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Analysis of *ras* Gene Mutation in Human Oral Tumours by Polymerase Chain Reaction and Direct Sequencing

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Abstract : Genetic alterations in proto-oncogenes or tumour suppressor genes are believed to be one of the key events in the multistage process of carcinogenesis. Activating point mutations occurring in either one of the three *ras* proto-oncogene families are common genetic alterations in human and animal neoplasms. However, the mechanisms leading to oral cancer are not completely understood. Activation of the *ras* oncogene in oral carcinogenesis, although absent or rare in the western world, accounts for up to 35% of all malignancies in India and South Asia. Recognised aetiological agents of oral cancer include tobacco and alcohol. Tobacco-associated compounds such as nitrosamines are linked with carcinogenesis in humans. In the present paper, point mutations of *ras* genes were analysed in human oral cancers. DNA obtained from the tissue was amplified by polymerase chain reaction and then analysed by direct DNA sequencing in order to detect possible mutations at codons 12, 13 and 61 of H-*ras*, K-*ras* and N-*ras*. The DNA sequencing analyses revealed that there were no mutations at the hotspots of the three *ras* genes. These results indicate that *ras* gene mutation may not play an important role in the development of oral tumours in western samples.

Key Words: Oral tumour, PCR, Direct sequencing, Tobacco-specific nitrosamines

Introduction

An increasing amount of evidence suggests that the multiple genetic events leading to carcinogenesis include the activation of oncogenes and inactivation of tumour suppressor genes. Oral carcinogenesis appears to be a complex phenomenon in which genetic events within signal trunsduction pathways governing normal cellular physiology are quantitatively or qualitatively altered (1). Cytogenic analyses have also shown that structural abnormalities often involve chromosomes 1, 3 (2, 3), 9 (4), and particularly 11 (5, 6), 13 and 14 (6) in oral carcinomas. It is possible that these abnormalities may affect genes involved in carcinogenesis such as oncogenes and tumour suppressors as well as genes controlling DNA repair or stability (7). It is also significant that viruses, most notably the human papilloma virus, have been associated with oral carcinogenesis (8).

Proto-oncogenes are genes that are present in normal cells, controlling cell growth, proliferation and differentiation. The *ras* gene is one of the most commonly detected mutated oncogenes in human cancers. The family of *ras* genes includes three well characterised genes, H-*ras*, K-*ras* and N-*ras*. All these three genes contain 4 coding exons that code for highly related proteins known as 21Kd proteins, which are composed of 188 or 189 amino acids. These genes are converted into active oncogenes by point mutations at one of the critical positions of 12, 13 or 61 (9).

Activation of *ras* genes by point mutations has been determined in a wide variety of human tumours. It has also been shown that the frequency of *ras* gene point mutations varies in different tumour types. Although there is a higher frequency of mutations in specific types of tumours, i.e., in 75-90% of pancreatic adenocarcinomas (10), 40-50% of colorectal carcinomas (11, 12), 30% of lung adenocarcinomas (13) and about 25% of acute myeloid leukaemias (14), the overall incidence of point mutations in *ras* genes in human tumours may be only about 10% (15). These point mutations are very rarely present in renal adenocarcinomas or in breast cancer, while there is lower prevalence in thyroid, testicular, skin, bladder, endometrial and liver cancers (16, 17).

Different results have been obtained in the study of mutational activation of the *ras* oncogene in oral carcinogenesis. In India (18) and Taiwan (19), *ras* gene activation has often been found in oral cancer patients, whereas studies in Caucasian populations have shown that *ras* gene activation is very rare (20-22). Epidemiological studies have demonstrated that the high incidence of oral cancer in India and Taiwan is closely associated with the habit of chewing tobacco or betel quid (19).

The most important risk factors for oral carcinomas remain tobacco and alcohol (23). Tobacco smoke contains more than 4000 compounds, of which at least 50 are carcinogenic (24). The major inducer of tobacco dependence for smokers and for tobacco chewers is nicotine. Cigarette smoking is a worldwide problem. Moreover, while cigarette smoking levels have declined during the past 20 years, the production and consumption of smokeless tobacco products, snuff and chewing tobacco, has increased significantly (25).

Chemical carcinogens may play an important role in inducing the *ras* oncogene in a variety of carcinogen-induced tumours in animal experiments. The *ras* oncogene activation that is observed in these experiments is carcinogen specific and suggests that *ras* oncogene activation by carcinogens plays an important role in chemical carcinogenesis (26). The complex nature of cigarette smoke precludes the assignment of its carcinogenic activities to any one compound or group of compounds. Among the well-established carcinogens found in tobacco products are tobacco-specific nitrosamines (TSNA), which chiefly consist of N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (27). The comparatively high concentrations of this group of compounds in tobacco make them prime candidates for explaining the carcinogenic activities of tobacco in smokers, snuff dippers and tobacco chewers.

In this study, we investigated the possible role of *ras* oncogene activation in oral cancer patients associated with tobacco smoking in the UK. The polymerase chain reaction followed by direct DNA sequencing methods was used for the analysis of the exon 1 and 2 regions of H-*ras*, K-*ras* and N-*ras* genes. The different form or mode of application of tobacco products was examined in order to explain the different prevalence of *ras* gene mutations in oral carcinogenesis in western and Asian samples.

Materials and Methods

Tumour Specimens

Human oral tumour specimens were obtained from patients undergoing surgery for the removal of oral squamous cell carcinomas by the West Glamorgan Health Service. Patient information is given in Table 1. DNA from placenta was used as the control. The tumour specimens were frozen and stored at -70ºC until DNA extraction. DNA was extracted by the phenol/chloroform method and was then quantitated by UV absorption.

Table 1. Smoking habits of patients analysed.

Patient number	Age	Sex	Smoking history*
8	49	M	>30/day
9	57	M	>30/day
11	48	M	>30/day
12	73	F	11-20/day
15	50	F	Ex (1)
16	61	M	Ex (12)
18	64	F	Ex (10)
21	50	F	11-20/day
26	81	M	>30/day
34	85	F	non
35	24	F	non
42	70	M	Ex (5)
44	54	M	10/day
45	39	M	non
47	57	F	25/day
51	66	M	Ex (8)
52	48	M	<10/day
53	67	M	non
55	68	M	>30/day
56	78	F	15/day
57	82	M	non
59	79	M	non

* For ex-smokers, years since cessation is in brackets.

Polymerase Chain Reaction

Oligonucleotide sequences were designed to amplify sequences around either codons 12 and 13 or codon 61 of the H-*ras*, N-*ras* and K-*ras* genes (Table 2). The selected oligomers (5'-biotin labelled) were synthesized on an automated DNA synthesizer (Applied Biosystems, Model 391) as instructed by the manufacturer, using standard cyanoethylphosphoramidite chemistry.

Table 2. Synthetic oligonucleotide sequences used as primers for human *ras* gene.

Gene	Priming region	Primer sequences (5' - 3')	
K-*ras*	Exon 1	TGACTGAATATAAACTTGTG	(sense)
	(codon 12/13)	TATTGTTGGATCATATTCGT	(antisense)
	Exon 2	TTCCTACAGGAAGCAAGTAG	
	(codon 61)	CACAAAGAAAGCCCTCCCCA	
H-*ras*	Exon 1	GGCAGGAGACCCTGTAGGAG	
	(codon 12/13)	GTATTCGTCCACAAAATGGTTCT	
	Exon 2	CTACCGGAAGCAGGTGGTCATT	
	(codon 61)	CGCATGTACTGGTCCCGCAT	
N-*ras*	Exon 1	GACTGAGTACAAACTGGTGG	
	(codon 12/13)	CTCTATGGTGGGATCATATT	
	Exon 2	TCTTACAGAAAACAAGTGGT	
	(codon 61)	ATACACAGAGGAAGCCTTCG	

PCR was performed with 10 µl (1µg) of genomic DNA solution, 40 pmol of each primer, 200 µM of deoxynucleotide triphosphates, 10 X PCR buffer (100 mM Tris-HCl, pH 8.8; 15 mM MgCl2; 500 mM KCl; 1% Triton X-100) and 2.5 U *Taq* polymerase in a final volume of 100 µl. The samples were overlaid with 70 µl mineral oil and subjected to cycles of PCR amplification using the Hybaid Omnigene HB-IR-CM DNA amplification machine according to the following thermocycling protocol: initial denaturation at 94ºC for 2 minutes, followed by 30-35 cycles at 94ºC for 1 minute, annealing for 30 seconds and extension at 72ºC for 30 seconds. Table 3 shows the optimum annealing temperatures and cycle numbers for each gene region.

Table 3. PCR parameters for *ras* gene primers.

Gene region	Annealing Temperature	Cycle number	Product length (bp)
K-*ras*			
Exon 1	55	35	107
Exon 2	60	35	128
H-*ras*			
Exon 1	56	35	145
Exon 2	59	30	102
N-*ras*			
Exon 1	59	30	109
Exon 2	59	35	130

After amplification, the DNA was resolved by electrophoresis in 6% of acrylamide gel and stained with silver nitrate to ensure the presence of 102 to 145 base-amplified DNA.

Direct Sequencing Method

Streptavidin coated magnetic beads (Dynabeads M-280, Dynal) were used as solid support in the purification and isolation of the single-stranded DNA of PCR products. The immobilised single-stranded DNA was sequenced using a Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemicals) according to the dideoxynucleotide chain termination method (28)

The sequencing reaction protocols were as follows: 2 µl of 5 X reaction buffer (200 mM Tris-HCl, pH 7.5; 250 mM NaCl) and 1 µl primer (2 pmol) were mixed in an Eppendorf tube with 7 µl single-stranded DNA. This mixture was incubated for two minutes at 65ºC using a water bath for annealing of the appropriate primer to the template DNA. The tube was cooled slowly to 35ºC over 15-30 minutes and the annealed template was placed on ice.

1 ml dithiothreitol (0.1 M DTT), 2 µl of diluted labelling mix, 0.5 µl of [35S] dATP (10 ci/ul), 1 µl manganase buffer (0.1 M MnCl2, 0.15 M sodium isocitrate) and 2 µl diluted sequenase polymerase were added to the ice-cold annealed DNA mixture and incubated at room temperature for 2-5 minutes. Then this mixture was divided into four tubes containing 2.5 µl of dideoxy A, C, G and T termination mixtures from the Sequanase Kit. After incubation at 37ºC for 5 min, 4 µl of the stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenolblue, 0.05% xylene cyanol FF) was added to the four individual reaction tubes. These samples were heated at 85ºC for 2 min and then subjected to electrophoresis on 8 M urea/6% acrylamide sequence gel. The gel was dried and exposed to hypaper-^{35}S sequencing film (Amersham) for 48 h.

Results

A total of 22 human oral tumour samples were examined by PCR and sequencing. In vitro amplification of the DNA by PCR followed by direct DNA sequencing methods were used for the analysis of possible mutations at codons 12, 13 or 61 of the H-*ras*, K-*ras* and N-*ras* genes.

PCRs of the tumour samples DNA were used to generate 107 bp and 128 bp fragments of the K-*ras* gene of exons 1 and 2, respectively. Similarly, 145 bp and 102 bp fragments, and 109 bp and 130 bp fragments were amplified for the H-*ras* gene and N-*ras* gene of exons 1 and 2, respectively. The PCR products were resolved on 6% polyacrylamide gels and visualized by silver staining.

Approximately 70 to 100 nucleotides around individual targeted genes were analyzed by the direct sequencing method. The analyzed nucleotide sequences of the tumour samples were in complete agreement with published nucleotide sequences of H-*ras*, K-*ras* and N-*ras* genes determined by conventional cloning and sequencing methods (29-31).

Point mutation analyses of codons 12, 13 and 61 of the three *ras* genes were performed on the 22 human oral tumour samples. Direct DNA sequencing analyses revealed that no mutations were present at codons 12, 13 and 61 of the H-*ras*, K-*ras* and N-*ras* genes in the DNA extracted from oral tumours.

Discussion

In this study, we used the sensitive technique of in vitro enzymatic amplification of target DNA sequences followed by direct DNA sequence analysis to study point mutations in *ras* genes in tobacco-smoking-related malignancies. PCR and direct sequencing were chosen as the methods for investigating *ras* genes mutations because dot blotting for point mutations is not as informative as direct sequencing. This method enabled the sequencing of virtually every nucleotide of the targeted *ras* genes. Thus, the results may be considered accurate and reliable, unlike those of the conventional cloning and sequencing methods, which take weeks to complete. The analysis of one oral tumour sample alone can take a couple of days with these methods.

We have extended (the whole *ras* gene family) and confirmed the observations of previous investigators (20-22) using direct DNA sequencing to show that mutations in the *ras* gene family are extremely rare or are absent in the pathogenesis of oral cancer in Caucasian patients. No mutations were observed in the H-*ras*, K-*ras* and N-*ras* genes in the 22 oral tumour samples. Our results and those of other investigators suggest that *ras* mutations are infrequent in western samples in contrast to the findings of some studies which were carried out on Indian and Taiwanese oral cancer patients. Saranath et al. (18) reported that a significantly high proportion of oral cancer patients (35%) have point mutations in codons 12, 13 or 61 of the H-*ras* gene in Indian patients. Mutations in primary tumours of the oral cavity from an eastern Indian population were observed in H-*ras* and K-*ras* genes at a frequency of 28% and 33%, respectively (32). H-*ras* oncogene activations have also been found at codon 59 in Indian oral cancer patients (33). Point mutations in K-*ras* codon 12 have also been reported in tumour samples obtained from Taiwan by Kuo et al. (19). In these studies, samples were collected from patients who had habitually chewed tobacco and betel quid together. Thus, the possible reason for the absence or low frequency of *ras* gene mutations in the development of oral cancer in western populations might be the differences in the mode of exposure to tobacco and tobacco products. This difference between smoking and chewing tobacco should be taken into consideration when explaining the occurrence of *ras* gene mutations in India and Taiwan.

In addition to tobacco, other principal risk factors, have been proposed. These are the patients' diet and the intake of certain nutrients such as iron and vitamin C, dental status and the patients' level of hygiene (34). It is disturbing to note that in the West the incidence of oral cancer has recently started to increase, particularly in young male cohorts who are exposed to traditional risk factors and low consumption of fruit and vegetables (35).

Tobacco contains several carcinogens and procarcinogens. The important relevant carcinogens and procarcinogens in tobacco and alcoholic beverages are nitrosamines, polycyclic aromatic hydrocarbons, arylamines, alkyl halides, ethanol and urethane (36). Dependence on or addiction to nicotine, which is a tertiary amine, is the main reason for the continued use of tobacco products. Tertiary amines and secondary amines (nornicotine, anabasine and anatabine) react with nitrosating agents to form stable chemicals known as N-nitrosoamines (37). Over 300 different nitrosamines have been shown to be carcinogenic in experimental animals (38). Nitrosation of nicotine with sodium nitrite gives nornitrosonornicotine (NNN) and 4-(methyl-nitroamino)-1-(3-pyridyl)-1-butanone (NNK) (39). NNN and NNK related nitrosamines are known as tobacco-specific nitrosamines.

Although early studies on the formation of tobacco-specific nitrosamines in tobacco demonstrated that it was produced during curing, subsequent investigations have shown that the type of post-harvest processing employed greatly influences the levels of tobacco-specific nitrosamines in tobacco (40, 41). The eventual levels of tobacco-specific nitrosamines can also be influenced by other factors such as tobacco genotype, soil type, climate, drying process and length and conditions of storage (40-42). These factors may also play a role in the differences in the development of oral tumours between western and Indian patients.

Nitrosamines require metabolite activation for binding to DNA and other cellular macromolecules and α-hydroxylation of nitrosamines is believed to be important in their metabolite activation to intermediates which bind to DNA (38). Of these intermediates, O^6-methlyguanine, 7-methylguanine and O^4-methylthymine, O^6-methylguanine has been conclusively shown to cause miscoding (43), and the resulting point mutations can lead to oncogene activation (44).

One important point is that the levels of tobacco-specific nitrosamines in tobacco products are notably higher than those of nitrosamines in cigarette smoke. Although in one study NNN levels were found to be 140-240 ng/cigarette in the smoke of a non-filter cigarette, high levels of NNN were determined in unburned tobacco such as 0.3-9.0 ppm in cigarette tobacco, 3.0-45.5 ppm in cigar tobacco, 3.5-90.6 ppm in chewing tobacco and 12.1-29.1 ppm in snuff (37). These levels are among the highest for environmental nitrosamine in terms of occurrence and human exposure. However, studies of NNN concentrations in mainstream and sidestream tobacco smoke found 0.066-1.01 µg/cigarette and 0.19-0.86 µg/cigarette respectively (27, 45). Another factor in exposure to TSNA is in-vivo formation of NNN in the use of tobacco products. When chewing tobacco was incubated with human saliva for 3 h at 37°C and the mixture analyzed for NNN, the concentration of NNN increased by 44% over that in the chewing tobacco, presumably as a result of further nitrosation (27). This additional exposure to TSNA in the use of tobacco products is also important. Three factors have been found to be significant in explaining the differences in oral carcinogenesis. These are: in vivo formation of TSNA, the high concentrations of TSNA and long exposure periods compared to smoking, which make smokeless tobacco products prime candidates as a cause of the development of oral malignancies due to the fact that the oral mucosa are exposed for a longer period to higher levels of tobacco carcinogens.

In conclusion, PCR/DNA sequence analysis would appear to contradict the theory that *ras* gene codon 12, 13 and 61 alterations play a role in human oral cancers. The findings suggest that the existence of genes (p53, c-myc, calcyclin, p16/CDKN2) or mechanisms (polymorphism in xenobiotic metabolizing enzyme such as CYP1A1, CYP1A2, CYP2A6, CYP2D6, CYP2E1 or GSTM1) other than mutations of *ras* genes might be responsible for the development of oral tumour.

One may speculate that tobacco-specific nitrosamines do not reach high enough concentrations to cause *ras* gene mutations in tobacco smoking when their concentrations are compared with those in smokeless products. The differences in *ras* oncogene activation between tobacco smoke and smokeless tobacco products may also be an important example of chemical carcinogenesis in terms of carcinogen quantities and possible response.

Although cessation of tobacco use is the only way to totally prevent exposure to carcinogenic TSNA, greater attention should be paid to smokeless tobacco products (snuff and chewing tobacco) than to tobacco smoke in the prevention of oral cancer. Alternatively, ways should be found to reduce nicotine and TSNA levels in tobacco through the selection of other tobacco species or modificcation of the fermentation and processing of tobacco.

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