計劃名稱：口腔鱗狀細胞癌症病人唾液中脫落細胞之 DNA **分析**

The analysis of saliva exfoliative cell's DNA in oral squamous cell Carcinoma patients

計畫編號：NSC 87-2314-B-040-016-

執行期限：86 **年** 8 **月** 01 **日至** 87 **年** 7 **月** 31 **日**

主持人：周明勇

執行機構及單位名稱：中山醫學院牙醫學院

摘要

本研究的目的是為了解 一、人類口腔鱗狀細胞癌症病人是否可以由唾液中脫落細胞分離出 DNA。 **二、口腔鱗狀細胞癌病人脫落上皮細胞** DNA p53 **基因失去異質性的研究及 三、**p53 **基因** exon 4、intron 6 **突變的發生情形。收集** 10 **位由中山醫學院附設醫院病理科診斷為口腔鱗狀細胞癌病人的唾液，對照中山醫學院牙醫系** 27 **位健康學生唾液來作分析。使用** 50 ml **的試管分別收集口腔鱗狀細胞癌病人及健康學生的唾液, 按照** DNA Purification System **步驟分離脫落上皮細胞的** DNA，**其分離後電泳於凝膠可見** Genomic DNA **者高達** 83.8% (31 /37)，**另三例樣本雖未見凝膠顯見** Genomic DNA **但在其後** PCR **反應均能有滿意的結果，唯有三例經** PCR **反應** p53 **基因** intron 6 **有反應，而** exon 4 **則無反應，總計能完全達到研究目的** DNA **高於** 91.9% (33/37)。**而對於** p53 **基因失去異質性研究的部份，**p53 exon 4 PCR **產物以** BstU1 **消化，而** p53 intron 6 PCR **產物以** Msp1 **消化, 由於避開同一口腔內取樣互相污染的可能，故其失去異質性的比率應大幅下降、而非一般同一口腔取樣之** 50 **至** 70%。**至於** p53 **基因突變的研究，經** ABI PRISM 377 Autosequencer **作** DNA **序列發現** p53 exon 4 **內** codon 63 **可能是個突變率很高的突變。**

Abstract

The purpose of this study are:1.whether we can extract saliva exfoliative cell's DNA from human squamous cell carcinoma patients. 2. the analysis of loss of heterozygosity of p53 gene exon 4 and intron 6. 3. The mutation of p53

gene within exon 4 and intron 6 areas. . In this study, twenty-seven normal healthy person's saliva and ten oral squamous cell carcinoma patient's saliva were collected. After DNA extraction and PCR amplification, more than 90% samples we can use successfully. For the loss of heterozygosity of p53 gene study, PCR-RFLP analysis was performed, p53 exon 4 PCR products digested with BstU1, p53 intron 6 PCR products digested with Msp1, for healthy saliva samples and tumor saliva samples the results are no significant different. Finally, the mutation of p53 exon 4 and intron 6 area study, probable hot-spots for the mutation induction was identified at codon 63.

Introduction

Most human squamous cell carcinomas in the oral cavity contain mutated p53 tumor- suppressor genes[1], the mutation includes point mutations, deletions, rearrangements, or allelic loss.[2] Besides oral cavity, these mutations have found in most human tumors, including colon, lung, liver, breast, esophageal, brain, and hematopoietic tissue cancers.[2..3] p53 gene compasses 16-20 Kb of DNA on the short arm of human chromosome 17 and contains 11 exons and the wild type p53 protein has been shown to be invol-ved in the regulation of cellular growth, and its expression suppresses the growth of transformed cells.[4,5] Recent evidence has shown that it is a DNA-binding pro-tein.[6] alterations in the tumor suppress-or gene p53 are the most frequently de-tected genetic abnormalities in human cancers, inactivated p53 gene (like other tumor suppressor genes) often is sugge-sted by loss of herterozygosity (LOH) studies. LOH in p53 was demonstrated in 52% of heterozygous individuals with primary esophageal neoplasmas.[7] and LOH in a p53 site is found in more than 70% of oral SCC from heterozygous individuals.[8] The results of LOH is depend on the samples they took in oral lesions and corresponding normal tissues, actually it always mixed between normal and tumor cells in the selected samples. Now we interest in the PCR-RFLP analysis in p53 exon 4 and intron 6 regions using separated samples. Also in this study, we use modern bio-molecular technique to sequencing p53 exon 4 and intron 6 areas,we have a great hope that we can find the differ-ences between tumor cell and normal cell and establish the diagnosis biomar-ker of oral squamous cell carcinoma.

Materials and methods

Samples collect

Twenty-seven normal person's saliva and ten oral squamous cell carcinoma patient's saliva were collected in 50 ml

centrifuge tube from Chung Shan Medical and Dental College Hospital.

DNA Extraction

DNA was extracted from the saliva using standard procedures described as Promega company Wizard Genomic DNA Purification protocol then store the DNA at 4°C.

PCR Amplifications

Extracted genomic DNA (0.2-1μg, depending on the total available extracted DNA), 30 pmol of each primer, all four deoxynucleotide triphospates (dNTP) (100 μM), reaction buffer, and 2.5 units of Taq polymerase were mixed and reacted in a total volume of 100μl. The primers used encompassed a 259 base pair region within exon 4 of p53 and a 107 base pair region in intron 6. the length was kept to 18-26 nucleotides, G C content was kept to 40-60%.the ampli -fication protocol for the exon 4 locus consisted of one cycle of 10 minutes at 95°C, 2 minutes at 57°C, and 30 seconds at 72°C, followedby 35 cycles of 1 minu -te at 95°C, 30 seconds at 57°C, and 30 seconds at72°C. The protocol for the intron 6 region was one cycle of 5 minutes at 95°C, 2 minutes at 60°C, and 1 mi -nute at 72°C, followed by 35 cycles of 1 minute at 95°C, and 1 minute at 72°C.

Enzyme digestion

PCR products were electrophoresed on 2% agarous gels to determine success of the reactions and nonelectrophoresed 30-70μl aliquots of the amplified exon 4 or the intron 6 region were digested with 30 units of Bst U1(New England Biolabs, Natick, MA) or 40 units of Msp1 (New England Biolabs, Natick, MA), respectively. Each amplimer contained only one polymorphic restriction enzyme site, the digested PCR products were run on 2% agarous gels, stained with ethidium bromide, and photographed, then the photographs were analyzed.

DNA Sequencing

ABI PRISM 377 Autosequencer was used and procedures described as follow: Prepare a loading buffer by combining deionized formamide and 25Mm EDTA (pH 8.0) containing 50 mg/mL Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran, resuspend the sample in the load- ing buffer, vortex and spin the sample, heat the sample at 90°C for two minutes to denature, then load sample.

Results

Ten oral squamous cell carcinoma patient's saliva and twenty-seven normal person's saliva were collected, genomic DNA extraction, p53 exon 4 PCR amplification, p53 intron 6 PCR amplification, p53 exon 4 PCR products digested with BstU1, p53 intron 6 PCR products didested wih Msp1 were made successfully (more than 90%). And PCR –RFLP analysis are: For p53 exon 4 region healthy specimens 7 of the 27 (25.93%) uncut, 4 of the 27 (14.81%) homozygous cut, 15 of the 27 (55.56%) exhibited heterozygous cut, 1 of the 27 (3.7%) fail to amplification and p53 in-

tron 6 region healthy specimens 23 of the 27 (85.19%) homozygous cut, 4 of the 27 (14.81%) exhibited heterozygous cut, and for p53 exon 4 region tumor specimens 1 of the 10 (10%) homozygous cut, 4 of the 10 (40%) heterozygous cut, 3 of the 10 (30%) uncut, and 2 of the 10 (20%) fail to amplification, for p53 intron 6 region tumor specimens 8 of the 10 (80%) homozygous cut, 2 of the 10 (20%) heterozygous cut , for total group: p53 exon 4 region : 5 of the 37 (13.51%) homozygous cut,19 of the 37 (51.35%) heterozygous cut, 10 of the 37 (27.03%) uncut, and 3 of the 37 (8.11%) fail to amplification, for p53 intron 6 region 31 of the 37 (83.78%) homozygous cut, 6 of the 37 (16.22%) heterozygous cut. And for p53 mutation study: at codon 63 GCT, in healthy samples only 19.2%(5/26) C deletion but in tumor samples, there is 62.5% (5/ 8) C deletion, significant different.

Discussion

DNA extraction from saliva exfoliative cell is reliable, it is more easy and harmless. PCR-RFLP analysis is used for detecting loss of herterozygosity, but in our study the three groups: healthy group, tumor group and total sample group are no significant different, so the articles claimed that high rate of LOH in p53 gene must be further study. And for p53 mutation study, cancer is considered to be a multi-hit process which involved a number of aberrant genetic events culminating in malignant transformation, probable hot-spots for the mutation induction in this study was identified at codon 63 C deletion.

References

1. Sakai E, Tsuchide N. Most human squamous cell carcinomas in the oral cavity contain mutated p53 tumor-suppressor genes.
Oncogene 1992; 7:927-33.

2. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutation in human cancers. Science 1991; 253:49-53.

3. J.K. Field Oncogenes and tumor-suppressor genes in squamous cell carcinoma of the head and neck. Oral Oncology, Eur J Cancer, Vol. 28B, No. 1, 67-76, 1992.

4. Robert A. Weinberg Tumor suppressor genes. Science 1991; 254 1138-46.

5. Warnakulasuriya KAAS. Johnson N W. Expression of p53 mutant nuclear phosphoprotein in oral carcinoma and proteinally malignant oral lesions. J oral path Med, 21: 404-408, 1992.

6.Cossman J, Schlegel R. p53 in the diagnosis of human neoplasms. J Natl Cancer Inst 1991; 83:980-1.

7. Kwok-Wai Lo, Sai-Wah Tsao et al p53 mutation in human nasopharyngeal carcinomas Anticancer Research 12: 1957-1964, 1992.

8. John S. Largey, David W. Archibald et al Loss of heterozygosity of p53 in oral cancers demonstrated by the polymerase chain reaction. Cancer 1993; 71: 1933-7.