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Pathology

LOSS OF HETROZYGOSITY AS A MARKER OF TUMOR PROGRESSION IN ORAL SQUAMOUS CELL CANCER

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ABSTRACT Cancer is the second leading cause of death globally. Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy. The survival rate is directly proportional to the stage of the disease. Genetic alterations are known to occur during carcinogenesis. Concept of LOH (Loss of heterozygosity) developed with the discovery of tumor suppressor gene.

METHODS: An observational study aimed at studying the LOH at 3p &9p by PCR in HNSCC

RESULTS: 30 cases were studied and LOH either at 3p or 9p was seen in all cases, but in varying stages.

INTERPRETATION: LOH can be used as a biomarker for studying disease progression in HNSCC

KEYWORDS : Loss of heterozygosity, oral squamous cell cancer, 9p locus, 3p locus

INTRODUCTION

Squamous cell carcinoma of head and neck is the sixth most common human malignancy although it accounts for 2% of all cancers in Western population. (1,2,3)

As per Indian data Cancers of oral cavity and lungs account for 25% cancer deaths in males and cancer of breast and oral cavity account for 25% cancers in females. (4).India has one third of oral cancers in the world (5). The immortalization of human cells is a critical step in multistep carcinogenesis [6]. Multiple and sequential genetic alterations occur in carcinogenesis and in progression of tumors [7,8]. Not many studies involving multiple genetic changes in relation to loss of heterozygosity are available from India. Study by G Mondal et al in India had shown high rate of chromosomal alterations at 11q21–24 in HNSCC and the presence of a putative tumor suppressor gene in this region [9]. Our study is similarly directed into acquiring further insight into predicting tumor progression. The genetic hypothesis of cancer implies that a tumor mass results from the clonal expansion of a single progenitor cell that has incurred genetic damage (i.e. tumor cells are monoclonal). Multiple genetic events lead to oral cancer, with around six to 10 genetic events believed to result in oral carcinogenesis. Genetic alterations known to occur during carcinogenesis including point mutations, amplifications, rearrangements, and deletions. Concept of LOH developed with the discovery of tumor suppressor gene. These genes regulate cell cycle and apply brakes to cell proliferation. Thus their function is to regulate cell growth and not to prevent tumor formation. "Loss of heterozygosity" defined as a loss of the only normal allele in a previously mutated heterozygous pair, thus resulting in two different dysfunctional alleles for a locus. In the series of sequential accumulation, the earliest to be lost are 9p and 3p followed closely by 17p and others in the more advanced lesions **(Fig 1**) Allelic imbalances have been reported in HNSCC also in chromosomes 4, 5, 6, 8, 10, 12, 13, 16, 18, 21 and 22. The most commonly reported chromosomal defect in HNSCC is loss of heterozygosity on 9p and LOH has been seen in at least five markers in 9p [3,10]. Loss of chromosomal region 9p21 is the most common genetic change that occurs early in the progression of these tumors [11,12]. The main effect of this loss is the inactivation of the p16 gene, an inhibitor of CDK that is important in regulating the cell cycle. The deletion map for 9P loss was given by Nakanishi Het et al in 1999 (**Fig 2).** LOH in oral cancers is especially concentrated between the D9S161 [9p21] and D9S156 [9p23-9p22] [13].

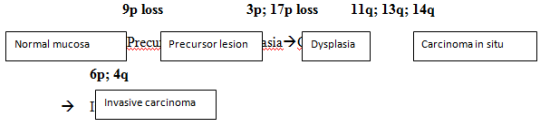

Fig 1: Model for molecular progression of carcinogenesis

Next in sequence is short arm of **chromosome 3 (3p)**, which is often deleted in head and neck squamous carcinoma. At least two or even three tumor suppressor genes may be involved on 3p. The region with the highest rate of allelic deletion is 3p21 and tumor suppressor genes at 3p21.2-p21.3 and 3p25 in particular may be implicated [14,15]. The other commonly involved genes include fragile histidine triad (FHIT) gene (3p14.2)

Common regions of LOH on chromosome 3 shown in **Fig 3.**

AIMS AND OBJECTIVES

To study loss of hetrozygosity in 3p and 9p chromosomes in head and neck squamous cell carcinomas in Indian population.

MATERIALS AND METHODS

Principle of the study technique

We used Polymerase chain reaction (PCR) as the baseline technique for studying LOH. The technique employed was to extract the DNA from the affected tissue, run a PCR for the required locus and compare a loss of

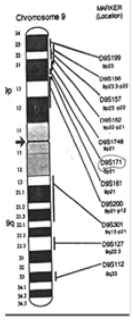

Fig 2: Chromosomal deletion map of 9p

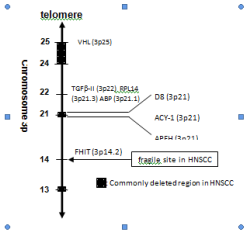

Fig 3: Common regions of LOH in chromosome 3p

intensity of bands along with a control and a cut off of > 50 % loss of intensity was taken as LOH..

I. **Source of case collection:** A total of 30 consecutive cases of histologically defined invasive squamous cell carcinoma of the head and neck regions diagnosed at a tertiary care hospital were studied over a period of 2 years SCC of the lip, tongue, buccal mucosa and retromolar trigone were included in the study.

Only those cases where hemi-mandibulectomy with RND was performed were taken for the study. Cases previously diagnosed and who had received CT/RT was not included in the study. Tissue samples from tumor tissue were obtained at the time of definitive surgery. A part of the tumor tissue was collected in sterile eppendorf tubes on ice, frozen immediately after careful removal from the surrounding normal tissues and stored at -70^{0}C until extraction of DNA was done.

The remaining resected sample was fixed in 10% buffered neutral formalin and routinely processed for histopathology.

Peripheral blood was also collected in EDTA vacutainers from all of the above patients for DNA extraction. This was meant to serve as control DNA for comparison for constitutive homozygosity while looking for loss of heterozygosity.

II. DNA EXTRACTION

DNA from fresh frozen sample was extracted by the standard Phenol chloroform extraction Method (Sambrook et al 1989).DNA from peripheral blood collected using EDTA as anticoagulant was extracted by a kit-based method (QIAamp DNA Blood Mini Kit, Qiagen, USA), following the manufacturers protocol. DNA extracted was quantified spectrophotometrically and 250 ng used as template for the polymerase chain reaction (PCR) amplification procedure [16,17]**.** The extracted DNA was stored at -20^{0}c.

Table 1

SN	Chromosome - Marker	Forward/ Reverse	Sequence
1	3p21 – D3S1284	F	5' GCC TTG GGG GTA AAT ACT CT 3'
		R	3' GGA ATT ACA GGC CAC TGC TC 5'
2	9p21 – D9S171	F	5' AGC TAA GTG AAC CTC ATC TCT GTC T 3'
		R	3' ACC CTA GCA CTG ATG GTA TAG TCT 5'

III. LOH-PCR

PCR was performed on all the DNA samples (i.e. fresh frozen tumor, and peripheral blood) for each case. Six sets of primers were used as microsatellite markers (**Table 1**).

Primers were obtained from Qiagen or Sigma. PCR amplification was performed in a total reaction volume of 50l, as described in literature. Amplicons were studied by gel electrophoresis using both 4% agarose gel and 12% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. The intensities of the signals in tumor DNA were compared with those of the corresponding normal (blood derived) DNA. **Loss of heterozygosity, if any, was defined as a reduction in signal intensity of at least greater than 50%.**

DATA ANALYSIS

Data on LOH was correlated with stage of tumor. The data was analyzed statistically by the Fischer test. Out of the 30 cases studied there were 04 (13.3%) cases in T1 stage, 14 (46.6%) in T2 and 12(40%) inT3 and distribution of LOH is as per the **given table 2.**

LOH AND TUMOR PROGRESSION

The aim of the study was to correlate LOH at 3p &9p chromosome in Head and Neck Squamous cell carcinoma (HNSCC) with tumor progression. In the study by Mao et al 60% of premalignant lesions with LOH at 3p &/or 9p developed HNSCC. It appears that LOH profiles can augment routine HPE evaluation of premalignant lesions. However we studied cases of Histologically proven SCC and grouped them in 3 stages based on TNM classification. In T1 cancer 3p &9p LOH was 75% & 50% respectively, in T2 cancers 3p & 9p loss being 35.6% &57.1 % respectively whereas T3 stage cancer showed 50% &66.6% respectively. Thus we see that as the tumor progresses to a higher stage there is a definite increase in LOH at 9p though this difference is not statistically significant (p value0.780). This could be only because of the small size of population we have studied. But LOH is definitely high in all the three stages of tumor corroborating with various studies which correlate LOH to tumor stage progression. The study by Kiyoto Shiga et al in 2004 studied 302 cases and found that LOH was significantly higher in stage T3 tumors. LOH in all the stages of lesion is consistent with the finding that it is an early event in carcinogenesis. Out of 30 cases we studied 11/30 i.e. 1 in T1, 07 in T2, 03 in T3 (total 36.6%) did not show LOH at any of the three sites studied. We could not find a probable answer to this but various studies done also have never been able to show 100% LOH.

CONCLUSIONS

There is a definite loss of hetrozygosity seen in Head and neck oral squamous cell carcinoma which is further substantiated by its presence is higher stages of the disease. A definite need is there to further analyse it where it can serve as a biomarker for disease progression in years to come.

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