Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 253 (2007) 282–290
CANCER Letters
www.elsevier.com/locate/canlet

Detection of non-diploid cells in premalignant and malignant oral lesions using combined morphological and FISH analysis – a new method for early detection of suspicious oral lesions

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Received 26 September 2006; received in revised form 2 February 2007; accepted 5 February 2007

Abstract

Alteration in DNA content is an early event in oral carcinogenesis. We have examined oral brush samples to detect non-diploid cells (NDC) using simultaneous morphological and cytogenetic analysis. The study included 8 oral squamous cell carcinomas (OSCC), 22 premalignant lesions (OPLs), and 25 control individuals. Slides stained with Giemsa followed by FISH using chromosome 2 centromeric DNA probe, were scanned and fluorescent signals were simultaneously analyzed in parallel with the morphology. The proportion of NDC increased with the severity of the diagnosis. In two control subjects, 1–1.5% of the examined cells were NDC. Over 2% NDC were present in all OSCC cases and in 11 of the OPLs, of which, in 8 the histologic diagnosis was either epithelial hyperplasia or mild dysplasia. A significant number of NDC had normal morphology when cytomorphology and FISH were compared. Two patients with OPLs developed OSCC these patients had a significant proportion of NDC.

We suggest that the combined morphological and cytogenetic analysis of cells collected by a non-invasive brush sampling can enhance early detection of potentially malignant cells.

Keywords: Ploidy; Aneuploidy; Leukoplakia; Oral cancer; FISH

1. Introduction

Carcinomas of the oral cavity (oral squamous cell carcinoma – OSCC) are among the 10 most

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doi:10.1016/j.canlet.2007.02.008

common cancers in the world, with smoking as the major risk factor. Despite significant advances in surgery and radiotherapy over the past decades, the 5-year survival rate of oral cancer patients has improved only moderately. Even when the surgical margins are diagnosed as tumor-free by histopathology, the local recurrence rate is still high [1,2]. Oral cancer is thought to progress through a series of well-defined clinical and histopathological stages in the form of premalignant lesions with different dysplastic changes. Several oral lesions, such as leukoplakia, erythroplakia, and lichen planus carry an increased risk for malignant transformation. Most oral premalignant lesions, however, are benign-appearing lesions that might have been either watched or ignored. Therefore, it would be advantageous to have effective and non-invasive diagnostic prognostic methods of monitoring available in the premalignant stage.

Treatment of an oral premalignant lesion is largely based on histological grading of epithelial dysplasia; premalignant lesions are classified histologically into stages with increasing risk of developing into invasive OSCC [3]. The subjectivity in assessing oral epithelial dysplasia has often been raised. This is largely due to the lack of well-defined criteria that can be used as guidelines for grading by reporting pathologists; therefore, leading to a wide variation in the degree of interexaminer agreement and intraexaminer reproducibility in the diagnosis [4].

Several recent studies provided evidence supporting the role of genetic tests in augmenting histopathological evaluation of oral premalignancies using microsatellite analysis to determine loss of heterozygosity (LOH) in chromosome regions mainly at 3p and/or 9p regions [5–7]. Nevertheless, caution is necessary in interpreting these data and the potential clinical implication, as these studies are retrospective, with unavoidable selection bias often making a final conclusion difficult. Furthermore, sample size is moderate in light of the requirement of analyses with many different LOH profiles [8].

Alteration in the number of chromosomes, defined as aneuploidy, stands out as the most consistent marker of malignancy, and is the earliest and most distinctive pre-neoplastic genotype [9–13]. Several tumors have shown the degree of malignancy to be directly proportional to the degree of aneuploidy.

The incidence of aneuploidy in head and neck squamous cell carcinomas (HNSCC) and particular in oral squamous cell carcinomas (OSCC) is high and occurs in up to 80% of cases [14–17]. In humans as well as in animal models, aneuploidy was found to be an early event in the development of oral cancer [18–22].

Most studies analyzing DNA content of oral lesions were based mainly on image cytometry [18–21]. In these studies, DNA content was measured by the use of Nuclear Integrated Optical Density which is equivalent and not identical with the actual nuclear DNA. In addition, the histomorphological characterization of the cells analyzed for DNA content is absent.

Recently, a multiparametric cell scanning system (Duet™, BioView Ltd., Rehovot, Israel) was introduced, which enables combined analysis of morphology and cytogenetic characteristics of single cells [23]. Several studies illustrate the power of this system [23–25], which is based on two important features: (1) an automated bright-field and fluorescence scanning to allow rapid and efficient identification of small residual populations of pathological cells, and (2) combined analysis of morphology and FISH on the same cell. Thus the combined analysis enables the distinction of small populations of non-diploid cells among overlapping diploid cells, thereby enhancing the specificity of pathological cell detection [26].

The aim of the study was to present the results of the combined morphological and FISH analysis in detecting non-diploid cells in premalignant and malignant oral lesions and to examine the power of the method in predicting the malignant potential of these lesions by comparing the results with the histopathologic diagnosis.

2. Materials and methods

2.1. Study group

The study group included patients admitted to the Oral and Maxillofacial Surgery Department at the Chaim Sheba Medical Center for evaluation of suspicious oral lesions: eight cases with a biopsy proven OSCC scheduled for surgery, 21 patients with oral premalignant lesions (OPLs – 22 lesions) presented as white patch (leukoplakia), red patch (erythroplakia) and mixed red and white (erythroleukoplakia). As a control, 25 individuals with no history of tobacco use or alcohol drinking were selected.

Consent was obtained from all patients in accordance with a protocol approved by the Institutional Review Board for Clinical Studies at the Sheba Medical Center, and by the Ministry of Health for the use of genetic material.

2.2. Sample collection from the oral cavity

Oral samples were collected using a disposable brush with hard nylon fibers, brushing the surface mucosa with rotation movements and applying light pressure to allow the harvest of cells from all epithelial layers. The brush was placed in RPMI 1640 medium containing 10% FCS (Gibco, Invitrogen, United States).

Two samples were taken from each patient: from the suspicious lesion and from normal looking mucosa at the opposite site. In the healthy controls, two samples were taken: from the posterior lateral border of the tongue and from the buccal mucosa.

In each suspicious lesion, a biopsy was taken for histopathologic diagnosis. In some cases the histological diagnosis was already established by a previous incisional biopsy.

2.3. Histopathologic grading of the biopsy samples

All biopsy samples examined were formalin-fixed paraffin-embedded tissues. Five microns thick slides were cut and stained with standard hematoxylin and eosin. The histopathologic diagnosis was confirmed for each lesion by an oral pathologist (AH) using criteria established by the WHO [3]: (1) hyperplasia: increased cell number with regular stratification; (2) mild dysplasia: architectural disturbances limited to the lower third of the epithelium accompanied by cytological atypia; (3) moderate dysplasia: architectural disturbance extending into the middle third of the epithelium; (4) severe dysplasia: architectural disturbances greater than two third of the epithelium with associated cytologic atypia; (5) carcinoma in situ: full thickness architectural abnormalities accompanied by pronounced cytologic atypia.

2.4. Combined analysis of morphology and I-FISH experiments

Combined analysis of morphology and I-FISH was performed using the Duet™ system (BioView Ltd., Rehovot, Israel). The technical procedures have been described in detail by Trakhtenbrot et al. [27]. Briefly, slides were prepared from oral samples by cytospin of the final cell suspension. Slides were air-dried and stained with May Grunwald-Giemsa (MGG) (Sigma, St. Louis, MO, USA).

Slides were scanned by the bright field mode of the system, based on a dual mode, fully automated microscope (Axioplan2, Carl Zeiss, Jena, Germany), an XY motorized stage with an accuracy of 0.2 nm (Marzhauser Wetzler Germany), a 3CCD progressive scan camera (DXC9000, Sony, Japan), and computer to control and analyze the data.

MGG was removed by methanol/acetic acid 3:1 and FISH performed on the same slide by standard procedures developed in our laboratory [28]. Probe denaturation was performed according to the manufacturer's instructions. Post-hybridization washings included $0.4 \times$ SSC (72 °C, 2 min) and 2 SSC/NP40 (RT, 3 min). DAPI/Antifade (Appligene, Oncor) mixture was used as a counter stain. The system enables observation of the fluorescent signals in the specific cells using 100× objective (magnification 1000×) simultaneous to the morphology of the same cells scanned previously. 50–500 cells with bright signals were scanned manually and captured as target nuclei. Chromosome #2 centromeric DNA probe CEP2 (D2Z1) directly labeled with SpectrumOrange (Vysis, Downers Grove, IL, USA) was used in all I-FISH experiments. We choose chromosome 2 because it has already been used successfully to detect polyploid cells in oral cancer and has been found to correlate with the DNA content in head and neck squamous cell carcinoma [29]. In normal nuclei the hybridization produces two signals, while in nuclei with abnormal DNA content (tetraploid/aneuploid) more than two signals are expected. The parallel analysis of morphology and FISH enables clear distinction between epithelial and inflammatory cells; only epithelial cells were analyzed. The percentage of cells with more than two signals from the entire cells population was calculated for each case. In addition, dual color FISH was performed with two centromeric DNA probes of Vysis (chromosome #2 labeled with SpectrumGreen and chromosome #8, labeled with SpectrumOrange).

2.5. Analysis of DNA content

DNA content was measured by determining DAPI intensity, which is directly proportional to the amount of nuclear DNA, on 3-dimensional images of nuclei with FISH signals. The Volocity Grid Confocal system was used including the OptiGrid, which eliminates all signals not within the plane of focus, and is controlled by the Volocity Acquisition software (Improvision Ltd., England). Since absolute measurements could not be used, because of differences in cell size between cases, cells with a normal hybridization pattern were used as internal controls for each case. The examined nuclei were divided into two classes depending on the hybridization pattern: class 1 – normal cells with two chromosome #2 signals, and class 2 – abnormal cells with more than two chromosome #2 signals. DNA index (DI) was defined as the mean value of the DAPI intensity of class 2 cells divided by the mean value of DAPI intensity of class 1 cells. The classifier was applied only on the DAPI channel and the intensity thresholds defining the boundary between the nucleus and background remained unchanged during all cell measurements from the same slide. All sections had the same thickness, 8 μm, a range that encompassed all fluorescent signals and therefore ensured high probability for inclusion of cell nuclei, with a maximum nuclear size of 6–7 μm. For DI calculation, all measurements not

within the confidence interval (higher or lower than means ± 3 SD) were excluded. All of these actions were implemented to ensure reliability, accuracy and reproducibility of measurements.

To examine the accuracy of measurements, DIs of the U266 and NCI-H929 cell lines with abnormal DNA content and known chromosome aberrations were determined. Both lines contain an IgH translocation and can be distinguished by using FISH with LSI IGH break-apart rearrangement probe (Vysis). These cell lines were mixed with normal blood cells in a 1:1 ratio and analyzed both by standard flow cytometry and by determining the DAPI intensity on 3D images of nuclei with FISH signals. DI of NCI-H929 deviated by only 3.2% and that of U266 by 3.6%. Therefore, it can be concluded that image processing is highly accurate to determine the DNA index.

3. Results

Tables 1 and 2 present the clinical and histopathologic diagnosis of the OSCC and OPLs cases, respectively. Histopathologically, the 22 OPLs were diagnosed either as epithelial hyperplasia or as hyperplasia with varying degrees of dysplasia. Patients 20 and 22 were followed for several years before brush samples were taken because of recurrent lesions. All cases with OSCC were males, while an equal gender distribution was found among OPLs patients. Most patients with OPLs were either current or past smokers, and none had a history of alcohol drinking. No correlation was found between gender and smoking habits and the proportion of non-diploid cells in patients with OPLs. The mean age of the patients with OPLs and OSCC was 62 and 59, respectively. However, the mean age of the patients with over 2% non-diploid cells was significantly higher than in those with diploid lesions (70 versus 54 years, respectively).

3.1. Correlation of chromosome 2 signal number with DNA cell content

Examination of the DNA content using the Volocity System was performed on eight cases, totaling 180 cells. DI of cells with three and four signals of chromosome 2 probe was 1.2 ± 0.12 and 2.03 ± 0.57, respectively. DNA content of cells with four signals differed significantly from normal diploid cells (2N) ($p < 0.001$) and equaled to the expected tetraploid state (4N) ($p = 0.85$). However, DNA content of cells with three signals significantly differed from both normal diploid cells and expected triploid state (3N), $p = 0.00007$ and $p = 0.00002$, respectively. It can be suggested that while four signals of chromosome 2 corresponded with a tetraploid state, a cell with three signals was more likely in a near-triploid state (aneuploid).

The use of dual color FISH showed a high correlation between the number of chromosomes 2 and 8 confirming the link of chromosome 2 with the ploidy level. Dual color FISH using chromosomes 2 and 8 was performed in 25 selected cases (Tables 1 and 2; Fig. 1), 7 with OSCC, and 18 with OPLs. A direct correlation between the two chromosomes in over 90% of the cells examined was found in 23 patients. A significant discrepancy between the two chromosomes was found in two patients (Nos. 16 and 21) when only non-diploid cells were examined. In patient 16, the biopsy performed one month following oral sampling revealed a carcinoma in situ; in patient 21,

Table 1
Clinical, histopathology and FISH results on eight cases with OSCC

Patient	Age/ sex	Tobacco	Diagnosis	No. of cells	Non-diploid %	3g/3r %	4g/4r %	5g/5r %	6r %	3g/2r %	4g/2r %	Stage	Follow up/ months
1	74/M	No	SCC	470	3.3	2.0	1.3					I–II	OK 5
			Normal	150	1.8	0.6	0.60				0.6		
2	82/M	Past (30y ago)	SCC	400	5	5						IV	OK 8
			Normal	250	5.2	4.5					0.7		
3	66/M	No	SCC	200	10	3.5	6.5					IV	Died 14
			Normal	100	1	1							
4	38/M	Yes	SCC	150	15	15						IV	Died 18
			Normal	100	0								
5	63/M	No	SCC	200	20	20						I	OK 23
			Normal	100	0								
6	40/M	Yes	SCC	210	54		50	3		1		II	Rec 20
			Normal	200	20		17.5			2.5			
7	61/M	Yes	SCC	50	64	24	24	16				I–II	OK 19
			Normal	100	0								
8	51/M	No	SCC	110	75	15	40	1	19			III–IV	OK 26
			Normal	150	0								

M, male; F, female; SCC, squamous cell carcinoma; Rec, local recurrence; g, chromosome 2 green signals; r, chromosome 8 red signals.

Table 2
Clinical, histopathology and FISH results on 22 OPLs

	Location	Age/ sex	Tobacco	Pathology	History/ years	Outcome months	No. of cells	Non-diploid %	Equal		Non-equal[b]
									3g 3r	4g 4r	
1	Fom	50/M	Yes	Hyperplasia			70	0			
2	Palate	61/F	No	Mild			500	0			
3	Cheek	53/F	Yes	Moderate			50	0			
4	Cheek	56/M	Yes	Hyperplasia			500	0			
5	Tongue	38/M	Yes	Mild			450	0			
6	Tongue	72/F	No	Mild			140	0			
7	Tongue	55/M	Yes	Hyperplasia			400	0			
8	Cheek	60/F	Yes	Hyperplasia			200	0			
9	Fom	61/F	Yes	Mild			200	0			
10	Cheek	43/M	Yes	Hyperplasia			400	0.25		0.25	
11	Tongue	43/M	Yes	Hyperplasia			300	0.7			0.7
12	Tongue	47/M	No	Hyperplasia			300	2		2	
13	Cheek	53/M	Yes	Mild			200	2			2
14	Cheek	69/M	Yes	Hyperplasia			150	3	3		
15	Tongue	75/F	No	Mild			50	4	4		
16	Tongue	71/F	No	Severe		CIS/1	520	8			8
17	Gingiva[a]	93/M	Past	Hyperplasia	Previous scc 1 year		90	9	9		
18	Fom	50/F	Yes	Mild			222	9.9	9	0.9	
19	Tongue	85/M	No	Moderate		Invasive SCC/6	80	20	10	10	
20	Alveolar ridge	79/F	No	Moderate/8 years previously		Invasive SCC/8	150	60.3	22.5	35.6	2.2
21	Gingiva[a]	93/M	Past	Hyperplasia	Previous scc 1 year		250	90	3		87
22	Tongue	82/F	No	Mild/4 years previously	Previous scc 6 years		150	100		100	

[a] The same patient with two separate locations.
[b] Non equal, non equal number of chromosome 2 and chromosome 8; M, male; F, female; Fom, floor of mouth; Mild, mild dysplasia; Moderate, moderate dysplasia; Severe, severe dysplasia; CIS, carcinoma in situ; SCC, squamous cell carcinoma; g, chromosome 2 green signals; r, chromosome 8 red signal.

an OSCC in the upper alveolar ridge was diagnosed one year previously, the brushing was from a lesion in the lower gingiva.

3.2. Combined FISH and morphological analysis

The proportion of non-diploid cells among the examined cells increased with the degree of the severity of the histopathologic diagnosis.

Control group: in each of the control subjects, 200–500 cells were examined, in two, 1% and 1.5% of the cells obtained from the lateral border of the tongue were non-diploid, i.e., a cut off value (mean values + 3 SD) of non-diploid cells was determined as 1.1%. To safeguard against error, 2% was chosen as a threshold value, therefore, only samples with minimum 50 cells were selected for analysis.

OSCC (Table 1): all patients with OSCC had over 2% non-diploid cells. In three patients (Nos. 6, 7, and 8), most of the examined cells were non-diploid. In two patients (Nos. 2 and 6), non-diploid cells over 2% were also found in the opposite normal looking site; one patient was a heavy smoker for over 20 years, while the other, an 82-year-old male stopped smoking about 30 years ago. The correlation with the clinical stage of the tumor was difficult to assess because of the short follow-up period (up to 26 months). Two patients died and in one patient, the tumor recurred several months after surgery.

OPLs (Table 2): non-diploid cells over 2% of the examined cells were found in 11 of the 22 samples. The proportion of non-diploid cells increased with the degree of dysplasia: 30% of the patients with epithelial hyperplasia, 50% of the mild dysplasia, 66% of the moderate dysplasia, and 100% of the severe dysplasia. Of the 11 lesions with over 2% non-diploid cells, eight were diagnosed histologically as either epithelial hyperplasia or as mild dysplasia. In three patients (Nos. 20, 21, and 22) cells were mostly non-diploid. These patients were in high risk to develop OSCC because of either a previously resected OSCC (Nos. 21 and 22), or recurrent lesions (diagnosed as proliferative verrucous leukoplakia, No. 20). Examined samples were taken from new lesions. In one patient (No. 18), 2% non-diploid cells were detected also in the opposite normal looking site; this patient smoked over 20 cigarettes for about 25 years.

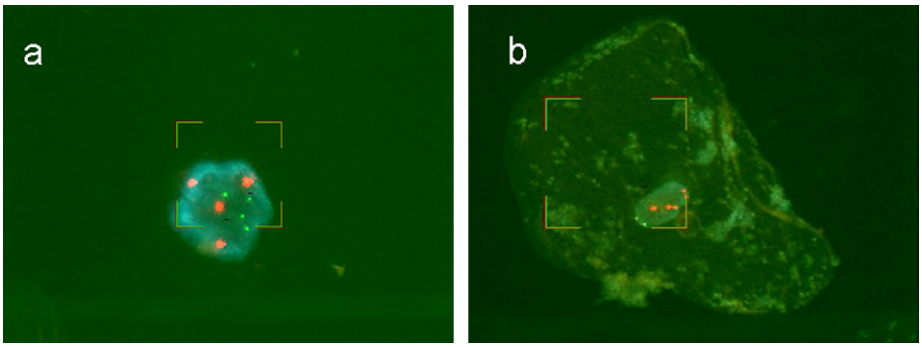

Fig. 1. Dual-color FISH analysis of brush collected cells (original magnification 1000×). Chromosome 2 probe is visualized with green signals and chromosome 8 with red signals. (a) A cell with four signals of both chromosomes. (b) A cell with an unequal number of chromosome 2 and chromosome 8.

A significant number of the examined non-diploid cells had normal morphology when cytomorphology was compared with FISH (Fig. 2).

3.3. Follow-up

Follow-up was between 4–26 months. Two patients with OSCC died 14 and 18 months after diagnosis. In one patient, the tumor recurred 20 months after diagnosis. Two patients with OPLs (Nos. 19 and 20) developed an invasive cancer 6 and 8 months after brush sampling. A carcinoma in situ was diagnosed one month after brush sampling in one patient (No. 16). It can be assumed that the carcinoma existed in the lesion and was not completely resected.

In two patients (Nos. 21 and 22), despite of the presence of a high proportion of non-diploid cells lesions were not completely resected. The oral lesions were watched and treated with local retinoids.

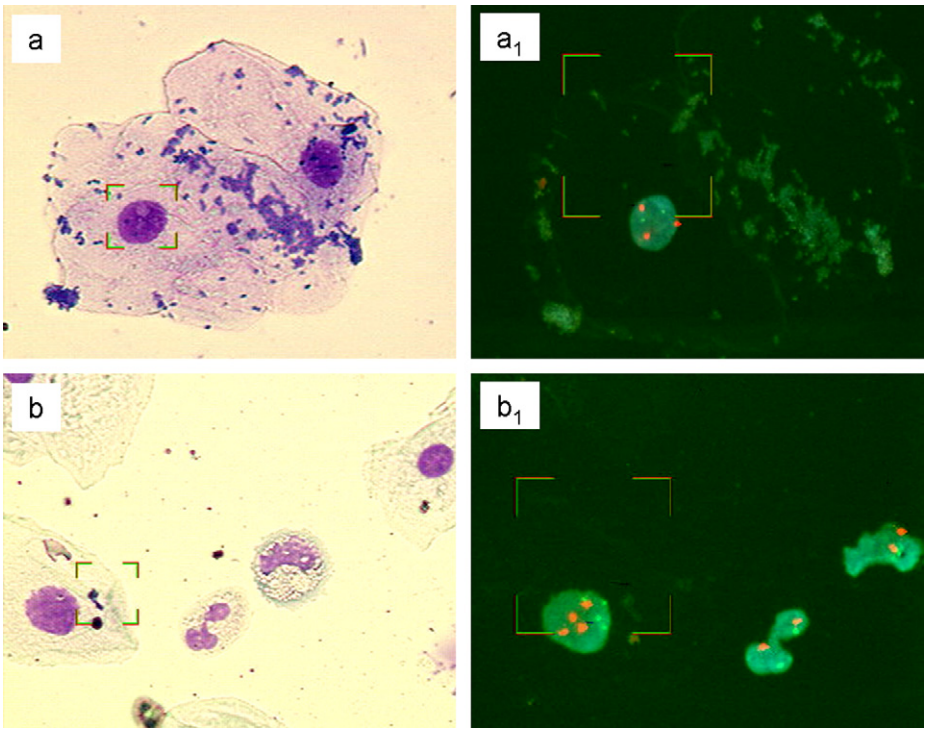

Fig. 2. A case with OPL (case 19). Combined analysis of morphology and FISH on the same cells examined. On the left, cells stained with Giemsa (a,b) and on the right the same cells with dual color FISH using chromosome 2 and 8 probes (a_1,b_1); note in (b) one epithelial cell and two inflammatory cells (May Grunwald-Giemsa stain and FISH using chromosome 2 centromeric probe labeled with SpectrumGreen and chromosome 8 centromeric probe labeled with SpectrumOrange, original magnification 1000×.

4. Discussion

Most oral premalignant lesions, mainly those diagnosed clinically as leukoplakia, are benign-appearing lesions that are either watched or ignored. Non-sampled lesions often undergo prolonged periods of observation and remain undiagnosed until clinical features of frank malignancy or advanced disease are displayed [30]. Even in leukoplakia cases that are speckled in their appearance and are clinically suspicious, incisional biopsy sampling may not be representative of the true nature of the lesion. The methods available for early monitoring of potentially malignant lesions, such as vital staining and the trans-epithelial brush biopsy with computer-assisted analysis, are insufficiently sensitive [31–33].

New techniques are required, therefore, to distinguish potentially malignant lesions from benign lesions by examining small numbers of cells collected by non-invasive procedures. Techniques based on total DNA, such as comparative genomic hybridization (CGH) and PCR-based LOH detection are not suitable for the detection of small populations of pathological cells. The multiparametric fluorescence-activated cell sorter (FACS) and polymerase chain reaction (PCR)-based techniques are highly sensitive for the detection of a small number of pathological cells, however, their false-positive rate is still high. Moreover, these techniques are not suitable for routine clinical settings.

DNA content analysis has been found to be a promising prognostic indicator for the study of cancer. Most studies of DNA content analysis of oral precancerous lesions were based mainly on image cytometry [18–21]. Determining DNA content by using image cytometry have well-known pitfalls and difficulties because the interpretation of DNA histograms is not always straightforward, i.e., there is a gray zone between a true diploid histogram and an aneuploidy pattern [34], which make these techniques not sensitive enough to guide treatment regimes for patients with cancerous and precancerous lesions in the oral cavity. In addition, most studies evaluating DNA content used archival material, mostly paraffin-embedded tissue samples that carry the inherent risk of tissue deterioration and a decrease in data resolution [35].

The present study suggests that the combined morphological and FISH analysis using brush sampling, is superior over the above mentioned methods for monitoring suspicious oral lesions in daily clinical setting; the cells are collected directly from the lesions and processed immediately, raising the credibility of the sample. The multiparametric analysis detects cell ploidy directly and provides phenotypic and genotypic information regarding a suspected cell, thus enhancing the specificity. This method is sensitive enough to detect single non-diploid cells among large populations of diploid cells. The parallel analysis enables clear distinction between epithelial cells and non-epithelial cells mainly inflammatory cells which are so common in oral lesions. In addition FISH analysis permits the distinction of non-diploid cells from proliferating mitotic cells; the hybridization pattern of non-diploid cells consists of several separate centromeric signals apart from each other while in proliferating cells, the signals are linked to each other.

The results clearly show that polyploidy is an early event in the process of oral carcinogenesis: 30% of the lesions diagnosed as epithelial hyperplasia contained non-diploid cells. The presence of increased DNA content during the various stages of oral carcinogenesis is consistent with previous findings in humans and in animal models [18–22,36,37] and are commonly observed in early stages of tumorigenesis in several other tumors, such as malignancies of the colon, esophagus, stomach, and cervix [12,13,38,39]. Surprisingly, non-diploid cells were detected in 2 of the 25 control subjects (8%), who did not have any known risk factors for oral cancer. Epithelial cells of the normal oral mucosa are postulated to contain normal diploid chromosome complement. Nevertheless, there are no large-scale studies regarding the genomic variations due to chromosomal instability in the normal oral mucosa and only few studies showed the prevalence of chromosomal instability in normal human tissues [40].

Three patients (2 with OSCC and 1 with OPL's), displayed non-diploid cells in the opposite normal looking site, two of the three patients were smoking for over 20 years. This finding shows that aneuploidy is a very early event in oral carcinogenesis supporting the concept of field cancerization which means that the epithelial surface of the upper airway exposed to the same carcinogens may possess an increased risk for cancer development [41–43]. Recent studies using microarray experiments have identified gene-expression changes in the apparently normal airway epithelium of smokers who have not yet developed cancer [44,45].

The frequency of non-diploid cells increased significantly with the degree of dysplasia. In three patients, cells were mostly non-diploid. These patients had previously resected OSCC and two were diagnosed clinically as having proliferative verrucous leukoplakia because of recurrent lesions, and therefore, are in high risk for developing a second primary tumor. Two patients with OPLs developed cancer. Both were diagnosed as moderate dysplasia with a significant number of non-diploid cells.

Of particular interest was the finding that 64% of the OPLs with over 2% non-diploid cells were diagnosed as epithelial hyperplasia or mild dysplasia. This evidence is of utmost importance. Such a diagnosis from an incisional biopsy of a benign looking lesion, in which most of the lesion is not removed, is usually only watched. A significant number of non-diploid cells in a lesion, which upon biopsy was not proven to be high grade dysplasia, show that these changes either represent an early event and harbor malignant potential, or that the biopsy specimen was either not representative or misdiagnosed. The supplement of a brush sample using combined analysis can increase the specificity in predicting the nature of the lesion and hence to guide treatment.

Combined analysis using a chromosome 2 probe is efficient in detecting the ploidy state of the cells examined, which correlates with the DNA content as analyzed using either dual FISH with additional chromosome probe (chromosome 8), or by determining the DAPI intensity on 3D images of nuclei with FISH signals using the Volocity system. The use of dual FISH may, however, raise the sensitivity of the analysis; in two patients (Nos. 16 and 21) unequal number of signals were found between chromosome 2 and chromosome 8, these patients were at high risk for developing oral cancer.

In conclusion, the combined simultaneous morphological and cytogenetic multiparametric analysis, which combines two known diagnostic methods, enhances early detection of potentially malignant cells usually overlooked by traditional histomorphological methods. The method is easy to perform, reproducible, and highly sensitive. Non-diploid cells can be detected in early stages of oral carcinogenesis, even in lesions diagnosed as epithelial hyperplasia or mild dysplasia with normal looking epithelial cells. The supplement of a brush sample and the combined morphological and FISH analysis increase the specificity in predicting the nature of a suspicious oral lesion to guide treatment and improve the outcome of these patients. Nevertheless, it should be reiterated that patient numbers in this study were relatively small, especially when the cohort was subdivided for analysis. As such, the observations made in this study should be confirmed with larger studies.

Acknowledgements

This work was supported by the Flight Attendants Medical Research Institute, and by the Israel Cancer Association.

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