ORIGINAL ARTICLE

Fluorescence *in-situ* hybridization technique as a diagnostic and prognostic tool in oral squamous cell carcinoma

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ABSTRACT

Background and Objectives: Early diagnosis and appropriate management are of prime importance for oral squamous cell carcinoma (OSCC) in the present scenario. Molecular changes in OSCC are well documented with the occurrence of a wide range of genetic damage. Identification of the genetic damage in OSCC using various diagnostic aids is mandatory, and one of the important advances in this field is cytogenetics using fluorescence *in-situ* hybridization (FISH). The aim of the present study is to analyze the genetic alteration in OSCC using FISH as a diagnostic aid. **Materials and Methods:** Peripheral blood was analyzed in 20 clinically and histopathologically proven OSCC cases and 10 healthy controls for chromosomal alteration under standardized conditions. **Results:** Of the 20 OSCC cases, 7 (35%) cases showed chromosomal alterations. No cases from the control group showed any chromosomal changes. Of the positive cases in OSCC, 30% cases showed increased copy number of cyclin D1 gene and 1 (5%) case showed positivity indicating extra copy of chromosome 11p11.11-q11 region. **Interpretation and Conclusion:** Increased genetic damage in OSCC which is a prominent feature can be identified by the use of FISH as seen from the present study. The findings suggest that FISH can be used as a diagnostic aid in the detection of genetic changes occurring in OSCC. The present study also suggests the importance of peripheral blood as a medium for assessing cytogenetic damage in OSCC.

Key words: **Fluorescence *in-situ* hybridization, oral squamous cell carcinoma, peripheral blood, cyclin D1**

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most frequent cancer in the world.[1] The development of OSCC can be due to genetic damage, which can alter cell growth, by the known etiologic factors, such as tobacco or excessive consumption of alcohol or both. The proliferative activity of the oral mucosa due to malignancy, are activated by the multiple mutations in growth regulatory genes.[2] The genetic changes occurring in OSCC have received the focus of attention in dentistry, especially in oral and maxillofacial pathology.

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Cytogenetics, the study of chromosomes entered into the area of cancer diagnosis only after early 1970s. Recently molecular cytogenetics has expanded rapidly and plays a major role in cancer disease diagnosis and management. Among the advanced molecular techniques, fluorescence *in-situ* hybridization (FISH) has a perfect balance of high specificity, sensitivity and rapidity, which is being used in routine clinical laboratory for genomic diagnosis.[3] The advantages of FISH over classical cytogenetics (karyotyping) are that it does not require, *in vitro* culture and metaphase preparation of the cells of interest and also its ability to study cells in interphase making it a better tool in advanced molecular cytogenetics.[4] In FISH, detection at a single cell level and simultaneous phenotypic analysis are possible, it can also be used in both archived and fresh specimens. Malignant cells do not grow well *in vitro* and therefore, karyotyping has limited application in cancer cytogenetics.[3] Among various approaches like southern blot hybridization, polymerase chain reaction, immunohistochemistry, FISH has an edge that it

needs less tumour tissue; it can be done rapidly and does not require radioactivity.[5]

Chromosome 11q13 region is frequently altered in OSCC. This region has been identified as frequent target for genetic alteration. Amplification of 11q13 region was one of the frequent abnormalities seen in the head and neck squamous cell carcinoma.[6] Aim of our study is to analyze the amplification of 11q13 region in the chromosome of OSCC patients by FISH with commercially available specific probe using peripheral blood.

MATERIALS AND METHODS

Clinically and histopathologically (Broder's classification) proven OSCC patients were included in the present study along with the control group. Detailed case history including systemic illness, medication and personal habits were recorded from both groups. Patients who were on systemic illness, long term medication or on antibiotics were excluded from the study. Consent of the patient and institutional ethical committee clearance were obtained for performing the study.

Peripheral venous blood was collected from the 20 OSCC patients and 10 controls from the brachial vein which was immediately transferred into a sterile, heparinized tube (vacutainer) and stored in the refrigerator.

Chromosome preparation and cytogenetic analysis were carried out by standard techniques as described previously.[7] 1-2 ml of peripheral venous blood was directly treated with 0.56% KCl for 30 min at 37°C to which cold fixative (3:1 methanol:Acetic acid) was added and centrifuged. Slides were prepared by adding 10 µl of the centrifuged cell pellet on to it, and hybridization areas were marked with a diamond tipped scribe. It was then transferred to coplin jar containing 2X sodium saline citrate (SSC) solution (Vysis cat no.: 32-804850) for 1 h at 37°C. Slides were then put through an alcohol gradient (freshly made each time) 70%, 85% and 100% for 2 min each and completely dried and denaturated using formamide + 2X SSC solution.

After slides were dehydrated with chilled ethanol series it was then placed in humidifying chamber. Freshly prepared probe locus specific cyclin D1 (CCND1) (11q13) (spectrum orange), and centromeric probe spectrum green (11p11.11-q11) (control probe)- Vysis mixture was added to one target area immediately and the cover slip was laid. Hybridization procedure was done overnight and then post hybridization washes were given with 2X SSC and Triton × 100 mixture.

Five microliter counterstain (DAPI diamidino-2- phenylindole-Vysis) was applied to the target area of the slide and coverslip was placed. The slides were viewed using a suitable filter set on a fluorescence microscope. Images were captured using cytovision software from applied imaging for documentation. On average 100 interphase cells were analyzed from each sample. The fields with high quality picture which showed abnormality were captured using cytovision software.

In a cell with normal copy number of the CCND1 gene (11q13 region) and chromosome 11 (11p11.11-q11), two red signal (CCND1) and two green signal (chromosome11 (11p11.11q11)) will be observed [Figure 1]. Abnormal copy number of CCND1 gene was indicated by 3 or more red signals. Simultaneously the copy number of chromosome 11 (11p11.11q11) can be quantified by enumeration of the green signal within the same cell.

RESULTS

Of the 20 OSCC cases, 7 (35%) showed chromosome alterations. In the cases showing chromosomal alteration, 6 (30%) cases showed three red signals [Figure 2] indicating increased copy no of CCND1 gene and 1 (5%) case showed three green signals [Figure 3] which indicate extra copy of chromosome 11p11.11-q11 region. But since red signal is not present in addition, it is probably due to partial duplication of chromosome 11 without CCND1 gene involvement.

DISCUSSION

FISH study revealed alteration in 6 (30%) cases in 11q13 region, control group being spared. Gebhart *et al*.,[8] found amplification of 11q13 region in 39% of patients and suggested 11q13 may be an important biologic marker indicating poor prognosis in OSCC. Supporting this Miyamato *et al*.,[5] using fine-needle aspiration biopsy samples found numerical aberration in 43% of patients with OSCC and also suggested chromosome11q13 alteration signifies worst prognosis. CCND1 gene is located on chromosome11q13. The CCNDI is a proto-oncogene which drives the cell from G1 into S phase of the cell cycle. Deregulation of this phase may lead to malignant tissue formation.[9] Ott *et al*.,[10] found 11q13 amplification in 8 of 20 tumor samples. Fortin *et al*.,[11] found gene amplification affecting the 11q13 band was lesser (11 of 50 (20%)) in oral and oropharyngeal carcinoma than HNSCC (19 of 31 (61%)). They also documented that epithelial cells from various sites of aerodigestive mucosa are not prone to or selected for the same type of genetic alteration following similar carcinogenic aggression. Breakage, fusion, and bridging cycles have been proposed to be an important mechanism of gene amplification.[12]

OSCC is the solid tumor, the genetic events from initiation till progression is multiple, unlike hematologic malignancies like leukemia where specific genes were altered. Among solid tumors chromosome11q13 is frequently altered, the cases which have not shown its change might have other genetic events. It was also noted from previous studies that chromosome 11q13 alteration has a poor prognosis which can be used as a prognostic indicator and with lesser invasive techniques like FISH. One case showed partial duplication of

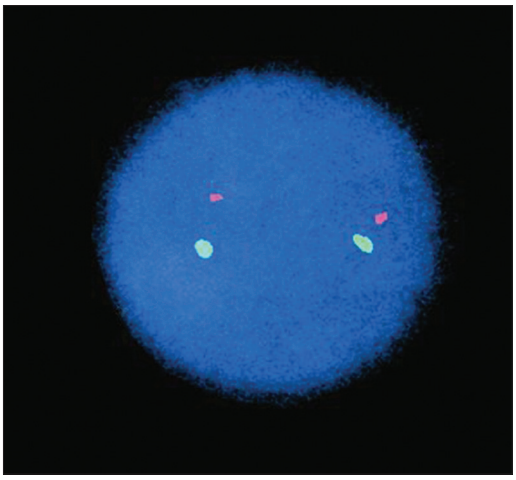

Figure 1: Normal FISH interphase showing two signals for each probe

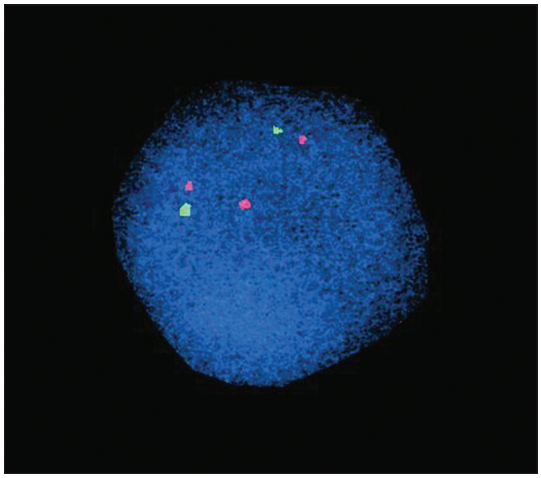

Figure 2: FISH showing three red signals and two green signals in interphase cell

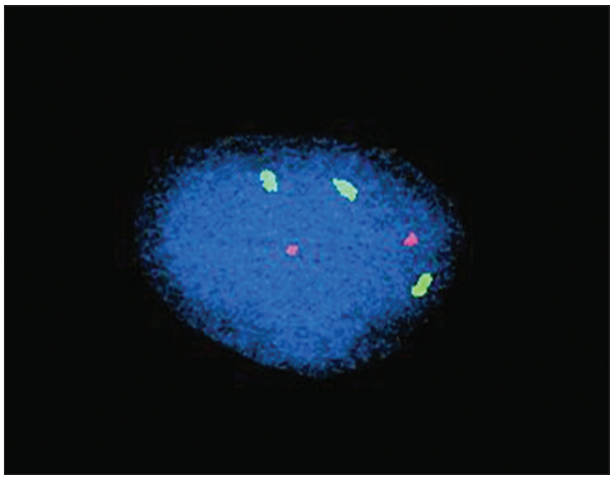

Figure 3: FISH showing three green signals and two red signals in interphase cell

chromosome 11, Wang *et al*.,[13] using 11q13 probe found out polysomy, greater than two copies of chromosomal 11 in two cases of 20 samples.

In most of the studies tissue samples were used for harvesting cultures. We used peripheral blood for our study, which gave promising results. This was based on the proposition made by Johanson *et al*., It states "heritable acquired characteristics of neoplastic cells brought about by changes in the genetic material, does not imply that their neighboring non neoplastic cells are without importance. Tumor cells face not only each other but also surrounding stromal tissue and the systemic antitumor response including the immune surveillance."[14] This proposition supports that even peripheral blood, which is a non-neoplastic tissue can be used for cytogenetics. Supporting this, chromosomal aberrations were also noted in peripheral lymphocytes of patients with breast cancer.[15] Circulating tumor cells are present in the peripheral blood of various carcinomas but are not present in patients with benign tumors.[16] This is another concept which supports the usage of peripheral blood. The usage of peripheral blood make the cancer diagnosis much easier and it will be very useful sample after surgery, chemotherapy and radiotherapy there by avoiding unnecessary surgery. The paramount importance in clinical oncology is to detect residual disease in solid malignancies.[17] FISH offers a good choice of investigating tumor cells in body fluids through non-invasive technique.

In the present study 11q13 region alteration was noted in 6 cases. The region of 11q13 can be studied in our population with larger samples to elucidate early changes in chromosomes there by preventing major fatalities. Various documented studies have proved that FISH can be used as diagnostic and prognostic indicator. The major limitation of the FISH is that it can identify only specific numerical or structural abnormality at a particular locus.[3] In spite of this limitation FISH can be used as a diagnostic aid to detect chromosomal alteration in OSCC and in addition a new molecular cytogenetic technology, generically termed multi-flurochrome FISH {M-FISH} is available which provides the means to directly examine the entire genome in one FISH experiment, thus allowing the elucidation of chromosomal rearrangements including complex structural alterations.[18]

From our experiment, we propose that by good standardization technique FISH can be used as diagnostic and prognostic tool in oral cancer treatment. Its application particularly in predicting the treatment response to therapy which involves less invasiveness by using peripheral blood as sample will be very useful in oral cancer disease management.

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