Ras mutations have been detected in a wide range of human tumours (Bos et al., 1989), and in the case of N-ras can be demonstrated to be important to the transformed phenotype (Paterson et al., 1987). Several studies have shown that the incidence of Harvey ras (H-ras) mutations in squamous cell carcinoma (SCC) of the head and neck (H & N) in Western Europe is low, but the role of ras mutations in the progression and development of SCC of the H & N is still unclear. H-ras point mutations were found by Saranath et al. (1991) in 35% of squamous cell carcinomas of the oral cavity in India and most of these patients chew tobacco which is a well described risk factor (Sankaranarayanan, 1990). Two ras mutations were found by Rumsby et al. (1990) looking at 37 SCCs of the H & N. However half of these patients and all their cell lines were treated with radiotherapy prior to analysis. Thus half of these were late-stage tumours or recurrent tumours by definition; the staging for the others was not given but is likely to be late-stage. Sheng et al. (1990) also found a low incidence of H-ras mutations in SCCs of the H & N (2 of 54). All their patients were untreated but only nine were possibly early stage and data was again not shown for the staging. More recently, Chang et al. (1991) reported the absence of ras mutations in 22 SCC biopsies from the H & N. In a study of human epidermal neoplasms however, 30% of a large series of benign epidermal keratoacanthomas were reported to contain ras mutations in contrast to only 13% of malignant SCC (Corominas et al., 1989). Similarly, in animal epidermal tumour models ras mutations were also shown to be more prevalent in benign lesions than malignant lesions (Balmain et al., 1984; Leon et al., 1988; Corominas et al., 1991; Brown et al., 1990). We therefore thought it possible that ras mutations are important for the initiation of SCC of the H and N but are eliminated at an early stage of progression.

The presence of a ras mutation in a human keratinocyte can render the cells more responsive to epidermal growth factor (Henrard et al., 1990) and can clearly give these cells a selective advantage in vivo (Corominas et al., 1989; Saranath et al., 1991). However, it has been suggested that in most circumstances, the presence of a ras mutation selects against further progression to SCC, due to its inability to amplify signals associated with terminal differentiation as well as proliferation (Corominas et al., 1989). Whilst this may be true, it is also likely that ras mutations lead to the secretion of transforming growth factor α (TGF-α) by the keratinocytes which possess them (Ozanne et al., 1980). These cells might then contribute to the clonal expansion of other malignant cells which have a greater potential to progress than those actually carrying the ras mutation. Indeed, benign mouse epidermal papillomas which are known to be polyclonal (Winton et al., 1989) do express high levels of TGF-α (Glick et al., 1991).

We were therefore concerned that an important function of ras mutations might have been missed because too few premalignant or early stage carcinoma lesions had been examined. We have extended the work of previous investigators and screened a number of lesions and cell lines at all stages of SCC development for H-ras mutations, using PCR followed by direct single-stranded sequencing. This allows the detection of all the known activating mutations of the H-ras gene (see Gibbs et al., 1985 for a review).

We have obtained results however, which show for the first time that H-ras mutations are also scarce in the early stages of H & N SCC development in Caucasian patients. These results are discussed in relation to the pathogenesis of SCC in Caucasian and Indian patients.

Materials and methods

Tumour samples

Twenty-six SCCs, five erythroplakias and six recurrent respiratory papillomas have been collected and snap frozen from Glasgow and Edinburgh. In addition 30 archival SCC embedded in paraffin blocks were obtained from Aberdeen. All the SCC were placed into the appropriate TNM tumour staging group (UICC 1987). Frozen and paraffin sections (5 μm thickness) were prepared by Ian McMillan (Department of Veterinary Pathology, University of Glasgow).

Cell lines and cell culture

The SCC cell lines have been described previously (Reinwald & Beckett, 1981). The new B.I.C.R. cell lines (BICR 3, 6, 7, 10, 16, 18, 19, 22, 31 and 56) were derived following explant culture of tumour fragments in DMEM plus 10% v/v FBS, 0.4 μg ml⁻¹ hydrocortisone and lethally irradiated 3T3 cells (Edington et al. – manuscript in preparation). The cells have now completed more than 100 population doublings and are considered to be established. The cells were grown initially on irradiated 3T3 feeder layers as originally des-
cribed by Rheinwald and Beckett (1981) in Dulbecco’s modification of Eagles Medium (DMEM), 10% (v/v) foetal bovine serum and 0.4 μg ml$^{-1}$ hydrocortisone. Forty eight hours before the DNA was isolated the medium was changed to MCDB 153 medium containing low concentrations (0.15 mmol l$^{-1}$) of calcium ions, 0.4% v/v bovine pituitary extract, 5 μg ml$^{-1}$ epidermal growth factor, 0.5 μg ml$^{-1}$ hydrocortisone and antibiotics (all from Clonetics Inc., San Diego USA). The low calcium medium facilitated the removal of the Swiss 3T3 feeder layer with 0.02% EDTA as described by Rheinwald and Green (1975) and ensured minimal (less than 1%) contamination of the cultures with mouse DNA (see also Parkinson & Newbold 1980; Alitalo et al., 1982). Normal human keratinocytes were used as a normal tissue control and were grown throughout on MCDB153 medium plus additives (see above). The human bladder carcinoma line EJ which is known to harbour a G$\rightarrow$T mutation at the second base codon 12 of the H-ras gene (Tabin et al., 1982) was used as a mutant control during the development of the direct sequencing techniques and was cultured in Special Medium (Life Sciences Technology, Paisley, Scotland) containing 10% v/v foetal bovine serum.

The erythroplakia keratinocytes were cultured in DMEM, 20% vol/vol. foetal bovine serum, 0.4 μg ml$^{-1}$ hydrocortisone, 10 ng ml$^{-1}$ cholera toxin (Sigma Chemical Co., Poole, UK) and will be described in detail elsewhere (Edington et al. – manuscript in preparation). Briefly, the erythroplakia cells are diploid but appear to differ from these cells that are known to be normal keratinocytes as assessed by involution staining, electron microscopy and response to suspension culture. The cells do eventually senesce, however, have normal keratinocyte growth requirements, and are not tumorigenic in nude mice. These properties are consistent with an abnormal premalignant population of human cells (see Paraskeva et al., 1984). The cells also have ultrastructural and immunochemical features of keratinocytes.

**DNA extraction**

DNA extraction was by a simplification of the method described by Gross-Bellard et al. (1973). Each 5 μm frozen section was lysed in 100 μl DNA lysis buffer (10 mmol l$^{-1}$ Tris-HCl, 10 mmol l$^{-1}$ EDTA, 10 mmol l$^{-1}$ NaCl) pH 8.0 plus 20 μl 20% sarcosine, 20 μl of 20 mg ml$^{-1}$ Proteinase K at 37°C overnight. The preparation was then extracted with 100 μl of phenol/chloroform and the supernatant precipitated with 10 μl of 3 M sodium acetate and 300 μl of 100% ethanol at −20°C overnight. The precipitates were then pelleted by centrifuging (14,000 g) for 30 min, washed in 80% ethanol, pelleted again by centrifuging at 8,500 g for 15 min, dried and resuspended in 10 μl of Tris-EDTA (10 mmol l$^{-1}$ Tris-HCl, 1 mmol l$^{-1}$ EDTA) pH 8.0. The cell cultures were treated as above except that DNA was spoiled by the method of Gross-Bellard et al. (1973) rather than being pelleted.

The paraffin-embedded sections were extracted twice with 1 ml xylene for 30 min with gentle tumbeling followed each time with a 5 min spin at 14,000 g (Dubeau et al., 1986). After the second extraction the tissues were rinsed with 500 μl of 100% ethanol and lysed in the same fashion as the frozen sections.

**PCR and direct single-stranded sequencing**

PCR primers were (reading 5’ to 3’):

- H ras codons 12, 13 a primer: AGG AGA CCC TGT AAG AGG AC
- d primer: AGC AGC TGC TGG CAC CTG GA
- H ras codons 59–63 a’ primer: CAG GAT TCC TAC CGG AGG CA
- c’ primer: CTG TAC TGG TGG ATG ATG TCC TCA

**Sequencing Primers were** (reading 5’ to 3’):

- H ras codons 12, 13 b primer: AGG CCC CTG AGG AGC GAT GA
- c primer: GGA TCA GCT GGA TGG TCA GC
- H ras codons 59–63 b’ primer: CGC ATG TAC TGG TCC CGC AT
- b’ primer: GAC GTG CCT GGT GGA CAT CC

The primers were synthesised on a standard Applied Biosystems machine with an elongated coupling time for the biotinylated group which is added at the 5’ end as the last step. The primers were purified using a desalting protocol with an oligonucleotide purification cartridge (Applied Biosystems, UK).

The PCR products were purified using magnetic streptavidin coated beads – Dynabeads M-280 Streptavidin (Dynal, UK Ltd.) and a magnetic separator MPC-E (Dynal, UK Ltd.). This enabled direct solid-phase sequencing of the PCR products to be undertaken (Hultman et al., 1991) and mutations or polymorphisms could be detected not only across the codons of interest but up to 200 base pairs away.

**Results**

**Sensitivity of the PCR/direct sequencing technique**

PCR and direct sequencing were chosen as the methods for investigating the possibility of ras mutations because dot-blotting for point mutations is not as informative as direct sequencing. All the regions of interest (codons 12 and 13 or codons 59–63) are seen with direct sequencing in addition to flanking sequences. Also it is not possible to obtain human cells and tissues carrying activating point mutations at codons 59 or 63 and it is arguable whether synthetic oligonucleotides represent an adequate control.

Many tumour biopsies are contaminated by normal cells. Therefore, to determine the sensitivity of the direct sequencing technique samples of EJ bladder carcinoma DNA were mixed at different ratios with normal human keratinocyte DNA, before subjecting the samples to PCR and analysis by direct sequencing. Figure 1 shows that the codon 12 ras mutation harboured by the EJ line is still easily detectable even when the mutant DNA represented only 15% of the sample. Examination of stained sections of all the tumour samples by a trained pathologist revealed that tumour tissue represented at least 45% of the cells in the section and therefore that a mutation would be detected in these cases.

![Figure 1](link-to-image)

**Figure 1** Sensitivity of PCR and direct sequencing in the detection of mutant Harvey ras genes. Normal HEK DNA (GGG) was mixed in different ratios with EJ Bladder carcinoma DNA (GTC) to determine the sensitivity of mutant ras detection. The T lane was clearly visible when the EJ DNA represented only 15% of the total DNA.
Absence of H-ras mutations at all stages of head and neck SCC development

Table I summarises all the benign, premalignant and SCC biopsies tested for H-ras mutations, and in some cases the keratinocytes cultured from them. No ras mutations were present in six benign papillomas, five premalignant erythroplakias, four T1, 16 T2, 12 T3 and 24 T4 and two spindle cell malignant SCC, all but five of which were untreated. Also, no mutations were detected in three SCC lymph node metastases. In addition cultures of four erythroplakia biopsies and 16 SCC cell lines all possessed normal H-ras genes.

Discussion

We have confirmed and extended the observations of previous investigators (Rumby et al., 1990; Sheng et al., 1990; Chang et al., 1991) to demonstrate that mutations in the H-ras proto-oncogene are extremely rare in the pathogenesis of H & N SCC in Caucasian patients. Extending the findings of others, we were unable to find H-ras mutations in 11 premalignant or benign lesions and 20 early stage (T1, T2) untreated SCC which were not studied by the earlier investigators. Similar findings were also reported recently by Yeudall et al. (1993) who could not detect ras mutations in any dysplastic oral biopsies from three patients. Taken together these results do not support the hypothesis that H-ras mutations are present during the early stages of H & N SCC development and are later selected against during tumour progression (see Introduction). The amount of tumour present in each biopsy should have been enough to render a mutation readily detectable by direct sequencing (see Figure 1). Also in several cases cultures of epithelial cells from the tumours or established tumorigenic cell lines were studied (Table I) and these were unlikely to be contaminated with normal cells. It is unlikely therefore that any mutations in the H-ras gene would have been missed because of excessive normal cell contamination.

The absence of H-ras mutations in Western samples of H & N SCC contrasts with the findings of Saranath et al. (1991) who reported finding H-ras mutations in 35% of Indian SCC samples collected from patients who had habitually chewed betel quid. These results do suggest therefore that ras mutations can give keratinocytes a selective advantage in H & N SCC sites and that they do persist throughout tumour progression (see also Corominas et al., 1989). It is also known that ras oncogenes can render human keratinocytes more sensitive to EGF in vitro and when grafted onto nude mice can regenerate an epithelium reminiscent of premalignant oral leukoplakia (Henrard et al., 1990). Interestingly, tobacco-related oral malignancies in India are usually preceded by premalignant lesions such as leukoplakia (Daftry, 1990) whereas oral malignancies in Western countries only rarely arise from premalignant lesions (Binnie, 1990).

It has been suggested that the absence of ras mutations in Western samples could be due to the different types of tobacco used in India and the UK (Chang et al., 1991) but since most UK H & N SCC are thought to have a tobacco smoking-related aetiology (Stell, 1972), and tobacco smoke contains several carcinogens which are known to activate the H-ras gene (IARC, 1986; Quintanilla et al., 1986; Brown et al., 1990), this does not seem a satisfactory explanation. Whilst it has still to be demonstrated that ras mutations are an early event in the pathogenesis of Indian SCCs (Saranath et al., 1991), it is possible that the betal quid contains tumour promoters which give the ras mutations a continuous selective advantage, analogous to the mouse two-stage epidermal tumorigenesis model (see Clark, 1993 for a review). It is known that the quid is held for long periods of time at the site of tumour induction (Chang et al., 1991; Saranath et al., 1991), and continuous exposure to tumour promoters is essential for their action (Boutwell, 1964).

It is clear that keratinocytes harbouring ras mutations can gain a continuous selective advantage during the pathogenesis of oral SCC, if the correct conditions for such selection prevail (Saranath et al., 1991). The exact nature of these conditions remains to be established but an answer should emerge when the molecular mechanisms of oral SCC induction in Caucasian and Indian subjects are understood in more detail.

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