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Methylation and Expression Status of *SOCS*1 and *SOCS*3 in Oral Lichen Planus

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Abstract

Oral lichen planus (OLP) is a disease of unknown etiology affecting oral mucosa by mediated chronic inflammation and is classified as a potentially malignant oral disorder. *SOCS*1 and *SOCS*3 in the *SOCS* family have been identified as negative regulators of the cytokine-activated JAK/STAT pathway responsible for inflammatory reaction. The DNA methylation in the promoter regions of *SOCS*1 and *SOCS*3 have been reported to correlate with carcinogenesis. In this study, we performed methylation-specific PCR (MSP) to investigate the methylation status of the promoter regions in *SOCS*1 and *SOCS*3 genes using biopsy samples from OLP and oral squamous cell carcinoma (OSCC) patients. *SOCS*1 was methylated in 14/29 (48.3%) cases with OLP and 7/15 (46.7%) cases with OSCC. At the same time, *SOCS*3 was methylated in 25/29 (86.2%) cases with OLP and 11/15 (73.3%) cases with OSCC. We didn't recognize any DNA methylation in *SOCS*1 or *SOCS*3 genes from the exfoliated cytological specimens of normal buccal mucosa. Furthermore, mRNA expression level was analyzed using real-time RT-PCR method to evaluate the correlation with DNA methylation status. DNA methylation status of *SOCS*1 seemed not to affect the expression level of *SOCS*1 mRNA. At the same time, DNA methylation status of *SOCS*3 was negatively correlated with the expression level of *SOCS*3 mRNA (p < 0.05). We posit frequent methylation of the *SOCS*3 gene promoter, theoretically resulting in the increase of cytokines expression, might be associated with the etiological mechanism of OLP.

Keywords

Oral Lichen Planus, *SOCS*3, *SOCS*1, Methylation

1. Introduction

Oral lichen planus (OLP) is a T-cell-mediated chronic inflammatory disease that occurs in 2.2% of the general population of women aged 30 - 80 years [1] [2]. Lesions are most commonly located in the buccal mucosa, tongue, and gingiva. The disease can appear in six different clinical forms: reticular, plaque, popular, erosive, atrophic, and bullous [3] [4]. An OLP of erosive or ulcerative type is accompanied with strong subjective symptoms and requires treatment although some cases of OLP may be asymptomatic [5]. The etiology of OLP remains uncertain. Previous studies have reported its cause to be a delayed type hypersensitivity reaction by external substances, including metal allergy [6], and the interaction between environmental factors, such as hepatitis C virus (HCV) infection or autoimmune disease [6] [7]. As the same time, OLP was classified by the World Health Organization (WHO) as a precancerous condition in 2005 [8] and as an oral potentially malignant disorder, like leukoplakia in 2017 [9]. The transformation rate into malignancy of OLP has been reported to range from 0.4% - 12.5%. Therefore, it requires continuous follow-up.

On histopathological examination, OLP lesions present as the hydropic degeneration in the basal cell layer of the epithelium, band-like lymphocyte infiltrate in the lamina propria, and Civatte bodies in the epithelium [10]. Previous studies have reported that lymphocytes in OLP are involved in cellular immunity because of CD4 and CD8 positivity. CD4-positive T cells are classified Th1 cell products of IFN-γ and Th2 cell products of IL-4 or IL-5 [7]. It has been deemed that OLP occurs due to the alteration of Th1/Th2 cytokine balance. However, in recent reports, the increased production of Th1 cytokines has been reported to be a key event. IFN-γ released primarily from activated T-lymphocytes plays a central role in local response in OLP [11].

The suppressors of cytokine signaling (*SOCS*) are inhibitors of cytokine signaling that function via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. To date, the cytokine-inducible SH2-domain-containing protein (CIS) and *SOCS*1-7 have been identified as members of *SOCS* family [12] [13]. *SOCS*1 is shown to inhibit signaling through the direct interaction with JAK and the suppression of IFN-γ or IL-17 cytokine signaling by activating STAT1; thus, *SOCS*1 is associated with inflammation, such as chronic hepatitis B [14] [15] [16] [17]. *SOCS*1 also regulates the expression of IL-6 and EGFR involved in tumor growth by activating STAT3 [18]. *SOCS*1 activation is induced by the phosphorylation of p53 and the proteasomal degradation of NF-κB [19] [20] [21]. *SOCS*3 can bind the cytokine receptor and JAK and is recruited to the tyrosine-phosphorylated receptor, facilitating JAK inhibition [12]. *SOCS*3 is a key regulator of IL-6 or IL-10 activated by Toll-like cell receptor stimulation, and it also suppresses cell growth and cell survival by controlling the activation of STAT3 [22] [23] [24]. STAT3 is correlated with the activation of NF-κB [23] [24]. The activation of STAT3 by cytokines thorough JAK/STAT signaling pathway has found earlier to induce inflammation and subsequent carcinogene-

sis [22] [23] [24] [25] [26].

Epigenetic mechanisms occur independent of changes in the primary DNA sequence. And they are involved in the development of individuals and tumors as well as chronic inflammation. Epigenetic processes have involved DNA methylation, posttranslational histone modifications, and noncoding RNAs, all of which are related to gene expression and chromatin structure [27] [28].

Due to DNA promoter methylation and the decreased protein expression of *SOCS*1 and *SOCS*3, cytokines are continuously released. Released cytokines are involved in the occurrence of chronic hepatitis B and allergic asthma [17] [29]. Regarding the relationship between carcinogenesis and the methylation of promoter region, *SOCS*1 methylation has been found to be frequent in hepatocellular and pancreatic carcinomas, whereas *SOCS*3 methylation has been shown to be frequent in lung cancer, hepatocellular carcinoma, and others [17]-[32].

There are only two reports of an aberrant methylation of the promoter region in OLP. One has demonstrated the hypermethylation of *miR*-137 and *p*16 associated with cell cycles, while the other has demonstrated the hypermethylation of the *ELANE* gene associated with immune response [33] [34].

We hypothesized that the methylation of *SOCS*1 and *SOCS*3 correlates with chronic inflammation and malignant transformation in OLP. In this study, we investigated the mRNA expression of *SOCS*1 and *SOCS*3 as well as their methylation status in OLP, SCC and normal oral mucosa.

2. Material and Method

This research has been approved by the Kyushu Dental University Ethics Committee (15-4-Nomal).

2.1. Patients Characteristics

Surgical specimens were collected from patients at the Department of Oral Surgery of the Kyushu Dental University, Fukuoka, Japan between 2011 and 2017. The study population consisted of 40 patients (15 males, 25 females; mean age 64.86 ± 13.490; range 41 - 83 years; 13 patients <60 years old; 27 patients ≥60 years) undergoing biopsies which were diagnosed with oral lichen planus (OLP). The OLP was located in the buccal mucosa in 24 patients, tongue in eight, gingiva in five, and palate in three. A total of 20 patients (13 males, 7 females; mean age 69.1 ± 13.80 years; range 40 - 82 years; five <60 years old; 15 ≥60 years old) had oral squamous cell carcinoma (OSCC). The OSCC was located in the buccal mucosa in three patients, tongue in 10, gingiva in five, and floor of mouth in two. After tissue collection, the biopsy specimens were fixed in 10% formalin and paraffin-embedded blocks were prepared. For control, 10 patients (five males, five females; mean age 36.4 ± 8.87 years; range, 27 - 55 years) underwent collection of normal oral mucosal exfoliated cells by scratching unilateral buccal mucosa 10 times each using an interdental brush.

OLP and OSCC were diagnosed by two oral pathologists (N.Y. and K.M.).

2.2. DNA Extraction, Bisulfate Treatment, and Methylation-Specific PCR (MSP)

Genome DNA of OLP and OSCC was extracted from tissue sections (4-μm-thick × 8 sheets) from paraffin blocks of those samples by a QIAampDNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany). On the other hand, the Cica geneusR total DNA prep kit (for tissue; Kanto Chemical, Tokyo, Japan) was used to extract cytological samples of normal buccal mucosa.

Bisulfite modification of DNA was done with the extraEZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). The bisulfate-modified DNA samples were amplified by the Epitect MSP kit (Qiagen) and primers specific for methylated and unmethylated sequences. The primer sequences for detecting methylated and unmethylated *SOCS*1 and *SOCS*3 are summarized in Table 1. PCR amplification conditions were followed by 40 cycles of 95˚C for 30 sec, 55˚C for 30 min and 72˚C for 30 sec. PCR products were analyzed in 2% agarose gel stained with ethidium bromide and visualized under UV illumination.

2.3. RNA Extraction and RT-PCR

Total RNA from paraffin blocks was isolated using the RNeasy® FFPE Kit (Qiagen). TotalRNA was reverse transcribed by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). mRNA levels of *SOCS*1 and *SOCS*3 were assessed by real-time RT-PCR using SYBR green chemistry.

Table 1. *SOCS*1 and *SOCS*3 primers of MSP and real-time RT-PCR.

Primer	Sequences	PCR products (bp)	Ref.
MSP			
*SOCS*1			
Unmethylated	F; TGAAGATGGTTTTGGGATTTATGA	184	[35]
	R; CACAACTCCTACAACAACCACACAC		
Methylated	F; TGAAGATGGTTTCGGGATTTACGA	183	
	R; ACAACTCCTACAACGACCGCACG		
*SOCS*3			
Unmethylated	F; TAGTGTGTAAGTTGTAGGAGAGTGG	134	[35]
	R; CTAAACATAAAAAAATAACACTAATCCAAA		
Methylated	F; GTAGTGCGTAAGTTGTAGGAGAG	139	
	R; GTAAAAAAATAACGCTAATCCGAA		
Real-time RT-PCR			
*SOCS*1	F; TGGTAGCACACAACCAGGTG	92	[36]
	R; GAGGAGGAAGAGGAGGAAGG		
*SOCS*3	F; CAAGGACGGAGACTTCGATT	110	[36]
	R; GGAGCCAGCGTGGATCTG		
GAPDH	F; TGCACCACCAACTGCTTAGC	87	
	R; GGCATGGACTGTGGTCATGAG		

F; forward, R; reverse.

Diluted cDNA was added to Ultra-Fast SYGR Green (Agilent Technologies, Santa Clara, California, USA) and each of forward and reverse primers, and performed using the Roche Applied Science 96cycler (Roche Diagnostics GmbH, Hilden, Germany). In each case, triplicate threshold cyclevalues were obtained and averaged, and expression levels were then evaluated by the ΔΔCT method. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control to normalize expression data, which were analyzed using then: $2^{-\Delta\Delta CT}$ where,

$$\Delta\Delta CT = \left(C_{\text{t-target}} - C_{\text{t-reference}}\right)_{\text{treated-sample}} - \left(C_{\text{t-target}} - C_{\text{t-reference}}\right)_{\text{calibrator-sample}}.$$ Primers of *SOCS*1, *SOCS*3, and GAPDH are shown in Table 1.

2.4. Statistical Analysis

Data were given as mean ± SD. The Mann-Whitney U test was performed and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Methylation Status of *SOCS*1 and *SOCS*3 Genes by Methylation-Specific PCR (MSP)

The results of MSP are summarized in Table 2. Association between clinicopathological findings and MSP results in OLP are summarized in Table 3, and typical gel pictures of MSP are shown in Figure 1.

*SOCS*1 and *SOCS*3 gene methylation could be detected in 29/40 (72.5%) cases of OLP, 15/20 (75.0%) of OSCC, and 10/10 (100%) of normal oral exfoliated mucosa by the MSP method. *SOCS*1 methylation in 14/29 cases (48.3%) of OLP and 7/15 (46.7%) of OSCC was observed. However, all normal mucosa cases showed *SOCS*1 unmethylation. In contrast, *SOCS*3 methylation was found in 25/29 (86.2%) of OLP and 11/15 (73.3%) of OSCC cases. All normal mucosa cases showed *SOCS*3 unmethylation. Among the OLP cases, *SOCS*1 gene methylation was detected in 4/10 (40.0%) smoking and 9/17 (52.9%) nonsmoking patients. On the other hand, *SOCS*3 gene methylation was detected in 8/10 (80.0%) and 15/17 (88.2%) patients, respectively. When the frequency of methylation was compared according to clinical pictures, *SOCS*1 gene methylation was

Table 2. Results of MSP.

Gene	Disorders	Detected cases of MSP	Methylated cases	Unmethylated cases	Rate of methylation (%)
	OLP ($N = 40$)	29	14	15	48.3
*SOCS*1	OSCC ($N = 20$)	15	7	8	46.7
	Normal mucosa ($N = 10$)	10	0	10	0
	OLP ($N = 40$)	29	25	4	86.2
*SOCS*3	OSCC ($N = 15$)	15	11	4	73.3
	Normal mucosa ($N = 10$)	10	0	10	0

Table 3. Association between clinicopathological findings and methylated cases of *SOCS*1 and *SOCS*3 in OLP.

Clinicopathological findings		$N = 29$	*SOCS*1 methylated cases (%) $N = 14$	*SOCS*3 methylated cases (%) $N = 25$
Sex	Male	10	5 (50.0)	10 (100.0)
	Female	19	9 (47.4)	15 (78.9)
Age	≤60 years	9	6 (66.7)	8 (88.9)
	>60 years	20	8 (40.0)	17 (85.0)
Location	Buccal mucosa	22	9 (40.9)	19 (86.4)
	Tongue	3	2 (66.7)	2 (66.7)
	Gingiva	2	2 (100.0)	2 (100.0)
	Palate	2	1 (50.0)	2 (100.0)
Smoking history	Smoking	10	4 (40.0)	8 (80.0)
	Non-smoking	17	9 (52.9)	15 (88.2)
	Unknown	2	1 (50.0)	2 (100.0)
Clinical feature of OLP	Reticular type	16	8 (50.0)	14 (87.5)
	Erosive type	13	6 (46.2)	11 (84.6)
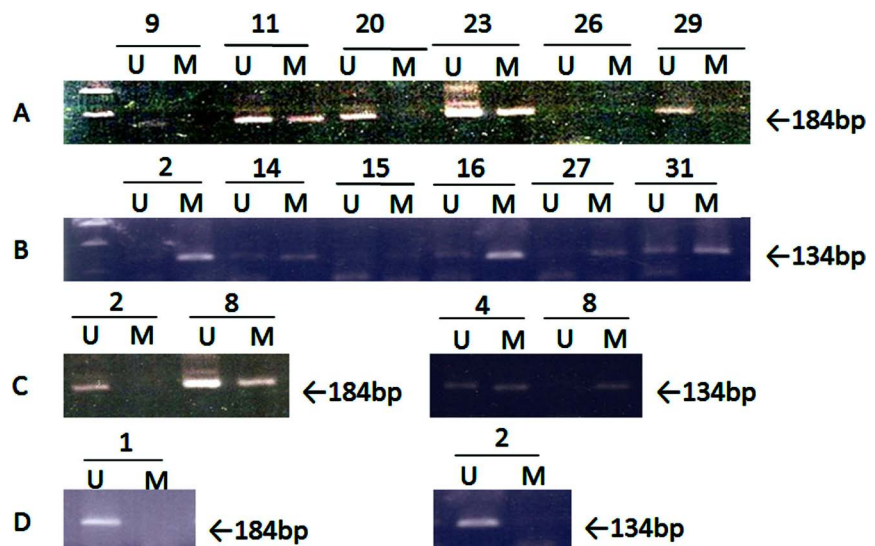
A: *SOCS*1 methylation status in OLP. Cases 9, 20, and 29 were found unmethylated; cases 11 and 23 showed partial methylation; case 26 was not detectable by MSP. B: *SOCS*3 methylation status in OLP. Cases 14, 16, and 31 showed partial methylation. Cases 2, 15, and 27 showed complete methylation. C: *SOCS*1 methylation status in OSCC. Case 2 showed unmethylation and case 8 showed partial methylation. D: *SOCS*3 methylation status in OSCC. Case 4 showed partial methylation. Case 8 showed complete methylation. E: *SOCS*1 methylation status in normal mucosa. Case 1 showed unmethylation. F: *SOCS*3 methylation status in normal mucosa. Case 2 showed unmethylation.

Figure 1. Results of MSP analysis.

found 8/16 (50.0%) reticular and 6/13 (46.2%) erosive types, whereas *SOCS*3 gene methylation was found in 14/16 (87.5%) and 11/13 (84.6%), respectively. Of three patients with HCV, two had *SOCS*1 and one had *SOCS*3 methylation.

Similarly, there was no significant difference in methylation status between *SOCS*1 and *SOCS*3 in sex, age, location and clinical features (Table 3).

3.2. *SOCS*1 and *SOCS*3 mRNA Expression by Real-Time RT-PCR

We detected mRNA expression levels of *SOCS*1 and *SOCS*3 genes in cases that were detectable by MSP. *SOCS*1 mRNA expression level in OLP was significantly higher than that in SCC and normal mucosa (p < 0.05, Figure 2). There were no significant differences in *SOCS*3 among the three groups, although OSCC showed a tendency for higher mRNA expression level than OLP and normal mucosa (Figure 3). In OLP, there were no significant differences between mRNA expression levels of *SOCS*1 and *SOCS*3 and clinicopathological findings (sex, age, location, smoking history, and clinical types; data not shown).
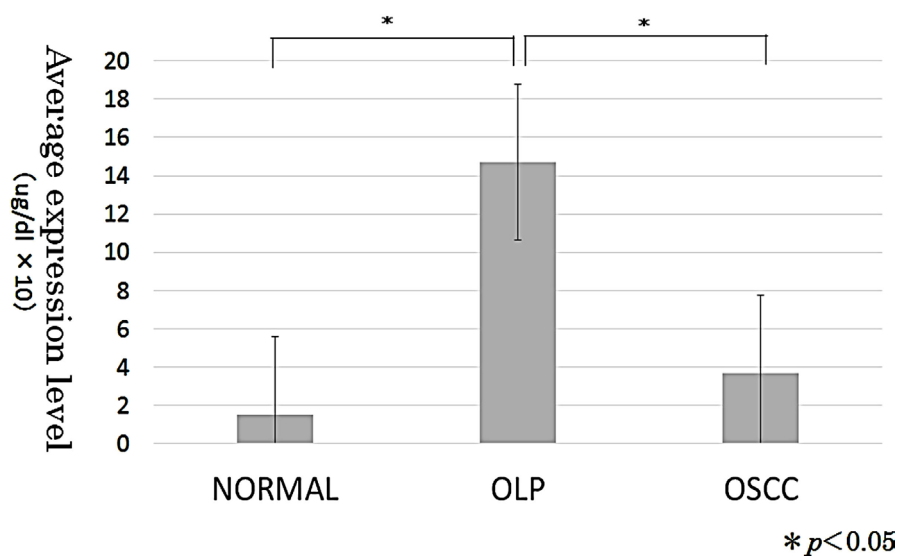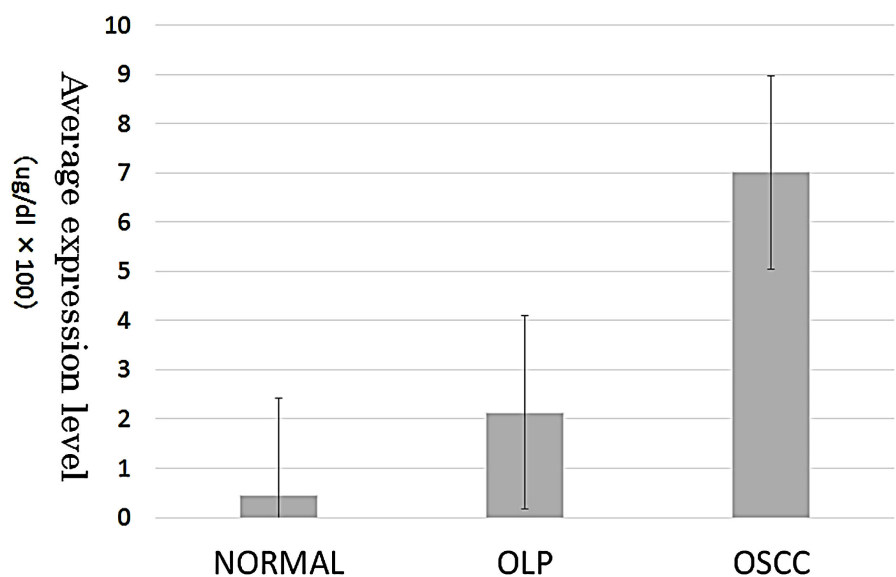

Figure 2. mRNA levels of *SOCS*1.

Figure 3. mRNA levels of *SOCS*3.

3.3. Correlation of *SOCS*1 and *SOCS*3 Methylation with mRNA Expression

There were no differences in *SOCS*1 mRNA expression level between DNA methylation and mRNA expression (Figure 4). *SOCS*3 mRNA expression level in OLP and OSCC methylated groups was significantly lower than that in normal mucosa. Moreover, it was significantly higher than that in unmethylated groups (Figure 5).

4. Discussion

The etiology and pathogenesis of OLP is not completely understood. Many studies have focused on the association with environmental factors, like metal allergy, HCV, autoimmune disease and others [6] [7]. Epigenetics contribute to chronic inflammation and the development of individuals and tumors by regulating gene
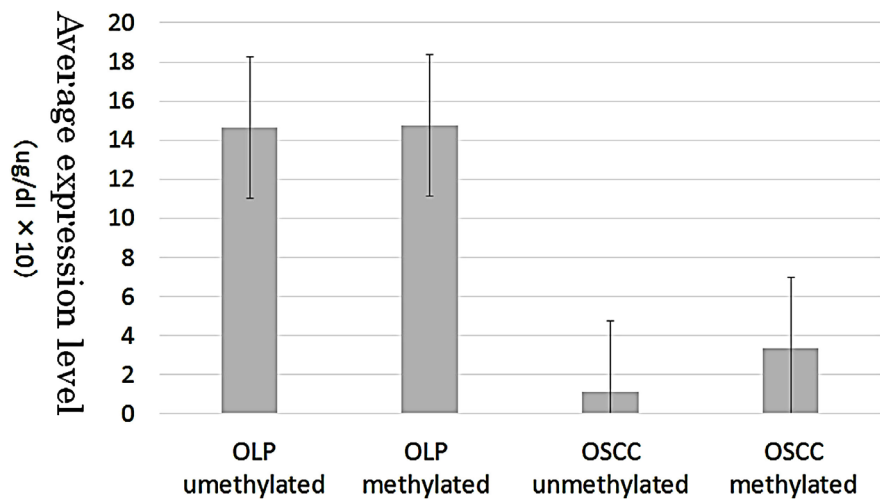

Figure 4. Correlation between methylation and mRNA expression level in *SOCS*1.
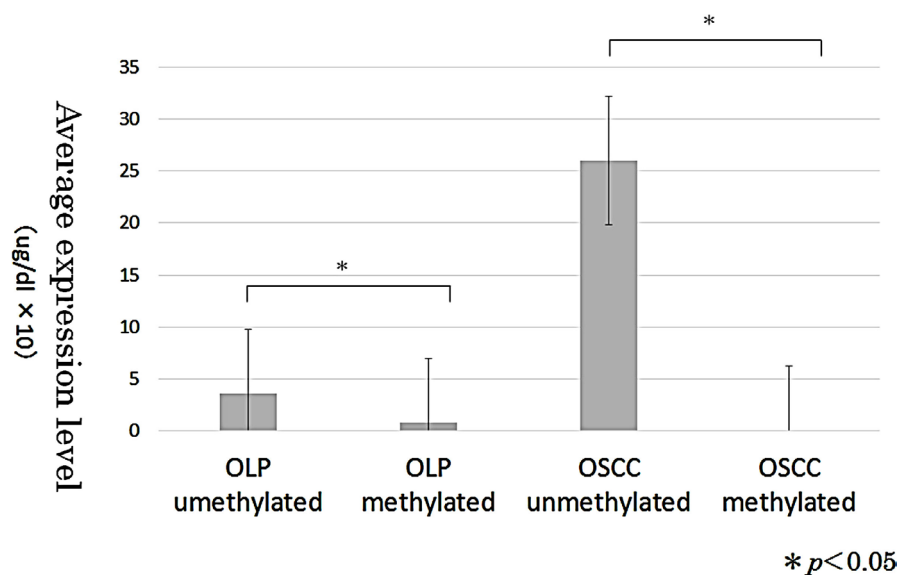

$* p < 0.05$

Figure 5. Correlation between methylation and mRNA expression level in *SOCS*3.

expression by acquired qualification by environment without dependency on nucleotide sequence. Epigenetics contribute to chronic inflammation and the development of individuals and tumors by regulating gene expression by acquired qualification by environment without dependency on nucleotide sequence. One of the epigenetic mechanisms is the DNA methylation of the promoter region [27] [28].

*SOCS*3 is involved in the negative regulation of inflammatory cytokines, such as IL-6 or IL-10, by interacting with the JAK/STAT pathway [22] [23] [24] [25] [26]. Furthermore, recent studies have shown that suppression of protein expression by aberrant methylation in the promoter region of the *SOCS*3 gene is associated with several types of malignancy, such as hepatocellular carcinoma, multiple myeloma, malignant melanoma, and colorectal carcinoma [32] [33] [34] [37] [38] [39]. In this study, *SOCS*3 methylation was found in 25/29 OLP (86.2%) and in 11/15 SCC (73.3%) cases. Moreover, it was found that the levels of mRNA for the *SOCS*3 genes were reduced in the methylated group compared to the unmethylated group in OLP and SCC. In addition, the level of mRNA expression in OLP was lower than that in SCC. In our study, we used the same primers as did Weber *et al.* [35], who designed primers of the promoter region of *SOCS*1 and *SOCS*3. They have reported that the inactivation of *SOCS*3 has observed frequently in head and neck SCCs is due mainly to 90% methylation, and *SOCS*3 is detected with lower staining in the methylated than unmethylated groups on the protein level. In the cell lines of the SCCs, *SOCS*3 transcripts have increased on treatment with demethylation [35].

*SOCS*3 is induced by tyrosine-phosphorylated STAT3 and terminates STAT3 phosphor ylation after initial exposure of cells to members of the IL-6 family of cytokines by binding cooperatively to the common receptor subunits gp130 and JAK1 and 2 [40]. Du *et al.* [41] have examined the immunoexpression and phosphorylation status of STAT3 of OLP using PathScan analysis and have found that pSTAT3 expression increased gradually in the normal mucosa, reticular OLP, and erosive OLP groups. They suggested that pSTAT3 might be involved in OLP development and progression [41]. On the other hand, a relationship has been reported between activation of STAT3 and Th17-induced cytokine, including IL-6 in malignant tumor, inflammatory bowel disease, and psoriasis [42] [43]. Th17-induced cytokines have activated STAT3 [42]. A previous study has shown that a new set, called Th17 cells, has been identified recently beyond the traditional Th1 and Th2 cells. Th17 cells produce IL-17A, IL-17F, IL-6, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-21, IL-26, and IL-22. Th17 cells have an important role in the protection against extracellular bacteria and fungi, in inflammation, and in the development of autoimmune diseases [44]. Th17-type molecules are increased in erosive OLP, whereas Th2-type molecules predominate in reticular OLP [44]. Activated STAT3 is interconnected with epidermal growth factor receptor (EGFR) and NF-κB, so that is related to tumor expression

[18] [23] [24]. Therefore, our research suggested cases of methylated status and low mRNA expression level of *SOCS*3 in OLP and SCC might be activated STAT3 and correlate with chronic inflammation and tumorigenesis. In addition, the occurrence of OLP and SCC might be controlled by the demethylation of *SOCS*3.

In this study, there is no statistical difference between methylation status and mRNA expression levels of *SOCS*1 gene in OLP and SCC. Previous studies of malignant tumors, such as hepatocellular carcinoma, and pancreatic carcinoma, have shown methylated *SOCS*1 and a decrease in mRNA levels and protein [14] [17] [30]. Our study used the *SOCS*1 primer similarly to those devised by Weber *et al.* Although they have reported that the methylation status of *SOCS*1 was detected in 10/94 head and neck OSCC samples (11%) [38], our study showed a high rate of the methylation status of *SOCS*1 in 7/15 OSCC (46.7%) and 14/29 OLP (48.3%) samples. The MSP method has detected a low concentration of methylated templates in an excess of unmethylated DNA. Consequently, the MSP method has a high analytical sensitivity [45]. This specificity is achieved by including many CpG in the primer sequences, preferably at or close to the 3' end, but it also facilitates amplification of incompletely converted sequences in the bisulfite-treated DNA and can be false positives [46] [47]. The possibility that *SOCS*1 methylation occurs at a high rate in this study because of incompletely converted molecules may mimic methylated sequences, although that confirms that it does not nonspecifically amplify with negative control. In our research, the reason for choosing the MSP method is highly sensitive method. Because OLP specimen is small size and DNA amount extracted formalin-fixed paraffin embedded tissue with bisulfate treatment is low concentration. Practically, DNA methylation of *SOCS*1 and *SOCS*3 were detected by the methylation-specific PCR (MSP) method in 29/40 (72.5%) cases with OLP in this study.

The mRNA expression level of *SOCS*1 is higher in OLP than in SCC in this study. Increased *SOCS*1 mRNA levels correlated with increased IL-10 expression levels in periapical granuloma [48], and with phosphorylated STAT1 expression levels in ligature-induced periodontitis in rats [49]. Furthermore, the expression of phosphorylated STAT1 is allowed in immunostaining of OLP [50]. In this study, increase of *SOCS*1 mRNA expression levels in OLP may be related closely to the activation of STAT1

5. Conclusion

Methylation of the *SOCS*3 promoter region resulted in the decreased expression levels of *SOCS*3 mRNA in OLP. Our data suggested that frequent methylation of the *SOCS*3 gene promoter, theoretically resulting in the increase of cytokines expression, might be associated with the etiological mechanism of OLP.

Acknowledgements

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