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Snail and Axin2 expression predict the malignant transformation of oral leukoplakia

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ABSTRACT

Objectives: Oral leukoplakia (OL) has a well-documented potential risk of malignant transformation into oral squamous cell carcinoma (OSCC), although biomarker(s) predicting malignant potential are limited in capability. The aim of this cross-sectional and retrospective cohort study was to investigate the predictive role of canonical Wnt genes Axin2 and Snail (*SNAI1*) expression in the malignant transformation of OL lesions.

Materials and methods: The expression of epithelial-mesenchymal transition (EMT) genes Snail and Axin2, which are regulated by the canonical Wnt pathway, were determined using immunohistochemical staining in an OL cohort consisting of 154 samples of patients with long-term follow-up and then evaluated as risk factors for malignant transformation of OL.

Results: Increased Axin2 and Snail abundance were found in 107 (69.5%) and 58 (37.7%) of OL patients, respectively. In a multivariate analysis using gender, age, lesion site, Axin2, and Snail as cofactors, both Axin2 and Snail were independent risk factors for malignant transformation with a hazard ratio of 7.47 (95% confidence interval, 2.23–25.02; P = 0.001) and 4.41 (95% confidence interval, 1.78–10.93; P = 0.001), respectively. A nomogram for predicting 5-, 10-, and 15-year cancer-free survival probability was developed in patients with OL by including gender, age, lesion site, Axin2, and Snail expression with a c-index of 0.760.

Conclusion: The increased abundance of Snail and Axin2 is highly correlated to malignant transformation of OL, making them novel biomarker(s) predicting oral cancer development.

Introduction

Oral leukoplakias (OL) are common whitish patches of the oral cavity having a well-documented potential risk of malignant transformation [1–3]. The prevalence of OL is approximately 1% in all ages, the annual rate of malignant transformation of untreated lesions having been reported as 2–3% [1]. Meanwhile, the risk of recurrence after the surgical treatment of non-homogeneous leukoplakia has been estimated at up to 20%, and oral squamous cell carcinomas (OSCC) developed from OL demonstrate poor clinical outcomes [2]. While many studies have sought prognostic markers of the malignant transformation of precancerous leukoplakia, the predictive values remain limited [4].

The well-known risk factors for malignant transformation of OL include old age, female gender, long duration of leukoplakia, large in size, past history of head and neck cancer, non-homogeneous clinical appearance, presence of dysplastic histologic features, and leukoplakia lesions on the tongue borders or mouth floor [1,2]. Additionally, aberrant expression of numerous molecular markers, including p16INK4a and Ki-67, chromosome instability, and loss of heterozygosity at 9p and mutated TP53 have been demonstrated as predicting factors [5–11]. However, predictive markers currently fall short in properly estimating the potential risk for developing OSCC in terms of high reproducibility and reliability.

The epithelial-to-mesenchymal transition (EMT) involves the alteration of cell states to invasive phenotypes by silencing epithelial traits and inducing mesenchymal phenotypes [12]. Snail, a

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zinc-finger transcriptional repressor, plays a critical role in EMT accompanying complementary morphogenetic programs in cancer [13]. Snail protein is stabilized by activation of canonical Wnt signaling through inhibition of serial phosphorylation by GSK-3 [14]. As a representative transcriptional target of TCF/LEF complex, the Axin2 scaffolding protein regulates a nucleocytoplasmic shuttle for GSK-3, resulting in the stabilization of nuclear Snail [12]. The co-expression of Axin2 and Snail in invasive breast and colon cancer is well-documented [12,15], while the expression and clinical significance of the canonical Wnt genes in OL are not well understood.

Although earlier EMT studies mainly focused on invasive and metastatic progression of human cancer, emerging evidence suggests an earlier activation of EMT genes in pre-neoplastic lesions and non-invasive tumors [16]. For example, a major pathogen of human stomach, *Helicobacter pylori* CagA (cytotoxin-associated gene A), strongly induces Snail-mediated EMT in a similar fashion to Axin [17], and Snail is upregulated in non-invasive endometrial carcinoma [18]. As downstream targets of the Wnt pathway, Axin2 and Snail are highly expressed in colorectal adenoma [19,20], suggesting a potential role of EMT genes in precancerous lesion. Furthermore, EMT features have been found to cause a higher risk of OL lesions during malignant transformation by exhibiting reduced expression of E-cadherin in moderate to severe dysplasia [21]. These observations suggest that expression of EMT genes in OL may help predict cancer progression.

The aim of this cross-sectional and retrospective cohort study was to investigate the predictive role of EMT genes in the canonical Wnt signaling pathway in OL. Immunohistochemical staining for Axin2 and Snail was performed using OL tissue samples, and the histologic patterns of Axin2 and Snail expression were analyzed and graded. Prediction accuracy was then investigated using the level of protein expression and clinicopathological parameters by constructing a graphical nomogram of a type which has been used to quantitatively evaluate the risk factors in several carcinomas [22–24]. We found that epithelial abundance of Axin2 and Snail, EMT genes involving the canonical Wnt pathway, are highly correlated to malignant transformation of OL, indicating their potential as biomarker(s).

Material and methods

Patients in the OL cohort

The OL specimens were obtained from the Department of Oral Pathology, Dental Hospital, Yonsei University Medical Center. Between 1995 and 2010, biopsy specimens were obtained from 183 patients with OL. Patients with a previous or concomitant diagnosis of OSCC (n = 16) or with inadequate tissue available for analysis (n = 17) were excluded from this study. In the OL cohort, a total of 154 patients (96 males and 58 females; median age of 55; age range of 13–89 years) with OL (median follow-up period: 130 months) were included. Among them, 22 patients progressed to invasive squamous cell carcinoma (median follow-up period: 40 months) and 132 patients did not progress to malignancy (median follow-up period: 142 months) during follow-up (Fig. 1A). The clinical characteristics of patients in the OL cohort are described in Table 1. The pathologic diagnoses of the sections were performed by two pathologists (HSK, JIY) independently. Additionally, normal oral mucosa tissues were obtained from 18 patients (6 males and 12 females; median age of 45; ages 11 to 53) during their third molar tooth extraction and used as normal control. This study was approved by the Institutional Review Board for Bioethics of Yonsei University College of Dentistry (IRB 2-2013-0045).

Immunohistochemical staining

After being deparaffinized with xylene and rehydrated with graded alcohol, the tissue sections were incubated with a mixture of H_2O_2 and methanol at a dilution of 1:40 at room temperature for 10 min to block endogenous peroxidase activity. Antigen retrieval

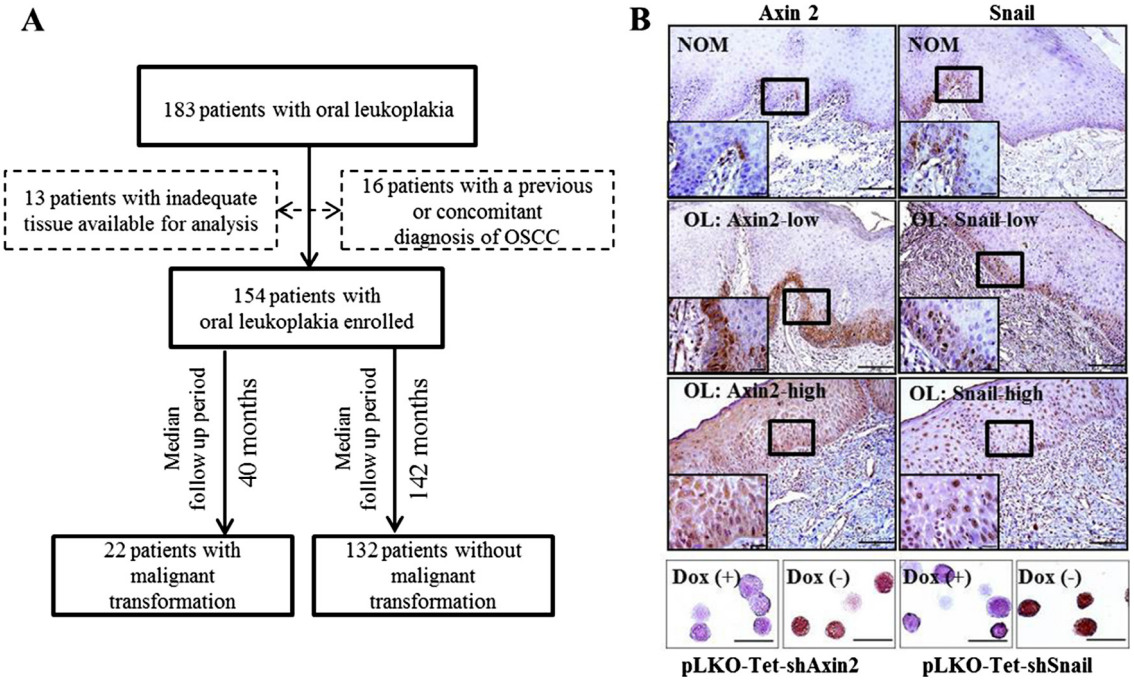

Fig. 1. Axin2 and Snail expression in normal oral mucosa (NOM) and OL tissue samples: (A) Flow diagram for selection and outcome of patients with OL. (B) Representative expression patterns for low or high level of Axin2 and Snail in NOM and OL tissues samples (original magnification, ×200; magnification for inset micrograph, ×1000; scale bar, 100 μm). Immunoreactivity of Snail and Axin2 antibodies were validated from SW-480 cell block with inducible knockdown of Snail and Axin2 (bottom panels, magnification, ×4000; scale bar, 100 μm).