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Molecular screening of oral precancer

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SUMMARY

Objectives: Early detection and treatment of high risk premalignant mucosal changes of the oral cavity, will expectedly improve survival and reduce treatment-related morbidity. Aims of this study were to evaluate a non-invasive screening approach and to assess the value of molecular markers to identify patients at risk for oral cancer.
Materials and Methods: Exfoliated cells and biopsies were obtained from oral leukoplakia lesions of 43 patients, of whom six developed oral cancer. All samples were investigated for loss of heterozygosity (LOH) at chromosomes 3p, 9p, 11q and 17p using microsatellite markers. On the biopsy specimen additional immunohistochemical staining for p53, *TP53* mutation analysis and histopathological grading were performed.
Results: The analytical sensitivity of the non-invasive assay using exfoliated cells to detect genetic changes present in the lesions was 45% (9 of 20), the specificity was 100% (19 of 19), and the positive predictive value was also 100% (9 of 9). LOH was present in 20 of 39 (51%) of the biopsies with uniformly LOH at 9p. Mutated *TP53* and LOH at 9p in the biopsy, as single markers and in combination, were significant risk factors for malignant progression of leukoplakia to oral cancer (Kaplan–Meier analysis, $p < 0.05$).
Conclusion: A non-invasive genetic screening approach using LOH in exfoliated cells has limited value for monitoring patients with leukoplakia. However, LOH at 9p, but also mutated *TP53* in biopsies of oral leukoplakia have a significant association with malignant transformation and are promising candidate biomarkers to predict the risk for malignant progression.

Introduction

It is generally accepted that most oral squamous cell carcinomas (OSCC) develop in a precancerous field of epithelial cells characterized by tumor-associated genetic alterations [1–3]. These fields can extend up to 10 cm in diameter, but are often not visible at clinical examination [2]. A minority of precancerous fields is clinically recognized as leukoplakia or erythroplakia [4]. Precancerous fields are a more promising target for screening than early stage tumors, as the time to malignant progression is usually measured in years instead of months. As part of the clinical work-up, these visible lesions are biopsied and graded as mild, moderate and severe dysplasia by histopathological examination. However, histological grading is somewhat subjective, and shows limitations in predicting the risk for malignant progression in individual cases [5]. Genetic analysis of precancerous lesions has shown to be more reliable in prediction of malignant transformation [6–9].

Recently it has been shown with autofluorescence imaging that precancerous fields can be larger than the visible leukoplakia lesion [10,11]. This observation fits with the clinical notion that visible lesions frequently recur after excision and that oral cancers may develop outside the visible lesion [7,12]. To assess the dimensions of a precancerous field outside the visible lesion, and to identify precancerous fields at other locations in the oral cavity, multiple biopsies need to be taken repeatedly and randomly at various locations at risk, and analyzed for histologic or genetic changes. However, such a screening approach is unpractical, and will cause serious discomfort to the patient. Non-invasive sampling would be an attractive alternative and allows repeated sampling at multiple sites without discomfort. By multiple brushings exfoliated

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cells of a relatively large mucosal area can be obtained, and the cytological samples can be analyzed by microscopy or genetic assays. Such an approach would not only be applicable to leukoplakia patients, but also to other high risk populations for oral cancer. These methods are, however, best validated on leukoplakia lesions as exfoliated cells and biopsy can be obtained at the same spot, with the biopsy data as gold standard.

In a previous study we presented a non-invasive screening method to detect precancerous fields in the oral cavity by detection of loss of heterozygosity (LOH) at chromosomes 3p, 9p, 11q, 17p in exfoliated samples [13]. The approach was tested in 25 leukoplakia patients and 20 control subjects. The data appeared to be promising: the exfoliated samples predicted aberrations in the biopsy with a specificity of 100%, a sensitivity of 78% and a positive predictive value of 100% [13]. However, a larger trial and association to malignant transformation is required to determine real clinical value of this non-invasive test. Remarkably, in these first series we found that only two of ten leukoplakia cases with LOH showed allelic losses at chromosome 17p21, the location of the *TP53* gene. This is not in line with the concept that *TP53* mutations are an early, if not the earliest, event in the development of the large majority of OSCC [3,14]. Small clusters of cells with *TP53* mutations have been found in biopsies of the normal mucosa of HNSCC patients, and were hypothesized as being the start of carcinogenesis [15]. Moreover, inactivation of *TP53* is a key step in the immortalization of primary keratinocytes [16]. Mutations in *TP53* might therefore be a more informative marker for prediction of malignant transformation of precancerous lesions than 17p LOH. In addition, missense mutations in *TP53* might be easily detected by immunohistochemistry (IHC) for p53 protein, and form a relatively simple alternative for mutation analysis [17,18].

In the present study, we have assessed the value of non-invasive molecular screening in patients with oral leukoplakia. In addition, we tested whether the sensitivity of field detection could be improved by using newly designed brushes, duplicate sampling and inclusion of *TP53* mutations as molecular marker for OSCC. Furthermore, the histopathological grading, the outcome of molecular marker detection in the brushed samples as well as the biopsies were investigated for their association with malignant transformation.

Materials and methods

Patients and samples

Between 2004 and 2009 we enrolled 43 patients with leukoplakia in the oral cavity who underwent incisional biopsy or excision of the lesion. The study was approved by the Institutional Review Board, and Informed Consent was obtained from all patients.

Before incisional biopsy or excision of the lesion, exfoliated cells were obtained from a surface of 0.5 × 1 cm of the leukoplakia using a small disposable brush (Omnident®, Dental Union, Nieuwegein, The Netherlands) The brush samples were collected in duplicate from the same area within the leukoplaka. In case of a leukoplakia lesion larger than 0.5 × 1 cm, multiple duplicate samples of the lesion were collected.

To improve the sampling we also tested a newly designed brush, the Orgenex® (Rovers Medical Devices, Oss, The Netherlands). These brushes (http://www.roversmedicaldevices.com) were designed to fit in 1.5 ml vials with screw cap (Sarstedt, Etten-Leur, the Netherlands) and when using 500 μl Cytolyt (Hologic Benelux, Almere, the Netherlands), a mild fixative and preservation medium, samples can be stored for days at room temperature and for weeks at 4 °C or for years at −20 °C without decrease of quality of DNA. Of all patients photographs were taken in which the sampling spot was indicated (Fig. 1). Reference DNA to score LOH was isolated from triplicate control brush samples collected from the contralateral cheek mucosa, being a low risk site for oral cancer. All brush samples were processed separately as described previously [8,13].

Of all patients follow-up data were collected. In case of suspected malignant progression, biopsies for routine histopathological analysis were collected. These biopsies were scored according to the standard criteria of the World Health Organization Classification international histological classification of tumors [19]. The main clinical characteristics of the 43 patients are summarized in Table 1.

Analysis of leukoplakia biopsies

The formalin-fixed paraffin-embedded (FFPE) biopsies and excision specimen were processed as follows. First two 5-μm sections were cut for routine hematoxylin-eosin (HE) staining and p53 IHC. Next twenty 10-μm sections were cut, and subsequently one 5-μm section for HE staining was obtained. The 10-μm tissue sections were placed on microscopic glass slides (Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany) and then deparaffinized in xylene. Subsequently, these were stained with 1% toluidine blue and 0.2% methylene blue in phosphate buffered saline (PBS) and manually dissected under a stereomicroscope to enrich for epithelial cells and stromal cells as source of normal reference DNA. Regions of dysplasia were marked and separately dissected.

For every biopsy, the grade and size of dysplasia was evaluated by an experienced pathologist (E.B.) according to the standard criteria of the World Health Organization as either no, mild, moderate or severe dysplasia [20]. To obtain an overall dysplasia-score per patient, dysplasia was labelled according to the highest grade of dysplasia scored in the biopsy.

DNA extraction

Dissected FFPE tissues were placed in 100 μl buffer containing 100 mM TRIS–HCl (pH 9.0), 10 mM NaCl, 1% sodium dodecyl sulfate, and 5 mM EDTA (pH 8.2) for 15 min. at 98 °C. Next the samples were cooled on ice for 15 min. Then tissue digestion was performed using 1 mg/ml of proteinase K for 16 to 24 h at 60 °C and replenished with another 1 mg/ml of proteinase K for another 16 to 24 h at 60 °C. Exfoliated cells were put in the same buffer, proteinase K added, and incubated overnight at 60 °C. Subse-

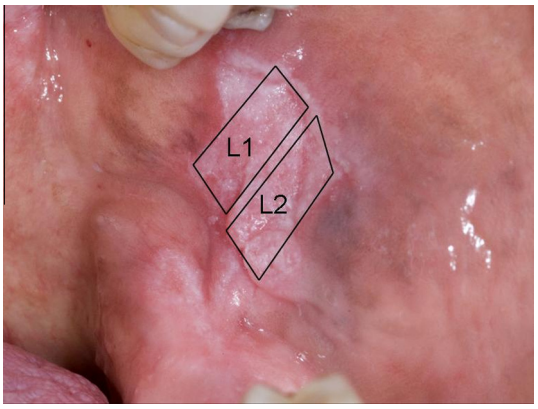

Figure 1. Brush sampling of a patient with a leukoplakia in the cheek (patient 22). Samples of the leukoplakia (L1 and L2) were collected in duplicate. In this case, no LOH was detected. P53 staining showed a p53 positive field, however, *TP*53 mutation analysis showed no mutation.