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ORIGINAL ARTICLE

The potential of interleukin 12 receptor beta 2 (IL12RB2) and tumor necrosis factor receptor superfamily member 8 (TNFRSF8) gene as diagnostic biomarkers of oral lichen planus (OLP)

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Abstract

Objective. This study evaluated the potential of interleukin 12 receptor beta 2 and tumor necrosis factor receptor superfamily member 8 as diagnostic biomarkers of oral lichen planus (OLP). ***Materials and methods.*** The mRNA expression of IL12RB2 and TNFRSF8 in FFPE OLP samples (OLP group, $n = 38$) were investigated with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis and compared to those of chronic non-specific mucositis (Non-OLP group, $n = 25$) and normal mucosa (Normal group, $n = 18$). Predictive modeling of the expression of IL12RB2 and TNFRSF8 was constructed using support vector machine (SVM), random forest (RF), linear discriminant analysis (LDA), neural network (NN) and naive Bayes (NB) methods. ***Results.*** Normalized expression of IL12RB2 in the OLP group (3.78 ± 1.67) was significantly higher than the Normal group (1.97 ± 1.12), but lower than the Non-OLP group (6.86 ± 1.67). TNFRSF8 gene expression in the OLP group (7.46 ± 1.51) was significantly higher than the Normal group (2.90 ± 1.61), but no significant difference was found between the OLP and Non-OLP groups. The ratio of IL12RB2/TNFRSF8 in the OLP group (0.52 ± 0.23) was significantly lower than the Normal group (0.74 ± 0.39) and the Non-OLP group (1.07 ± 0.38). In the predictive modeling, the area under receiver operating characteristic (ROC) curves (AUC) ranged from 0.83–0.92 and their accuracy was higher than 0.75 in all methods. ***Conclusions.*** The IL12RB2/TNFRSF8 ratio can be a useful diagnostic tool for OLP.

Key Words: *oral lichen planus (OLP), biomarker, predictive modeling, IL12RB2, TNFRSF8, formalin-fixed, paraffin-embedded (FFPE)*

Introduction

Oral lichen planus (OLP) is a common inflammatory disease of the oral mucosa, which is thought to arise from a T-cell-mediated autoimmune response in which autocytotoxic $CD8^+$ T-cells trigger apoptosis of the oral epithelial cells [1]. In spite of some controversies, OLP is regarded as a pre-malignant condition because OLP patients showed a significantly increased risk of oral cancer [2–4]. However, because its clinical and histopathological features are not pathognomonic [5], it is difficult to differentiate OLP from other white lesions, especially oral lichenoid lesions [6] and also results in the lack of consistency and reliability of diagnosis [7].

To overcome these limitations, novel biomarkers for OLP have been investigated, but this approach also had a limitation. Gene expression analyses in a clinical setting are limited by the freshness of tissue samples and difficulty in large-scale studies. Formalin-fixed and paraffin-embedded (FFPE) tissue specimens have been proposed as an alternative, but they are not routinely used because the extracted RNA is not considered of sufficiently high quality for genetic analysis. In recent years, however, some researchers reported successful extraction of RNA

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from old FFPE samples and they were suitable for molecular analyses [8].

In the present study, interleukin 12 receptor beta 2 (IL12RB2) and tumor necrosis factor receptor superfamily member 8 (TNFRSF8) genes were selected as candidate biomarkers because (1) these genes are inflammatory cytokines and associated with various kinds of disease, including malignancies [9], and (2) our previous study identified their potential as biomarkers of oral squamous cell carcinoma (OSCC). In that study, we performed microarray analysis of tumor and normal tissues from OSCC patients. With four biostatistical methods (the permutation test, local pooled error [LPE], Student's paired *t*-test and significance analysis of microarray [SAM]), 15 genes were screened. After literatures analysis, four genes (IL12RB2, TNFRSF8, ADAM15 and CDC7) were selected for candidate biomarkers. Their validation was performed with quantitative reverse transcriptase polymerase chain reaction. Consequently, these four 74 genes (ADAM15, CDC7, IL12RB2 and TNFRSF8) seemed to have a potential as the novel biomarkers for OSCC (publication in progress) [10].

IL12RB2 is a sub-unit of heterodimeric receptor of interleukin 12 (IL-12), the main cytokine that regulates T helper type 1 (Th1) differentiation [11] which in turn produces interferon gamma (IFN-γ) and promotes cell-mediated immunity and is known to be related to allergic asthma [12] and autoimmunity [13]. TNFRSF8 (CD30), a member of the TNF receptor family, encodes a cell-surface protein up-regulated on activated T-cells and its up-regulation was reported in several inflammatory diseases [14–16].

The purpose of this study was to evaluate the potential of IL12RB2 and TNFRSF8 as diagnostic biomarkers of OLP by investigating their expression in formalin-fixed paraffin-embedded (FFPE) OLP samples.

Materials and methods

Study design and samples

The FFPE samples of OLP and chronic non-specific mucositis were collected from the archives of the Department of Oral Pathology, Seoul National University Dental Hospital. Normal mucosal FFPE samples were obtained from patients who underwent orthognathic surgery or surgical extraction of an impacted 3rd molar after informed consent. According to the criteria proposed by van der Meij et al. [6], all OLP slides were reviewed and re-confirmed by two qualified pathologists. All FFPE samples from patients with a diagnosis of chronic non-specific mucositis, but not OLP were assigned to the Non-OLP group. These OLP and Non-OLP groups were classified by the same pathologists according to the degree of inflammation (mild or mild-to-moderate, moderate-to-severe and severe) in both groups. Clinical characteristics of the patients were retrieved by reviewing medical records. This study was approved by the Institutional Review Board at Seoul National University Dental Hospital (CRI12002G) and conducted in full accordance with the Declaration of Helsinki.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from FFPE samples using the RNeasy® FFPE Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instruction. Quantification and integrity analysis of the RNA were performed using a 2100 Bioanalyzer®

Table I. Biological properties and primary sequences of the target genes.

	IL12RB2	TNFRSF8
Protein name	Interleukin-12 receptor, beta 2	Tumor necrosis factor receptor superfamily, member 8
NCBI reference sequence	NM_001258216.1	NM_001243.3
Biological role	Differentiation of T helper 1 cells and proliferation of T cells as well as natural killer cells	Positive regulator of apoptosis Limits the proliferative potential of autoreactive CD8 effector T cells and protects the body against autoimmunity
Related diseases	Allergic asthma [12,43] Autoimmunity [13] Burkitt's lymphoma [44]	Hodgkin's lymphoma [45] Atopic dermatitis [14] Allergic rhinitis [15]
Primer sequences (5'–>3')	Forward : ACT GGA GCC TCA GCA CAT CT Reverse : AGC CTC ACC ACT CAG AGC AT	Forward : GCT CAG ATG TTT TGG GGA AA Reverse : AGA CAC CCA CTC CAT CCT TG

*Biologic information of these genes were obtained from www.genecards.org.

(Agilent Technologies Inc., Santa Clara, CA) and reverse-transcribed with 10 μg of total RNA in the presence of an oligo(dT) T7 primer (iScript™ Select cDNA Synthesis Kit; Bio-Rad Laboratories Inc., Hercules, CA). This cDNA was used for *in vitro* transcription amplification in the presence of biotinylated nucleotides.

Target and housekeeping gene sequences were retrieved from GenBank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) and applied primers were designed manually using the Primer-BLAST tool (National Centre for Biotechnology Information, National Institutes of Health). Biologic properties and primer sequences of the target genes (IL12RB2 and TNFRSF8) are described in Table I. RNA mixtures were subjected to first-strand cDNA synthesis using forward primers. The endogenous housekeeping gene, β–actin (NM_001101.3), was used for data normalization and relative quantification was performed by relative standard curve analysis using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) with SYBR® Green I (Bio-Rad Laboratories Inc.) detection. cDNA (10 ng) was dissolved in iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.). For each cDNA sample, qRT-PCR was performed in quadruplicate and negative controls with no template were included for each primer pair. Threshold cycle (Ct) values and target gene expression levels were calculated using CFX Manager™ Software (Bio-Rad Laboratories Inc.). The fold change of target gene expression in each treated sample relative to the control sample (normalized expression) was derived from the negative Ct value: Ct (β–actin) – Ct (target gene).

Statistical analysis

Normalized expression of IL12RB2 and TNFRSF8 were expressed as mean ± SD. The statistical significance of differences in qRT-PCR results between groups was analyzed by one-way ANOVA followed by Tukey's *post-hoc* analysis using Statview® software version 5.0.1 (SAS Institute Inc., Cary, NC). *P*-values less than 0.05 were considered significant.

Predictive modeling

To assess the probability of OLP, predictive modeling with support vector machine (SVM), random forest (RF), linear discriminant analysis (LDA), neural network (NN) and naive Bayes (NB) methods and their receiver operative characteristic (ROC) curves were made. The following parameters were set as

Table II. Demographic data and severity of inflammation of the patients.

Characteristics	Non-OLP (n = 25)	OLP (n = 38)	Normal (n = 18)
Age (years, mean ± SD)	55.04 ± 18.78 (range: 14–85)	48.27 ± 10.82 (range: 22–68)	26.80 ± 10.62 (range: 13–52)
Gender			
Male	7 (28.0%)	14 (36.8%)	7 (38.9%)
Female	18 (72.0%)	24 (63.2%)	11 (61.1%)
Clinical form			
Erythematous/ulcerative	10 (40.0%)	2 (5.2%)	
Reticular	5 (20.0%)	8 (21.1%)	
Erythematous with reticular striae	3 (12.0%)	28 (73.7%)	
Other	7 (28.0%)	0 (0%)	
Location of lesion			
Buccal mucosa	12 (48.0%)	33 (86.8%)	10 (55.6%)
Tongue	4 (16.0%)	1 (2.6%)	
Other (gingiva, vestibule, palate etc.)	9 (36.0%)	4 (10.5%)	8 (44.4%)
Comorbidity			
Diabetes	5 (20.0%)	2 (5.3%)	
Hepatitis	1 (4.0%)	5 (13.2%)	
Cutaneous lesion	1 (4.0%)	2 (5.3%)	
Severity of inflammation			
Mild or mild to moderate	16 (64.0%)		
Moderate to severe	7 (28.0%)	38 (100%)	
Severe	2 (8.0%)		

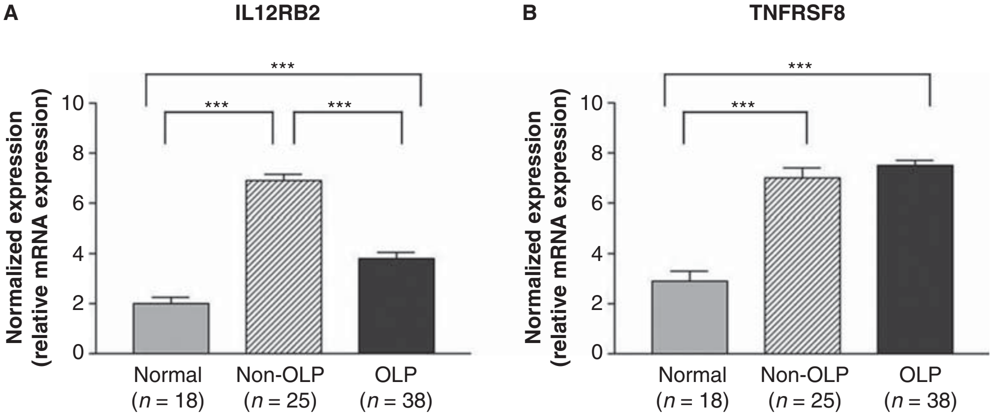

Figure 1. Comparison of normalized expression (relative mRNA expression) of IL12RB2 (A) and TNFRSF8 (B) between Normal, Non-OLP and OLP groups.

covariates for these analyses: age, gender, clinical form, location of lesion, presence of cutaneous lesion, comorbidity (diabetes mellitus and hepatitis), normalized expression of IL12RB2 and TNFRSF8 and severity of inflammation. All analyses were conducted using R statistical software version 2.15.0 (from http://www.r-project.org).

Results

Clinical characteristics of the patients

A total of 81 FFPE samples (M:F = 28:53), comprising 38 OLP, 25 non-OLP and 18 normal patients, were included in this study (Table II). The majority of cases in the OLP group were erythematous with reticular striae and were found in the buccal mucosa, while the common clinical form in the Non-OLP group was erythematous/ulcerative and found in the buccal mucosa. All OLP samples were categorized as moderate-to-severe inflammation.

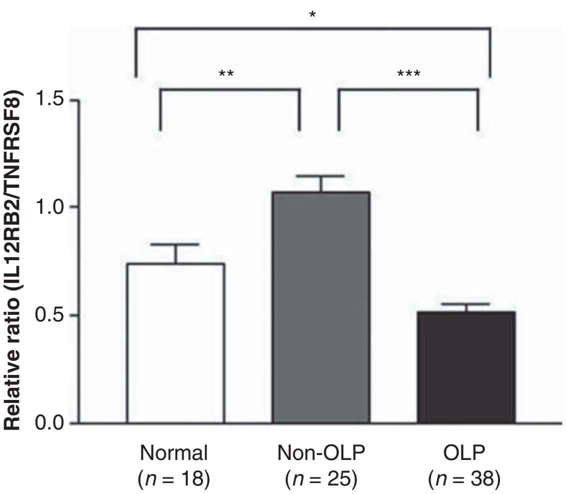

Figure 2. Comparison of the relative ratio of normalized expression of IL12RB2 and TNFRSF8 (IL12RB2/TNFRSF8) between Normal, Non-OLP and OLP groups.

Expression of IL12RB2 and TNFRSF8 mRNA in the normal, non-OLP and OLP groups

As described in Figure 1A, the normalized expression of IL12RB2 in the OLP (3.78 ± 1.67) group was significantly higher than the Normal group (1.97 ± 1.12, $p < 0.001$), but significantly lower than the non-OLP (6.86 ± 1.47, $p < 0.001$). In aspect of TNFRSF8 (Figure 1B), its expression in both OLP (7.46 ± 1.51) and Non-OLP groups (6.99 ± 1.96) were significantly higher than the Normal group (2.90 ± 1.61, $p < 0.001$), but there was no significant difference between the non-OLP and OLP groups ($p = 0.311$). The expression ratio of IL12RB2/TNFRSF8 in the OLP group (0.52 ± 0.23) was significantly lower than that of the Normal group (0.74 ± 0.39, $p < 0.05$) and the Non-OLP group (1.07 ± 0.38, $p < 0.001$; Figure 2).

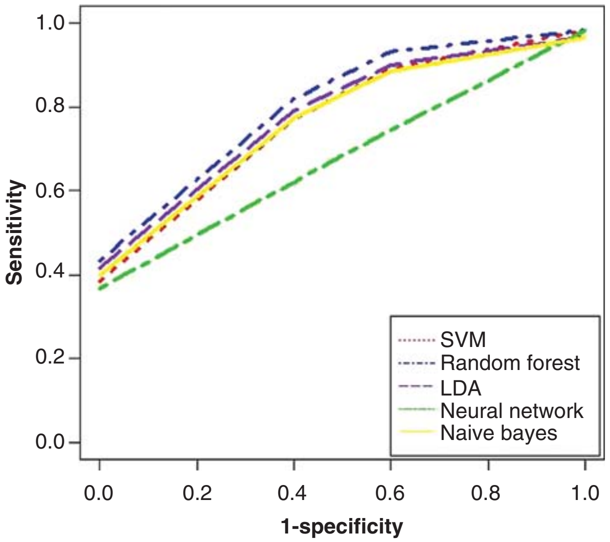

Figure 3. Receiver operating characteristic (ROC) curves of multiple prediction models. All ROC curves are approximate except for the NN curve. (SVM, support vector machine; LDA, linear discriminant analysis; RF, random forest; LDA, linear discriminant analysis; NN, neural network; NB, naive Bayes).

Predictive modeling

All ROC curves were approximated, with the exception of the NN method (Figure 3). The area under ROC curves (AUC) ranged from 0.87–0.92, but that of NN (0.83) was slightly lower than other methods. Accuracy was higher than 0.75 in all methods.

Discussion

As there was a close relationship between Th1/Th2 (T helper type 2) imbalance and pathogenesis of autoimmune diseases including Behcet's disease (Th1 dominant response) [17] and Sjögren syndrome (Th2 dominant response) [18], several recent studies have shown that Th1/Th2 imbalance also plays an important role in OLP pathogenesis [19,20]. Th1 cells primarily secrete interleukin 2 (IL-2), IL-12 and IFN-γ. A recent study reported IL-12p40 and IFN-γ proteins were significantly up-regulated in OLP [21]. In Th1 cells, IFN-γ stimulation up-regulates the transcription factor T-bet, which in turn maintains IL12RB2 chain expression [22,23]. Meanwhile, TNFRSF8 is expressed at high levels by activated cells in autoimmune and chronic inflammatory diseases, such as rheumatoid arthritis (RA) [24] and systemic lupus erythematosus (SLE) [25], which have similarities to OLP. It is preferably expressed on CD4 and CD8 T cell clones that produce Th2 cytokine [26,27].

Sugerman et al. [1] concluded that OLP was a Th1 cytokine biased disease based on previous studies describing the expression pattern of different cytokines/chemokines involved in Th1 and/or Th2 polarization. However, Rhodus et al. [19] suggested that OLP is Th2 dominant, based on the result of tissue transudates from OLP lesions. In addition, Liu *et al.* [20] reported that salivary IFN-γ (Th1 cytokine) levels decreased significantly compared with the control group. In a recent study, the authors reported that OLP patients showed a significantly higher level of serum IL-10 and lower IL-2 level compared to normal patients and concluded that a Th2 dominant response might shift the balance of Th1/Th2 in OLP [28]. In our study, IL12RB2 expression of the OLP group was significantly lower than that of the Non-OLP group. Considering that increased expression of IL12 and its receptors might enhance Th1 cytokine production [29–31], it might be acceptable that IL12RB2 (Th1-related) expression was decreased in Th2-dominant OLP. In respect of TNFRSF8, previous studies reported that its increased level played a role in the activation of Th2 cytokines-mediated immune response [26,32,33], which coincides with higher level of TNFRSF8 in Th2-dominant OLP. In the OLP group, IL12RB2 (numerator) expression was similar to that of other groups, but TNFRSF8 (denominator) expression was higher than that of other groups. As a result, the IL12RB2/TNFRSF8 ratio of the OLP group was significantly decreased. However, further investigations using other Th1 and Th2 cytokines would be warranted to clarify these findings.

Table III. Comparison of multiple prediction models for oral lichen planus (OLP) and corresponding receiver operating characteristic (ROC) curve values.

	SVM	RF	LDA	NN	NB
AUC	0.87	0.92	0.88	0.83	0.87
Sensitivity	0.77	0.90	0.87	0.79	0.77
Specificity	0.75	0.80	0.75	0.77	0.85
Accuracy	0.76	0.86	0.82	0.78	0.80

AUC, area under the ROC curve; SVM, support vector machine; RF, random forest; LDA, linear discriminant analysis; NN, neural network; NB, naive Bayes.

Although fresh tissue specimens are desirable for genomic study, the use of fresh tissue can be difficult due to issues with collecting sufficient tissue samples for the proposed studies and the need to design the collection of tissues in a prospective manner. In contrast, FFPE tissue blocks are routinely preserved and stored after pathological diagnosis and, therefore, represent invaluable resources for large-scale and multi-national gene expression studies.

Despite the merits of FFPE samples for large-scale and multi-national studies, their use for genomic analysis has been limited because the process of formalin fixation and paraffin embedding affects RNA quality, thus RNA degrades continuously over time. Fortunately, recent technical advances could enable successful RNA extraction [34–36]. Although RNA from FFPE is not appropriate for whole genome profiling because monomethylol groups cannot be removed completely, it is still suitable for analysis of the expression of specific genes and these are suitable for analysis of specific genes expression [8]. Hence, qRT-PCR is an optimal analysis tool for FFPE samples. To the best of the author's knowledge, this is the first study to perform qRT-PCR analysis using RNA extraction from FFPE samples, as well as to compare OLP and chronic mucositis to investigate biomarkers of OLP.

Since a single biomarker alone rarely provides enough information, a combination of IL12RB2 and TNFRSF8 was integrated in this study. Additionally, in order to minimize statistical errors and maximize the accuracy and diagnostic power, predictive modeling was performed with five prediction models, which have been shown to accurately predict clinical outcomes [37–41]. As demonstrated in Table III and Figure 3, all models showed acceptable sensitivity and specificity. They were regarded as 'moderately-to-highly' accurate tests according to the guidelines proposed by Greiner et al. [42].

However, in spite of meaningful outcomes, additional studies based on fresh specimens should be performed to confirm our results and validate these novel biomarkers in larger data-sets. For their clinical application, threshold levels (both for OLP and differential diagnoses) should be determined through large-scale, multi-center studies. With this approach, following such validation, use of these novel biomarkers could facilitate diagnosis of OLP in those cases where diagnostic uncertainty exists.

In conclusion, our study demonstrated that the IL12RB2/TNFRSF8 ratio has potential as a useful diagnostic tool for differentiating between OLP and chronic non-specific mucositis.

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