

Changes in Biomarkers after Initial Periodontal Treatment in Gingival Crevicular Fluid from Patients with Chronic Periodontitis Presenting with Drug-Induced Gingival Overgrowth

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Received 18 January 2016; accepted 26 February 2016; published 29 February 2016

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Abstract

Calcium channel blocker-induced gingival overgrowth (CCB-GO) is increasing in elderly patients who have been prescribed medication for hypertension for years. The purpose of the present study was to analyze the comprehensive protein expression levels of candidate biomarkers in gingival crevicular fluid (GCF) from CCB-GO patients. Eleven GO patients (10 males and one female, mean \pm SD: age: 64.4 \pm 14.0 years) who had been systemically prescribed CCBs, either amlodipine or nifedipine, for hypertension for at least 12 months were recruited. Before (baseline) and 4 weeks after initial periodontal treatments, subgingival plaque and GCF samples were taken from two sites per patient: sites affected by CCB-GO and chronic periodontitis. Measurement of clinical parameters and quantitative analysis of periodontopathic bacteria using real-time PCR were performed. Biomarkers/cytokines in GCF were examined using multiplex bead immunoassays. The Mann-Whitney *U* test was used to compare the collected data between groups. The correlations between pairs of biomarkers were assessed using the Spearman correlation relationship. Levels of two of the 14 biomarkers, interleukin (IL)-1 β and transforming growth factor (TGF)- β , were significantly decreased in CCB-GO sites after initial periodontal therapy. The intragroup comparison

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at baseline showed that counts of *Treponema denticola* in the GO group were significantly higher than those in the chronic periodontitis group ($P < 0.05$). The results confirmed that initial periodontal therapy effectively reduces pro- and anti-inflammatory cytokines such as IL-1 β and TGF- β in CCB-GO patients. These factors are involved in initiation and progression of GO as well as periodontitis.

Keywords

Gingival Overgrowth, Gingival Crevicular Fluid, Multiplex Bead Immunoassay, Periodontitis

1. Introduction

Drug-induced gingival overgrowth (GO), a side effect of calcium channel blockers (CCB), immune suppressors, and anti-seizure drugs, is characterized by fibrosis with inflammation of the gingivae. In particular, CCB-GO has been increasing in Japan as well as internationally due to the rise in the use of medication in patients with hypertension. CCB such as nifedipine and amlodipine have been widely used to treat hypertension and/or angina. GO has been observed in 6% - 30% of patients taking these drugs [1] [2]. Multiple risk factors for CCB-GO have been suggested including age, sex, genetic pre-disposition, duration of therapy, gingival inflammation, and concomitant medications, such as cyclosporine A [3].

GO results from the deposition of excessive extracellular matrix, reflecting both overproduction of collagen and reduced levels of collagen breakdown [4]-[6]. We have previously reported that matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play a role in the pathogenesis of periodontitis [7]-[10] as well as GO [11] [12] by controlling extracellular matrix metabolism.

We have also reported the specific transcription/transcriptome in GO-affected gingival tissues with microarray analyses [13]. Oral hygiene may play a key role, as it exacerbates the expression of drug-induced GO. In a particular clinical case of GO, it is often observed gingival inflammation with deep-pocket depth as well as periodontitis, sharing common characteristics including patient age and oral hygiene levels [14].

Periodontitis is diagnosed almost entirely based on an array of clinical attachment level (CAL), bleeding on probing, and radiographic findings. However, probing pocket depth (PPD) and CAL measurements using periodontal probes and radiographic bone levels provide information about past periodontal tissue destruction but do not elucidate the current state of disease activity or prognosis. Therefore, identification of a method for predicting the progression of periodontal diseases as well as GO or at least for declaring the current state of disease activity is necessary. Numerous previous studies have evaluated the association between periodontal diseases and factors found in gingival crevicular fluid (GCF) [15] [16]. Identification of biomarkers in GCF is considered important for the diagnosis and treatment of periodontal diseases [17] [18]. Thus, the relationships between the subgingival microbiota and host mediators should be examined by collecting subgingival biofilm and GCF samples from the same periodontal sites. Only a few investigators have attempted to measure the levels of GCF biomarkers and microbiological data obtained from the same sites [19] [20].

Recently, multiplex immunoassays have been used for samples with limited volumes such as GCF. We also previously reported the simultaneous, successful detection of levels of various types of cytokines and chemokines in GCF from periodontitis patients by using multiplex immunoassays [15].

The aim of the present study was to simultaneously quantify the levels of 14 biomarkers in GCF samples from sites with both GO and chronic periodontitis (CP) by multiplex immunoassays [15]. In addition, we examined the relationship between the subgingival microbiota and GCF biomarkers before and after therapy.

2. Materials & Methods

2.1. Study Design

Eleven GO patients (10 males and 1 female, mean \pm SD: age: 64.4 \pm 14.0 years) who had been systemically prescribed a CCB, either amlodipine or nifedipine, for hypertension treatment for at least 12 months were recruited. Before (baseline) and 4 weeks after initial periodontal treatments including plaque control, scaling, and root planing, GCF samples were taken from two sites per patient: GO-affected and periodontitis-affected peri-

odontal pockets. GO sites were selected as those that were at least GO grade 2 according to GO grades defined by Eva Ingles *et al.* [21].

Informed consent was obtained prior to the study from all subjects in accordance with the Helsinki Declaration, and the study protocol was approved by the Institutional Review Board of the Faculty of Dentistry, Niigata University. All patients were told that they had the right to withdraw from the study at any time.

2.2. Clinical Assessments

A thorough medical and drug history was obtained for each patient. Smoking habits were also recorded (number of cigarettes/day, years of smoking). For more than 1 month before the study, all subjects received standard oral hygiene instructions and underwent full-mouth supragingival scaling and eventually showed a plaque control record of <20%. One week before the start of the study, a full-mouth periodontal examination was performed. The following clinical parameters were recorded: PPD, CAL, and bleeding on probing. They were recorded at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) with a periodontal probe (CP-12 Color-Coded Probe; Hu-Friedy, Chicago, IL, USA) [22]. The sites with the most severe periodontal condition based on clinical findings were selected for sampling.

2.3. Sample Collection

A GO (PPD ≥ 5 mm) and a CP site (PPD ≥ 4 mm) were sampled in each subject. Before sampling, the individual tooth site was isolated with cotton rolls, supragingival plaque was removed carefully, and the site was gently air-dried with an air syringe. Subgingival plaque samples were taken before GCF sampling. The subgingival plaque samples were collected using paper points (10 s \times two times), and the samples were placed in separate microcentrifuge tubes. Then, paper strips were placed into the pocket until mild resistance was sensed and left in place for 30 s to obtain GCF samples. Samples visually contaminated with blood or diluted with saliva during sampling were discarded. The procedure was repeated four times at the same site using new strips each time. After recording the measurements, they were converted into actual GCF volumes using a calibration graph. The GCF samples were placed in tubes with a transport medium containing 200 μ l phosphate-buffered saline without calcium chloride or magnesium chloride and with 0.5% bovine serum albumin. After shaking for 15 min, the eluates were centrifuged for 10 min at 12,000 $\times g$ to remove plaque and cellular elements, and the strips were removed. The samples were frozen at -80°C until further analysis.

The multiplex bead immunoassay used in this study allows a more comprehensive assessment of mediators within a single sample and is efficient and cost effective compared with traditional methods. The results obtained with the multiplex bead immunoassay were not compared with other conventional assays, such as an enzyme-linked immunoassay (ELISA). However, a recent study comparing 14 different immunoassays with different detection platforms (including a traditional solid-phase sandwich ELISA and immunobead-based assays using the fluidics Luminexfluorometry platform) reported interassay disparities as well as significant interlaboratory variation, suggesting that studies using a given assay cannot be directly extrapolated or compared with studies utilizing another assay [15]. This pilot study used a multiplex antibody capture and detection platform to quantitatively assess the effects of initial periodontal therapy on a comprehensive panel of cytokines and MMPs in GCF.

2.4. Cytokine and Chemokine Analysis

The following cytokines and chemokines, which are involved in inflammation and fibrosis, were selected as candidate GCF biomarkers according to our previous study [14] and analyzed: interleukin (IL)-1 α , IL-1 β , IL-6, IL-8, MMP-1, MMP-3, MMP-12, MMP-13, transforming growth factor (TGF)- β , intercellular adhesion molecule (ICAM)-1, tumor necrosis factor (TNF)- α , basic fibroblast growth factor (FGF-b), platelet-derived growth factor (PDGF)-bb, and vascular endothelial growth factor (VEGF)-A. The levels in GCF (pg/30 s \times four times) were determined using a commercial multiplexed fluorescent bead-based immunoassay (Panomics[®], Fremont, CA, USA) in a Bio-Rad instrument (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Bacterial Analysis

Two paper points were collected in a sterile plastic tube and stored at -30°C . Universal primers targeting the 16S

ribosomal RNA gene, which is expressed and conserved in many types of bacteria, were used to estimate the total number of bacteria [23]. The presence and quantity of *Porphyromonas gingivalis*, (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), and *Treponema denticola* (*T. denticola*) from the subgingival plaque were determined using real-time PCR analysis according to the method reported by Lyons *et al.* [24]. The number of bacteria was represented by the estimated number of cells per 10 μ l plaque suspension. Each ratio was calculated as the number of each type of bacteria to the total bacterial counts.

2.6. Statistical Analysis

Total cytokine levels (pg/30 s \times four times; concentration of biomarkers in GCF collected four times) were analyzed and reported for each cytokine. Non-parametric approaches were used based on the distribution of the data. The statistical significance of the differences in clinical parameters, GCF volumes, and cytokine and chemokine levels in the GCF samples between the two groups was determined using the Mann-Whitney *U* test. In each case, the level of significance was set at $P < 0.05$. The correlations between pairs of biomarkers were assessed using the Spearman correlation relationship.

3. Results

The clinical parameters of the GO and CP sites selected for sampling are shown in **Table 1**. The GO grade and the PPD in the GO group were significantly decreased after initial periodontal treatment. In addition, the PPD, CAL, and gingival index in the CP group were also significantly decreased.

In total, 12 of the 14 evaluated biomarkers were detectable within the range of the assay and were present in both GO and CP sites (**Table 2**). Two of the 14 biomarkers (FGF-b, PDGF-bb) were partially or completely undetectable because their levels were too low for detection by the assay.

The markers detectable in both GO and CP sites were: the cell adhesion factor, ICAM-1; the cell growth factor, VEGF-A; and the inflammatory factors, MMP-1, MMP-3, MMP-12, MMP-13, IL-1 α , IL-1 β , IL-6, IL-8, TGF- β , and TNF- α (**Table 2**). IL-1 β and TGF- β in the GO group were significantly decreased after adjustment for multiple comparisons using the Wilcoxon signed-rank test ($P < 0.05$). No significant differences were found for any of the biomarkers in the CP group. No significant differences in biomarkers were observed at baseline with intragroup comparison (**Table 2**).

At baseline, IL-1 α , IL-1 β , and IL-8 levels in GO sites were relatively higher than in CP sites. However, no significantly different markers were detected between before treatment for GO and before treatment for CP. The correlations between pairs of protein levels are displayed in **Table 3**. Thirteen of 66 possible combinations showed a tendency for a strong correlation: IL-1 α /IL-1 β , IL-1 β /IL-8, IL-1 β /MMP-3, IL-1 β /TGF- β , IL-6/MMP-1, IL-6/MMP-3, IL-6/TNF- α , IL-8/MMP-3, IL-8/TNF- α , MMP-1/MMP-3, MMP-1/TGF- β , MMP-1/TNF- α , and MMP-3/TGF- β (**Table 3**).

Table 4 shows inter- and intragroup comparisons of subgingival bacterial counts. The *T. forsythia* ratio (to

Table 1. Clinical assessments in periodontitis patients before and 4 weeks after treatments (mean \pm SD).

	GO			CP		
	Baseline	After	P-value	Baseline	After	P-value
GO Grade	2.1 \pm 0.7	1.4 \pm 0.7	0.03	-	-	
PPD (mm)	6.0 \pm 1.4	4.0 \pm 1.7	0.02	5.0 \pm 0.7	3.0 \pm 1.0	0.01
CAL (mm)	5.0 \pm 1.1	4.0 \pm 1.5	0.23	5.0 \pm 1.1	4.0 \pm 1.1	0.03
GI	1.0 \pm 0.5	1.0 \pm 0.6	0.29	1.0 \pm 0.6	0.0 \pm 0.5	0.03
BOP (% positive)	54.5 \pm 52.2	36.4 \pm 50.5	0.32	36.4 \pm 50.5	27.3 \pm 46.7	0.99
Mo	0.2 \pm 0.4	0.2 \pm 0.4	0.99	0.0 \pm 0.0	0.0 \pm 0.0	0.99
BL (%)	27.5 \pm 34.3	-		15.8 \pm 21.5	-	

Before vs. after: Wilcoxon signed-rank test; level of significance, $P < 0.05$. GO grade defined by Ingles *et al.* (New clinical index for drug-induced gingival overgrowth. Quintessence Int. 1999; 7: 467-73). PPD: Probing pocket depth; CAL: Clinical attachment level; GI: Gingival index; BOP: Bleeding on probing; Mo: Mobility of tooth; BL: bone loss by radiographic examination.

Table 2. Protein levels (pg/ml) of GCF biomarkers before and 4 weeks after treatment (median IQR).

	GO			CP			*Intragroup comparison at baseline
	Baseline	After	P-value	Baseline	After	P-value	P-value
IL-1 α	799.1 [1191.2]	683.4 [629.7]	0.25	332.7 [795.7]	370.7 [607.1]	0.48	0.38
IL-1β	73.9 [120.0]	53.8 [68.2]	0.02	46.3 [78.8]	23.6 [33.6]	0.79	0.18
IL-6	2.0 [2.1]	1.2 [1.7]	0.21	1.8 [3.2]	1.8 [0.6]	0.42	0.67
IL-8	218.8 [155.0]	222.0 [112.7]	0.66	161.1 [171.8]	106.5 [94.1]	0.33	0.97
ICAM-1	2007.3 [2856.1]	2157.3 [557.7]	0.42	2255.0 [3246.7]	2038.8 [2310.5]	0.92	0.72
MMP-1	71.0 [92.5]	108.1 [76.8]	0.48	69.3 [52.0]	63.6 [90.2]	0.66	0.92
MMP-3	214.4 [395.9]	206.4 [326.5]	0.48	202.5 [178.1]	91.4 [108.7]	0.13	0.20
MMP-12	142.1 [338.6]	322.5 [370.9]	0.13	231.2 [377.0]	109.1 [162.3]	0.51	0.40
MMP-13	611.6 [3143.6]	1444.0 [2742.7]	0.79	1073.4 [1780.8]	811.9 [1582.1]	0.29	0.72
TGF-β	4.4 [5.6]	3.1 [3.1]	0.01	4.1 [5.1]	1.7 [1.3]	0.29	0.49
TNF- α	1.3 [0.6]	1.8 [1.2]	0.79	1.8 [2.4]	1.4 [1.2]	0.88	0.53
VEGF-A	6.6 [9.9]	7.1 [8.1]	0.25	6.3 [6.7]	6.7 [4.9]	0.33	0.41

before vs. after: Wilcoxon signed-rank test; level of significance, $P < 0.05$, GO-before vs. CP-before: Mann-Whitney U test; level of significance, $P < 0.05$, IQR = Interquartile range; Not detectable: FGF-b, PDGF-bb; Values are the median IQR.

Table 3. Correlations between pairs of GCF biomarkers.

Variable	IL-1 α	IL-1 β	IL-6	IL-8	MMP-1	MMP-3	MMP-12	MMP-13	TGF- β	TNF- α	VEGF- β	ICAM-1
IL-1 α		0.66	0.18	0.18	0.05	0.19	-0.41	-0.56	0.41	-0.13	0.25	-0.05
IL-1 β			0.11	0.62	0.32	0.62	-0.50	-0.53	0.74	0.28	0.25	-0.82
IL-6				0.55	0.62	0.67	0.39	0.22	0.10	0.69	-0.30	0.10
IL-8					0.64	0.82	-0.25	0.11	0.41	0.63	-0.20	0.25
MMP-1						0.72	0.38	0.37	0.62	0.62	-0.01	0.36
MMP-3							0.03	-0.11	0.62	0.54	0.03	0.07
MMP-12								0.55	-0.27	0.33	-0.16	0.37
MMP-13									-0.54	-0.52	0.10	0.67
TGF- β										-0.11	0.65	-0.31
TNF- α											0.65	0.50
VEGF- β												-0.71
ICAM-1												

Statistics: Spearman correlation relationship. Bold: significant correlations.

total bacteria) in the CP group was significantly decreased after 4 weeks compared to baseline ($P < 0.05$). The intragroup comparison at baseline showed that the counts of *T. denticola* in the GO group were significantly higher than that of the CP group ($P < 0.05$).

4. Discussion

In the present study, we demonstrated that two biomarkers, IL-1 β and TGF- β , in GCF were significantly decreased in GO patients after initial periodontal treatment. The periodontal pathogenic bacteria *T. denticola* were significantly higher in GO patients presenting with periodontal pockets and were decreased after treatment.

Table 4. Multiple logistic regression analysis for progression of chronic periodontitis.

	GO			CP			Intragroup comparison
	Baseline	After 4 weeks	P-value	Baseline	After 4 weeks	P-value	P-value [*]
Total bacterial amount	5.3 (0.6)	4.8 (0.6)	0.17	4.8 (0.4)	4.6 (0.5)	0.31	0.43
<i>P. gingivalis</i> (log ₁₀)	0.5 (1.8)	0.5 (0.0)	0.66	0.50 (0.0)	0.50 (0.0)	0.08	0.55
<i>T. forsythia</i> (log ₁₀)	2.50 (2.9)	2.10 (2.5)	0.37	2.40 (2.0)	0.50 (1.0)	0.08	0.21
<i>T. denticola</i> (log ₁₀)	2.10 (1.8)	1.70 (1.6)	0.52	0.50 (0.9)	0.50 (0.0)	0.16	0.04
<i>P. gingivalis</i> ratio (%)	0.00 (0.0)	0.00 (0.0)	0.66	0.00 (0.0)	0.00 (0.0)	0.08	0.66
<i>T. forsythia</i> ratio (%)	0.41 (0.78)	0.16 (0.31)	0.32	0.08 (0.29)	0.00 (0)	0.04	0.35
<i>T. denticola</i> ratio (%)	0.04 (0.08)	0.04 (0.02)	0.65	0.00 (0)	0.00 (0)	0.25	0.28

Before vs after: Wilcoxon signed-rank test; before-GO vs before-CP: Mann-Whitney U test; level of significance, $P < 0.05$, IQR = Interquartile range. The ^{*} represents the intragroup comparison between baseline in GO and CP before treatments.

The levels of IL-1 β and TGF- β in GO-GCF were significantly decreased 4 weeks after initial conventional periodontal treatments including scaling and root planing. The decreased IL-1 β and TGF- β levels seemed to be associated with levels of gingival enlargement and fibrosis [25] [26].

IL-1 β stimulates the proliferation of fibroblasts, keratinocytes, and endothelial cells and enhances the synthesis of type 1 procollagen, hyaluronate, and fibronectin [13]. As seen in periodontitis, the pathogenesis of GO is also modified by proliferation, growth factors, and MMPs [13]. Lu *et al.* reported that nifedipine and IL-1 β play important roles in GO via androgen receptor up-regulation that results in collagen accumulation [27]. Similar pathogenesis was also reported in cyclosporine A- and phenytoin-induced GO [28]-[31]. Recently, GCF IL-1 β levels in patients with CCB-GO were reported to be significantly decreased after periodontal treatment in GO patients, which is in agreement with our results. Thus, IL-1 β may play a role in GO pathogenesis.

TGF- β is widely recognized as a key fibrogenic cytokine [32]. TGF- β 1 is also a multifunctional cytokine with pleiotropic properties, exhibiting both pro-inflammatory and anti-inflammatory effects that modulate cell proliferation and differentiation by inhibiting the destruction of extracellular matrix proteins through the suppression of matrix-degrading proteinases [19]. Kim *et al.* demonstrated that nifedipine up-regulates periostin in human gingival fibroblasts in a TGF- β -dependent manner [33]. The present study demonstrated significantly decreased TGF- β levels in GO-GCF after initial periodontal treatments, implying a similar relationship between TGF- β levels and the degree of fibrosis in GO patients. Wright *et al.* also reported that subjects with high levels of TGF- β may be at greater risk for cyclosporine-induced gingival overgrowth [19]. Findings including ours suggested that TGF- β levels could contribute to the fibrotic changes in gingival connective tissues in patients with CCB-GO as well.

The etiology of CCB-GO appears complex, similar to periodontitis [34]. Gingival tissues of CCB-GO patients are often seen with clinically mixed features of GO and CP [14]. Correlation analysis revealed that IL-8 and MMP-3, IL-1 β and TGF- β , and MMP-1 and MMP-3 showed strong correlations. These associations could be related to the pathogenesis of the mixed features of GO.

Multiplex immunoassays have the advantage of the capability of analyzing samples with limited volumes such as GCF. We previously reported simultaneous successful quantification of various cytokine and chemokine levels in GCF from periodontitis patients [15]. The present study compared the levels of 12 biomarkers at both GO and CP sites in each patient with GO-mixed periodontitis presenting with GO as well.

GCF biomarkers are considered to be important for clarifying the mechanism of gingiva inflammation [15]. GCF itself is considered to be important for diagnosis of periodontal diseases because inflammation of periodontal tissues can be measured in this fluid. The diagnostic potential of GCF has been extensively investigated because of the possibility of non-invasive collection and a rich array of cellular and biochemical molecules associated with disease activity [35].

However, because of the small sample volumes, evaluating the relationships among the various types of biomarkers is sometimes difficult. The present study successfully compared the levels of 12 biomarkers at both GO and CP sites in each subject with periodontitis. Our findings suggest that the cytokine expression profiles, espe-

cially levels of IL-1 β and TGF- β , in GCF could be markers of the GO status and are detectable with the multiplex bead immunoassay.

Gingival inflammation induced by periodontopathic bacteria increases the risk of occurrence of GO [36]-[38]. The interaction between nifedipine and bacterial lipopolysaccharide may contribute to CCB-GO [39]. Patients with GO present with increased prevalence of *P. gingivalis*, *T. denticola*, and *T. forsythia* (red complex) as well as significantly higher levels of IL-1 β than those without GO [36]. Periodontal GO patients that carry the CD14-260 genotypes CT + TT with allelic T have higher frequencies of *P. gingivalis*, *T. denticola*, and *T. forsythia* compared to those carrying the CC genotype [40]. Counts of *P. gingivalis* and *T. denticola* in periodontal pockets of cyclosporine A-GO patients are significantly higher than those of CP patients [40]. However, our results demonstrated that only *T. denticola* was significantly higher. Certain mechanisms of drug-induced GO by periodontopathic bacteria such as *T. denticola* may be involved. The total bacterial counts tended to be decreased, though it did not reach the significant levels. It could be due to the individual variation of the microbial flora and the plaque control levels. According to some previous reports and the present study, CCB-GO could be effectively prevented by controlling microbial plaque factors, nevertheless CCB were continued in the patients [41]. Thus the supportive periodontal therapy (SPT) must be one of the most important ways to preventing GO recurrences.

In the future, a comprehensive study of bacterial adhesions and virulence factors, their molecular targets, and host proteases using proteomic and peptidomic approaches will likely be valuable for identifying molecular disease progression and for discriminating among the various manifestations of GO.

Consequently, the ease, efficiency, and expansiveness of multiplex technology may lead to a better understanding of not only the differences in cytokine profiles in periodontal disease and GO states but also the impact of periodontal therapy on inflammatory mediators. Periodontal pathogens may be related to the initiation and development of periodontal treatment for GO. Inflammatory cytokines (IL-1 β and TGF- β 1) may also be considered contributing factors.

5. Conclusion

In conclusion, the significant GCF biomarkers, IL-1 β and TGF- β 1 were detected in sites with GO periodontal pockets by multiplex bead immunoassay and decreased by periodontal initial treatments. Simultaneously the presence of *T. denticola*, could also be involved in the initiation and the progression of peiodontitis presenting with GO.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (No. 26861809) from the Japanese Ministry of Education, Culture, Sports, Science and Technology of Japan.

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