Frequency of Satellite Associations of Acrocentric Chromosomes in Oral Squamous Cell Carcinoma Patients after 5-FU and Cisplatin Treatments

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Abstract Oral squamous cell carcinoma is one of the most prevalent diseases worldwide. Acrocentric (D and G groups) satellite associations are known to play important role in the pathogenesis of diseases including cancers. The present work was aimed to study the frequency of satellite association (SA) in human peripheral blood lymphocytes of freshly diagnosed oral squamous cell carcinoma patients and comparison will be made with in vitro combined treatments of 5-Fluorouracil (5-FU) and Cisplatin to observe the changes in frequency of SA. Results revealed a significant increase in SA after combined treatments of chemotherapeutic agents on the frequency and pattern of satellite associations.

Keywords SA- Satellite association; SCC- Squamous cell carcinoma; 5-FU- 5 Fluorouracil; AC-Acrocentric chromosome

Introduction
Squamous cell carcinoma of the head and neck (SCCHN) and its subset, oral squamous cell carcinoma (Oral SCC), arise through an accumulation of genetic alterations. The nucleolar organizer regions (NORs) of human chromosomes, located on the short-arm of chromosome numbers13-15 and 21-22, which are the sites of the 18s and 28s ribosomal RNA (rRNA) genes. The phenomena of satellite association (SA) involving a specific position of the acrocentric chromosomes with their acrocentric directed towards each other was first observed in mitotic and meiotic human chromosomes (Ferguson et al., 1961). The formation of SA's has often been attributed to the involvement of acrocentric chromosomes in nucleolus formation. The sticky nucleolar material would have a tendency to hold the associated chromosomes together through mitosis. The fusion of two or more nucleoli mechanically stretches the nucleolar forming chromosome segment with risk of breakage. If breaks occur in more than one of the chromosomes involved, the closeness of the broken ends would predispose to translocations and the satellite associations would thus be active between acrocentric chromosomes. A high frequency of SA has often been considered as predisposing to an increased tendency of nondisjunction for instance in Down’s syndrome. On the other hand, a significant decrease in frequency of SA was observed in patients of Alzheimer disease in comparison to age-matched controls (Migliore et al., 1999).

The aim of present investigation was to analysed frequency and patterns of SA in lymphocyte cultures of freshly diagnosed oral squamous cell carcinoma patients who has not undergone the chemotherapeutic treatments in comparison to patients with combined in vitro treatments of 5-FU and Cisplatin. 5-FU is considered to be purely an S phase dependent agent and known to cause DNA damage especially of double strands (and single-strand) breaks due to the unavailability of FdUTP.

On the other hand, Cisplatin is a potent anti-tumour agent being cytotoxic to tumour cells via DNA–protein and DNA–DNA interstrand and intrastrand crosslinks. The drug may also induce apoptosis (programmed cell death). Both drugs in combination are given to oral SCC patient as part of the treatment regime. Therefore, it is interesting to know the actions of these drugs to patient survival rate in oral squamous cell carcinoma. It is also essential to find out whether or not SA be used as a prognostic biomarker.
Materials and Methods

Lymphocyte Culture:
Lymphocyte cultures were set up by routine method of Hungerford (Hungerford, 1965) with slight modifications (Gadhia et al., 2004). Heparinized whole blood (0.5 ml) was added to a mixture containing 5 ml of culture medium RPMI 1640 and 0.1 ml phytohemagglutinin (Lectin). Then the culture vials were kept in HERA cell 150 CO2 incubator for 69 hours. at 37°C with 5% CO2. At 69 hours. Colcemid was added and kept for 2 hours. The cells were collected by centrifugation, resuspended in a pre-warmed hypotonic solution (KCL, 0.075 M) for 20-25 minutes and fixed in chilled methanol/ acetic acid (3:1 v/v) solution. The drops of cell suspension were allowed to fall from at least 2.5 feet height on pre chilled chemically cleaned slides. These slides were air dried on a hot plate at 50-60 C. All slides were blind coded and labelled soon after assuring about well spread chromosome.

Nucleolar Organizing Regions staining by AgNO3:
Nucleolar Organizing Regions (NOR) staining was performed according to the silver nitrate (AgNO3) method of Verma and Babu (Verma and Babu, 1995). AgNO3 changes was prepared by mixing 4 g AgNO3 in 8 ml distilled water and stored light protected at 4 C. Few drops of silver nitrate solution were applied on slide along with 2 % gelatine solution mixed with formic acid. Heat was applied till brownish colour appeared. Prepared slides were blind coded and scored for observations of NORs.

Criteria for consideration of satellite associations (SA):
- All chromosomes associated were confirmed to be acrocentric.
- All associated chromosomes were inter-oriented (close proximity).
- Distance between their centromeres was not more than the thickness of chromatids of the same.

Experimental protocol:
Total of 30 peripheral blood Lymphocyte cultures (PBLC) of oral squamous cell carcinoma patients (Blood was collected from Lions Cancer Detection Centre, Surat) were studied along with 30 age and sex matched controls. Written informed consent of patients was obtained. All controls and patients were divided in 4 groups.

Group A1 and Group A2:
Total 15 PBL cultures of healthy individuals (Group A1) and 15 that of freshly diagnosed oral SCC patients (Group A2) were cultured without 5-FU and cisplatin.

Group B1 and Group B2: Total 15 PBL cultures of control (Group B1) and 15 of oral SCC patients (Group B2) were exposed to combination of 5-FU and cisplatin drugs at the concentration of 30 ng/30µl and 15ng/15µl respectively after 24 hours of initiation of cultures.

Slides were prepared from all the cultures, blind coded and scored. Results were analyzed statistically using student ‘t’ test with aid of SPSS (version 10) software.

Result
Distribution of satellite association (SA) in various study groups is shown in Table-1 and Figs 1 and 2. Group A1 and A2 represent control and patients without chemotherapeutic treatments. The percentage value of SA was found to be 7.47% for control and 17.6% for oral SCC without treatment. It is interesting to note that the pattern of association between 2 chromosomes was significantly (P<0.005) higher in D and G groups than association involvement between 3 and more than 3 chromosomes (Figure.1).

On the other hand, effects of combination of drugs namely 5-FU and cisplatin showed increased in SA as 29.6% in oral SCC and 15.0% in control group which was statistical significant at P<0.005 level (Table-1). Further combined treatments also showed higher percentage of association between 2 groups of chromosomes (P<0.005) as compared to 3 and more than 3 groups of chromosomes (Figure. 2). However, Figure 3 represents SA of acrocentric chromosomes between D/D and D/G groups of chromosomes (marked).
Table 1: Satellite Associations of D & G groups chromosomes in various study groups.

<table>
<thead>
<tr>
<th>Groups (No. of individuals)</th>
<th>Total No. of Metaphases analyzed</th>
<th>Total No. of S.A Found</th>
<th>Association between 2 chromosomes</th>
<th>Association between 3 chromosomes</th>
<th>Association of more than 3 chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Without chemotherapeutic treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A1 Control (15)</td>
<td>1484</td>
<td>111</td>
<td>7.47</td>
<td>93</td>
<td>6.26</td>
</tr>
<tr>
<td>Group A2 (Oral SCC) (15)</td>
<td>1479</td>
<td>261</td>
<td>17.6*</td>
<td>236</td>
<td>15.95*</td>
</tr>
<tr>
<td>After 5-FU and cisplatin combined treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B1 Control (15)</td>
<td>1206</td>
<td>181</td>
<td>15.00</td>
<td>161</td>
<td>13.34</td>
</tr>
<tr>
<td>Group B2 (Oral SSC) (15)</td>
<td>1189</td>
<td>352</td>
<td>29.60*</td>
<td>301</td>
<td>25.31*</td>
</tr>
</tbody>
</table>

*Significantly different at (P<0.005) from control

Discussion

There are five pairs of acrocentric chromosomes in human karyotype. The nuclear organizer region (NOR) represents site for ribosomal RNA genes located at secondary constriction of acrocentric chromosomes. The short arm of these chromosomes is often located in mutual aggregation called a satellite association (SA). There have been few available reports on frequency of satellite associations in healthy individuals (Kumagai, 1982); (Melaragno et al, 1990). In addition, the frequency of satellite associations was studied in 40 -60 years men (Vormittag, 1980); (Lezhava, 1984). There is a paucity of information on the frequency of satellite association with special reference to oral cancers (Guleria et al., 2005). In present study, we have selected Oral SCC because the prevalence of these cancers is higher in region of south Gujarat (Gadhia et al., 1995).

Since few years there was introduction of genetic tests for detecting activities of mutagenic and/or carcinogenic of chemicals which are harmful. Among them, satellite associations were most appropriate test to assess toxic effects of carcinogens like tobacco. The sticky NOR has tendency to hold the associated chromosomes together in mitosis. It is reported that higher frequency of SA was observed in mothers using oral contraceptives than in control mothers. It is also reported that mothers of Down’s syndrome have higher frequency of SA in comparison to control mothers suggesting that drugs intake and chromosomal anomalies predispose to SA (Hansson and Mikkelsen, 1974); (Anuradha et al., 2002).
The analysis of SAs has known popularity as biomarker in humans for drugs toxicity. An increase in the frequency of SA has been reported with cigarette and bidi smokers and increased cytogenetic damage has been observed in peripheral blood lymphocytes and exfoliated buccal mucosa cells to pan masala (Farred et al., 2011). On the contrary, Zivkovic et al. (2010) have reported a significant decrease in frequency of SA between Alzheimer disease (AD) patients and controls which could be related to etiology or pathology of genome instability in AD.

**After 5-FU and cisplatin combined treatment**

<table>
<thead>
<tr>
<th></th>
<th>Group B1 (control)</th>
<th>Group B2 (Oral SCC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.6*</td>
<td>15</td>
<td>25.31*</td>
</tr>
<tr>
<td>Association between 2 chromosomes</td>
<td>13.34</td>
<td>16.9</td>
</tr>
<tr>
<td>Association between 3 chromosomes</td>
<td>4.04</td>
<td>6.6</td>
</tr>
<tr>
<td>Association of more than 3 chromosomes</td>
<td>0.25</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Figure 2 Percentage of satellite association in Group B1 and Group B2 (*significant at P <0.05)

Figure 3 Showing a G-banded metaphase with D/D and D/G satellite associations (circle)

In the present study, we found higher frequency of SA with combined in vitro treatments of 5-FU and cisplatin in comparison to non-treated oral squamous cell carcinoma. This could be due to the actions of anti-cancer drugs on the selective D & G groups of chromosomes and/or there is a selective inhibition of r-RNA synthesis especially on
acrocentric chromosomes which might lead to high frequency of SA after treatments. However, more such studies are required before we arrive to a meaningful conclusion.

Conflict of interest
There is no conflict of interest.

Author Contributions
The work was carried out in collaboration between two authors. BD designed the study, wrote the protocol and conducted the experiments. PG has carried out literature survey, wrote first draft and done data analyses. Both authors read and approved final manuscript.

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