Mutations, expression and genomic instability of the H-ras proto-oncogene in squamous cell carcinomas of the head and neck

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Summary Mutation and overexpression are the main activating mechanisms for the ras family of genes in human cancer and the variable tandem repeat (VTR) located at the 3’ end of H-ras has been associated with this risk. In the present study, we have analysed the relative levels of expression of H-ras mRNA in 26 samples of squamous cell carcinomas of the head and neck (SCCHN) by competitive reverse transcription–polymerase chain reaction (competitive RT–PCR) and also investigated whether there is an association between ras expression and alterations in the 3’-VTR region. In addition, we have studied the incidence of point mutations in codon 12 of H-ras, codons 12 and 13 of K-ras and codon 61 of N-ras in 120 SCCHN samples. Our results indicate that only two samples carry mutations, both of which are located in codon 12 of K-ras, but that overexpression of the H-ras proto-oncogene is a frequent event in SCCHN (54% (14/26)) and is associated with a favourable prognosis: 3 of 14 patients with H-ras overexpression have died, whereas 9 of 12 patients with low levels of H-ras expression have died. We have also undertaken an analysis of these results together with our previous investigations on microsatellite instability and loss of heterozygosity in SCCHN, but no associations were found. We therefore conclude that ras mutations are an infrequent event in the progression of the SCCHN in the Western world, whereas overexpression of the H-ras proto-oncogene is a common event.

Keywords: ras mutations; H-ras expression; squamous cell carcinoma of the head and neck; oral cancer

Genetic alterations in oncogenes and tumour-suppressor genes have been implicated in the initiation, promotion and progression of cancer. The ras family of genes (H-, K- and N-ras) encode a 21 kDa membrane protein (p21) which possesses GTPase activity and participates in a signal transduction pathway (Boguski and McCormick, 1993). Hotspots for ras mutations are found in codons 12, 13 and 61, causing the mutant protein to lose its ability to exchange GTP with GDP; thus, it binds GTP with higher affinity and remains activated (Barbacid, 1987). Mutational activation of the ras family genes has been reported in a wide range of human malignancies: 90% of pancreatic and 40% of colonic carcinomas harbour a mutation in K-ras and 50% of bladder carcinomas have H-ras mutations (Bos, 1989). Overexpression of the normal alleles of ras genes can also transform cell lines in vitro (Spandidos and Wilkie, 1984) and have been reported in a number of cancers, such as breast and colon cancer and squamous cell carcinoma of the head and neck (SCCHN) (Field and Spandidos, 1990). High levels of p21 have been postulated to activate other genes (including oncogenes such as myc, jun and fos) that are functionally downstream of ras and regulate normal cell growth and differentiation.

In addition to mutations and overexpression of ras genes, other genetic alterations have been described and shown to be associated with the development of human tumours. Loss of heterozygosity (LOH) of the H-ras locus has been reported in a number of carcinomas (Shiraishi et al., 1987; Garcia et al., 1989; Vachterheim et al., 1994), including SCCHN (Howell et al., 1989; Sheng et al., 1990; Kiaris et al., 1994), and it has been proposed that the normal H-ras gene may possess tumour-suppressor as well as oncogene functions (Spandidos et al., 1990). Recently, we reported that genetic instability of a repetitive element which is located within intron 1 of H-ras is associated with the nodal status of patients with SCCHN (Kiaris et al., 1994). These observations indicate that ras genes play a complex role in carcinogenesis and may have a number of different functions during the development of neoplasia.

The ras family of genes has been studied in SCCHN by several investigators, mainly at the level of mutations, mRNA and protein expression. A high incidence of mutations in ras genes has only been reported in SCCHN patients from India (Saranath et al., 1991) and is most likely to be associated with the chemotherapy used. Although high ras mutations are considered to be rare events in the Western world (Field, 1992, for a review), overexpression of ras family genes has been reported by a number of investigators. Spandidos et al. (1985) demonstrated elevated levels of H-ras and K-ras mRNA in all of the 14 SCCHN samples studied, but no correlation was found with clinicopathological parameters (Field et al., 1986) while Sheng et al. (1990) reported elevated levels of H-ras mRNA in lymph node metastases and primary tumours from SCCHN. An immunohistochemical analysis has been undertaken by Field et al. (1992), who demonstrated that levels of expression of ras p21 in 69 SCCHN patients correlated with a favourable clinical outcome. However, two Japanese studies were at variance with these results. Azuma et al. (1987) found that high levels of H-ras correlated with poor prognosis in SCCHN patients, and Tsuji et al. (1989) failed to find any association between the overexpression of H-ras and the clinical outcome. The results of these two Japanese groups are not in agreement with those from the UK, a difference that may be attributed to different environmental risk factors. Recently, the levels of H-ras p21 in primary laryngeal cancers were studied by an Italian group using Western blotting and, even though elevated levels were found, no association with any clinical parameters was reported (Scambia et al., 1994).

In the present study, we have analysed the overexpression of H-ras mRNA in SCCHN using competitive reverse transcription PCR (Foley et al., 1993; Kotsinas et al., 1994). Briefly, this technique consists of the reverse transcription of specific mRNAs and their co-amplification by PCR with an internal control which shares the same primer sites within the
target cDNA, a process that results in increased sensitivity and accuracy. We have also investigated the incidence of mutations in ras genes by PCR followed by restriction fragment length polymorphism (RFLP) in 120 SCCHN patients. The results of this study were also analysed with relation to our previous findings of loss of heterozygosity and genetic instability of the H-ras gene.

Materials and methods

Specimens

A total of 120 tumour specimens were collected from carcinomas of the head and neck at the Department of Otorhinolaryngology, Royal Liverpool University Hospital, and Maxillofacial Unit, Walton Hospital, Liverpool. Tumour samples obtained from surgical specimens were frozen in liquid nitrogen and stored at \(-70^\circ\text{C}\).

DNA and RNA extraction

Genomic DNA and total RNA were extracted from tumour specimens using the Nucleon II DNA extraction kit (Scotlab) and the TRIzol reagent (Gibco BRL), respectively, following the manufacturer's instructions. Genomic DNA samples were stored at 4\(^\circ\text{C}\) and total RNA samples at \(-20^\circ\text{C}\).

Oligonucleotide primers and PCR amplification

The oligonucleotide primers used for amplification of codon 12 of H-ras, codons 12 and 13 of K-ras and codon 61 of N-ras are shown in Table I. PCR reactions were performed in a final volume of 50 \(\mu\text{L}\) and contained 35 mm magnesium chloride, 100 mM Tris–HCl pH 8.3, 500 mM potassium chloride 0.1% gelatin, 200 mM of each dNTP, 200 ng of DNA, 100 ng of each primer and 1.25 units of Taq polymerase (Advanced Biotechnologies). The amplification conditions for each pair of primers were as follows:

(a) H-ras. Denaturation at 95\(^\circ\text{C}\) for 40 s, primer annealing at 61\(^\circ\text{C}\) for 45 s and extension at 72\(^\circ\text{C}\) for 45 s; 28–30 cycles; PCR product size 419 bp.

(b) K-ras. Denaturation at 94\(^\circ\text{C}\) for 40 s, primer annealing at 60\(^\circ\text{C}\) for 45 s and extension at 72\(^\circ\text{C}\) for 50 s; 28–35 cycles; PCR product size 157 bp.

(c) N-ras. Denaturation at 94\(^\circ\text{C}\) for 30 s, primer annealing at 58\(^\circ\text{C}\) for 40 s and primer extension at 72\(^\circ\text{C}\) for 30 s; 28–35 cycles, PCR product size 65 bp.

All PCR reactions were initially denatured for 5 min at 95\(^\circ\text{C}\).

RFLP analysis of ras mutations

(1) H-ras codon 12: 10–20 \(\mu\text{L}\) of the amplification product was digested overnight with 40 units ofMspI.

(2) K-ras codons 12 and 13: 10–20 \(\mu\text{L}\) of the amplification product was digested for 3 h with 20 units of BstNI (codon 12) and another aliquot of 20 units ofHphI (codon 13, Gly to Asp).

(3) N-ras codon 61: 10–20 \(\mu\text{L}\) of the amplification product was digested overnight with 40 units ofMsal.

The incubation temperatures were 60\(^\circ\text{C}\) forBstNI and 37\(^\circ\text{C}\) for the remaining enzymes. The digestion products were 354 bp and 385 bp for H-ras codon 12 and 113 bp and 142 bp for K-ras codon 12, normal and mutant alleles respectively. The normal codon 61 N-ras allele produced a 44 bp band, while the mutant allele remained 65 bp in size and the mutant (Gly to Asp) codon 13 K-ras allele produced a 115 bp band compared with a normal PCR product of 157 bp. Restriction enzymes were supplied by New England Biolabs. The digestion products were analysed on 2% agarose gels (H-ras codon 12, K-ras codon 12 and 13) or on 10% polyacrylamide gels (N-ras codon 61) and visualised under UV illumination after staining with ethidium bromide.

Competitive reverse transcription PCR (RT–PCR) and RNA quantitation

This procedure has been described in detail elsewhere (Kotinis et al., 1994). Briefly, the competitor sequence (cloned within a plasmid) has been derived by an internal deletion of the PCR product of the same set of primers, applied to genomic DNA. This results in a fragment which is smaller than the genomic DNA product, but larger than the cDNA product; thus, it shares the same primer sites and serves in the PCR reaction as a competitor. A 200 ng aliquot of total RNA was reverse transcribed in a 50 \(\mu\text{L}\) reaction (10 mM Tris–HCl pH 8.3, 50 mM potassium chloride, 1 mM magnesium chloride, 200 mM dNTP, 200 ng of antisense primer and 2.5 unitsTth polymerase) for 15 min. PCR amplification of cDNA was then performed by adding 50 \(\mu\text{L}\) of 75 mM Tris–HCl pH 9.0, 20 mM ammonium sulphate, 1.5 mM magnesium chloride, 0.01% Tween 20 (w/v), 0.75 mM EGTA and 200 ng of sense primer and using the PCR programmes previously described for 28 cycles. The number of cycles was decided after preliminary experiments showed that the PCR reaction remained in the exponential phase at this time. A standard curve was derived from serial dilutions of total RNA (0.1 \(\mu\text{g}\), 0.2 \(\mu\text{g}\), 0.5 \(\mu\text{g}\), 1.0 \(\mu\text{g}\) and 2.0 \(\mu\text{g}\)) mixed with 50 \(\mu\text{g}\) of competitor plasmid (Figure 1). The PCR products were analysed in a 6% polyacrylamide gel stained with either silver or ethidium bromide. The quantity and quality of target RNA was determined by amplification of \(\beta\)-actin.

![Figure 1](image)

**Table I** Primers used for ras analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-ras codon 12</td>
<td>5’-GACCGAATTAAGCTGTTGG3’&lt;br&gt;3’-TAACTACCCCTCTGACCGGA5’</td>
</tr>
<tr>
<td>K-ras codons 12 and 13</td>
<td>5’-ACTGAATATAAACCTCTTGATGTTGGACCT3’&lt;br&gt;5’-TCAAAAGAATGTTGCCTGGAC3’</td>
</tr>
<tr>
<td>N-ras codon 61</td>
<td>5’-GACATACTGGAATAACGCTGC3’&lt;br&gt;5’-CCTGTCCTGATTTGTC3’</td>
</tr>
<tr>
<td>VTR region</td>
<td>5’-GAGCTAGACGGGCTATCGCGC3’&lt;br&gt;5’-AGCAAGTGTTGAAAGGCCG3’</td>
</tr>
<tr>
<td>(\beta)-actin mRNA</td>
<td>5’-GTTGGGCGCCACAGGCAA3’&lt;br&gt;5’-CTCCCTTAAATGTCAAGCAGATTTTC3’</td>
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mRNA (548 bp) using the reaction mixture described above and the following amplification conditions: 1 min at 95°C, 1 min at 58°C and 1 min at 72°C for 28 cycles. The quantity of RNA used in all experimental amplifications of H-ras mRNA was thus previously normalised by the prior amplification of β-actin mRNA from each sample. The interpretation of the expression levels of H-ras using competitive RT-PCR (using the aforementioned competitor plasmid) is calculated using the following ratio: (target vs competitor in the tumour tissue) to (target vs competitor in the normal tissue). Amplification and quantitation of H-ras mRNA was performed at least twice for each sample and produced similar results each time.

Results

Among the 120 samples analysed, only two were found to contain a ras mutation, both of which were in codon 12 of K-ras (Figure 2). No mutations were found in codon 13 of K-ras, codon 12 of H-ras, or codon 61 of N-ras. These results are in agreement with previous studies, which show less than 5% ras mutations in SCCHN in the Western world (Tables II and III). As there were only two positive samples with mutations out of 120 SCCHN tested, no analysis with the clinico pathological parameters was undertaken.

Twenty-six SCCHN samples (five oropharynx, 11 hypopharynx, five oral, five larynx) were analysed for aberrant expression of H-ras mRNA. Twenty of the 26 SCCHN samples exhibited elevated expression of H-ras in the tumour tissue, ranging from 1.1-to 8.1-fold expression (Table IV, Figures 3 and 4). Since tumour cells are characterised by high rates of proliferation, small increases in the level of expression of the H-ras gene would be expected as being representative of a cell kinetic system in which the ras signal transduction pathway is activated. However, four samples (184, 360, 225 and 1092) showed slightly lower levels of the H-ras mRNA in their tumour tissue. Thus, we arbitrarily divided

<table>
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<th>Table II Cumulative results on ras mutations in SCCHN</th>
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<td>K-ras-12</td>
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<tr>
<td>Western world</td>
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<td>N-ras-61</td>
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Figure 2  PCR–RFLP assay for the detection of K-ras codon 12 mutations in SCCHN. Lane 1, marker pUC18/HaeIII; lane 2, undigested PCR product; lane 3, control DNA sample from cell line SW 480 which harbours a homozygous mutation at codon 12 of K-ras; lanes 4, 7 and 8, normal samples; lanes 5 and 6, mutant samples.

Table III Cumulative results of ras mutations in SCCHN in the Western world

<table>
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<tr>
<th></th>
<th>H-ras 12</th>
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<th>61</th>
<th>K-ras 12</th>
<th>13</th>
<th>61</th>
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<td>190</td>
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<td>Number of mutations</td>
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<td>Per cent mutations</td>
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Table IV H-ras expression in SCCHN

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<tr>
<th>Sample</th>
<th>H-ras mRNA levels</th>
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<th>Site*</th>
<th>Histology*</th>
<th>TNM stage*</th>
<th>Nodes at pathology</th>
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<tr>
<td>338</td>
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<td>184</td>
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<td>HP</td>
<td>PD</td>
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</table>

*Levels of H-ras mRNA are expressed as the ratio of the levels in the tumour vs the normal tissue. **OP, oropharynx; HP, hypopharynx; O, oral; L, larynx; WD, MD and PD, well, moderately and poorly differentiated SCC respectively. *TNM staging (UICC, 1978). ND, no data.

the tumour samples by their H-ras mRNA levels of expression into two groups; patients in the first and second groups exhibited <1.5- and ≥1.5-fold expression of H-ras, respectively, compared with normal tissue. Thus 14 of the 26 (54%) SCCHN specimens tested showed overexpression of H-ras mRNA, but no association was found with site, histology, TNM staging or positive nodes at pathology (Table IV). An association was found, however, between the levels of expression of H-ras and the clinical outcome; 9 of the 12 patients with no evidence of H-ras overexpression have died, whereas 3 of the 14 patients with elevated expression of the H-ras proto-oncogene are dead (log-rank analysis, \( P = 0.05 \)). This result should be treated with caution as a number of these patients have been followed up for under 1 year (median 13 months, range 1–87 months).

PCR amplification of the 3'-VTR region was only possible in 14 of the 26 patient samples owing to technical difficulties with some of the specimens. In 5 of 14 (36%) SCCHN paired samples, a different pattern was observed in the tumour specimens compared with their normal tissue counterparts, indicating genetic instability of the VTR region. (Some of these results have been previously reported in Kiaris et al., 1994.) No association with clinicopathological parameters was demonstrated. An interesting observation was that, among the five SCCHN tumour specimens with VTR genetic instability, four had altered levels of H-ras mRNA compared with their normal tissue; two had lower levels (0.8 and 0.9) and two had higher levels (7.5 and 3.3). This may indicate that, at least in a certain number of patients, deregulation in the expression of H-ras is due to destabilisation of the VTR region of the gene.

Discussion

Head and neck cancers are the sixth commonest cancers in the world but have a wide geographical variation which is most likely due to specific environmental risk factors (Vokes et al., 1993). This is reflected in the different incidence of ras mutations in the Western world compared with SE Asia and India. In the Western world, ras mutations in SCCHN are very rare (<5%), whereas in India 35% of SCCHN patients harbour a mutation in H-ras, and this has been associated with tobacco chewing (Sarath et al., 1991). In Taiwan, 18% of oral cancer patients investigated were found to have a K-ras mutation, and these patients chew betel quid but do not use tobacco (Kuo et al., 1994). In this study, 120 samples were analysed for mutations in codon 12 of H-ras, codons 12 and 13 of K-ras and codon 61 of N-ras, but only two of these samples (1.8%) contained ras mutations, both of which were found in codon 12 of K-ras. This is the largest analysis to date of ras mutations in SCCHN and the results confirm that ras mutations in SCCHN are rare in the Western world (Table III).

The expression of H-ras mRNA in SCCHN was investigated by competitive RT–PCR, which is a fast and sensitive technique for the interpretation of the levels of specific transcripts in cells or tissues. This is the first report to our knowledge that employs competitive RT–PCR for the investigation of H-ras mRNA expression in tumour tissues. In the past, Northern blot and dot blot hybridisation techniques have been used, but they have the disadvantages of being time-consuming and requiring the use of radioactivity. The primers used for H-ras cDNA amplification lie in adjacent exons of the H-ras gene, and thus it is possible to dis-
criminate against contamination of the target RNA with genomic DNA. This particular feature of the amplification reaction and the co-amplification of the internal control (pGEM2Z0H-ras) produces increased specificity and accuracy of results (Kotsinas et al., 1994).

Significantly elevated levels of H-ras mRNA were found in 14 of the 26 (54%) SCCHN specimens tested, and in four (15%) patients the levels of H-ras mRNA were lower in the tumour than in the normal tissue. No association was found between the levels of expression of H-ras and the site of the tumour, TNM staging, histology or the nodal status of the patients, suggesting that, although overexpression of the H-ras proto-oncogene is associated with the development of SCCHN, alterations in other oncogenes or tumour-suppressor genes (TSGs) may be more important for the progression of this disease. The four TNM stage I tumours investigated in this present study all exhibited elevated levels of H-ras mRNA (> 1.5-fold), which may indicate that the overexpression of this proto-oncogene is important in the early stages of the disease. In vitro studies have suggested that H-ras overexpression is associated with morphological transformation, but tumorigenicity is also greatly increased when it is accompanied by the overexpression of K-ras and N-ras (Coleman et al., 1994). The association of H-ras overexpression in SCCHN with a favourable prognosis is in agreement with an earlier immunohistochemical analysis of ras p21 in 69 SCCHN samples (Field et al., 1992).

We have previously reported LOH at the H-ras locus (Kiaris et al., 1994). Three of the specimens that exhibited LOH were used in the present study, but no association between LOH at H-ras and overexpression of the gene was found. This result is in agreement with Sheng et al. (1990), who also demonstrated no association between overexpression of mRNA and LOH at the H-ras locus in 11 SCCHN specimens. However, the transcriptional activation of H-ras still remains a puzzle. The lack of a TATA box and the presence of a CAAT box in the GC-rich promoter at the 5' end of the gene reveals similarities with other housekeeping genes (Breathnach and Chambon, 1981; Honkawa et al., 1987). If overexpression of H-ras was due solely to the high proliferative status of cancer cells, then normalisation of the quantity of H-ras mRNA with actin mRNA (which is also a housekeeping gene) would equalise any differences between normal and tumour tissue from the same patient. However, the relative levels of expression ranged from 0.8 to 8.1, and this probably indicates that specific genetic alterations in particular tumour samples activate or inactivate H-ras proto-oncogene expression.

The VTR region in the 3' end of H-ras possesses differential enhancer activity (Spandolis and Holmes, 1987) and may divide the SCCHN population into certain subgroups which have an increased risk of developing cancer (Krontiris et al., 1993). Since we have already reported that instability occurs in a repetitive element (HRMS) located in intron 1 of H-ras (Kiaris et al., 1994), we considered that genetic instability in the 3'-VTR region of H-ras may also be a potentiality, thus resulting in the generation of new VTR alleles within the tumours of patients. This may explain, at least in a certain number of samples, the deregulation of H-ras gene. Indeed, we did find that 5/14 (36%) patients exhibited 3'-VTR instability, confirming the suggestion that the H-ras VTR is a potential target for instability. However, the levels of H-ras mRNA were not associated with instability of the 3'-VTR region; two tumours exhibited overexpression (7.5 and 3.3 times) and two had relatively lower levels of H-ras mRNA (0.8 and 0.9 times). This may indicate that instability of the 3'-VTR region could influence the regulation of the H-ras gene in vivo, as previously proposed at an in vitro experiment (Krontiris, 1993). However, a combined analysis of H-ras expression and instability of the VTR region in a larger number of patients is required to extend this observation.

Our study confirms the proposal that ras gene mutations are rare in SCCHN in the Western world but that overexpression of H-ras is associated with the development of SCCHN, however, the interpretation of this finding remains unclear. Further investigations should aim to clarify the significance of elevated transcription of the H-ras proto-oncogene and also to distinguish the mechanism(s) that results in the aberrant expression of H-ras in SCCHN. Finally, the present study illustrates the power of the competitive RT-PCR as a fast and sensitive technique for screening a relatively large number of samples for specific mRNA expression.

References


