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1. Introduction

Epidemiologic and experimental evidence indicate that oral cancer originates and progresses with the contribution of carcinogen exposure, mainly from tobacco smoking (IARC, 1986, 2004). This is thought to contribute to DNA damage within the mucosa and, in particular, to subsequent gene mutations, chromosomal instability and aneuploidy, resulting in an increased risk of developing oral cancer. Accumulation of genetic/genomic aberrations over time leads to a multi-step process of carcinogenesis in which the functions of genes which control the cell cycle (proliferation and apoptosis), chromosome stability, angiogenesis, invasion and metastasis, become aberrant (Califano et al., 1996; Hanahan & Weinberg, 2011; Martorell-Calatayud et al., 2009). Chromosomal aberrations in oral cancer are located, in particular, at 9p21, 17p13, 3q26, 11q13, 3p21, 14q32 (Forastiere et al., 2001; Gollin, 2001) corresponding to several putative tumor suppressor genes and oncogenes including p16 at 9p21 and TP53 at 17p13.

Studies aimed at elucidating the steps of transition between the oral precursor lesions and oral cancer and, in particular, the transition from visually normal appearing non-dysplastic oral mucosa to precursor lesions are potentially very informative. Such investigations address the theory of “field cancerization” (Slaughter et al., 1953) and its more recent genetic explanation (Braakhuis et al., 2003; Tabor et al., 2001). These studies have led to a genetic progression model of oral cancer (Braakhuis et al., 2004). A critical step in this model is the conversion of a patch, in which stem cells share genetic/genomic aberrations, into an expanding field in which many more aberrations occur and which sometimes becomes visible as leukoplakias and erythroplakias (Braakhuis et al., 2002; Reid et al., 1997; Van Houten et al., 2001). The role of chromosomal instability during the genesis and progression of oral cancer has clearly been indicated by several studies but still our understanding of the molecular mechanisms is relatively poor. Analyses were performed with the use of different techniques including loss of heterozygosity (Braakhuis et al., 2004; Bremmer et al., 2008; Graveland et al., 2011; Lydiatt et al., 1998; Mithani et al., 2007; Partridge et al., 1997; Tsantoulis et al., 2007), comparative genomic hybridization and gene expression array (Bremmer et al., 2008; Cha et al., 2011; Garnis et al., 2009; Liu et al., 2006, 2011; Smeets et al., 2009; Snijders et al., 2005; Squire et al., 2002), in situ hybridization (Nees et al., 1993; Voravud et al., 1993), immunohistochemistry (Nees et al., 1993), multiplex ligation-
dependent probe amplification (Bremmer et al., 2008; Cha et al., 2011; Liu et al., 2006), DNA image cytometry (Bremmer et al., 2008; Diwakar et al., 2005) and DNA flow cytometry (DNA FCM) (Donadini et al., 2010; Hemmer, 1990, 1997; Pentenero et al., 2009; Saito, 1998, 1991, 1995; Seoane et al., 1998). DNA FCM was often adopted as a useful technique for detecting the presence of DNA aneuploid sublines in several human predisposing and preneoplastic lesions such as Barrett’s esophagus (Reid et al., 2000), ulcerative colitis (Rabinovitch et al., 1999; Risques et al., 2008), colorectal adenomas (Giaretti et al., 1994) and oral lesions (Donadini et al., 2010; Pentenero et al., 2009; Saito, 1998, 1991, 1995; Seoane et al., 1998).

2. High resolution DNA FCM of oral lesions and visually normal non-dysplastic mucosa

The DNA FCM data provided so far for the human oral precancerous lesions were mainly derived from paraffin-embedded material of dysplastic oral potentially malignant lesions (OPMLs). In order to better investigate early oral fields of carcinogenesis and to separate them from later progression steps, we have included the analysis of non-dysplastic “oral clinically normal appearing mucosa sited in OPML and OSCC distant fields within the same subsites” (ODFs; \( n = 122 \)). In addition, we have analyzed multiple samples from OPMLs without and with dysplasia at histology, including also the lesion margins. OPMLs in our series of cases were clinically identified mainly as white lesions of the oral mucosa or leukoplakias \(( n = 235 \)). Further, we analyzed, though in a relatively small number of cases, oral verrucous carcinomas (OVCs; \( n = 9 \)) and oral squamous cell carcinomas (OSCCs; \( n = 32 \)). In all cases the multiple samples were only from fresh/frozen material. Patients were recruited in three different medical centers: the Oral Medicine and Oral Oncology Section of the University of Turin, the Department of Otolaryngology, “S. Martino Hospital” in Genoa and the National Institute for Cancer Research in Genoa. Patient written consent was obtained in every case according to the Institutional Ethic Committees. Diagnosis in every case was obtained from the Pathology Departments of the same Institutions. In particular, the diagnosis of OPMLs, using both incisional biopsies and/or microbiopsies as previously detailed (Navone et al., 2008) was based on internationally accepted criteria with levels of diagnostic certainty C3-C4 (Van der Waal et al., 2009). The assessment of the degree of dysplasia was carried out by a specially trained pathologist according to the WHO guidelines (IARC, 2005). Tissue fragments were minced on Petri dishes using scalpels and collected in 2 ml detergent solution \((0.1 \text{ M citric acid, } 0.5\% \text{ Tween-20})\) (Otto, 1994) and then submitted to mechanical disaggregation in a disposable 50 \(\mu\text{m}\) Medicon using a Medimachine (DAKO, Copenhagen, Denmark). Nuclei suspensions were obtained and filtered over a 50 \(\mu\text{m}\) nylon sieve (CellTrics, Partec GmbH, Muenster, Germany). An absolute count of the nuclei in suspension was performed by FCM (CyFlow® ML, Partec GmbH (Shapiro, 2003)) after 1 to 10 dilution in water. The final volume was calculated to obtain the concentration of 600,000 nuclei/ml. One volume (1/7 of the final volume) of detergent solution was first added followed by 10 min incubation and gentle shaking. Finally, 6 volumes (6/7 of the final volume) of staining solution \((0.4 \text{ M Na2HPO4, } 5 \mu\text{M DAPI in water})\) were added. Samples were kept on dark for a minimum of 15 min incubation before filtering and FCM analysis. Excitation of DAPI was provided with an UV mercury lamp (HBO-100 W, Partec GmbH) and the emitted blue fluorescence was collected.
using a 435 nm long-pass filter. Measurements of DNA content histograms were performed with a high resolution DNA FCM (CyFlow® ML, Partec GmbH (Shapiro, 2003)) according to quality controls and analysis consensus criteria (Ormerod et al., 1998). Only samples with at least 2 separate G0–G1 peaks were considered DNA aneuploid. Sex specific human lymphocytes and “true normal mucosa” from healthy donors were used as DNA diploid controls. DNA Index (DI) values were evaluated as the ratio of the mean channel number of the DNA aneuploid G0–G1 peak to the mean channel number of the DNA diploid G0–G1 peak. Thus, DNA diploid and aneuploid sublines have values respectively DI =1 and DI ≠ 1. The CV values of the G0–G1 peaks for the DNA diploid normal mucosa samples from healthy donors were used as a measure of accuracy (DNA resolution): a mean CV= 1.88±0.26% was obtained by a Gaussian curve fitting method (FloMax Software 3.0b4 2001, Partec GmbH). The mean CV value using human lymphocytes from sex specific healthy donors was 1.2 ± 0.2%. Data collection, management and analyses were done using Microsoft Office Excel and the SPSS 16.0 software package (Apache Software Foundation, Chicago, IL, USA). The association between two variables in 2 x 2 contingency tables was evaluated with the Fisher exact test. A p-value < 0.05 was taken as statistically significant.

Table 1a shows the prevalence of DNA aneuploidy for 7 subgroups of oral mucosa/lesions: “true normal mucosa” from healthy donors (n = 36), non-dysplastic ODFs corresponding to OPMLs (n = 105), and OPMLs without (n = 208) and with dysplasia (n=27) were DNA aneuploid respectively in none of the cases, in 12/105 (11.4%), 37/208 (17.8%) and in 10/27 (37%). The samples relative to advanced cancer, respectively OVCs and OSCCs, were DNA aneuploid in 6/9 (66.7%) and 25/32 (78.1%) cases. ODFs corresponding to OSCCs were DNA aneuploid in 3/17 (17.6%) cases. Two or more DNA aneuploid sublines were detected in none of the ODFs, in 5/37 (13.5%) of the OPMLs without dysplasia, in 2/10 (20%) of the OPMLs with dysplasia. OVCs and OSCCs presented respectively multiple DNA aneuploid sublines in 2/6 (33.3%) and in 13/25 (52%) cases.

<table>
<thead>
<tr>
<th>Oral mucosa/lesion groups</th>
<th>N. cases within groups</th>
<th>N. DNA aneuploid cases</th>
<th>One DNA aneuploid subline</th>
<th>Two or more DNA aneuploid sublines</th>
</tr>
</thead>
<tbody>
<tr>
<td>“True normal mucosa” from healthy donors</td>
<td>36</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ODFs corresponding to OPMLs</td>
<td>105</td>
<td>12 (11.4%)</td>
<td>12 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>OPMLs without dysplasia</td>
<td>208</td>
<td>37 (17.8%)</td>
<td>32 (86.5%)</td>
<td>5 (13.5%)</td>
</tr>
<tr>
<td>OPMLs with dysplasia</td>
<td>27</td>
<td>10 (37.0%)</td>
<td>8 (80.0%)</td>
<td>5 (20.0%)</td>
</tr>
<tr>
<td>OVCs</td>
<td>9</td>
<td>6 (66.7%)</td>
<td>4 (66.7%)</td>
<td>2 (33.3%)</td>
</tr>
<tr>
<td>ODFs corresponding to OSCCs</td>
<td>17</td>
<td>3 (17.6%)</td>
<td>3 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>OSCCs</td>
<td>32</td>
<td>25 (78.1%)</td>
<td>12 (48.0%)</td>
<td>13 (52.0%)</td>
</tr>
</tbody>
</table>

Abbreviations: ODFs = non-dysplastic oral clinically normal appearing mucosa sited in OPML and OSCC distant fields within the same anatomical subsites; OPMLs = Oral Potentially Malignant Lesions (mainly leukoplakias); OVCs = Oral Verrucous Carcinomas; OSCCs = Oral Squamous Cell Carcinomas.

Table 1a. DNA aneuploidy by high resolution DNA FCM among 7 different groups of oral lesions and non-dysplastic normal appearing mucosa. The last 2 columns report the cases with single and multiple DNA aneuploid sublines.
All the DI aneuploid sublines ($n = 126$) were subdivided in 2 classes: DNA near-diploid (DI ≠ 1 and <1.4) and DNA high aneuploid (DI ≥1.4) (Table 1b). ODFs and OPMLs without dysplasia were characterized by near-diploid sublines respectively in 12/12 (100%) and in 38/43 (88.4%) of the cases. OVCs had a significantly higher frequency of DNA near-diploid aneuploid cases than OSCCs (respectively, 87.5% and 33.3%; $p=0.006$). In contrast, OPMLs with dysplasia and OSCCs had high aneuploid sublines respectively in 5 out of 12 (41.7%) and in 32 out of 48 (66.7%) of the cases. The prevalence of high aneuploidy in OPMLs with dysplasia was statistically significantly higher than in OPMLs without dysplasia ($p = 0.03$).

<table>
<thead>
<tr>
<th>Oral mucosa/lesion groups</th>
<th>N. DNA aneuploid sublines</th>
<th>N. DNA near-diploid aneuploid sublines (DI≠1 and DI&lt;1.4)</th>
<th>N. DNA high aneuploid sublines (DI≥1.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;True normal mucosa&quot;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ODFs corresponding to OPMLs</td>
<td>12</td>
<td>12 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>OPMLs without dysplasia</td>
<td>43</td>
<td>38 (88.4%)</td>
<td>5 (11.6%)</td>
</tr>
<tr>
<td>OPMLs with dysplasia</td>
<td>12</td>
<td>7 (58.3%)</td>
<td>5 (41.7%)</td>
</tr>
<tr>
<td>OVCs</td>
<td>8</td>
<td>7 (87.5%)</td>
<td>1 (12.5%)</td>
</tr>
<tr>
<td>ODFs corresponding to OSCCs</td>
<td>3</td>
<td>2 (66.7%)</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td>OSCCs</td>
<td>48</td>
<td>16 (33.3%)</td>
<td>32 (66.7%)</td>
</tr>
</tbody>
</table>

Abbreviations: ODFs = non-dysplastic oral clinically normal appearing mucosa sited in OPML and OSCC distant fields within the same anatomical subsites; OPMLs = Oral Potentially Malignant Lesions (mainly leukoplakias); OVCs = Oral Verrucous Carcinomas; OSCCs = Oral Squamous Cell Carcinomas.

Table 1b. Presence of DNA near-diploid (DI≠1 and DI<1.4) and high aneuploid (DI≥1.4) sublines among 7 different groups of oral mucosa/lesions. DNA aneuploidy was measured by high resolution DNA FCM.

3. Discussion

The incidence of DNA aneuploidy by FCM reported in the literature ranges from about 10% to 40% for dysplastic OPMLs (Donadini et al. 2010; Pentenero et al. 2009; Saito et al., 1998, 1991, 1995; Seoane et al., 1998) and up to about 80% for OSCCs (Donadini et al., 2010; Pentenero et al. 2009; Hemmer, 1990, 1997). These values may strongly depend on the tissue material type (paraffin embedded or fresh-frozen) and DNA FCM resolution. In the present study, partly based on a previous data set that was already published (Donadini et al., 2010), we have performed FCM measurements at optimized conditions (fresh-frozen material, concentration of 600,000 nuclei/ml, DAPI staining in nuclei suspensions, UV incident light, the use of a dedicated instrument). Correspondingly, the CV values of the G0–G1 peaks of human normal control DNA diploid nuclei were commonly near 1%, while a minimum DNA change of 2.4% was detected (Figure 1). It is likely that DNA FCM at lower resolution and higher CV values would not allow separating DNA near-diploid aneuploid sublines with only slight DNA changes above/below DNA diploidy.

The present data set confirmed in a larger number of cases that, while “true normal oral mucosa” and human lymphocytes of healthy donors were DNA diploid in all cases, non-dysplastic “clinically normal appearing mucosa fields of the oral cavity” (ODFs) in patients with OPMLs already contained DNA aneuploid sublines in a subgroup of cases (12/105, 11%).
Moreover, it was found that OPMLs that could be clinically identified mainly as white lesions of the oral mucosa (leukoplakias) and classified without dysplasia at histology, contained already DNA aneuploid sublines in 37/208 (18%) of the cases. These data appear in agreement with the concept of field effect in oral carcinogenesis (Braakhuis et al., 2003; Bremmer et al., 2008; Leemans, 2011; Tabor et al., 2001; Van der Waal, 1997). The data obtained for the non-dysplastic OPMLs, in particular, were in agreement with previous literature reports including two studies from our group using an independent patient population (Donadini et al., 2010; Pentenero et al., 2009; Saito, 1995). These data were, however, in contrast with other studies, which did not detect DNA aneuploid sublines in such lesions (Kahn et al., 1992; Saito, 1998). The present study has additionally highlighted that ODFs and OPMLs without dysplasia were characterized by single near-diploid DNA aneuploid sublines. On the contrary, OPMLs with dysplasia contained high DNA aneuploid sublines (DI $\geq 1.4$) in slightly less than half of the cases (42%). High DNA aneuploid sublines were predominant (67%) for the OSCCs, which were in addition characterized by the presence of multiple DNA aneuploid sublines in 52% of the DNA aneuploid cases. In contrast, OVCs were characterized by DNA near-diploid aneuploid sublines in 67% of the cases in agreement with previously published data (Pentenero et al., 2011). Overall, the present data support a previous model of aneuploidy genesis and evolution (Giaretti, 1994). Accordingly, a transition from DNA diploidy to near-diploid aneuploidy would be an early step of the natural history of OPMLs, while high DNA aneuploidy (likely to derive from the endoreduplication of a DNA hypo-diploid or hyper-diploid near-diploid cell) would frequently occur as a later event in OPMLs with dysplasia and OSCCs (Figure 1). From the clinical point of view, one can speculate that the detection of DNA content genomic aberrations in oral fields, which appear visually and histologically normal, and in OPMLs may have profound implications for improvement of the present patient management by identifying individuals at high risk to develop cancer (Brennan et al., 2007; Dakubo et al., 2007; Lodi et al., 2006).

Moreover, it is possible, though still unproved, that the OSCC group in which near-diploid aneuploid DIs remained “frozen” during time are at better prognosis compared to OSCCs with multiple DNA aneuploid sublines with high DNA aneuploidy. Interestingly, OVCs that were mainly characterized by DI values in the near-diploid region are known to be less aggressive and at better prognosis than OSCCs.

Clearly, what is still strongly needed in the model system of oral preneoplasia and neoplasia is a better understanding of the origin and dynamic evolution of chromosomal instability, chromosomal aberrations and aneuploidy (Albertson et al., 2003; Asteriti et al., 2010; Compton et al., 2011; Geigl et al., 2008; Giet et al., 2005; Kops et al., 2005; Lingen et al., 2011; Sieber et al., 2003; Suijkerbuijk & Kops, 2008; Thompson et al., 2010; Viet & Schmidt, 2010). In other models of cancer genesis and progression, like the colorectal adenoma-carcinoma sequence, the Barrett's esophagus and the ulcerative colitis transition to carcinoma, the role of APC and TP53 has been highlighted (Fodde et al., 2001; Giaretti et al., 2004; Rabinovitch et al., 2004). A role of TP53 in oral cancer chromosomal instability (Negrini et al., 2010) is also likely to occur due to different sources of TP53 inactivation including HPV infection in different sites of the oral cavity (Leemans, 2011; Klingelhutz et al., 2005; Tsantoulis et al., 2007). Studies that linked the genome-wide integrity analysis with gene expression profiles have provided powerful indications that chromosomal instability and aneuploidy massively deregulate the cellular transcriptome (Albertson et al., 2003). Future studies coupling both
these techniques are likely to contribute to discover specific recurrent genomic aberrations, which encompass specific genes with a potential role in the genesis of chromosomal instability and aneuploidy. The functional consequences of specific DNA gains/losses are, however, not only involving oncogenes and tumor suppressor genes. More subtle and complex mechanisms are present since many aberrations span large chromosomal regions including normal genes, which coordinately and cooperatively may influence important cell functions as proliferation, differentiation, apoptosis and DNA repair.

Fig. 1. Examples of DNA content histograms from oral fresh/frozen mucosa/lesions as obtained by low background and high resolution DNA FCM. A model of DNA aneuploidization and evolution.

Single DNA aneuploid sublines in the DNA near-diploid aneuploid region (DI=1 and DI<1.4) are shown in A and B for two OPMLs. Sex specific human lymphocytes (Ly) and true normal mucosa of healthy donors were used as DNA diploid controls (DI=1.0). Multiple DNA aneuploid sublines for an OSCC with DI values respectively of 1.12 and 2.18 are shown in C. This example illustrates a model of DNA aneuploidisation as previously reported (Giaretti, 1994; Donadini et al., 2010). The key mechanism of DNA aneuploidisation appears related to a loss of symmetry of DNA content during an abnormal mitotic division in which the two daughter cells loose or gain respectively a small amount of DNA and generate DNA near-diploid aneuploid sublines (see examples A and B). A second
The step of DNA aneuploidy evolution appears to be due to the endoreduplication of the DNA near-diploid aneuploid cells sublines (see example C). The large G0-G1 subpopulation of cells with DI= 2.18 is characterized by a relatively large CV value (about 3% with respect to 2% of the G0-G1 DNA diploid peak), which is indicative of chromosomal instability and potential loss of DNA. The DI= 2.18 value of the high DNA aneuploid G0-G1 peak was likely originated from an initial DI value of 2.24 (twice as much of the original near-diploid DI=1.12 ) with the loss of 2.7% DNA.

An example of DNA histogram with a small G0-G1 DNA aneuploid peak (with 5% of the total number of nuclei in this case) and with DI= 1.24 is shown in D to illustrate that such a small percentage of DNA aneuploid nuclei can be sufficient to be detected by our high resolution and low background DNA FCM measurements. This approach was characterized by the use of fresh/frozen tissue material, nuclei suspensions at the fixed concentration of 600,000 nuclei/ml, DAPI staining and a dedicated FCM instrument (see details in the text; Donadini et al., 2010; Shapiro, 2003).

4. Acknowledgements
This study has been supported by “Compagnia di San Paolo - Programma Oncologia” and by Philip Morris International. Thanks are due, in particular, to A. Donadini, M. Maffei and P. Castagnola for critically reading the manuscript.

5. References


Oral cancer is a significant public health challenge globally. Although the oral cavity is easily accessible, early diagnosis remains slow compared to the enhanced detection of cancers of the breast, colon, prostate, and melanoma. As a result, the mortality rate from oral cancer for the past four decades has remained high at over 50% in spite of advances in treatment modalities. This contrasts with considerable decrease in mortality rates for cancers of the breast, colon, prostate, and melanoma during the same period. This book attempts to provide a reference-friendly update on the etiologic/risk factors, current clinical diagnostic tools, management philosophies, molecular biomarkers, and progression indicators of oral cancer.

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