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ANATOMICAL PATHOLOGY

Chromosome instability predicts progression of premalignant lesions of the larynx

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Summary

The histopathology of premalignant laryngeal lesions does not provide reliable information on the risk of malignant transformation, hence we examined new molecular markers which can easily be implemented in clinical practice. Dual-target fluorescence *in situ* hybridisation (FISH) for chromosome 1 and 7 centromeres was performed on tissue sections of laryngeal premalignancies in 69 patients. Chromosome instability was indicated by numerical imbalances and/or polysomy for chromosomes 1 and 7. Additionally, immunostainings for p53, Cyclin D1 and (p)FADD expression were evaluated. Malignant progression was recorded. Eighteen patients with carcinoma *in situ* (CIS) were treated after diagnosis and excluded from follow-up. Chromosome instability was strongly associated with a high risk of malignant transformation, especially in lower grade lesions (hyperplasia, mild and moderate dysplasia; odds ratio = 8.4, $p = 0.004$). Patients with lesions containing chromosome instability showed a significantly worse 5-year progression-free survival than those with premalignancies without chromosome instability ($p = 0.002$). Neither histopathology nor the protein markers predicted progression in univariate analysis, although histopathological diagnosis, p53 and FADD contributed positively to chromosome instability in multivariate analysis. Chromosome instability is associated with malignant progression of laryngeal premalignancies, especially in lower grade lesions. These results may contribute to better risk counselling, provided that they can be validated in a larger patient set.

Key words: Chromosome instability, cyclin D1, FADD, FISH, head and neck, larynx, p53, premalignant.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world with an estimated 500,000 new cases annually and a poor 5-year survival rate of approximately 50%.[1] The predominant aetiological risk factors for HNSCC are alcohol consumption and tobacco smoking.[1–3] Important factors which influence prognosis are locoregional recurrence in up to 30% of patients, originating from minimal residual disease,[4,5] and the development of new or progression of existing precursor lesions.[5,6] Furthermore, molecularly targeted therapies are directed toward activated oncogenes and do not influence inactivated tumour suppressor genes, which are frequently found in HNSCC.[7] This makes early detection of potentially malignant head and neck precursor lesions extremely important.

A diagnostic dilemma is to decide which precursor lesions are potentially malignant. However, histopathological examination is troubled by inter-observer variability.[8–10] Therefore, we need more reliable, sensitive, fast, and easy to interpret molecular-based detection methods to identify potentially malignant precursor lesions, which can be easily implemented in clinical diagnostics.

The histomorphological transition from normal mucosa to cancer is driven by the accumulation of genetic changes, resulting in chromosome instability,[1,5,11] which can already be detected in premalignant lesions using different molecular biological approaches. The relationship of chromosome instability with cancer development has been extensively studied in oral lesions. First, loss of heterozygosity (LOH) on chromosome 9p21 ($p16^{INK4A}$ and $p14^{ARF}$ locus) and possibly also 3p correlates with cancer development in half of the cases, and is associated with an 11- to 52-fold increase in relative cancer risk if detected together with LOH at 4q, 8p, 11q, 13q, 14q and/or 17p.[12–15] Second, DNA flow and image cytometry have demonstrated an association between aneuploidy and malignant outgrowth of oral premalignant lesions.[16,17] Third, array comparative genomic hybridisation (CGH) has also shown accumulation of chromosomal alterations in association with disease progression.[18] Fourth, *in situ* hybridisation (ISH) analyses have revealed that numerical chromosomal aberrations can predict progression.[4,19,20] Numerical aberrations for chromosomes 1 and 7 were detected in dysplasia and carcinoma *in situ* (CIS) and not in histologically normal mucosa and the majority of hyperplastic lesions.[21] A 10-year translational study showed that LOH for 3p and 9p, p53 protein overexpression, and polysomy for chromosomes 9

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and 17, strongly predicted cancer development in oral leukoplakia.[12] The use of chromosome 7 alterations to assess the aggressiveness of potentially malignant oral lesions has been demonstrated by Poh *et al.*[22] This study also showed that polysomy for chromosome 9 seems less suitable for this purpose due to loss of chromosome 9 sequences.

Relatively few studies have assessed the clinical value of chromosome instability to predict malignant development of laryngeal precursor lesions. DNA ploidy analysis showed that 77% of the lesions which preceded carcinoma (*in situ*) showed aberrant DNA ploidy results.[23] Furthermore, microsatellite and single-target brightfield ISH analysis showed that LOH at 9p21, 17p13 and 18q23 correlated with numerical abnormalities for chromosomes 1, 7, 9, 17 and 18 in high risk premalignant lesions.[24] Because of the limited size of laryngeal biopsies, variations in the number of (pre)malignant cells and loss of tissue architecture during processing, microsatellite, array CGH and DNA content analyses are not ideal. Therefore, we applied ISH in an earlier retrospective study on chromosome instability detection (indicated by the presence of imbalances in chromosome 1 and 7 centromere copy numbers and/or polyploidisation). This study showed that chromosome instability predicted malignant progression in premalignant laryngeal lesions.[25]

Other potential molecular markers of progression include the cell cycle proteins p53, Cyclin D1, Fas-associated Death Domain (FADD) and its phosphorylated isoform pFADD. Multiple studies have shown that accumulation of p53 occurs more often in high grade than in low grade precursor lesions, although a strong association with malignant progression has not been identified.[12,26,27] Amplification of the 11q13 region, including the Cyclin D1 and FADD genes, is frequently found in HNSCC.[28,29] Particularly, Cyclin D1 and FADD proteins are accumulated in tumour cells, which is indicative of a worse cancer free survival.[30,31] Furthermore, dysregulated Cyclin D1 expression has been associated with progression in premalignant head and neck lesions.[29,32] High levels of FADD and pFADD have been associated with a poor disease-specific survival in laryngeal and pharyngeal carcinomas, and pFADD overexpression with a better response to taxol-based chemotherapy.[30,33] Its value as a predictor for progression of premalignant laryngeal lesions has not as yet been investigated.

In this study we examined a new collection of premalignant laryngeal lesions for the presence of chromosome instability as well as p53, Cyclin D1, FADD and pFADD and analysed their value to predict malignant progression.

MATERIALS AND METHODS

Patient data and tissue material

Of 180 patients with laryngeal dysplasia that had undergone a laryngeal biopsy during microlaryngeal surgery between 1986 and 2001 in the Maastricht University Medical Centre, we included 69 premalignant laryngeal (5 supraglottic, 64 glottic) lesions of 69 patients (57 male, 12 female) in this study (referred to as Group 1). The other patients were excluded due to (1) a follow-up time shorter than 6 months prior to malignant progression, (2) a lack of sufficient patient material, (3) a radically performed biopsy, e.g., vocal cord ‘stripping’, or (4) previous inclusion in a previous study from our institute.[25] In case of multiple available biopsies, the last biopsy preceding squamous cell carcinoma (SCC) or CIS was chosen, taking into account the 6 month period as a minimum. The material was treated according to the Code for Proper Secondary Use of Human Tissue (Federation of Medical Scientific Societies, The Netherlands, 2003) and the study protocol was approved by the institutional ethical committee.

The histopathological assessment of the lesions was performed using the World Health Organization (WHO) classification system and thoroughly reviewed (FJB). Eighteen cases diagnosed as CIS were treated by radiotherapy or (laser-assisted) microlaryngeal surgery after diagnosis, and therefore excluded from follow-up and survival analyses, resulting in a follow-up group of $n = 51$ (referred to as Group 2). Because of the current discussion on whether a distinction between severe dysplasia and CIS can be made, possibly resulting in the treatment of these lesions, we also defined a third group (referred to as Group 3): lesions with hyperplasia, mild or moderate dysplasia only, severe dysplasia excluded.

Follow-up information of the 69 patients was collected from the medical records. Lesions were classified as ‘progressive’ when there was development of CIS or SCC at the same localisation as the primary biopsy site.

Four-μm thick formalin fixed, paraffin embedded tissue sections of all 69 specimens (further referred to as Group 1) were subjected to dual-colour FISH analysis using centromere-specific DNA probes for chromosomes 1 and 7. A control group consisting of 10 tissue samples of normal squamous head and neck epithelium was also subjected to fluorescence *in situ* hybridisation (FISH) analysis. On subsequent tissue sections of the 51 cases which were submitted to follow-up and survival analyses (Group 2), immunostainings were performed for p53, Cyclin D1, FADD and pFADD in 51, 50, 48 and 48 specimens, respectively. Missing cases were due to insufficient patient material.

Data concerning tobacco smoking and alcohol consumption were available in 52 of the 69 patients. Patients were divided into (1) non-smokers (never smoked or ceased smoking for at least 20 years), and (2) smokers (current smokers or ceased smoking for less than 20 years). Alcohol consumption was categorised as (1) non-user, i.e., $\leq$2 alcohol units (AU)/day, and (2) user, i.e., $>$2 AU/day.

Detection of chromosome 1 and 7 alterations by FISH

FISH was performed on 4 μm thick tissue sections as described previously.[4,34] Briefly, sections were deparaffinised, pretreated with 85% formic acid / 0.3% H_2O_2, 1 M NaSCN and 4 mg/mL pepsin in 0.01 M HCl, post-fixed in 1% formaldehyde in phosphate-buffered saline (PBS), dehydrated in an ethanol series and hybridised with a mixture of digoxigenin-labelled human centromere 1-specific and biotin-labelled centromere 7-specific DNA probes [1 ng/μL 60% formamide, 2x sodium-saline citrate (SSC), 10% dextran sulphate and 50x excess salmon sperm carrier DNA]. After hybridisation the preparations were washed stringently in 2x SSC at 42°C (twice for 5 min) and 0.1x SSC at 60°C (twice for 5 min). The probes were detected by application of (1) mouse anti-digoxin (Sigma, USA) / avidin fluorescein (Vector Laboratories, USA), (2) rabbit anti-mouse rhodamin (Dako, Denmark) / biotinylated goat anti-avidin (Vector Laboratories), and (3) swine anti-rabbit rhodamin (Dako) / avidin fluorescein. Preparations were mounted in Vectashield (Vector Laboratories) containing 4,6-diamidino-2-phenyl indole (Sigma; 0.2 μg/mL).

Microscope images were recorded with the Metasystems Image Pro System (black and white charge coupled device camera; Metasystems, Germany) mounted on top of a Leica DM-RE fluorescence microscope (Leica, Germany) equipped with fluorescein-, rhodamine- and DAPI-specific filter sets for single-colour analysis and a double bandpass filter set (fluorescein, rhodamine) for simultaneous dual-colour analysis.

Controls and evaluation of FISH results

Hybridisations on tissue sections with proven aneusomies (monosomy, disomy, trisomy, and tetrasomy) as well as on 10 normal squamous tissue sections were used as a control group to exclude hybridisation artefacts and interpretation problems. Evaluation of the FISH results was carried out by two investigators (AH and EJMS) according to our earlier described and validated protocol.[25,34] FISH signals were scored per colour, and nucleus for the presence of aberrant copy numbers of chromosomes 1 and 7. The highest copy number per nucleus was determined and set if $\geq$20% of the nuclei (with a minimum of 50 nuclei and a maximum of 500 nuclei, depending on the size of the lesion) showed this number of FISH signals. Based on this evaluation, the lesions were categorised as either monosomic, disomic, trisomic, tetrasomic or polysomic ($>$4 signals per nucleus) for the respective probe targets.

After this evaluation, the lesions were divided into (1) a group of lesions without chromosome instability, i.e., no evidence of numerical chromosomal alterations (diploid) or an altered but balanced chromosome 1 and 7 status (trisomy or tetrasomy for both chromosomes, indicating tri- or tetraploidisation), and (2) a group of lesions containing chromosome instability, i.e., showing

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