck squamous cell carcinomas; SCLC, small-cell lung carcinoma; NSCLC, non-small-cell lung carcinoma; LOH, loss of heterozygosity; FISH, fluorescence *in situ* hybridization; RT–PCR, reverse transcription–PCR; UPCI, University of Pittsburgh Head and Neck Tissue Culture Laboratory.

DNA Sequencing. DNA bands corresponding to the normal and abnormal sized *FHIT* transcripts were excised from the gel and the DNA purified from the agarose using the QIA quick gel extraction kit (Qiagen, Chatsworth, CA). Depending on the amount recovered, the DNA fragment was either directly sequenced, using the primer 5U1, or was cloned in the vector, pCRII (Invitrogen), and individual clones sequenced. Sequencing was by dideoxynucleotide termination reaction chemistry for sequence analysis on the Applied Biosystems models 373A and 377 DNA sequencers.

Southern Blot Hybridization. Genomic DNA was purified by standard phenol/chloroform extraction; 8 μg of DNA was digested with *Bam*HI, resolved on a 0.8% agarose gel, and transferred to membrane by standard protocol (25). The cosmids B4, c76, c63, and 05#1, covering portions of the *FHIT* locus (21), were labeled by random primer extension (Stratagene), preannealed to sheared human genomic DNA, and hybridized to the filters, as described (22).

Cell Lines. Cell lines were developed in the University of Pittsburgh Head and Neck Tissue Culture Laboratory (UPCI) (J. K. Reddy, K. M. Rossie, C. M. Lese, R. L. Wagner, J. T. Johnson, and S.M.G., unpublished data). Briefly, primary tumors biopsies were minced and plated in minimal essential medium supplemented with penicillin, streptomycin, neomycin, L-glutamine, nonessential amino acids, and 10–15% fetal bovine serum. The individual passages of the cell lines are specified in the text.

Fluorescence *in Situ* Hybridization (FISH) Analysis. Cells were harvested for molecular cytogenetic studies by standard techniques. Briefly, cell cultures were incubated in the presence of 10–50 μg/ml Colcemid from 5 to 16 hr at 37°C in a 5% CO_2 incubator. The cells were trypsinized, washed in Hank's balanced salt solution, and incubated in a hypotonic 0.075 M KCl solution for 18 min at 37°C. Cells were fixed in a 3:1 methanol/acetic acid solution at room temperature for 20 min, followed by two washes in the same solution. Fixed cell pellets were stored at −20°C until use. The cosmid c76, covering exon 5 and flanking regions, and cosmid 05#7, spanning exons 9 and 10, were labeled by nick translation with biotin-16-dUTP and digoxigenin-11-dUTP, respectively. The FISH probe nick translation, chromosomal *in situ* suppression hybridization, and detection reactions were carried out as described (26), except that unincorporated nucleotides were removed from the probe by ethanol precipitation. In addition to protease pretreatment, the slides were aged in 2× standard saline citrate (SSC) for 30 min at 37°C, and treated with RNase A for 30 min at 37°C. Posthybridization washes were carried out three times for 3 min each in 50% formamide/2× SSC at 45°C, and 2× SSC at 37°C. Detection was accomplished using 5 μg/ml of fluorescein isothiocyanate-avidin and rhodamine-conjugated anti-digoxigenin. Approximately 200 nuclei were counted for each sample, along with a concurrent normal peripheral blood leukocyte control slide. Only subpopulations that represented at least 10% of the total number of nuclei were considered in the evaluation.

RESULTS

Aberrant Transcripts in HNSCC Cell Lines. Twenty-six cell lines established from HNSCC tumors were analyzed by RT–PCR for the presence of aberrant *FHIT* transcripts. Cell lines were all at relatively early passage in culture, mostly within the first 10–20 passages. Sites and stages of individual tumors are given in Table 1. RNA was isolated, reverse transcribed, and amplified by PCR using primers derived from exons 1 and 10 followed by amplification with nested primers in exons 3 and exon 10 of the *FHIT* gene (21, 22). Aberrant transcripts were observed in 15 of 25 (55%) of the cell lines (Table 1); one cell line, SCC084, gave no RT–PCR product. Amplified products from most of the cell lines with aberrant transcripts (12/15) displayed both normal and abnormal sized products with some lines showing multiple abnormal sized products. The *FHIT* gene is composed of 10 exons with the protein coded by exons 5–9. Sequence analysis of the aberrant

Table 1. Tumor characteristics, patient history, and *FHIT* transcripts

Cell line, UPCI no.	S/A	Tumor site	Stage	*FHIT* transcripts	Aberrant products, missing exons/insertions
SCC032	+/+	RT	T2 N2b MX	Normal	None
SCC036	+/+	Tonsil	T3 N1 MX	Normal plus aberrant	Exon 4–8
SCC056	+/+	Tongue	T3 N2b MX	Aberrant	Exons 5–7
SCC065	+/+	Tongue	T2 N0 MX	Normal	None
SCC066	+/+	Mandible	T1 N0 MX	Normal	None
SCC070	+/+	RT	T2 N2b MX	Normal plus aberrant	NA
SCC072	+/+	BOT	T3 N2b MX	Normal plus aberrant	Exons 5–6 and exons 4–8
SCC074	−/−	Mandible	T4 N1 MX	Normal plus aberrant	NA
SCC075	+/+	Tongue	T3 N2b MX	Normal	None
SCC077	+/+	FOM	T2 N2 MX	Normal plus aberrant	Exons 4–7 and exons 4–8
SCC078	+/+	FOM	T2 N0 MX	Normal plus aberrant	Exons 4–8
SCC081	+/−	AR	T4 N0 MX	Normal	None
SCC084	+/+	RT	T2 N2B MX	ND	None
SCC089	+/+	Tonsil	T4 N2b MX	Aberrant	Exons 5–7
SCC103	+/−	Tongue	T1 N0 MX	Normal plus aberrant	Exon 8
SCC104	+/+	FOM	T4 NX MX	Normal plus aberrant	Exons 4–8
SCC105	+/+	FOM	T2 N0 MX	Normal plus aberrant	Exons 5–7 and exons 5–8
SCC111	+/−	FOM	T1 N1 MX	Normal	None
SCC114	+/+	FOM	T2 N0 MX	Aberrant	Exon 4
SCC116	−/+	AR	T2 N0 MX	Normal	None
SCC121	+/+	RT	T1 N0 MX	Normal plus aberrant	72-nt insertion at exon 5
SCC125	+/+	FOM	T4 N2b MX	Normal plus aberrant	Exon 8 + 6 nt insertion
SCC131	+/+	FOM	T2 N2 MX	Normal	None
SCC136	−/−	RMT	T3 N2 MX	Normal plus aberrant	Exon 4
SCC142	+/+	FOM	T4 NX MX	Normal plus aberrant	NA
SCC122	+/+	Tongue	T1 N1 MX	Normal	None

S/A, smoking/alcohol history; RT, retromolar trigon; BOT, base of tongue; FOM, floor of mouth; AR, alveolar ridge; NA, not analyzed; ND, not detected.

RT–PCR products showed that absence of exon 4 or 5 through exon 8 was the most common abnormality, accounting for 7 of the 14 aberrant products sequenced, as summarized in Table 1. In these cases, the protein coding region is absent except for exon 9, which does not contain a translation start codon (21). Therefore no portion of the protein could be synthesized from such transcripts. Aberrant RT–PCR products from cases SCC103 and SCC125 showed absence only of exon 8 and the SCC125 product also contained a 6-nt insertion between exons 5 and 6, resulting in the insertion of two amino acids. Absence of exon 8 is probably crucial, since it contains the highly conserved histidine triad motif and is essential for the function of the protein (27). Two cell lines, SCC114 and SCC136, showed absence of exon 4, leaving the starting ATG in exon 5 untouched, resulting in transcripts with the potential to produce intact Fhit proteins. Similarly, an aberrant transcript from SCC121 contained an additional 72 nt inserted between exons 4 and 5, leaving the protein coding region intact. The effect of alterations in the 5′ untranslated region is not clear, but could possibly affect translation. Interestingly, in sample SCC114 the product lacking exon 4 is the only one observed. Furthermore this cell line shows a homozygous deletion of a portion of intron 5, as discussed below.

Deletion of the *FHIT* Gene. To look for deletions or rearrangements of the *FHIT* gene, Southern blot analysis of DNA from 22 of the cell lines was carried out using four *FHIT* locus cosmids: B4 and c76 covering part of intron 4, all of exon 5, and a portion of intron 5; c63 covering a portion of intron 5; and cosmid 05#1 covering exons 6 and 7 and flanking regions (see Fig. 2*A*). Genomic DNA was digested with *Bam*HI and the blots were sequentially hybridized with the individual cosmids using supressive competition to eliminate hybridization to repetitive sequences. An example of a Southern blot with DNA from 10 of the cell lines is shown in Fig. 2*B*. Three cell lines showed homozygous deletions within the *FHIT* gene: SCC056 DNA did not give a hybridization signal when hybridized with cosmids B4 and c76 indicating deletion of exon 5 and flanking intron 4 and 5 sequences; SCC089 DNA showed no hybridization to cosmids c63 and 05#1 indicating deletion of exons 6 and 7 and flanking intron sequences, especially a large portion of intron 5; SCC114 also showed deletion of a portion of intron 5 when hybridized with cosmid c63. In summary, 3 of 22 cell lines showed homozygous deletions within the *FHIT* gene by Southern analysis, indicating that in at least 12% of the cases, both copies of the *FHIT* gene are partially deleted. This is a minimum estimate, because only a portion of the *FHIT* locus was covered by these cosmids. These data were in agreement with results of the RT–PCR analysis, in which all three samples, SCC056, SCC089, and SCC114, did not exhibit a normal amplified *FHIT* cDNA (Fig. 1); interestingly the aberrant SCC089 transcript lacked exons 5, 6, and 7, but at the genomic level exon 5 was present and exons 6 and 7 were deleted, suggesting that downstream deletion may affect the correct splicing of exon 5. PCR of single FHIT exons from

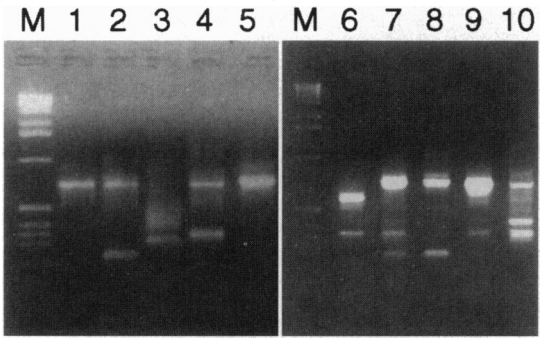

FIG. 1. RT–PCR of *FHIT*. RNA from the UPCI HNSCC cell lines was used for nested RT–PCR with *FHIT* primers. Lanes: 1, SCC032; 2, SCC036; 3, SCC056; 4, SCC74; 5, SCC122; 6, SCC072; 7, SCC077; 8, SCC78; 9, SCC104; 10, SCC105.

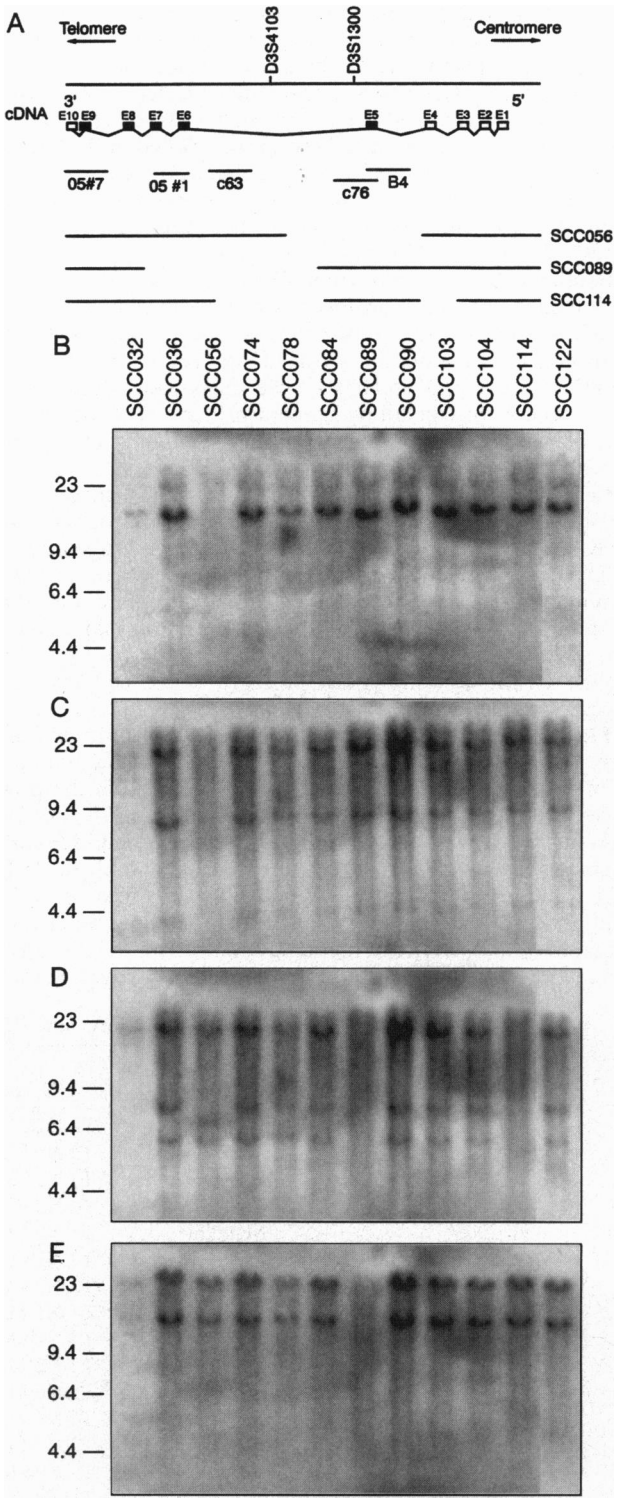

FIG. 2. Genomic alteration detected by Southern blot hybridization with cosmid probes. (*A*) Schematic presentation of the *FHIT* gene organization showing the position of the internal microsatellite markers, the FRA3B represented by the hybrid cl3 break, and individual cosmids encompassing portions of the *FHIT*. The lower lines are a schematic presentation of the estimated extent of the homozygous deletions observed in the UPCI cell lines: SCC056, SCC089, and SCC114. (*B–E*) Southern blot hybridization of *Bam*HI-cleaved DNA from HNSCC cell lines with cosmid B4, c76, c63, and 05#1, respectively.

Table 2. FISH analysis of HNSCC cell lines

Cell Line (UPCI)	Population 1	Population 2	Population 3	Population 4
Control	88% 2RG Normal			
SCC072 (P5)	70% 2RG Normal			
SCC074 (P5)	74% 2RG Normal			
SCC077 (P5)	66% 2RG Normal			
SCC114 (P29)	82% 2RG Normal			
SCC116 (P7)	73% 2RG Normal			
SCC131 (P3)	75% 2RG Normal			
SCC136 (P2)	84% 2RG Normal			
SCC036 (P5)	48% 2RG Normal	18% 1RG, 1R Loss of exon 5		
SCC056 (P9)	66% 1R Loss of exon 10, deletion of exon 5	29% 0RG Deletion exons 5 and 10		
SCC081 (P2)	62% 2RG Normal	17% 1RG, 1R Loss of exon 5	10% 1RG Loss of exons 5 and 10	
SCC084 (P1)	72% 1RG Loss of exons 5 and 10	10% 2RG Normal		
SCC089 (P32)	35% 2RG Normal	25% 1RG, 1G Loss of exon 10	14% 1RG Loss of exons 5 and 10	
SCC104 (P24)	60% 2RG Normal	13% 1RG, 1G Loss of exon 10		
SCC111 (P33)	59% 2RG Normal	10% 0RG Deletion of exons 5 and 10		
SCC121 (P14)	35% 2RG Normal	22% 2G Deletion of exon 10	17% 1RG, 1G Loss of exon 10	15% 1RG Loss of exons 5 and 10
SCC065 (P20)	48% 2RG Normal	17% 3RG Gain of exons 5 and 10		
SCC066 (P31)	52% 2RG Loss of exons 5 and 10*	13% 3RG Loss of exons 5 and 10*		
SCC075 (P4)	80% 2RG Normal	11% 4RG Gain 2 copies of exons 5 and 10		
SCC103 (P41)	56% 3RG Gain of exons 5 and 10	29% 2RG Normal		
SCC105 (P11)	50% 2RG Normal	16% 1RG Loss of exons 5 and 10	13% 2RG, 1R Gain of exon 10	

P, passage number; RG, red/green (exon 10/5) signal doublet; R, red exon 10 signal; G, green exon 5 signal.
*Based on tetraploid background.

genomic DNA demonstrated that sample SCC056 does retain exon 4, centromeric to the homozygous deletion, and sample SCC089 retains exon 8, telomeric to the homozygous deletion (data not shown), indicating that all three deletions are internal to the *FHIT* gene. The extent of the observed deletions is shown in Fig. 2*A*.

FISH Analysis. FISH analysis was carried out on interphase nuclei from 20 of the cell lines. Two cosmids were used concomitantly, c76 and 05#7, covering exon 5 and exons 9 and 10, respectively (Fig. 2*A*). The cosmid c76 was detected with fluorescein tagged avidin to give a green signal and 05#7 with rhodamine-conjugated anti-digoxigenin to give a red signal. The results are summarized in Table 2 and show that most (65%) of the cell lines examined displayed multiple cell populations with diverse regions of deletion of portions of the *FHIT* gene; 6/9 of the cell lines which showed multiple *FHIT* transcripts also displayed multiple cell populations. This observation may explain the amplification of both normal and aberrant transcript(s) by RT–PCR. The majority of the cell lines (16/20) showed a mayor population, defined as "normal," displaying signals for both exon 5 and exon 9/10, but could be missing any of the intermediate exons not tested by FISH analysis, as for example the cell lines SCC114 and SCC089, which we have shown by Southern analysis to exhibit homozygous deletion of exon 4 and exons 6 and 7, respectively. Cell line, SCC056, did not display the presence of a normal population, and no signal was detectable for exon 5, confirming the results obtained by RT–PCR and Southern analysis; sample SCC084, in which an RT–PCR product was not detectable, showed 72% of the cells with copy number loss for both probes. Four of the cell lines, SCC065, SCC066, SCC075, and SCC103, presented a subpopulation with a gain of both markers; three of these cell lines reveal only normal *FHIT* transcript by RT–PCR and the fourth, SCC103, has both aberrant and normal forms. It is possible that the apparent gain reflects the ploidy level of the specific cell line. As for SCC066, which is tetraploid (S.M.G., unpublished data), the population with 2 signals by FISH may actually

reflect a loss. Karyotypes of the other cell lines presenting gains are not available. FISH analysis revealed the presence of populations with deletion of exons 9 and 10. Aberrant transcripts originating from these populations would not be detected by RT–PCR since the primers used for amplification are in exon 10. In summary, FISH analysis revealed the presence of multiple cell populations within the majority of cell lines. We believe that they represent populations present in the original tumor sample since the number of *in vitro* subcultures was relatively low (Table 2), sometimes only two or three passages, and comparison of other cytogenetic markers between primary tumors and the cell lines suggested that the cell lines are reflective of the heterogeneous cell populations within the tumors. Furthermore two groups of investigators have previously observed loss of different 3p alleles at different sites of the same lesion, indicating the presence of multiple cell populations in primary oral dysplastic lesions (18, 19).

DISCUSSION

The *FHIT* gene was recently isolated from chromosome band 3p14.2, which is often deleted in epithelial cancer cell lines (21). Furthermore the gene is disrupted by chromosome translocation, t(3;8)(p14;q24), which is transmitted in an Italian-American family and segregates in the family with the early onset of clear cell renal carcinoma. The gene has been reported to be abnormally transcribed in primary tumors of the digestive tract and lung (21, 22). LOH at 3p14 has been observed frequently in squamous cell carcinomas of the head and neck (3, 5, 19, 28); in this study we report the detection of abnormal *FHIT* RT–PCR products in 55% of our HNSCC cell lines. The occurrence of *FHIT* gene abnormalities was studied by RT–PCR, Southern blot analysis, and interphase FISH. In 12% of the cell lines, both copies of the gene sustained deletions; in at least two other cases (SCC072 and SCC105) the normal *FHIT* transcript was barely detectable by RT–PCR; in all remaining samples both normal and abnormal *FHIT* transcripts were observed. The occurrence of normal and aberrant transcripts in the same cell line might be explained by the presence of several distinct cell populations, which would obscure the detection of homozygous deletions in a subpopulation; in this regard, FISH analysis showed at least two more cell lines, SCC111 and SCC121, contained subpopulations with nullisomy of exon 5, and/or exons 9 and 10. This may be an underestimation since only 20 of the 26 cell lines were analyzed by FISH and only probes for exons 5 and 9/10 of the *FHIT* coding region were utilized in the FISH analysis.

The multiple genetic lesions within the *FHIT* gene may be explained by the location of the gene within a fragile site, by definition highly susceptible to breakage induced by carcinogens (29). This is important, since HNSCCs have been recognized to be caused by etiological agents such as alcohol and tobacco.

The finding that 55% of HNSCC cell lines express aberrant *FHIT* transcripts and that one or both *FHIT* alleles are partially deleted in many of these cell lines indicates that loss of *FHIT* function is likely to be important for the development of the tumorigenic phenotype, as reported for SCLC, NSCLC (22), tumors of the digestive tract (21), Merkel cell carcinoma (30), and breast tumors (31), although the biological function of the Fhit protein, an Ap_3A hydrolase (21, 27), is not known. The clarification and understanding of the function of the Fhit protein in the cell and its mechanism of action may provide important information toward the understanding of the biological mechanisms underlying many different types of human cancer.

We thank Ms. Seia Comsa and Ms. Jaya Reddy for developing and culturing the cell lines, Dr. Karen Rossie for specimen triage and histopathologic diagnoses, and Ms. Robin Wagner, Ms. Kim Kachman, and Drs. Jonas T. Johnson and Eugene N. Myers for clinical coordination of the studies at the University of Pittsburgh. This work was supported in part by National Cancer Institute Outstanding Investigator Grant CA 39860 to C.M.C., by Grant CA 21124 to K.H., and by Natiional Institutes of Health Grant DE10513 to S.M.G.

1. Parker, S. L., Tong, T., Bolden, S. & Wingo, P. A. (1996) *Cancer J. Clin.* **65,** 5–27.
2. Johnson, N. W. (1991) in *Oral Cancer*, ed. Johnson, N. W. (Cambridge Univ. Press, Cambridge, U.K.), Vol. 2, pp. 3–26.
3. Ah-See, K. W., Cooke, T. G., Pickford, I. R., Soutar, D. & Balmain, A. (1994) *Cancer Res.* **54,** 1617–1621.
4. Nawroz, H., van der Riet, P., Hruban, R. H., Koch, W., Ruppert, M. J. & Sidransky, D. (1994) *Cancer Res.* **54,** 1152–1155.
5. Ishwad, C. S., Ferrel, R. I., Rossie, K. M., Appel, B. N., Johnson, J. T., Myers, E. N., Law, J. C., Srivastava, S. & Gollin, S. M. (1996) *Int. J. Cancer* **69,** 1–4.
6. Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K. & Carson, D. A. (1994) *Nature (London)* **368,** 753–756.
7. Cairns, P., Polascik, T. J., Eby, Y., Tokino, K., Califano, J., *et al.* (1995) *Nat. Genet.* **11,** 210–212.
8. Zhang, S., Klein-Szanto, A. J., Sauter, E. R., Shafarenko, M., Mitsunaga, S., Nobori, T., Carson, D. A., Ridge, J. A. & Goodrow, T. L. (1994) *Cancer Res.* **54,** 5050–5053.
9. Kok, H., Osinga, J., Carrit, B., Davis, M. B., van der Veen, A. J., Landsvater, R. M., de Leij, L. F. M. H., Berendsen, H. H., Postmus, P. E., Popema, S. & Buys, C. H. C. M. (1987) *Nature (London)* **330,** 578–581.
10. Naylor, S. L., Johnson, B. E., Minna, J. D. & Sakaguchi, A. Y. (1987) *Nature (London)* **329,** 451–454.
11. Yokota, A., Wada, M., Shimosato, Y., Terada, M. & Sugimura, T. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9252–9256.
12. Yokoyama, S., Yamakawa, K., Tsuchiya, E., Murata, M., Sakiyama, S. & Nakamura, Y. (1992) *Cancer Res.* **52,** 873–877.
13. Sato, T., Akiyama, F., Sakamoto, G., Kasumi, F. & Nakamura, Y. (1991) *Cancer Res.* **51,** 5794–5799.
14. Jones, M. H. & Nakamura, Y. (1992) *Oncogene* **7,** 1631–1634.
15. Southerland, G. R. (1991) *Genet. Anal. Tech. Appl.* **8,** 161–166.
16. Smeets, D. F. C. M., Scheres, J. M. J. C. & Hustinx, T. W. J. (1986) *Hum. Genet.* **72,** 215–220.
17. Yunis, J. J. & Soreng, A. L. (1984) *Science* **226,** 1199–1204.
18. Roz, L., Wu, C. L., Porter, S., Scully, C., Speight, P., Read, A., Sloan, P. & Thakker, N. (1996) *Cancer Res.* **56,** 1228–1231.
19. Mao, L., Lee, J. S., Fan, Y. H., Ro, J. Y., Batsakis, J. G., Lippman, S., Hittelman, W. & Hong, W. K. (1996) *Nat. Med.* **2,** 682–685.
20. Kastury, K., Baffa, R., Druck, T., Ohta, M., Cotticelli, M. G., Inoue, H., Negrini, M., Rugge, M., Huang, D., Croce, C. M., Palazzo, J. & Huebner, K. (1996) *Cancer Res.* **56,** 978–983.
21. Ohta, M., Inoue, H., Cotticelli, M. G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., Croce, C. M. & Huebner, K. (1996) *Cell* **84,** 587–597.
22. Sozzi, G., Veronese, M. L., Negrini, M., Cotticelli, M. G., Inoue, H., Tornielli, S., Pilotti, S., De Gregorio, L., Pastorino, U., Pierotti, M., Ohta, M., Huebner, K. & Croce, C. M. (1996) *Cell* **85,** 17–26.
23. Franceschi, S., Talamini, R., Barra, S., Baron, A. E., Bidoli, E., Serraino, D. & La Vecchia, C. (1990) *Cancer Res.* **50,** 6502–6507.
24. Wynder, E. L. & Stellman, S. D. (1977) *Cancer Res.* **37,** 4608–4622.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
26. Lichter, P. & Ried, T. (1994) *Methods Mol. Biol.* **29,** 449–478.
27. Barnes, L. D., Garrison, P. N., Siprashvili, Z., Guranowski, A., Robinson, A. K., Ingram, S. W., Croce, C. M., Ohta, M. & Huebner, K. (1996) *Biochemistry*, in press.
28. Wu, C. H., Sloan, P., Read, A. P., Harris, R. & Thakker, N. (1994) *Cancer Res.* **54,** 6484–6488.
29. Glover, T. W., Coyle-Morris, J. F., Brown, R. S., Berger, C., S., Gemmill, R. M. & Hecht, F. (1988) *Cancer Genet. Cytogenet.* **31,** 69–73.

30. Sozzi, G., Alder, H., Tornielli, S., Corletto, V., Baffa, R., Veronese, M. L., Negrini, M., Pilotti, S., Pierotti, M., Huebner, K. & Croce, C. M. (1996) *Cancer Res.* **56,** 2472–2474.

31. Negrini, M., Monaco, C., Vorechovsky, I., Ohta, M., Druck, T., Baffa, R., Huebner, K. & Croce, C. M. (1996) *Cancer Res.* **56,** 3173–3179.