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Anatomic Site Based Ploidy Analysis of Oral Premalignant Lesions

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Abstract The location of oral leukoplakia correlates strongly with the probability of finding dysplastic or malignant alterations at biopsy. It is well established that early detection can dramatically improve the 5-year survival rates for oral squamous cell carcinomas. Since aneuploidy is predictive of future conversion to malignancy, we hypothesized that dysplastic lesions from high-risk sites (floor of mouth, tongue and lips) would exhibit greater aneuploidy than low-risk sites (palate, gingiva and buccal mucosa). Epithelial sections from 60 archival samples diagnosed as mild dysplasia (36 females, 20 males) from various high/low risk locations were stained with Blue Feulgen Stain for DNA Ploidy Analysis (Clarient, Aliso Viejo, CA) and ploidy was analyzed using a ChromaVision ACIS II (Clarient, ALiso Viejo, CA) Image cytometry system. A DNA histogram was generated using an image analyzing software that evaluated the amount of Feulgen stain which is proportional to the amount of nuclear DNA. An ANOVA analysis followed by the Student's '*t*' test revealed significant differences between means ($P \leq 0.05$). Lesions originating from lateral/ventral tongue (85%), floor of mouth (50%) and soft palate (44%) exhibited a higher frequency of aneuploidy than lesions from gingiva (22%) and lower lip (25%). This pilot study demonstrates that dysplastic lesions from high-risk sites such as the floor of the mouth and lateral/ventral tongue have higher frequency of aneuploidy.

Keywords Ploidy · Aneuploid · Oral leukoplakia · Dysplasia

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Introduction

Oral squamous cell carcinoma (SCC) is often preceded by readily detectable mucosal changes in the oral cavity. These are most often white (leukoplakia) and/or red (erythroplakia) patches. Leukoplakia is defined by the World Health organization as a "white patch or plaque that cannot be characterized clinically or pathologically as any other disease" [1]. A recent review 'In the Recommendations from the Fourth World Workshop on Oral Medicine', stated that non-cancerous leukoplakias have a greater than normal malignant transformation potential ranging from 0.4% in non-dysplastic lesions to 9% in dysplastic lesions [2].

Depending on the study, the frequency of dysplastic or malignant alterations in leukoplakia generally varies between 15.6 and 39.2% [3–8]. The location of the lesion correlates with the probability of finding dysplastic or malignant changes at biopsy with the floor of mouth usually being the location of greatest risk (42.9%) followed by the tongue (24.2%) and lip (24%) [7, 8]. Oral cancers, however, are most frequently found on the tongue, floor of the mouth and lower lip [7, 8]. The lateral aspect of the soft palate is also at risk with the gingiva as the least common location for oral SCC [5–8].

Although common risk factors (tobacco/alcohol) for oral SCC have been identified and potentially pre-malignant lesions may be visible in the oral cavity, the clinical behavior of an individual lesion may not be easily predicted. Despite the advances in molecular characterization of oral SCC, the diagnosis and characterization of putatively pre-malignant lesions is purely histological. Also because the 5-year survival rate is related to the stage at diagnosis and only a minority of clinical leukoplakias and even histopathologically diagnosed dysplasias become malignant [2–4, 7–9] it is expected that early detection strategies could increase survival by allowing oral cancer to be discovered at a preliminary stage. As such, investigators are actively seeking tools that can differentiate lesions having a higher risk of conversion to malignancy compared to lesions having a relatively lower-risk. In this context one study concluded that the anatomic location of mild and moderate oral dysplasias may be important as lesions from high-risk sites have a higher tendency to exhibit genetic alterations associated with elevated risk of malignant progression [9].

Several laboratories have focused on using DNA content or ploidy to differentiate low-risk from high-risk lesions. A provocative series of retrospective studies seemed to firmly establish a link between aneuploidy and future development of oral cancer, but this work has been discredited thereby throwing the entire field into disarray [10]. Further investigation in this direction is warranted because those articles were subsequently promptly removed and a retraction and expression of concern provided [10]. This has naturally lead investigators to question the link between DNA ploidy and the clinical behavior of leukoplakic lesions [10]. Hence, we hypothesized that if aneuploidy was predictive of future conversion to malignancy, then we would predict that samples obtained from high-risk anatomical sites (floor of mouth, tongue, lips) would have a higher frequency of aneuploidy than samples obtained from low-risk anatomic sites (palate, gingiva). Indeed, the data presented herein show that in a small group of samples, lesions obtained from the floor of mouth and tongue had a higher frequency of aneuploidy than lesions collected from other anatomical sites within the oral cavity.

Materials and Methods

Study Population

This was a retrospective study using archival pathological specimens that were collected before 1995. Specimens of epithelium exhibiting mild epithelial dysplasia were chosen using computer queries of diagnostic codes. Sixty specimens that were obtained from 56 individuals (36 females; 20 males) were included in the study. Ten specimens from each anatomical site (gingiva, floor of mouth, soft palate, lower lip, and tongue) were initially chosen. Nine specimens were subsequently excluded from the study due to mislabeling of the specimen ($n = 1$) or insufficient material for DNA content analysis ($n = 6$). The relatively uninvolved epithelium covering benign fibromas ($n = 10$) served as a control for diploidy. The diagnosis of all specimens was confirmed to be mild epithelial dysplasia by two independent oral pathologists prior to inclusion in the study and each was blinded to the others diagnoses. The risk factors available for the study subjects were tabulated and are presented in Table 1. The WHO histological category of epithelial dysplasia was used. The University of Florida Institutional Review Board approved this study.

Feulgen Staining

The archival formalin-fixed paraffin-embedded blocks were cut into 7 μM sections that were affixed to glass slides. The sections were stained using the Blue Feulgen Stain Kit for DNA Ploidy Analysis (Clarient). Briefly, the tissue sections were hydrolyzed in 5N Hydrochloric acid (HCl) for 60 min and then rinsed in deionized water. The slides were stained for 60 min in freshly prepared stain. The slides were then rinsed thrice in deionized water and thrice more in freshly prepared rinse solution followed by a

Table 1 Table showing risk factor in the study samples

Risk factors	Control	Gingiva	Floor of mouth	Soft palate	Lower lip	Tongue
Unknown	3	4	2	1	3	4
No known habits[a]	3	3	2	2	2	1
Alcohol[b]	3	0	3	3	1	2
Smoking	3	2	3	4	2	2
Chewing tobacco	0	0	0	0	1	1
Total samples	10	9	8	9	8	7

[a] None provided to clinician

[b] Only one patient admitted to regular and heavy use, all others report casual and occasional alcohol consumption

final three rinses in deionized water. The sections were then dehydrated in absolute methanol followed by two changes of xylene. Finally, cover slips were affixed using Permount.

DNA Ploidy Analysis

DNA ploidy was analyzed using a ChromaVision ACIS II (Clarient, San Juan Capistrano, CA), Image cytometry system. In this assay, the amount of Feulgen stain is proportional to the amount of nuclear DNA. The ACIS II, which consists of a microscope equipped with digital chips, a monitoring screen, and a computer, quantitates the amount of blue stain. The image analysis software uses this information to create a DNA histogram.

The mean integrated optical density of normal cells within each specimen was assigned a DNA index (DI) = 1. This served as an internal control for cells having a diploid (2 N) DNA content with a primary G0/G1 peak and, thus, provided an internal reference for the DI of the dysplastic cells. Lesions having a DI < 1.1 were considered diploid, whereas lesions having a DI > 1.2 were defined as aneuploid.

Statistical Analysis

The data were analyzed using JMP (SAS Institute) Statistical Discovery Software Version 4. The difference between means was determined using a one-way analysis of variance (ANOVA) followed by the Student's *t* test for each pair. Differences were considered significant where $P \leq 0.05$.

Results

The resultant data show that lesions originating from the tongue (85%), floor of mouth (50%), and soft palate (44%) exhibited a higher frequency of aneuploidy than lesions obtained from the gingiva (22%) or lower lip (25%). As expected, 100% of the tested fibromas displayed diploid DNA content (Fig. 1).

When the DNA indexes for the series of cases were considered, the control epithelia had a mean DNA index of 1.07 (range 1.0–1.1). As expected, dysplastic lesions had higher DNA indices on average than benign fibromas (Table 2). However, most oral lesions are diploid by standard criteria, because most of the cells within them have no chromosomal instability. Thus, the DNA index of the G0/G1peak should remain constant. Dysplastic lesions originating from the tongue and floor of mouth had mean DNA indexes of 1.37 (range 1.1–1.5) and 1.31 (range 1.0–2.3), respectively. These means were significantly different

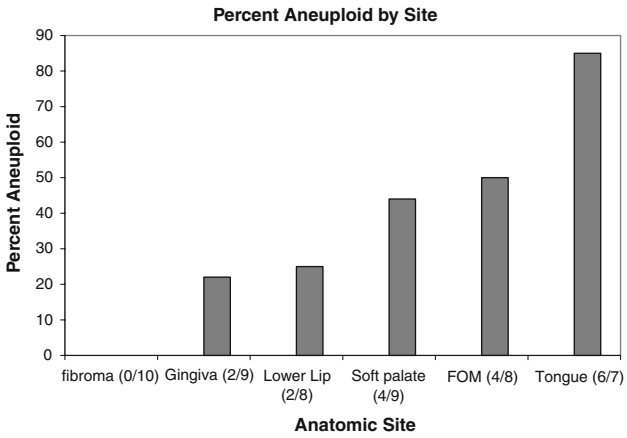

Fig. 1 Histogram showing the oral site of mild dysplasia along the *X*-axis in relation to the percentage aneuploidy along the *Y*-axis

Table 2 Mean DNA index by site

Site	Mean (range)	Standard error
Control	1.07 (1.0–1.1)	0.07
Gingiva	1.15 (1.0–1.6)	0.07
Floor of mouth	1.31 (1.0–2.3)*	0.09
Soft palate	1.20 (1.0–1.5)	0.07
Lower Lip	1.21 (1.0–1.5)	0.09
Tongue	1.37 (1.0–1.5)*	0.09

The DNA index (DI) for each specimen was determined as described in methods. The mean and standard errors were calculated for each site. An analysis of variance was performed and the differences between means were determined using the Student's *t*-test

* Significantly greater than control (fibroma) ($P < 0.05$)

($P < 0.5$) than the control epithelia. Although dysplastic lesions originating from the gingiva (1.15), soft palate (1.20), and lower lip (1.21) had higher mean DNA indices than benign fibromas, the differences were not significant from fibromas or each other (Table 2). Overall, these data show that in this small series of cases, dysplastic lesions obtained from the floor of the mouth and tongue have a higher frequency of aneuploidy and a higher mean DNA index than benign fibromas and lesions obtained from other anatomical sites located within the oral cavity.

Discussion

Despite significant advances in molecular characterization of oral SCC, the diagnosis and characterization of leukoplakia remains largely histological. Dysplastic lesions are at higher risk for malignant transformation than non-dysplastic lesions, but the behavior of an individual lesion is unpredictable. As such, efforts have focused on predicting the risk of malignant transformation of a given white/red

lesion [7, 8, 11]. Past studies have investigated convincingly, loss of heterozygosity, use of fluorescence and molecular markers such as telomerase, [12] epidermal growth factor receptor [13], survivin [14], hypermethylation of the promoters of specific genes [15], and p53 expression [16] to assess the probability of conversion of oral lesions to cancer. Over the years, analysis of DNA content (DNA ploidy) has been suggested to be an important predictor of malignant potential of leukoplakia or erythroplakia [17–22].

Normally, a non-dividing somatic cell has a diploid DNA content containing 23 pairs of chromosomes. This DNA content is doubled prior to cell division and if the cell does not divide, the amount of DNA is tetraploid. These are thought to be metastable cells intermediate between normal and cancer-associated aneuploidy [23–25]. If one or more pairs of chromosomes are not evenly distributed to the daughter cells or if pieces of chromosomes are missing, this unbalanced DNA content is aneuploidy [16]. Aneuploidy is commonly observed in human cancers and evidence suggests that mutations in genes controlling chromosome segregation during mitosis and centrosome abnormalities play a role in causing the genetic instability found in cancer cells [23, 24, 26]. Because aneuploidy is indicative of genetic instability, it is reasonable to hypothesize that aneuploidy may predict the malignant potential of leukoplakia and/or erythroplakia [19].

Ploidy can be measured by flow cytometry of propidium iodide-stained nuclei or by image cytometry of Feulgen-stained nuclei. Image cytometry is the preferred method of analyzing ploidy of solid cancers because the nuclei are mounted on a glass slide enabling them to be directly visualized [27]. This method is more sensitive than flow cytometry, which uses a laser to analyze DNA content in a population of nuclei. Flow cytometry tends to underestimate ploidy [27]. Image cytometry is suitable for analysis of small samples such as fine needle aspirates in contrast to flow cytometry, which requires large numbers of cells [27].

Indeed, some studies using image cytometry have shown that aneuploidy can be predictive of future conversion of oral lesions to malignancy [18, 21, 22, 27]. One study showed that 73% of dysplastic lesions were non diploid/aneuploid and that 86% of the subsequent invasive carcinomas were non diploid/aneuploid, whereas 81% of hyperplastic or inflammatory lesions were diploid [18]. The same group later demonstrated that oral lesions that developed into cancer had a higher rate of DNA aberration than lesions not becoming cancerous [18]. Retrospective studies conducted by other laboratories have shown that DNA ploidy can predict future conversion to malignancy up to 1 year [21] or even 15 months prior to the appearance of histological malignancy [22]. However, these studies relied on small sample numbers.

A provocative series of retrospective studies seemed to firmly establish a link between aneuploidy and future development of oral cancer, but this work has been discredited thereby throwing the entire field into disarray [10]. The articles were subsequently promptly removed and a retraction and expression of concern was provided. However, since many human cancers are aneuploid [23–26], it is reasonable to assume that aneuploidy of early non-malignant lesions precedes the development of frank cancer. However, some oral cancers are diploid, so it is reasonable to assume that diploid cancers are preceded by diploid pre-malignant lesions [20]. As such, DNA analysis of at least some pre-malignant lesions would be expected to yield false negative results.

We hypothesized that if aneuploidy was predictive of future conversion to malignancy, dysplastic oral lesions from higher-risk anatomical sites would show a higher frequency of aneuploidy than lesions from lower-risk anatomical areas. In this work, image cytometry was used to measure ploidy in mild dysplastic lesions originating from the tongue, floor of mouth, soft palate, gingiva, and lower lip. The data presented herein show that lesions originating from the tongue and floor of mouth, the two areas most likely to develop SCC had the highest frequency of aneuploidy and greater DNA indices than control specimen or lesions obtained from the soft palate, gingiva, and lower lip.

The floor of mouth, tongue, and lips are characterized as higher-risk sites with 42.9, 24.2, and 24%, respectively, of leukoplakias from these areas being dysplastic or malignant, whereas the palate and gingiva are lower-risk sites [8]. As such, with the exception of the lower lip, the data described herein show a correlation between DNA index and theoretical risk of converting to malignancy. These observations are consistent with those of Kim et al. [19], who showed that dysplastic lesions from high-risk sites exhibited greater levels of chromosome polysomy than lesions obtained from low-risk sites.

Conclusions

In summary, our data shows a convincing correlation between DNA index and the anatomic location of oral lesions. These results, however, should be interpreted in light of the small sample size and the fact that some chromosomal aberrations are below the limit of detection of image cytometry. We do not have follow-up information on this set of patients. Nonetheless, this pilot study suggests that lesions obtained from higher-risk anatomical sites (tongue, floor of mouth) have higher DNA indices than lesions obtained from low-risk anatomical sites (palate, gingiva). Although a consensus does not yet exist, the

available data suggest that dysplastic aneuploid lesions should be regarded as having a high-risk of future conversion to malignancy [17], but diploid lesions present prognostic uncertainty.

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