Mutation Analysis of *RAS* Oncogenes in Oral Squamous Cell Carcinoma

Li-Wan Lee, Kung-Shi Yang, Kun-Tu Yeh[1], Nancy M Wang[2], Ting-Hsu Lin[2], Jan-Gowth Chang[2]

Department of Dentistry, Taipei Municipal Jen-Ai Hospital, Taipei, Taiwan; [1]Department of Pathology, Changhua Christian Hospital, Changhua, Taiwan; [2]Department of Medical Research, China Medical College Hospital, Taichung, Taiwan, R.O.C.

Background. The mutations of *RAS* oncogenes have been found in a number of cancers and play an important role in oncogenesis. However, only a few studies have analyzed the mutations of N-, H-, and K- *RAS* in oral squamous cell carcinoma.

Methods. Hotspot mutations of N-, H-, and K-*RAS* oncogenes were analyzed by the amplified created restriction site method (ACRS), polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and direct sequencing analysis.

Results. Out of the 20 squamous cell carcinoma (SCC) specimens we studied, four had a GGC to AGC change at codon 12, which corresponds to an amino acid glycine to serine mutation of the H-*RAS* oncogene. No mutation was found in K- or N-*RAS* oncogenes.

Conclusions. Our results differed from Kuo et al, which may be due to different chemical components in betel quid or to different patient populations studied. Further analysis is needed.

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Key words

RAS gene, mutation analysis, PCR-RFLP, direct sequencing

INTRODUCTION

Squamous cell carcinoma (SCC) of the oral cavity is a major cause of mortality in several developing countries, comprising 40% to 50% of all malignancies in parts of India and South East Asia [1-3]. This high prevalence is in contrast to 2% to 4% of the total malignancies in developed Western countries [4-5]. In Taiwan, the prevalence is 2% to 3% of the total malignancies, which is close to that in Western countries. There is an unequivocal relationship between betel quid (BQ) chewing and oral cancer [6]. In Taiwan, BQ preparation differs from that in other parts of the world; most people consume BQ by cutting the fresh betel nut into halves and sandwiching them with a piece of the inflorescence of Piper betle and lime paste. No tobacco is added to the BQ.

Oncogenes of the *RAS* family have been implicated in the pathogenesis of oral SCC [7]. Three of these genes have been characterized: H-*RAS*, K-*RAS* and N-*RAS* [8]. These genes code for proteins p21 which possess guanosine triphosphate (GTP)-binding activities, and are immunologically related to one another [8]. Mutations in the *RAS* genes have been shown to occur predominantly in codons 12, 13 and 61 [9,10] and may represent direct effects of exposure to carcinogens [9,10]. These mutations are known to produce *RAS* proteins that are less able to hydrolyze GTP, resulting in altered signal transmission from the plasma membrane to cellular targets that subse-

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Address reprint requests to : Jan-Gowth Chang, Division of Molecular Medicine, Departments of Laboratory Medicine and Medical Research, China Medical College Hospital, No 2, Yuh-Der Road, Taichung 404, Taiwan, R.O.C.

quently affect growth and differentiation [8,9].

The prevalence and type of *RAS* mutation varies widely among neoplasms [9,11,12]. For example, the K-*RAS* gene is often mutated in colorectal, pancreatic and lung adenocarcinomas [13-15], whereas H-*RAS* and N-*RAS* genes are often mutated in uro-epithelial neoplasms and in hematopoietic malignancies, respectively [16,17]. Thyroid neoplasms appear to contain mutations in all three *RAS* genes with equal frequency [18]. Mutations in N-*RAS*, on the other hand, are rarely observed in esophageal, gastric and anal carcinomas [19-21]. For oral SCC, H-*RAS* is the most commonly mutated oncogene [22-28]. In Taiwan, however, it has been shown that K-*RAS* is the most commonly mutated gene in oral SCC [29]. In this study, we investigated this discrepancy by using ACRS and PCR-RFLP methods followed by direct sequencing to analyze 20 cases of BQ related oral SCC.

MATERIALS AND METHODS

Subjects

A total of 20 oral tumor specimens were obtained from surgical procedures at the Changhua Christian Hospital, Changhua, Taiwan. All the specimens were SCC, and they were kept frozen after surgical removal from the oral cavity. DNA was extracted by a standard method [30].

DNA Amplification and Restriction Enzyme Analysis

Primers with 3' or 5' terminal base alterations were used to generate polymerase chain reaction (PCR) products with artificially created substitutions, either adjacent to or within the mutated region. For the detection of K-*RAS* mutations in codons 12, 13 and 61, we used the method previously described [30]. For detection of codon 12 mutations of H-*RAS*, a mismatch base "A" at the second base of the 3' end of the antisense primer (5'-CAGCGCACT CTTGCCCACAAC-3') was used to destruct a potential *Msp* I site located on codon 13; that is, GGT changed to GTT, constructing the authentic *Msp* I site in the region of codons 11 and 12. After PCR with sense primer 5'-CTTGGCAGGTGGGGCAGGA-3', a normal *Msp* I site was created. If any combinations of mutations occurred at the first two bases of codon 12 (GG), the restriction site was lost. For detection of codon 13 mutations of H-*RAS*, a mismatch base "C" at the last base of the 3' end of the upstream primer was used to destruct the authentic *Msp* I site in the region of codons 11 and 12, creating a new *Msp* I with the first two bases of codon 13 after PCR. For detection of codon 61 mutations of H-*RAS*, two primers (5'-GATTCTACCGGAAGCAGGTG-3' and 5'-CTGTACTGGTGGATGTCCTCA-3') were used to amplify this region, followed by direct sequencing of the PCR products. For detection of codons 12, 13 and 61 mutations of the N-*RAS* gene, we used the method described by Todd et al [31]. Direct sequencing of the PCR products for detection of K-, H- and N-*RAS* was performed as described [32].

RESULTS

All 20 cases of oral SCC were analyzed for hotspot mutations in H-*RAS*, K-*RAS* and N-*RAS* using PCR-RFLP. There were no hotspot mutations in K-*RAS* or N-*RAS* after PCR-RFLP analysis (data not shown). For H-*RAS* mutations, the PCR products for the detection of codon 12 of H-*RAS* were digested with restriction enzyme *Msp* I and electrophoresed on a 4% agarose gel. A normal codon 12 revealed three fragments: 56bp, 37bp and 25bp (poor visualization) (Fig. 1 lanes 1-4). Homozygous mutation of codon 12 resulted in two fragments: 81bp and 37bp, whereas the heterozygous mutation had 4 fragments: 81bp, 56bp, 37bp and 25bp (Fig. 1 lanes 5 and 6). Four out of 20 oral SCC cases showed a picture of partial digestion, and all were heterozygous. Further confirmation of the mutation by direct sequencing analysis revealed that these 4 cases all had a GGC to AGC mutation which changed the encoded amino acid from glycine to serine (Fig. 2). No mutations were found in codons 13 or 61 of H-*RAS* in these 20 oral SCC specimens.

DISCUSSION

The amplified created restriciton sites

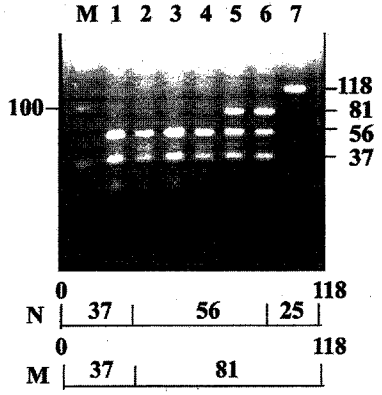

Fig. 1 The representative results of PCR-RFLP for codon 12 mutation of H-*RAS* are shown. After *Hpa* II digestion of PCR amplified codon 12, wild type H-*RAS* had 25bp, 37bp and 56bp fragments (lanes 1-4), and heterozygote mutation had 25bp, 37bp, 56bp and 81bp fragments (lanes 5-6). Lane M: marker, 100bp ladder. Lane 7: uncut control.

(ACRS) method has been used to detect mutations of the *RAS* oncogene, and a number of diseases, including glucose-6-phosphate-dehydrogenase deficiency, and β-thalassemia [30,33,34]. Because this method incorporates two independent steps, an assay and an examination of the generated DNA fragments, its accuracy is reliable. Only two primers are needed in each mutation and there is no need for radioactive oligonucleotides. Furthermore, most of the restriction enzymes have very high fidelity. This method is simple, rapid, and accurate for screening mutations. Hemi-nested PCR and restriction enzyme digestion, followed by sub-cloning of the PCR product and sequencing of the cloned plasmid was used by Kuo et al. The procedure is much more complicated and risks of contamination and artifact mutations are high.

The incidence of *RAS* mutations in oral SCC in this study was in agreement with many previous reports, with the exception of Kuo et al [29]. In their study, 18% of betel quid chewing-related oral SCC were found to have a K-*RAS* mutation in codon 12. In contrast, we only found mutation of H-*RAS* in codon 12. This discrepancy needs further study. All the mutant cases found in this study had a GGC to AGC alteration at codon 12 of the H-*RAS* gene, which was different from tobacco-related oral SCC in India (codon 12 GGC→

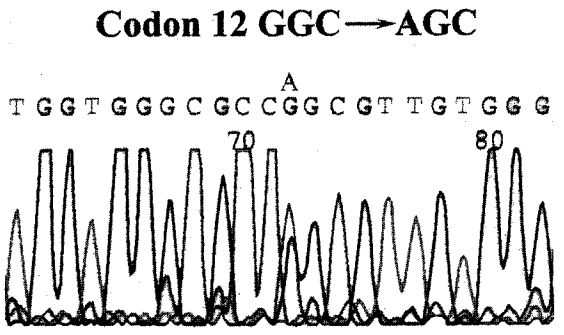

Fig. 2 The results of direct sequencing for mutants. A GGC to AGC change in codon 12 of H-*RAS* was found after sequencing the above heterozygous mutation (Lanes 5 and 6).

GTC and codon 61 CAG→CGG for most cases, and higher mutation rates at codon 61) [28]. This result may be due to various types of chemicals in BQ causing different mutations of the *RAS* gene.

In conclusion, we analyzed K-, H- and N-*RAS* hotspot mutations in 20 oral SCC specimens. Although similar populations were used, our results differed from those of Kuo et al. Several possible factors may have caused this discrepancy. The chemical components of BQ in central Taiwan may be different from those in northern Taiwan used in Kuo's cases. Although the people from the North and Center are similar, there are some differences. The people of northern Taiwan have a much higher population originating from northern mainland China than the people from mid-Taiwan. Personal habits, such as smoking and alcohol intake may be different between the people in these two areas as well. In addition, the environment, temperature, and humidity also differ in these two regions. These factors may also play a role in the development of mutations of *RAS* oncogenes. Further study is needed to confirm this discrepancy.

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*RAS*致癌基因在口腔癌的表現

李麗莞　楊恭熙　葉坤土[1]　王妙媛[2]　林亭旭[2]　張建國[2]

台北市立仁愛醫院 牙科　彰化基督教醫院 病理部[1]　中國醫藥學院附設醫院 醫學研究部[2]

背景　*RAS*致癌基因的突變在很多腫瘤均可發現，它在腫瘤的形成過程佔有相當重要的角色。目前爲止，只有少數的幾篇研究同時探討三種*RAS*致癌基因在口腔上皮癌的突變情形。

方法　我們利用ACRS（或PCR-RFLP）及DNA定序的方法，分析N-, H-, K-*RAS*致癌基因的常見突變。

結果　20例口腔上皮癌的病人中有4例有H-*RAS*基因codon 12 GGC變成AGC的突變。並沒有其它*RAS*的突變。

結論　我們的研究結果與大多數的報告類似，但與國內Kuo等的研究有些出入，因此須要更進一步收集更多的病例，才能得到更明確的結論。（**中台灣醫誌 2001;6:69-73**）

關鍵詞

*RAS*致癌基因，熱點突變，聚合酶鏈鎖反應及內切酶切段片多形性變化，直接定序

聯絡作者：張建國

地　　址：404台中市北區育德路2號

中國醫藥學院附設醫院 醫學研究部

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