PERGAMON

European Journal of Cancer 38 (2002) 1585–1592

European Journal of Cancer

www.ejconline.com

Frequent 3p allele loss and epigenetic inactivation of the *RASSF1A* tumour suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma

R.P. Hogg[a,1], S. Honorio[a,1], A. Martinez[a], A. Agathanggelou[a], A. Dallol[a], P. Fullwood[a], R. Weichselbaum[b], M.J. Kuo[c], E.R. Maher[a], F. Latif[a,*]

[a]*Department of Paediatrics and Child Health, Section of Medical and Molecular Genetics, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[b]*Department of Radiation Oncology, University of Chicago Medical Center, Chicago, IL 60637, USA*
[c]*Department of Otolaryngology and Head and Neck Surgery, Queen Elizabeth Hospital, Queen Elizabeth Medical Centre, Edgbaston, Birmingham B15 2TT, UK*

Received 30 July 2001; received in revised form 31 October 2001; accepted 5 December 2001

Abstract

Studies of allelic imbalance and suppression of tumourigenicity have consistently suggested that the short arm of chromosome three (3p) harbours tumour suppressor genes (TSGs) whose inactivation leads to the development of various types of neoplasia including head and neck squamous cell carcinoma (HNSCC). Previously, we defined a critical minimal region of 120kb at 3p21.3 that contains overlapping homozygous deletions in lung and breast tumour lines and isolated eight genes from the minimal region. Mutation analysis in a large panel of lung and breast cancers revealed only rare mutations, but the majority of lung tumour lines showed loss of expression for one of the eight genes (*RASSF1A*) due to hypermethylation of a CpG island in the promoter region of *RASSF1A*. We found *RASSF1A* to be methylated in the majority of lung tumours, but to a lesser extent in breast and ovarian tumours. In order to define the role of 3p TSGs, in particular *RASSF1A* in HNSCC, we (a) analysed 43 primary HNSCC for allelic loss in regions proposed to contain 3p TSGs (3p25-26, 3p24, 3p21-22, 3p14 and 3p12), (b) analysed 24 HNSCC for evidence of *RASSF1A* methylation and (c) undertook mutation analysis of *RASSF1A* in HNSCC. We found that 81% of HNSCC showed allele loss at one or more 3p markers, 66% demonstrated loss for 3p21.3 markers and 56% showed allelic losses at 3p12 loci. Thus, 3p loss is common in HNSCC and extensive 3p loss occurs even in early stage tumours. *RASSF1A* promoter region hypermethylation was found in 17% (4/24) of the sporadic HNSCC, but *RASSF1A* mutations were not identified. Furthermore, we found *RASSF1A* methylation to be significantly higher in poorly differentiated then in moderate to well differentiated HNSCC ($P=0.0048$). Three of the four tumours showing *RASSF1A* methylation also underwent 3p21.3 allelic loss, hence *RASSF1A* behaves as a classical TSG (two hits, methylation and loss). One tumour with *RASSF1A* methylation had retention of markers at 3p providing further evidence of specific inactivation of *RASSF1A* as a critical step in some HNSCC. Although the frequency of 3p21.3 allele loss was substantially higher than that of *RASSF1A* methylation this does not necessarily suggest that other genes from 3p21.3 are also implicated in HNSCC, as 3p21.3 LOH was invariably found with LOH at other 3p loci. Thus, the presence of 3p21.3 allele loss without *RASSF1A* methylation might reflect a propensity for 3p21.3 loss to occur as a secondary consequence of large 3p deletions targeted at other 3p TSG regions. Furthermore, in the presence of homozygous inactivation of other 3p TSGs, *RASSF1A* haploinsufficiency might be sufficient to promote tumourigenesis in many HNSCC.

Keywords: Tumour suppressor gene; Allele loss; *RASSF1A*; HNSCC

* Corresponding author. Tel.: +44-121-627-2741; fax: +44-121-627-2618.
E-mail address: flatif@hgmp.mrc.ac.uk (F. Latif).
[1] These authors contributed equally.

0959-8049/02/$ - see front matter
PII: S0959-8049(01)00422-1

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a common neoplasm associated with exposure to tobacco and alcohol. It accounts for up to 5% of all malignancies in the Western world [1]. There are major geographical differences in the incidence of HNSCC, in the Far East and the Indian subcontinent up to 40% of malignancies occur in the head and neck region [2]. Recent evidence from epidemiological studies suggest an increased incidence and mortality of head and neck cases particularly in developed countries with young males being the worst affected. Changes in tobacco and alcohol habits seem to be the most likely explanation for the trend.

The activation of oncogenes and inactivation of tumour suppressor genes (TSGs) are mechanisms that play pivotal roles in the multistep molecular process of tumourigenesis. Chromosomal arms 1p, 3p, 4, 2q, 8p, 9p, 11q, 13q, 17p, 18q ([3–7], reviewed in Ref. [8]) show high levels of allelic losses in HNSCC. These regions of high levels of loss of heterozygosity (LOH) have been corroborated by cytogenetic and comparative genome hybridisation studies in many cases.

Deletions of 3p are also frequent in many other common sporadic cancers including lung, breast, kidney, cervical and ovarian cancer (reviewed in Ref. [9]). Regions which have been consistently implicated as harbouring 3p HNSCC TSGs mapped by LOH to a number of loci—namely 3p25, 3p21.3, 3p14.2 and 3p12 [7,10] and the multiplicity of sites implies the possibility of multiple TSGs which may either operate singly or in concert, depending on the carcinogen involved. These regions are in part concordant with some other sites of intensive investigation in lung, breast and other cancers. The von Hippel–Lindau (*VHL*) TSG at 3p25 [11,12] has been excluded as a candidate HNSCC TSG using mutation and methylation studies [7,13]. The region at 3p21.3 is thought to harbour at least two TSGs. One region (LCTSGR1) is defined by overlapping homozygous deletions in a breast tumour and in three small cell lung cancer tumour lines with a 120kb minimal commonly deleted region [14–16]. Eight genes have been isolated from the minimal region, but none shows frequent mutations in lung or breast tumours [16]. Very recently, we and others have shown that one of the eight genes from the 120kb minimal region (*RASSF1A*) is silenced by hypermethylation of a CpG island in its promoter region in lung and breast tumours and tumour lines [17–19]. The other region is telomeric to the first region and contains a 800kb homozygous deletion in one lung tumour line [20]. *FHIT* [21], a putative TSG which resides at the 3p14.2 FRA3B fragile site, has for a while been thought of as a strong candidate in multiple cancer types and in HNSCC has been found to demonstrate homozygous deletions and aberrant transcripts [22] in cell lines. In addition, a 3p12 region (LCTSGR2) is implicated as a candidate TSG interval by the finding of overlapping homozygous deletions in two small cell lung cancer lines and in one breast tumour line and contains the *DUTT1* candidate gene [23].

The functional relevance of 3p in HNSCC is demonstrated by suppression of tumorigenicity studies which have suggested 3p21.3 and 3p21.3-p21.2/3p25 as regions containing TSGs in nasopharyngeal carcinoma [24] and in oral cancer [25], respectively. Studies examining the relationship between tumour stage and grade with LOH rate have suggested an accumulation of aberrations with clinical progression and 3p loss has also been shown to be an early event in oral tumorigenesis [26,27]. Similar findings have been published for lung cancer [28]. The fact that smoking is a risk factor of such dominant importance in both HNSCC and lung cancer means that lung cancer TSG regions should be carefully tested to determine whether they harbour genes that are critical in both of these tumour types.

In order to investigate the role of 3p TSGs in the pathogenesis of HNSCC, we performed detailed studies of 3p allelic loss in primary HNSCC using microsatellite markers spread across 3p and including recently developed markers from within regions LCTSGR1 at 3p21.3 and LCTSGR2 at 3p12, regions that have been implicated in other common cancers including lung. We also analysed the methylation and mutation status of a newly identified TSG from region 3p21.3 (*RASSF1A*), which we have previously reported to be hypermethylated in the majority of lung tumours.

2. Patients and methods

2.1. Clinical material

Tumour tissue samples and patient-matched normal blood or mucosa were collected at the Queen Elizabeth Medical Hospital, Queen Elizabeth Medical Centre, Edgbaston, Birmingham, UK. Amongst the HNSCC, there were 23 laryngeal, 13 pharyngeal, five from the oral cavity and two paranasal sinuses. Histological grade: eight were well differentiated, 19 were moderately differentiated, 13 were poorly differentiated and for three HNSCC the grade was unknown. Clinical Stage: one was stage 1, nine were stage 2, 10 were stage 3, 20 were stage 4 and three were of unknown stage. Dr Weichselbaum from the University of Chicago provided the cell lines. Genomic DNA was extracted using a proteinase K digestion, phenol/chloroform extraction and subsequent ethanol precipitation.

2.2. Loss of heterozygosity analysis

Twenty-three microsatellite repeat polymorphic markers were selected for use in our array spanning the regions of interest on chromosome 3p, all are available through the Genoma Database (GDB) database. The

microsatellite analysis has been previously described in Ref. [29]. Briefly, the forward primers were 5′ end-labelled in a 10 μl reaction volume containing 2 pmol of primer per reaction, 1×T4 polynucleotide Kinase (PNK) buffer (MBI Fermentas, Vilnius, Lithuania), 10 units of T4 polynucleotide kinase (MBI Fermentas), and 30 μCi of [γ-^{32}P] adenosine triphosphate (ATP) (3000 Ci/mmol) (Amersham Life Science, Amersham Pharmacia Biotech, Buckingham, UK). This reaction was then incubated at 37 °C for 30 min, and 94 °C for 5 min. Polymerase chain reaction (PCR) was performed in a 10 μl reaction volume containing 100 ng of genomic DNA, 1×PCR buffer (Gibco-BRL, Paisley, UK), 100 μM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), 0.05% (v/v) W1 (Gibco-BRL), 3 mM $MgCl_2$, 0.5 units of DNA Taq polymerase (Gibco-BRL), and 2 pmol of reverse primer. PCR cycling conditions were as follows: an initial 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at the appropriate annealing temperature and 30 s at 72 °C, with a final soak at 72 °C for 10 min. The reaction products were then diluted 1:1 with a solution of 95% (v/v) formamide, 10 mM ethylene diamine tetraacetic acid (EDTA), 10% (w/v) bromophenol blue and 10% (w/v) xylene cyanol. After heating for 5 min at 94 °C, 3.5 μl was loaded onto a 6% (v/v) polyacrylamide denaturing gel (Sequagel-6; National Diagnostics, Hessle, Hull, UK). Gels were electrophoresed at 90 W constant power to achieve adequate separation of alleles and then dried at 80 °C and autoradiographed (Fuji RX film, Japan).

LOH was scored based on a >50% loss of allele intensity in tumour-derived DNA upon comparison to the normal tissue-derived DNA.

2.3. Bisulphite modification, direct sequencing and restriction enzyme digestion

Bisulphite DNA sequencing was performed as previously described in Refs [19,30]. Briefly, 0.5–1.0 μg of

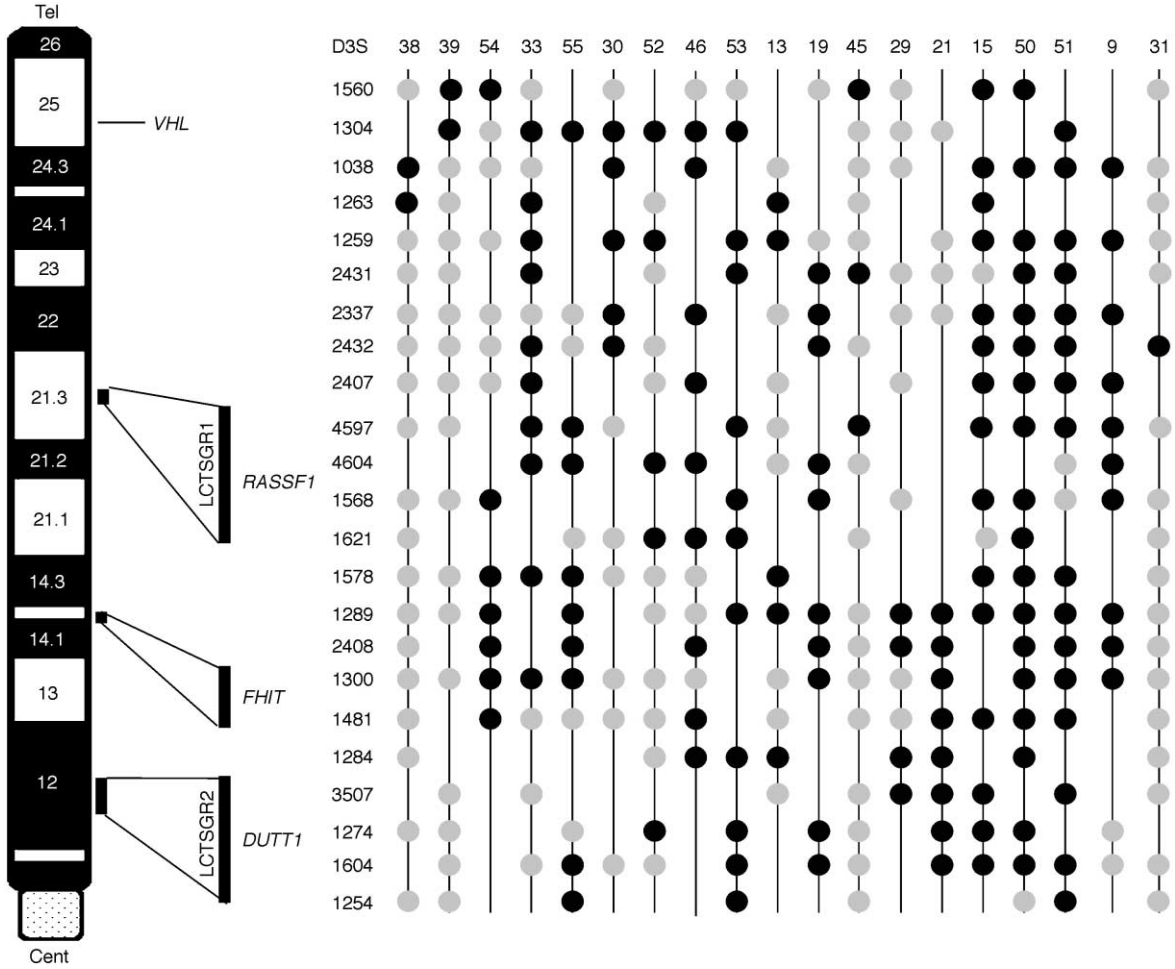

Fig. 1. Pattern of loss of heterozygosity (LOH) for 19 head and neck squamous cell carcinoma (HNSCC) showing partial loss of 3p. Each column represents a tumour and each row represents a 3p microsatellite marker listed in descending order from telomere (D3S1560) to centromere (D3S1254). LCTSGR1 at 3p21.3 is represented by markers D3S4597, D3S4604, D3S1568 and D3S1621. LCTSGR2 at 3p12 is represented by markers D3S3507, D3S1274, D3S1604, D3S1254. The status of each 3p locus is indicated as follows: black circles represent loss, grey circles represent retention and no symbol represent uninformative loci. Tel, telomere; Cent, centromere.

genomic DNA was denatured in 0.3 M NaOH for 15 min at 37 °C and then unmethylated cytosine residues were sulphonated by incubation in 3.12 M sodium bisulphite (pH 5.0) (Sigma, Dorset, UK)/5 mM hydroquinone (Sigma) in a thermocycler (Hybaid) for 30 s at 99 °C/15 min at 50 °C for 20 cycles. The sulphonated DNA was recovered using the Wizard DNA clean-up system (Promega, Madison, USA) in accordance with the manufacturer's instructions. The conversion reaction was completed by desulphonating in 0.3 M NaOH for 10 min at room temperature. The DNA was ethanol precipitated and resuspended in water.

DNA sequences specific for the *RASSF1A* promoter region were amplified using primers and conditions already described in Ref. [19]. Methylated cytosine residues were identified either by direct sequencing of PCR products using a dRhodamine cycle sequencing kit (PE Applied Biosystems, Warrington, UK) or by restriction enzyme digestion. Briefly, 16 μl of the 204bp PCR product was incubated with 20 units of *TaqI* (Roche Diagnostics, Lewes, UK) or *BstUI* (New England BioLabs, Hitchin, UK) in separate reactions for 2 h at 65 or 60 °C, respectively. To ensure that restriction enzyme was present in all incubations, a master mix containing *TaqI* or *BstUI* was prepared and aliquoted into sample and control PCR products. The size of the *TaqI* restriction enzyme digestion products possible are 173, 112, 92, 81 and 31bp; and the size of the *BstUI* digestion products are 172, 121, 89, 83 and 32bp (Fig. 2). The restriction enzyme digestion products were then visualised by separation in a 3% (w/v) agarose gel or an 8% (w/v) polyacrylamide gel stained with ethidium bromide.

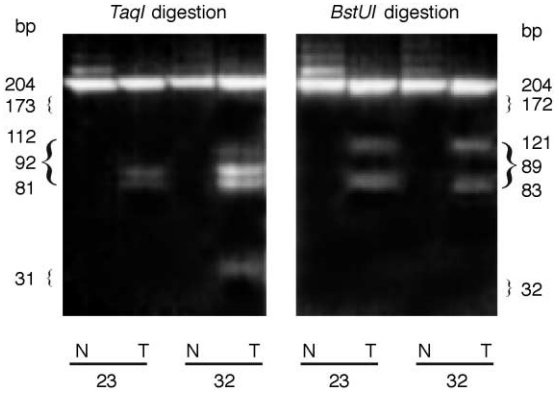

Fig. 2. Representative results of bisulphite-Polymerase Chain Reaction (PCR) methylation analysis in head and neck squamous cell carcinoma (HNSCC) of the 5′ region of *RASSF1A*. Tumour and normal DNA was treated with bisulphite, amplified using specific primers described in Ref. [19] and digested using restriction enzymes that recognise methylated alleles only, bands were separated on 3% agarose gel stained with ethidium bromide. The methylated alleles were cleaved with the restriction enzymes (*TaqI* and *BstUI*). The sizes of the digested products are against the brackets. The 204bp undigested product is seen in each lane.

2.4. Mutation analysis of RASSF1A in head and neck tumours

Mutation analysis on 10 HNSCC normal/tumour pairs was performed using PCR-Single Strand Conformation Polymorphism (SSCP) as previously described in Ref. [19] using intronic primers to amplify each exon, any aberrantly migrating bands were sequenced on an ABI 377 automated sequencer.

2.5. Cloning and sequencing of PCR products

The PCR products were purified using High Pure PCR product purification kit (Roche) and ligated into the pGEM-T easy vector system (Promega) according to the manufacturer's instructions. Several clones were then isolated and sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA).

2.6. Statistical analysis

Comparisons were made by Fisher's Exact test and Chi-square test as appropriate. P values of <0.05 were taken as statistically significant.

3. Results

3.1. 3p LOH analysis in head and neck tumours

We determined the frequency, extent and patterns of 3p loss in 43 HNSCC using 23 polymorphic microsatellite markers spanning 3p, but targeted to regions containing known or putative 3p TSGs (Fig. 1). One tumour showed microsatellite instability for the majority of the 3p markers analysed. 34 tumours (81%) showed LOH for at least one of the 3p loci tested. 15 tumours showed LOH of all informative markers (indicating a complete deletion of 3p), eight tumours showed no loss for any 3p informative loci and 19 tumours demonstrated some degree of interstitial microdeletion within an otherwise intact 3p (Fig. 1). The pattern of allele loss in the majority of these tumours was complex, showing discontinuous LOH with several regions of 3p allele loss separated by intervening regions of heterozygosity. 3p21.3 allele loss was found in 66% (27/41) tumours informative for the 3p21.3 markers (D3S4597, D3S4604, D3S1568 and D3S1621). However, loss at 3p21.3 was always accompanied by allelic losses at other 3p regions, such as 3p12, p14, p24-26. Six tumours informative for at least one 3p21.3 marker demonstrated loss in other 3p regions, but not at 3p21.3 (tumours 38, 39, 30 and 31 showed 3p LOH at loci distal to 3p21.3, tumour 29 underwent allelic loss at more proximal loci including 3p12, whilst tumour 13 had losses at both distal and proximal loci).

3p12 allele loss (D3S3507, D3S1274, D3S1604 and D3S1254) was found in 56% (22/39) tumours, again 3p12 loss, like the losses at 3p21.3, was always accompanied by allele loss at other 3p loci. There were eight tumours informative for at least one 3p12 marker that showed allele loss at distal 3p markers, but no loss for 3p12 loci.

3.2. Methylation analysis of a CpG island in the promoter region of RASSF1A

We analysed the methylation status of a CpG island in the promoter region of a recently identified TSG (*RASSF1A*) from LCTSGR1 at 3p21.3 in 24 of the above HNSCC by PCR amplification of bisulphite modified DNA followed by restriction digest with a methylation-sensitive restriction enzyme (*TaqI* and *BstUI*) (see Materials and methods and Ref. [19]). *RASSF1A* methylation was found in four (17%) primary HNSCC (Fig. 2, Table 1), whilst none of seven HNSCC cell lines demonstrated *RASSF1A* hypermethylation. As none of the tumour samples was microdissected, the unmethylated allele (204bp) was seen in all samples. None of the matched normal DNA (either from blood or normal mucosa) showed *RASSF1A* methylation. We also cloned the PCR products from two HNSCC showing *RASSF1A* methylation to determine the precise methylation patterns of the CpG dinucleotides. As for lung tumours, the majority of the 16 CpG dinucleotides in the fragment were methylated in each tumour (Fig. 3, HNSCC 32, 13 of the 16 CpGs were methylated).

Although *RASSF1A* mutations are rare in lung tumours [16] in which inactivation by methylation is common, we proceeded to analyse 10 head and neck tumours that showed allelic losses for 3p21.3 markers, but had no *RASSF1A* methylation. No inactivating mutations were found, but one previously described frequent polymorphism (A/C in exon 1A) [18,19] was found in one normal/tumour pair (Table 1).

3.3. Clinical-pathological parameters and 3p LOH and RASSF1A methylation in HNSCC

The frequency of 3p LOH in early stage tumours (TNM 1 and 2: 80% (8/10)) was similar to that in the advanced stage tumours (TNM 3 and 4: 80% (24/30)) ($P = 1.0000$). 3p LOH for the majority of the loci analysed was found even in the two TNM stage 1 tumours, indicating that LOH at multiple 3p loci is an early event in HNSCC tumourigenesis.

Whilst 13 of the 24 HNSCC analysed for the *RASSF1A* methylation status had allelic losses at 3p21.3 only three of the 13 showed *RASSF1A* methylation, whilst the remaining HNSCC with *RASSF1A* methylation retained all 3p loci (but had allele loss for 8p markers; M.J. Kuo, Queen Elizabeth Medical Centre, Birmingham, UK). All of the tumours that had 3p21.3 loss and no *RASSF1A* methylation had allelic

Table 1
Clinical data and *RASSF1A* methylation and mutation status in head and neck squamous cell carcinoma (HNSCC)

Tumour number	Site	Histological Grade	Clinical Stage	Methylation	3p21.3 LOH	Mutation
1	Glottis	MD	3	−ve	+ve (LOH)	−ve
2	Glottis	MD	3	−ve	−ve	na
4	Supra glottis	PD	4	+ve	+ve (LOH)	na
8	Glottis	MD	2	−ve	+ve (LOH)	−ve
9	Glottis	MD	3	−ve	+ve (LOH)	−ve
10	Glottis	PD	3	−ve	+ve (LOH)	−ve
13	Supra glottis	MD	4	−ve	−ve	na
14	Glottis	PD	3	−ve	+ve (LOH)	−ve
15	Tonsil	MD	2	−ve	+ve (LOH)	−ve
19	Hypopharynx	MD	1	−ve	+ve (LOH)	−ve
21	Hypopharynx	MD	3	−ve	Uninformative	na
22	Paranasal sinus	WD	2	−ve	−ve	na
23	Hypopharynx	PD	3	+ve	+ve (LOH)	na
26	Tongue	PD	UN	+ve	+ve (LOH)	na
30	Supra glottis	MD	2	−ve	−ve	na
31	Tonsil	WD	4	−ve	−ve	na
32	Hypopharynx	PD	4	+ve	−ve	na
34	Glottis	WD	4	−ve	−ve	na
37	Oral	MD	4	−ve	−ve	na
38	Subglottis	UN	4	−ve	−ve	na
39	Tongue	MD	3	−ve	−ve	na
54	Glottis	WD	4	−ve	+ve (LOH)	−ve
55	Nasopharynx	PD	4	−ve	+ve (LOH)	Germline missense substitution (Lys 21Gln)
57	Hypopharynx	UN	UN	−ve	+ve (LOH)	−ve

PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; UN, unknown; na, not available.

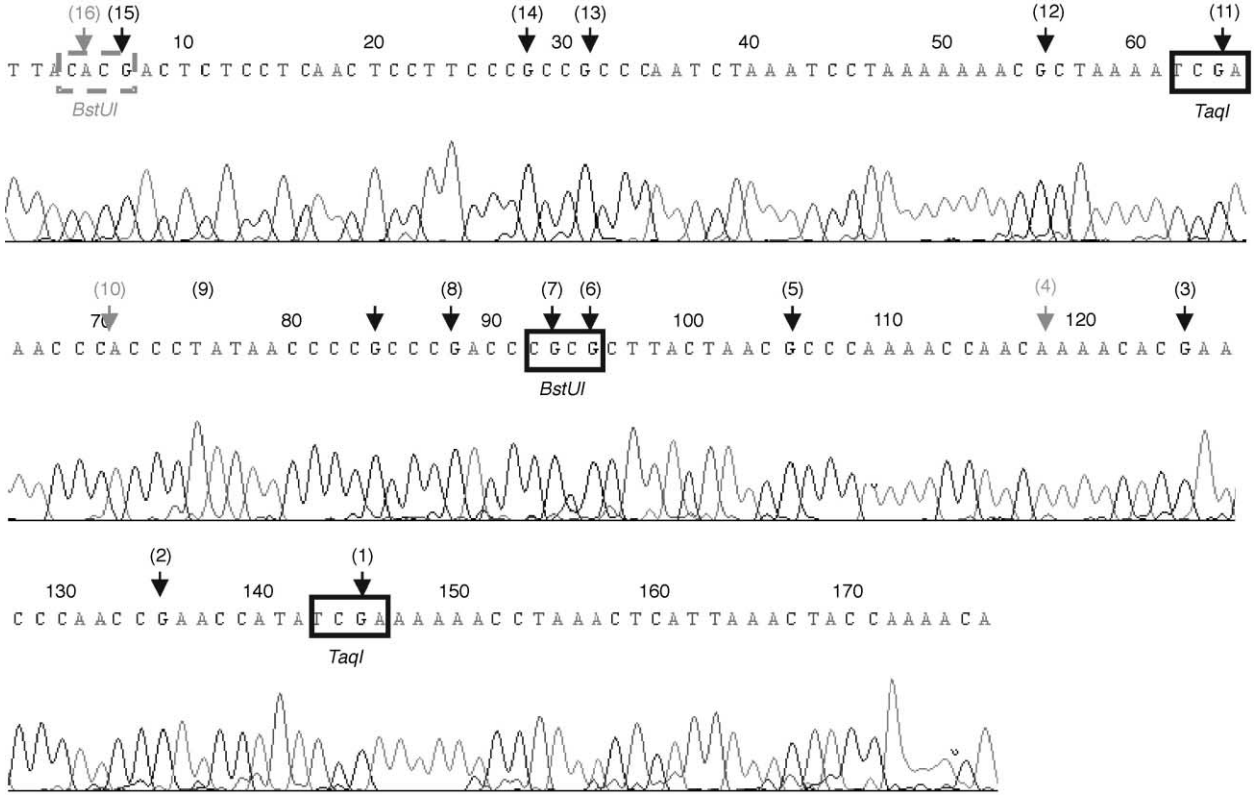

Fig. 3. Sequence of a cloned PCR product from bisulphite-treated DNA of the head and neck squamous cell carcinoma (HNSCC) tumour 32. Methylated cytosines appear as a G signal in the complementary strand. The number in parentheses indicates the position of the 16 CpGs analysed, the restriction sites for *TaqI* and *BstUI* (bold boxes) are also shown. Methylated CpGs are indicated with a black arrow, whilst the unmethylated are indicated by a grey arrow.

losses at other candidate 3p TSG regions (3p25, p24, p14, p12) (see Fig. 1). Three out of 17 stage 3 and 4 tumours were methylated for *RASSF1A*, whilst none of the five stage 1 and 2 HNSCC underwent *RASSF1A* methylation ($P=1.000$) (Table 1). We also noticed that four out of seven poorly differentiated tumours underwent *RASSF1A* methylation, whilst zero out of 11 moderately and zero out of four well differentiated HNSCC demonstrated *RASSF1A* methylation (Table 1) ($P=0.0048$; poorly versus moderately and well differentiated).

4. Discussion

Loss of 3p occurs commonly in head and neck tumours. Previous studies in HNSCC (and comparable studies in lung cancer) have consistently indicated that this loss is maximal at 3p25, 3p21.3, 3p14.2 and 3p12 [7,10,31]. In order to further define the pattern and extent of 3p loss, we performed fine deletion mapping in a large set of head and neck primary tumours by using 23 microsatellite markers encompassing the entire 3p arm, but focusing on regions that have previously been localised as strong candidate regions for TSGs in a variety of common sporadic tumours including head and neck, lung and breast. We also analysed in the HNSCCs the methylation status of a recently identified TSG from region 3p21.3, which in previous studies we and others have shown to be methylated in the majority of lung and, to a lesser extent, in breast and ovarian tumours. We found 3p LOH to be very frequent in HNSCC (any 3p loss 81%, 3p21.3 loss 66%, 3p12 loss 56%) regardless of tumour stage, hence 3p loss is an early event in the development of HNSCC. Although the pattern of allele loss in tumours showing partial deletion of 3p was complex with several regions of 3p allele loss separated by intervening regions of heterozygosity (discontinuous regions of LOH), indicating multiple separate 3p regions harbouring HNSCC TSGs. Furthermore, we determined that 17% of the primary HNSCC are hypermethylated for a CpG island in the promoter region of *RASSF1A*, a gene residing within LCTSGR1. Interestingly, we found that poorly differentiated HNSCC were more commonly methylated for *RASSF1A* than moderately and well differentiated HNSCC ($P=0.0048$). Although larger studies are required to substantiate this finding, the data suggest that *RASSF1A* methylation in HNSCC may be used as a prognostic factor.

TSGs are currently being sought at 3p21.3 and 3p12 regions in a number of tumour types. One specific 3p21.3 region (LCTSGR1) is thought be critical because of the detection of multiple overlapping homozygous deletions

in lung and breast cancer cell lines. LCTSGR1 is defined by overlapping homozygous deletions in small cell lung cancer cell lines NCI-H740, NCI-H1450 and GLC20, and the breast cancer cell line HCC1500, giving a 120kb minimum common deleted region [16]. Many genes have been isolated in the former region, but none are found to be mutated in lung cancer with any significant frequency. The detection of novel microsatellite repeats with accurate relative positions from LCTSGR1 at 3p21.3 has meant that the region can be more densely mapped by the LOH approach. In the current study, D3S4604 and D3S4597 are derived from this computational analysis [32]. The second region which is studied in greater detail in this study is at 3p12 (LCTSGR2) which contains four overlapping deletions in three SCLC and one breast tumour line. The region is covered in a 8 MB YAC/PAC contig, so far only one gene, *DUTT1*, has been isolated from the minimal deletion region at 3p12 [23].

Although no frequent mutations have been found in the eight genes (*CACNA2D2-PL6-101F6-NPRL2-BLU-RASSF1-FUS1-HYAL2*) residing in the 120kb minimal region at 3p21.3, very recently, we and others have shown that one of the genes (*RASSF1A*) from the 120kb minimal region at 3p21.3 is hypermethylated in the majority of lung and, to a lesser extent, in breast tumours, this methylation is associated with loss of expression in tumour cell lines and expression is restored after treatment with 5′-aza-2′ deoxycytidine. In addition, *in vivo* and *in vitro* studies have demonstrated that *RASSF1A* suppresses the growth of tumour cell lines [17–19]. *RASSF1* has several major isoforms due to alternative splicing and promoter usage. *RASSF1C* is the shorter isoform (32 KDa predicted peptide), and is well expressed in lung tumour lines, whilst *RASSF1A* the longer isoform (39 KDa predicted peptide) expression is lost or downregulated in many lung tumour lines [16]. *RASSF1* contains a RAS association domain (RAD) by Simple Modular Architecture Research Tool (SMART) and Protein Families Database of Alignments and HMMs (PFAM) analysis, and was recently shown to be an effector of RAS both *in vitro* and *in vivo* studies [33]. Kim and colleagues [34] recently reported several putative *in vitro* phosphorylation targets for *ATM*, including *RASSF1* (PTS in Ref. [34]). In addition, *RASSF1A* contains a predicted diacylglycerol (DAG) binding domain also found in a related mouse gene *NORE1*. Interestingly, *NORE1* was recently identified as a potential new Ras effector in a yeast two-hybrid screen [35]. In this report, we show that 17% of the primary HNSCC undergo hypermethylation of a CpG island in the promoter region of *RASSF1A*. Although many HNSCC without *RASSF1A* methylation demonstrated 3p21.3 allele loss, we suggest that this does not necessarily imply that further HNSCC 3p21.3 TSGs remain to be identified. Thus, if another 3p21.3 TSG was implicated we might have expected to find many tumours without *RASSF1A* methylation in which 3p allele loss was limited to 3p21.3. However, almost invariably HNSCC with 3p21.3 allele loss and no *RASSF1A* methylation also showed allele loss in other candidate regions. Thus, in these tumours the presence of the 3p21.3 allele loss might not be a marker for homozygous inactivation of another 3p21.3 TSG, but a consequence of the tendency of large chromosomal deletions to occur as a 'second hit' in HNSCC such that in addition to loss of the region containing a specific TSG (e.g. 3p14), adjacent regions (e.g. 3p21) might also be lost. In addition, if *RASSF1A* haploinsufficiency provided a further advantage for tumourigenesis under such circumstances, it would not be necessary to invoke the existence of further 3p21.3 HNSCC TSGs. In a previous study, we showed that 72% of small-cell lung carcinoma (SCLC) and 34% of non-small cell lung carcinoma (NSCLC) were methylated for *RASSF1A* and that the majority of SCLC, but not NSCLC that were methylated for *RASSF1A* had 3p21.3 allele loss [19]. These results suggest that *RASSF1A* inactivation by two hits (methylation and loss) is a critical step in SCLC tumorigenesis, but that TSGs in other regions of 3p have a relatively greater role in the pathogenesis of NSCLC and HNSCC than in SCLC. It is interesting to note that while all three tumour types are associated with tobacco smoking, the frequency of *RASSF1A* methylation differs depending on the lineage of the cell, suggesting that there could be a tumour type-specific inactivation of this gene. As most early HNSCC demonstrated extensive 3p LOH, elucidating the relationship between tumorigenesis and inactivation of specific 3p genes may be difficult (especially if haploinsufficiency effects are significant for some genes). It was therefore of interest that one tumour without 3p LOH demonstrated *RASSF1A* methylation. Mutation and epigenetic analysis of candidate 3p TSGs in HNSCC tumours without 3p allele loss may better elucidate the role of specific genes in tumour initiation and progression than studies of HNSCC with extensive 3p allele loss.

Note: While this work was in progress Lo and colleagues [36] reported a high percentage of *RASSF1A* methylation in nasopharyngeal carcinomas (NPC). Amongst our series of HNSCC there was only one NPC, this NPC was not methylated for *RASSF1A*, although it underwent allelic loss for many of the 3p markers analysed. Hence epigenetic inactivation of *RASSF1A* plays an important role in the development of the majority of NPC, but it is less important in other types of HNSCC, where genes on 3p other than *RASSF1A* may play a greater role.

Acknowledgements

This work is supported, in part, by the Association for International Cancer Research, Cancer Research

Campaign and Get A-Head charity. R.P.H. is a recipient of a fellowship from Royal College of Surgeons of England (Newman fellowship). M.J.K. is a recipient of a Medical Research Council/Royal College of Surgeons of England Joint Special Training Fellowship. S.H. is supported by Portuguese Foundation for Science and Technology. A.M. is supported by the University of Antioquia, Medellin, Colombia, and a fellowship from COLCIENCIAS.

References

1. Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. *N Engl J Med* 1993, **328**, 184–194.
2. Saranath D, Bhoite LT, Deo MG. Molecular lesions in human oral cancer: the Indian scene. *Eur J Cancer B Oral Oncol* 1993, **29B**, 107–112.
3. Li X, Lee NK, Ye YW, *et al*. Allelic loss at chromosomes 3p, 8p, 13q, and 17p associated with poor prognosis in head and neck cancer. *J Natl Cancer Inst* 1994, **86**, 1524–1529.
4. Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carcinoma. *Cancer Res* 1994, **54**, 1115–1152.
5. Field JK, Kiaris H, Risk JM, *et al*. Allelotype of squamous cell carcinoma of the head and neck: fractional allele loss correlates with survival. *Br J Cancer* 1995, **72**, 1180–1188.
6. Pershouse MA, El-Naggar AK, Hurr K, Lin H, Yung WK, Steck PA. Deletion mapping of chromosome 4 in head and neck squamous cell carcinoma. *Oncogene* 1997, **14**, 369–373.
7. Partridge M, Emilion G, Pateromichelakis S, Phillips E, Langdon J. Location of candidate tumour suppressor gene loci at chromosomes 3p, 8p and 9p for oral squamous cell carcinomas. *Int J Cancer* 1999, **83**, 318–325.
8. Sidransky D. Molecular genetics of head and neck cancer. *Curr Opin Oncol* 1995, **7**, 229–233.
9. Kok K, Naylor SL, Buys CH. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. *Adv Cancer Res* 1997, **71**, 27–92.
10. Maestro R, Gasparotto D, Vukosavljevic T, Barzan L, Sulfaro S, Boiocchi M. Three discrete regions of deletion at 3p in head and neck cancers. *Cancer Res* 1993, **53**, 5775–5779.
11. Latif F, Tory K, Gnarra J, *et al*. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 1993, **260**, 1317–1320.
12. Kaelin Jr, WG, Maher ER. The VHL tumour-suppressor gene paradigm. *Trends Genet* 1998, **14**, 423–426.
13. Waber PG, Lee NK, Nisen PD. Frequent allelic loss at chromosome arm 3p is distinct from genetic alterations of the Von-Hippel Lindau tumor suppressor gene in head and neck cancer. *Oncogene* 1996, **12**, 365–369.
14. Wei MH, Latif F, Bader S, *et al*. Construction of a 600kb cosmid clone contig and generation of a transcriptional map surrounding the lung cancer tumour suppressor gene (TSG) locus on human chromosome 3p21.3. *Cancer Res* 1996, **56**, 1487–1492.
15. Sekido Y, Ahmadian M, Wistuba II, *et al*. Cloning of a breast cancer homozygous deletion junction narrows the region of search for a 3p21.3 tumor suppressor gene. *Oncogene* 1998, **16**, 3151–3157.
16. Lerman MI, Minna JD, for the International Lung Cancer Chromosome 3p213 Tumor Suppressor Gene Consortium. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. *Cancer Res* 2000, **60**, 6116–6133.
17. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 2000, **25**, 315–319.
18. Burbee D, Forgacs E, Zöchbauer-Müller S, *et al*. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 2001, **93**, 691–699.
19. Agathanggelou A, Honorio S, Macartney DP, *et al*. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene* 2001, **20**, 1509–1518.
20. Murata Y, Tamari M, Takahashi T, *et al*. Characterization of an 800 kb region at 3p22-p21.3 that was homozygously deleted in a lung cancer cell line. *Hum Mol Genet* 1994, **3**, 1341–1344.
21. Ohta M, Inoue H, Cotticelli MG, *et al*. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 1996, **84**, 587–597.
22. Virgilio L, Shuster M, Gollin SM, *et al*. FHIT gene alterations in head and neck squamous cell carcinomas. *Proc Natl Acad Sci USA* 1996, **93**, 9770–9775.
23. Sundaresan V, Chung G, Heppell-Parton A, *et al*. Homozygous deletions at 3p12 in breast and lung cancer. *Oncogene* 1998, **17**, 1723–1729.
24. Cheng Y, Poulos NE, Lung ML, *et al*. Functional evidence for a nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3. *Proc Natl Acad Sci USA* 1998, **95**, 3042–3047.
25. Uzawa N, Yoshida MA, Hosoe S, Oshimura M, Amagasa T, Ikeuchi T. Functional evidence for involvement of multiple putative tumor suppressor genes on the short arm of chromosome 3 in human oral squamous cell carcinogenesis. *Cancer Genet Cytogenet* 1998, **107**, 125–131.
26. Wu CL, Sloan P, Read AP, Harris R, Thakker N. Deletion mapping on the short arm of chromosome 3 in squamous cell carcinoma of the oral cavity. *Cancer Res* 1994, **54**, 6484–6488.
27. Roz L, Wu CL, Porter S, *et al*. Allelic imbalance on chromosome 3p in oral dysplastic lesions: an early event in oral carcinogenesis. *Cancer Res* 1996, **56**, 1228–1231.
28. Wistuba II, Behrens C, Milchgrub S, *et al*. Sequential molecular abnormalities are involved in the multistage development of squamous cell lung carcinoma. *Oncogene* 1999, **18**, 643–650.
29. Fullwood P, Marchini S, Rader JS, *et al*. Detailed genetic and physical mapping of tumor suppressor loci on chromosome 3p in ovarian cancer. *Cancer Res* 1999, **59**, 4662–4667.
30. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, **93**, 9821–9826.
31. Wistuba II, Behrens C, Virmani AK, *et al*. High resolution chromosome 3p allelotyping of human lung cancer and preneoplastic/preinvasive bronchial epithelium reveals multiple, discontinuous sites of 3p allele loss and three regions of frequent breakpoints. *Cancer Res* 2000, **60**, 1949–1960.
32. Fondon 3rd JW, Mele GM, Brezinschek RI, *et al*. Computerized polymorphic marker identification: experimental validation and a predicted human polymorphism catalog. *Proc Natl Acad Sci USA* 1998, **95**, 7514–7519.
33. Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *J Biol Chem* 2000, **275**, 35669–35672.
34. Kim ST, Lim DS, Canman CE, Kastan MB. Substrate specificities and identification of putative substrates of ATM kinase family members. *Biol Chem* 1999, **274**, 37538–37543.
35. Vavvas D, Li X, Avruch J, Zhang XF. Identification of Nore1 as a potential Ras effector. *J Biol Chem* 1998, **273**, 5439–5442.
36. Lo KW, Kwong J, Hui AB, *et al*. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. *Cancer Res* 2001, **61**, 3877–3881.