Oral Oncology 72 (2017) 132–139

Contents lists available at ScienceDirect

Oral Oncology

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

Nomogram for risk prediction of malignant transformation in oral leukoplakia patients using combined biomarkers

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ARTICLE INFO

Article history:
Received 11 November 2016
Received in revised form 12 April 2017
Accepted 14 July 2017

Keywords:
Oral leukoplakia
Nomogram
Risk prediction
P53
Carbonic anhydrase (CA9)

ABSTRACT

Objective: Squamous cell carcinomas (SCC) are the most common malignancies in the oral mucosa; these carcinomas have been preceded by potentially malignant oral disorders (PMODs), mostly oral leukoplakia (OL). No specific biomarker has been widely accepted for predicting the risk of malignant transformation of PMODs. The aim of this study was to develop an accurate prediction model for the malignant transformation of OL using clinical variables and candidate biomarkers.

Materials and methods: To achieve this goal, 10 candidate biomarkers that had previously been reported as useful molecules were investigated: P53, Ki-67, P16, β-catenin, c-jun, c-met, insulin like growth factor II mRNA-binding protein (IMP-3), cyclooxygenase (COX-2), podoplanin (PDPN) and carbonic anhydrase 9 (CA9). For this study, malignant transformed (n = 22, median interval of malignant conversion: 3.3 years) and untransformed (n = 138) OL specimens with median follow-up period of 11.3 years (range: 4.6–23.2 years) were immunohistochemically stained.

Results: Using univariate Cox regression analysis, all biomarkers were proven to be significant for predicting malignant transformation in OL. To reach the highest prediction accuracy, the repeated simulation was performed, revealing that the combination of P53 and CA9 with the clinical factors including age and degree of dysplasia achieved the highest prediction accuracy. We constructed a nomogram with the identified prognostic factors for predicting the 5-, 10-, and 15-year progression free survival of OL.

Conclusions: The proposed nomogram may be useful for the accurate and individual prediction of the transformation to SCC in OL patients and may help clinicians offer appropriate treatments and follow up.

Introduction

The International Agency for Research on Cancer in 2014 reported that an estimated 529,000 new cancer cases in the oral cavity, lip and pharynx occurred worldwide in 2012, with 292,000 deaths. When the main subsites are examined separately, they do not rank highly; combined, however, they would rank as the seventh most frequent type of cancer and the ninth most common cause of cancer death [1]. The most common oral cancer, oral squamous cell carcinoma (OSCC) may arise from potentially malignant oral disorders (PMODs) such as oral leukoplakia (OL) and oral submucous fibrosis, with a variable transformation rate [2]. In india, approximately 80% of OSCC were developed from OL [3]. The fact that OSCC occurs from PMODs, which are clinically easily accessible, enables oncologists to use biomarkers for the early detection of high-risk lesions and to conduct chemoprevention trials for arresting or removing the lesions. Once identified, the high-risk individuals could be offered more aggressive treatment options and more intensive follow-up [4].

The histologic assessment of epithelial dysplasia has been the gold standard for detecting high-risk lesions. Considering diagnostic discrepancies for the presence and severity of epithelial dysplasia [5,6], a low- and high-grade binary system has been proposed, with an 82% accuracy rate for predicting malignant transformation [7,8]. Despite the significance of the degree of dysplasia, it has been claimed that patients with mild dysplasia or even without evidence of dysplasia can develop OSCC [9]. There-

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http://dx.doi.org/10.1016/j.oraloncology.2017.07.015
1368-8375/

fore, there has been a great effort to search for a better objective biomarker for assessing high-risk PMOD.

In the present study, we investigated 10 candidate biomarkers that had previously been reported as possible predictors for malignant transformation of PMODs. The most commonly studied biomarker is the proliferating index by examining Ki-67 expression, which is detected in the nuclei of proliferating cells in all active phases of the cell cycle [10]. Ki-67 immunolabeling could distinguish dysplastic lesions from OL, by estimating a higher proliferative rate of OL [11]. P53, the *p53* tumor suppressor gene product, deserves particular attention because mutation of the *p53* gene has been found in 35% of OSCCs, suggesting that its function has an active role in OSCC carcinogenesis [12]. The homozygous deletions that eliminate the *p16 INK4* locus, another tumor suppressor gene, have been described previously in 33% of head and neck tumors [13]. Loss of p16 expression has been reported in 38% of oral premalignant lesions [14]. In addition, a panel of biomarkers has been widely investigated to evaluate their usefulness as predictable biomarkers, such as β-catenin [15,16], c-jun [17], c-met [18], insulin-like growth factor II mRNA-binding protein (IMP-3) [19], cyclooxygenase (COX-2) [20], podoplanin (PDPN) [21] and carbonic anhydrase 9 (CA9) [22]. No specific molecular biomarkers, however, have been globally acceptable to assess high-risk lesions.

The purpose of this study was to assess the predictive impact of biological markers in OL transformation to OSCC and to develop an accurate risk prediction model for malignant conversion of OL using clinical variables and biological markers. To achieve this goal, this study first investigated a wide spectrum of previously reported molecular biomarkers. The implemented prediction model was represented in the form of a diagram, referred to as a nomogram. A nomogram is a graphical representation of a statistical model and provides the probability of a particular clinical outcome, such as death or recurrence [23,24]. The constructed nomogram was evaluated using the concordance index (c-index) [25]. This nomogram can serve as an objective guideline to assess high-risk OL. With the identification of high-risk individuals, more efficient chemoprevention trials can be designed to reduce the incidence of OSCC.

Material and methods

Patient selection

In this study, we retrospectively reviewed the archived files of 160 OL patients at Dental Hospital, Yonsei University Medical Center, Seoul, Korea, from 1994 to 2009. All clinical information and follow-up data were obtained from the records. The following clinical data of the patients were recorded at the initial diagnosis of OL: age, gender, lesion site, history of smoking and alcohol intake, previous history of OL or lichen planus, and follow-up period. The clinical factors such as history of smoking and alcohol uptake, as well as previous history of OL or lichen planus were excluded from this study because of the missing entries more than 50%.

The patients with oral white plaque were clinically diagnosed as OL and were histologically confirmed by showing hyperkeratosis and hyperplasia with or without epithelial dysplasia. Patients with epithelial dysplasia were classified into low and high grade according to the histological patterns of epithelial dysplasia [7] and were confirmed by two pathologists. The tissue samples of OL were obtained from incisional biopsy of the lesions, and none of the lesions had been previously treated.

Normal oral mucosa (NOM) tissues were obtained from 18 patients with third molar extractions, who otherwise did not have any infective or inflammatory oral lesion. This study was approved by the Institutional Review Board (IRB) for Bioethics of Yonsei University College of Dentistry (IRB 2-2013-0045).

Immunohistochemical staining

Immunohistochemical staining was performed in paraffin-embedded archival OL tissue samples with primary antibodies against 10 candidate molecular markers that had previously been reported as possible predictive biomarkers for malignant transformation of OL. They were COX2 (1:50, Abcam, Cambridge, UK), c-Met (1:100, Santa Cruz Biotechnology, Texas, USA), β-catenin (1:100, Abcam), c-Jun (1:100, Abcam), IMP3 (1:100, Dako, Carpinteria, CA, USA), CA9 (1:100, Abcam), Ki-67 (1:150, Dako), P16 (1:100, Dako), P53 (1:150, Dako), and PDPN (1:100, Dako). The real Envision HRP Rabbit/Mouse detection system (Dako) was used as the secondary antibody. All of the sections were developed with 3,3'-diaminobenzidine and counterstained with hematoxylin. Mouse IgG (Dako Cytomation, Denmark) or rabbit IgG (R&D, Germany) was used as the negative control. Based on the expression patterns of NOM, we divided the OL expression pattern into low and high expression. The absence or restriction of positive cells in the lower third of the epithelium was considered low expression, and the presence of positive cells in the upper two-thirds of the epithelium was considered high expression.

Statistical analysis

The expression of biomarkers in each group of tissue samples was compared with the Chi-square test. The Cox proportional hazard model was performed to assess the effects of various factors on the prediction of the transformation to cancer. Cox proportional hazard model is a survival model which considers time to event. The nomogram for predicting the risk of malignant transformation was created with the selected significant variables and evaluated using the concordance index (c-index). The c-index is a measure for predictive accuracy of cox regression model, which considers censored data. The log-rank test was used to compare the risk of malignant transformation between patient groups with different total scores, which were calculated by the nomogram. And logistic regression was used to calculate sensitivity and specificity, which considers only status, not time to event. All statistical analyses were performed using R (version 3.1.1) with the rms library.

Results

Patient characteristics

One hundred and sixty patients with OL were included in this study for whom a median follow-up of 11.3 years (range, 4.6–23.2 years). Histologically confirmed SCC cases transformed from OL occupied 22 (13.8%) patients with a median interval of progressing OSCC of 3.3 years (range, 1–10.4 years). They occurred at the same or adjacent region with OL in each patient. In 138 (86.2%) patients, no evidence of malignant transformation was noted during the follow-up period. There were 60 (37.5%) females and 100 (62.5%) males, with a median age of 54 (range, 13–89) years at initial diagnosis. The average age was 51.9 and the distribution of age was left skewed. So, the patients were divided into two groups according to the median age for analysis. The gingiva was affected in 72 (45.0%) patients, followed by the buccal mucosa (27.5%) and tongue (27.5%) (Table 1). According to the Chi-square test, the clinical factor of age ($P = 0.007$) and the degree of dysplasia ($P < 0.001$) showed significant association with malignant transformation of OL, but gender and lesion site were not (Supplementary Table 1).