Genetic Heterogeneity in Adjacent Normal Mucosa of Oral Squamous Cell Carcinoma is a Marker of Poor Prognosis

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Oral squamous cell carcinoma (OSCC) is the most frequent oral cancer, however, despite much effort, mortality therefrom has not fallen substantially. Recent studies have focused on tumor heterogeneity as a possible cause of therapeutic resistance.
Intratumoral Heterogeneity in Recurrent Metastatic Squamous Cell Carcinoma of the Oral Cavity: New Perspectives Afforded by Multiregion DNA Sequencing and mtDNA Analysis

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Purpose: Improvements in sequencing technologies have shown that genetic differences among neoplastic cells can reflect clonal expansion. Intratumor heterogeneity (ITH) has been suggested to explain differences in prognosis and treatment response, indicating that personalized medicine is the goal of the future. This study evaluated ITH in 5 patients with recurrent metastatic oral squamous cell carcinoma (OSCC) and tracked the evolution from non-neoplastic tissue to neoplastic events developing after primary tumor formation.

Patients and Methods: Representative regions were macrodissected from specimens obtained from patients with OSCC of the tongue (n = 4) and floor of the mouth (n = 1). ITH and tumor evolution were explored by analyzing DNA mutations disclosed by next-generation sequencing of specific driver genes combined with changes in the mtDNA D-loop hypervariable region. Phylogenetic trees were generated employing MAFFT tool with UPGMA/Jukes-Cantor serving as the substitute model.

Results: High levels of heterogeneity were observed within and among tumors. ITH emerged as metastatic and recurrent events progressed, but the evolutionary patterns differed. In some patients, specific subclones persisted during tumor relapse. Neighboring tissue also was heterogeneous at the premalignant level.

Conclusions: A multiregion approach yielded more representative data than did single samples when tumors were subjected to molecular investigation. Persistent mutations that might be targeted by individ-
mtDNA phylogenetic tree
Genetic heterogeneity displayed by tumour cells (intratumoural heterogeneity, ITH) represents a diagnostic challenge when assessing tumour mutational profile.

A multiregion approach yielded more representative data than did single samples (small biopsy) when tumors were subjected to molecular investigation.

mtDNA is a useful adjunct tool when studying the phylogenetic evolution of subclones.
In OSCC, ITH may be found both in tumour cells and in adjacent mucosa. Genetic heterogeneity of the adjacent mucosa can be interpreted as evidence of the field cancerization (field heterogeneity, FH).

The aim of this study was to investigate the impact of intratumoural and intra-field heterogeneity on loco regional control.
Patients enrolled in this study:

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Site</th>
<th>TNM</th>
<th>Histology</th>
<th>Recurrent Events</th>
<th>Follow Up (Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>32</td>
<td>Cheek</td>
<td>T4N0M0</td>
<td>Epidermoidal Well Differentiated</td>
<td>2(24 M-12y)</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>57</td>
<td>Gum</td>
<td>T4N0M0</td>
<td>Squamous Moderately Differentiated</td>
<td>2(1 M-18m)</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>57</td>
<td>Tongue</td>
<td>T3N0M0</td>
<td>Squamous Moderately Differentiated</td>
<td>1(12m)</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>67</td>
<td>Fom</td>
<td>T2N0M0</td>
<td>Squamous Poorly Differentiated</td>
<td>5(10m-5m-20m-10m-10m)</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>52</td>
<td>Tongue</td>
<td>T4N0M0</td>
<td>Squamous Well Differentiated</td>
<td>2(6m-110m)</td>
<td>130</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>59</td>
<td>Gum</td>
<td>T3N0M0</td>
<td>Squamous Moderately Differentiated</td>
<td>No</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>46</td>
<td>Tongue</td>
<td>T3N0M0</td>
<td>Squamous Moderately Differentiated</td>
<td>No</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>55</td>
<td>Gum</td>
<td>T4N0M0</td>
<td>Squamous Well Differentiated</td>
<td>No</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>43</td>
<td>Tongue</td>
<td>T4N0M0</td>
<td>Squamous Well Differentiated</td>
<td>No</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>66</td>
<td>Tongue</td>
<td>T3N0M0</td>
<td>Squamous Well Differentiated</td>
<td>No</td>
<td>96</td>
</tr>
</tbody>
</table>
Group 1 (recurrent OSCC):
2 patients were males and 3 patients were females (mean age 53±12.9 years)
Tumours were located in the cheek, maxillary gingiva, tongue (2 cases) and floor of the mouth; they were G1 (2), G2 (2), G3 (1).
The non-neoplastic oral mucosa adjacent to OSCC presented mild to moderate oral epithelial dysplasia in 4 cases and severe dysplasia in the remaining case.
One patient had one recurrent event, three patients had two recurrent events and one patient had 5 events. Mean follow up duration in these 5 patients was 88±49.82 months.
**Group 2 (non recurrent OSCC):**

4 patients were males and 1 patient was female (mean age 53±9.2 years)

Tumours were located in the tongue (3 cases) and mandibular gingiva (2 cases).

Tumours were G1 (3) and G2 (2). Mean follow up duration in these 5 patients was 59.2±24.39 months.

The non-neoplastic oral mucosa adjacent to OSCC presented mild to moderate oral epithelial dysplasia in 1 case.
Intratumoural heterogeneity (ITH) and Field Heterogeneity (FH) were calculated as the ratio between unshared somatic mutations over the total amount of mutations deriving sequencing of two distant areas of the tumour and adjacent mucosa respectively.

\[
ITH = \frac{\text{unshared Mutations in OSCC}}{\text{total Mutations in OSCC}}
\]

\[
FH = \frac{\text{unshared Mutations in non neopl area}}{\text{total Mutations in non neopl area}}
\]

Cao et al. Oncogenesis 2015
The figure illustrates rates of genetic heterogeneity in primary tumours (ITH) and related adjacent mucosa (Field Heterogeneity, FH) in the groups of recurrent OSCC (blue boxes) and not recurrent OSCC (red boxes). *P* values refer to Mann-Whitney U test for independent samples results.
The figure illustrates the Kaplan-Meier curves for loco-regional control cumulative survival rates according to FH.

The green curve represents patients with values of Field Heterogeneity < 50% whereas the red curve describes patients with Field Heterogeneity > 50%.

\(P\) values refer to Log-Rank test among the two groups. Values of FH > 50% exhibit worse loco regional control.
An index of the genetic heterogeneity within different tumour specimens was determined also by evaluating the VAF (Variant Allele Frequency), assuming that a clonal tumour population exhibits a VAF of more than 30% (Kitukake et al. Oncotarget 2018).

High genetic heterogeneity = high mutations with VAF<30%

\[ \text{VAF Heterogeneity} = \frac{\text{mutations with VAF} < 30\%}{\text{All mutations}} \]
VAF Heterogeneity comparing Recurrent and non Recurrent Group by Mann-Whitney U test:

Non-neoplastic samples of Group 1 vs Group 2 p-value = 0.0006

Tumour samples of Group 1 vs Group 2 p-value = 0.0048

All together of Group 1 vs Group 2 p-value = 0.00001
In summary, the prognostic implication of studying Field Heterogeneity (FH) is here documented for the first time.

Apparently, the biology of adjacent mucosa seems to influence more deeply local outcome of OSCC than the clonal architecture of the bulk of the tumour.
Prognostic impact of intra-field heterogeneity in oral squamous cell carcinoma

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Received: 24 May 2019 / Revised: 29 July 2019 / Accepted: 22 August 2019
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Abstract
Genetic heterogeneity displayed by tumour cells (intratumoural heterogeneity, ITH) represents a diagnostic challenge when assessing tumour mutational profile. In oral squamous cell carcinoma (OSCC), ITH may be found both in tumour cells and in adjacent mucosa. Genetic heterogeneity of the adjacent mucosa can be interpreted as evidence of the field cancerization (field heterogeneity, FH). The aim of the study was to investigate the impact of intratumoural and intrafield heterogeneity on locoregional control. Ten OSCC patients (5 recurrent and 5 nonrecurrent) were studied. Multiple areas were sampled from the bulk of the tumour and the adjacent nonneoplastic mucosa. A panel of 10 tumour-specific OSCC driver genes was analysed for each sample and was used to calculate heterogeneity. Values were compared among recurrent and nonrecurrent OSCC. Mutational analysis highlighted that a single tumour sample has limited accuracy in assessing the genetic profiles of tumours. High values of ITH considering shared mutations between specimens were found in both recurrent and non-recurrent OSCC ($p = 0.095$). On the contrary, the intrafield genetic heterogeneity was significantly less frequently in the non-recurrent OSCC group ($p = 0.032$). Heterogeneity within each specimen calculated with variant allele frequency confirmed that there was better discrimination between recurrent and nonrecurrent groups using nonneoplastic adjacent mucosa than tumour tissue ($p$ value 0.0006 and 0.0048 respectively). In agreement with the theory of field cancerization, intrafield genetic heterogeneity correlates with a higher risk of developing loco-regional recurrences and second primaries. In order to reduce the ITH effects, analysis of multiple tumour areas should be encouraged.

Keywords Oral squamous cell carcinoma · Intratumoural heterogeneity · Field cancerization · Prognosis