Oral Oncology 49 (2013) 1121–1128

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Chromosome instability predicts the progression of premalignant oral lesions

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ARTICLE INFO

Article history:
Received 26 June 2013
Received in revised form 29 August 2013
Accepted 7 September 2013
Available online 26 September 2013

Keywords:
Chromosome instability
Ploidy analysis
FISH
Oral premalignancies
Leukoplakia
Oral cancer
Head and neck cancer

SUMMARY

Objectives: One of the main problems in reducing the incidence of oral squamous cell carcinoma (OSCC) is the inability to appropriately deal with leukoplakia. Accurately identifying lesions which will progress to malignancy is currently not possible. The present study aims to establish the value of chromosome instability (CI) detection by DNA image cytometry and FISH analysis for prognosis and monitoring of oral leukoplakia.

Materials and methods: For this purpose, we included from our archives 102 oral leukoplakia cases, which had been diagnosed between 1991 and 2008. Patient follow-up data were collected and the histopathological diagnosis was revised. CI assessment was carried out on paraffin-embedded tissue sections using both DNA image cytometry (ICM) and dual target FISH for chromosomes 1 and 7.

Results: 16 of 102 Patients developed carcinoma in situ or OSCC. Both detection methods were found to yield prognostic information independent of the histopathological diagnosis. CI was a strong individual marker of progression, with hazard ratios (HRs) of 7.2 and 6.8 for ICM and FISH respectively. Moreover, this approach seems suitable for monitoring lesions over time (especially ICM). Combining histopathology and CI enables subdivision of patients into three risk groups, with different probabilities of malignant progression.

Conclusion: CI detection seems a reliable method for risk assessment of oral premalignancies and its application may contribute to a better risk-counselling and appropriate treatment regimen or watchfull-waiting approach of patients.

Introduction

Oral cancer is the sixth most common malignancy worldwide, with a yearly incidence of about 274.000 cases [1]. Most oral tumours are squamous cell carcinomas (SCCs) with smoking and alcohol consumption as major risk factors [1,2]. In contrast to cancers of the breast, colon, prostate and melanoma that are showing a significantly better prognosis due to improvements in early detection and therapy [3], the survival rate of patients with oral squamous cell carcinomas (OSCCs) has not increased substantially over the last decades [2,4]. The oral cavity and oropharynx are easily accessible for visualization, which may facilitate early diagnosis of (pre)malignant lesions. Nevertheless, in spite of advances in surgical and other treatment modalities, the 5-year survival rate of OSCC remains only approximately 50% [2,3]. This lack of progress can partially be explained by our inability to adequately recognize early developed OSCC as well as precursor lesions at risk for progression [3].

Oral leukoplakia and, to a lesser degree, erythroplakia are relatively common lesions of the oral mucosa, some of which are at risk for malignant transformation [5]. In particular, the borders of the tongue and the floor of the mouth have been mentioned as high-risk sites [6]. Whereas oral erythroplakia show malignant transformation in almost all cases, oral leukoplakia eventually progress to malignancy in an estimated 1.1–17.5% [7]. Therefore, especially discrimination between potentially malignant leukoplakias and non-harmful lesions is of key importance.

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http://dx.doi.org/10.1016/j.oraloncology.2013.09.006

Unfortunately, current histopathological examination of precursor lesions (i.e. dysplastic and non-dysplastic) is insufficiently accurate to predict their malignant potential [8]. In addition, histopathological classification is hampered by observer variability [9,10]. and sampling variability [11]. Therefore, more effective methods for the assessment of the malignant potential of oral leukoplakia are urgently needed [12]. It is widely believed that instability of the genome is the driving force behind the development of the majority of human epithelial cancers [13]. CI may be detected by different molecular biological approaches. DNA content analysis using image cytometry (ICM) or flow cytometry (FCM) are frequently used techniques [4,14–17]. Three recently published retrospective studies have shown that ICM is able to identify patients with dysplasias that are more likely to progress to OSCC [14,16,17]. FCM displays an increasing degree of aneuploidy in the spectrum of lesions ranging from healthy oral mucosa, non-dysplastic and dysplastic leukoplakia to OSCC [15]. Alternatively, copy number alterations of individual chromosome loci can be studied using fluorescence in situ hybridization (FISH) analysis [18–21]. Several studies on FISH for chromosomes 1 and 7 revealed that numerical chromosome aberrations have a predictive value in the progression of oral precursor lesions to SCC [18,19,22].

The aim of the present study was to establish the value of CI detection by means of both ICM and FISH in comparison with routine histopathological assessment for the risk assessment of oral precursor lesions in a series of 102 leukoplakia. In addition, we evaluated both essays for their reliability to monitor possible progression of premalignant lesions over time.

Materials and methods

Patient selection

This retrospective study included patients who were admitted to the department of Oral and Maxillofacial Surgery at the Radboud University Nijmegen Medical Centre (RUNMC), The Netherlands, between 1991 and 2008 for evaluation of suspicious lesions of the oral mucosa. The minimal follow-up time was 6 months. Patients with oral hyperplastic or dysplastic lesions were identified using the Dutch Pathology diagnosis and registration system (PALGA). Patients who had been previously treated for malignancy as well as patients with a histologically confirmed carcinoma in situ at first biopsy were excluded. Clinical data including age, etiological factors, localization, type of treatment (surgery, laser ablation or expectative policy) were obtained from the patients' medical charts. Only patients with a complete data-set were included, resulting in 102 specimens (of 102 patients). Data collection was performed in accordance with local ethical guidelines.

ICM and FISH analyses were performed on biopsy specimens obtained at the time of the first diagnosis of leukoplakia and on specimens from eventually developed carcinoma (carcinoma in situ or invasive carcinoma). The time between consecutive lesions was set at a minimum of 6 months in order to exclude synchronous lesions. To assess the value of ICM and FISH for the monitoring of lesions over time, subsequent premalignant lesions and recurrent malignancies were analysed.

For each case of leukoplakia and SCC first a 4 μm thick slide was cut from a paraffin-embedded specimen for Haemotoxylin-eosin (HE) analysis followed by three 50 μm and two 4 μm sections for ICM and FISH respectively and ending again with one 4 μm slide for HE analysis. The first and last slides of the initial leukoplakia were haematoxylin-eosin stained and revised by an experienced pathologist (PJS). In this manner it could be assured that both ICM and FISH analysis were performed on representative areas, to enhance the accuracy of the test.

DNA ploidy measurement of isolated nuclei from tissue sections

Two to four 50 μm thick paraffin-embedded sections were used to isolate cell nuclei according to well-established procedures [10]. A thickness of 50 μm was shown to minimize the number of artefacts in DNA histograms [23]. Sections were deparaffinised and rehydrated as follows: xylene (three times 15 min), 100% ethanol (twice), 96% ethanol (twice), 70% ethanol, 40% ethanol, distilled water and phosphate-buffered saline (PBS) at least 10 min in each solution. The tissue sections were transferred into a centrifuge tube and incubated with 0.5% pepsin (Sigma Aldrich, St Louis, MO, USA) in PBS (pH 1.50) at 37 °C, for 60 min. After enzymatic digestion, 4 ml of ice-cold PBS was added to stop the reaction. Next the tissue suspension was filtered through a CellTrics filter with 50 μm pore size to separate nuclei from tissue debris. After centrifugation (1000 rpm, 10 min), 2 ml PBS was added and the amount of nuclei was counted with a cell-counting device (Z1 Coulter Particle Counter®, Beckman Coulter Inc., Woerden, The Netherlands). The cell suspension was diluted until the right concentration, (20,000 cells) was reached, to form a monolayer in a cytospin-centrifuge (10 min 700 rpm), air dried, and fixed in Böhm fixative for 1 h, hence a mixture of 85% methanol absolute, 10% formaldehyde (37%), and 5% acetic acid (96%). Slides were air dried after twice rinsing in methanol absolute.

Feulgen staining

First the cell preparations were hydrolysed in 5 M HCl under controlled temperature at 25 °C for 1 h, after which the process was stopped using distilled water. Thereupon the isolated nuclei were stained by the Schiff method [10] (Merck®, Darmstadt, Germany) for 1 h at room temperature. Next the slides were rinsed in streaming tap water for 20 min, dehydrated in increasing concentrations of ethanol, xylol and mounted in Permount® (Thermo Fisher Scientific, Landsmeer, The Netherlands).

Measurement of DNA content

All measurements were performed using the Leica QPath Image Cytometry Workstation (Leica Microsystems GmbH, Wetzlar, Germany) according to an established protocol [10]. Slides of stained cell nuclei were analysed using a microscope equipped with a 550-nm green filter and a computer controlled stage. Each monolayer contained at least 1500 sampled nuclei. The QPath software measures the Feulgen integrated optical density (IOD) of individual nuclei while avoiding the measurement of overlapping, folded, or clumped cells. ICM analysis of all histological specimens was performed in a blinded fashion at the RUNMC. DNA ploidy histograms were visually classified independently by two experienced observers (JvdL; IOH). In all cases of discrepancy, a consensus classification was reached by the same two observers.

In the present study, a diploid histogram is defined by a single dominant 2c peak (representing G_0/G_1 phase cells) and possibly a 4c peak (G_2 phase cells) not exceeding 10% of the total number of nuclei (Fig. 1A). If the number of nuclei with a DNA content >5c exceeded 1% of the total number of cells, the histogram was classified as aneuploid. Also, a histogram was classified as aneuploid if there was a clear and distinct peak outside the 2c/4c regions (Fig. 1B). A lesion was defined as tetraploid if the number of 4c nuclei exceeded 10% of the total number of epithelial cells and a 8c peak was observed. The measurements were performed according to previous published guidelines [24]. Because in some cases the coefficient of variation (CV: standard deviation of the 2c peak divided by the mean, given in percent) slightly exceeded the advised threshold of 5%, CVs up to 6% were allowed in the present study.