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Lipase-a single-nucleotide polymorphism rs143793106 is associated with increased risk of aggressive periodontitis by negative influence on the cytodifferentiation of human periodontal ligament cells

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## Abstract

**Background and objective:** Aggressive periodontitis (AgP) is characterized by general health and rapid destruction of periodontal tissue. The familial aggregation of this disease highlights the involvement of genetic factors in its pathogeny. We conducted a genome-wide association study (GWAS) to identify AgP-related genes in a Japanese population, and the lipid metabolism-related gene lipase-a, lysosomal acid type (*LIPA*), was suggested as an AgP candidate gene. However, there is no report about the expression and function(s) of *LIPA* in periodontal tissue. Hence, we studied the involvement of how *LIPA* and its single-nucleotide polymorphism (SNP) rs143793106 in AgP by functional analyses of *LIPA* and its SNP in human periodontal ligament (HPDL) cells.

**Materials and methods:** GWAS was performed using the genome database of Japanese AgP patients, and the GWAS result was confirmed using Sanger sequencing. We examined the mRNA expression level of *LIPA* and the protein expression level of the encoded protein lysosomal acid lipase (LAL) in periodontium-composing cells using conventional and real-time polymerase chain reaction (PCR) and western blotting, respectively. Lentiviral vectors expressing *LIPA* wild-type (*LIPA* WT) and *LIPA* SNP rs143793106 (*LIPA* mut) were transfected into HPDL cells. Western blotting was performed to confirm the transfection. LAL activity of transfected HPDL cells was determined using the lysosomal acid lipase activity assay. Transfected HPDL cells were cultured in mineralization medium. During the cytodifferentiation of transfected HPDL cells, mRNA expression of calcification-related genes, alkaline phosphatase (ALPase) activity and calcified nodule formation were assessed using real-time PCR, ALPase assay, and alizarin red staining, respectively.

**Results:** The GWAS study identified 11 AgP-related candidate genes, including *LIPA* SNP rs143793106. The minor allele frequency of *LIPA* SNP rs143793106 in AgP patients was higher than that in healthy subjects. *LIPA* mRNA and LAL protein were expressed in HPDL cells; furthermore, they upregulated the cytodifferentiation of HPDL cells. LAL activity was lower in *LIPA* SNP-transfected HPDL cells during cytodifferentiation than that in *LIPA* WT-transfected HPDL cells. In addition, ALPase activity, calcified nodule formation, and calcification-related gene expression levels were lower during cytodifferentiation in *LIPA* SNP-transfected HPDL cells than those in *LIPA* WT-transfected HPDL cells.

**Conclusion:** *LIPA*, identified as an AgP-related gene in a Japanese population, is expressed in HPDL cells and is involved in regulating cytodifferentiation of HPDL

cells. *LIPA* SNP rs143793106 suppressed cytodifferentiation of HPDL cells by decreasing LAL activity, thereby contributing to the development of AgP.

**Keywords:** genome-wide association study (GWAS); aggressive periodontitis; lipase a (*LIPA*); human periodontal ligament cells

## 1. Introduction

In 2017, the European Federation of Periodontology (EFP) and the American Academy of Periodontology (AAP) have announced a new classification of periodontitis. According to the new classification, aggressive periodontitis (AgP) is defined as Stage III or IV and Grade C periodontitis. AgP refers to the rapid destruction of periodontal tissue, including alveolar bone resorption and loss of attachment. AgP is characterized by familial aggregation and a young age of onset, from adolescence until the late twenties<sup>1</sup>. This clinical definition strongly suggests the involvement of genetic risk factors in its pathogenesis.

We performed a genome-wide association study (GWAS) using DNA isolated from Japanese AgP-patients and identified genetic risk factors for AgP, such as sphingomyelin phosphodiesterase 3 (*SMPD3*)<sup>2</sup> and paraoxonase 1 (*PONI*)<sup>3</sup>. Functional analyses of the single-nucleotide polymorphisms (SNPs) in *SMPD3* (rs145616324) and *PONI* (rs854560) that were associated with AgP revealed that these SNPs affected the cytodifferentiation of periodontal ligament cells and disturbed the homeostasis of the periodontal tissue, thereby contributing to the pathogenesis of AgP. Interestingly, both of these genes identified are lipid metabolism-related genes and had never been considered to be involved in the pathophysiology of AgP. Hence, it is possible that lipid metabolism-related genes may play important roles in the homeostasis of periodontal tissue.

In this study, we performed a GWAS using the previously established database of Japanese AgP patients and the integrative Japanese Genome Variation Database (*iJGVD*). We identified lipase a, lysosomal acid type (*LIPA*), a lipid metabolism-related gene, as another AgP-related gene in the Japanese population. *LIPA* encodes lysosomal acid lipase (LAL), which catalyzes the hydrolysis of cholesteryl esters and triglycerides in lysosomes, thereby producing cholesterol, free fatty acids, and glycerol<sup>4</sup>. Loss-of-function mutations in the *LIPA* gene cause Wolman's disease, a rare genetic disease characterized by abdominal distention, hepatosplenomegaly, and adrenal calcification<sup>5</sup>. Additionally, *LIPA* has been reported as an atherosclerosis-related gene associated with ectopic calcifications in sclerotic lesions<sup>6</sup>. Based on these studies, we presumed that *LIPA* was associated with calcification in the periodontal tissue. However, there are no studies that have reported the involvement of *LIPA* in the calcification and homeostasis of periodontal tissue. Therefore, in this study, we analyzed the expression and function(s) of *LIPA* and its SNP rs143793106 in periodontium-composing cells,

especially focusing on the cytodifferentiation and calcification of human periodontal ligament (HPDL) cells.

## **2. Materials and Methods**

### **2.1. GWAS for AgP in a Japanese cohort**

To identify the genetic risk factors for AgP in a Japanese population, we performed a GWAS on Japanese AgP patients from the DNA Data Bank of Japan at the National Institute of Genetics (JGAS 00000000024 and JGAS 00000000040)<sup>7</sup>. The Japanese patients were diagnosed based on the 1999 classification of periodontal diseases and condition described in detail by the American Academy of Periodontology<sup>8</sup>. Briefly, the common phenotypes of AgP are based only on clinical evidence, including systemic health, rapid progression and familial aggregation. Localized AgP was characterized by clinical attachment loss of the first molars or incisors only, and generalized AgP was characterized by clinical attachment loss of at least three teeth as well as the first molars and incisors.

The sequencing reads were aligned to the human reference genome (UCSC build HG19/HG18, <http://genome.ucsc.edu>) using BWA software. Variants were called using GATK and SAMtools and annotated with the dbSNP database and 1000 Genomes Project database. Variants were included in the analysis based on the following criteria: (1) variants with minor allele frequency (MAF)  $\leq 0.05$  in the 1000 Genome database, (2) variants with read depths  $\geq 10$ , (3) variants that were present in four or more patients, (4) variants that caused protein structural and functional changes according to the results of *in silico* bioinformatics analysis using SIFT and Polyphen2. The MAF of the AgP-related candidate genes was analyzed, and the MAF in the AgP group and the control group was compared. Data from the *iJGVD*, a gene reference library database for healthy Japanese individuals, were used for the control group. The study was approved by the Osaka University Research Ethics Committee (approval no. 629) and informed consent was obtained from all patients.

### **2.2. Sanger sequencing**

To confirm the MAF of the *LIPA* SNP rs143793106 in the 44 Japanese AgP-patients used for the GWAS, we performed Sanger sequencing (Macrogen Japan, Tokyo, Japan) using specific primers (forward: 5'-TCCAGGCCCAAATGAAGTC-3', reverse: 5'-

TGCAACTTGAAAAGATACTCAAAGA-3') (Fasmac, Kanagawa, Japan). The results were analyzed and compared with the human reference genome (Hg19/Hg18).

### **2.3. Reagents and cell lines**

Anti- $\beta$ -actin was purchased from Sigma-Aldrich (St Louis, MO). Anti-LAL antibody (ab154536) and goat anti-rabbit IgG H&L (ab205718) antibodies were purchased from Abcam (Cambridge, MA). Sheep anti-mouse IgG horseradish peroxidase (HRP)-linked antibodies were purchased from Cytiva (Tokyo, Japan). HPDL cells from donors #1 and #3 were purchased from Lonza (Basel, Switzerland) and ScienCell Research Laboratories (Waltham, MA), respectively. HPDL cells from donor #2 were isolated from a healthy first premolar extracted from a patient for orthodontic treatment and cultured using the outgrowth and limiting dilution method<sup>9</sup>. HPDL cells were cultured and maintained in  $\alpha$ -modified Eagle's medium ( $\alpha$ MEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) and 60  $\mu$ g/ml kanamycin (Wako). To induce mineralization of HPDL cells, we used mineralization medium,  $\alpha$ MEM supplemented with 50  $\mu$ g/mL L-ascorbic acid (Wako) and 5 mM  $\beta$ -glycerophosphate (Wako). Human gingival fibroblasts (HGF) cells<sup>10</sup> and human gingival epithelial (HGE) cells<sup>11</sup> were established in our laboratory and cultured, as previously described.

### **2.4. RNA isolation, reverse transcription (RT), conventional polymerase chain reaction (PCR) and real-time PCR**

Total RNA was extracted from cells ( $1 \times 10^6$  cells per well) using the Maxwell RSC RNA Cells/Tissue Kit (Promega, Madison, WI) and treated with DNase I (Takara Bio, Shiga, Japan). To synthesize cDNA, the isolated RNA was reverse transcribed using a High Capacity RNA-to-DNA kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The conventional PCR was performed using a Mastercycler nexus X2 (Eppendorf, Hamburg, Germany) with AmpliTaq Gold DNA polymerase (Applied Biosystems) and specific PCR primers (Supporting material Table 1). The amplification conditions consisted of an initial incubation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1 min. The PCR products were evaluated using agarose gel electrophoresis. Real-time PCR was performed using the StepOnePlus Real-time PCR System (Applied Biosystems) with Power PCR SYBR Master Mix (Applied Biosystems) and specific PCR primers (Supporting material Table 1) (Takara Bio and

Fasmac). The expression levels of each gene were calculated as relative ratios to the expression level of hypoxanthine phosphoribosyltransferase (*HPRT*), which was used as an internal control gene.

## **2.5. Western blotting**

Cells ( $1 \times 10^6$  cells per well) were lysed with RIPA buffer (Millipore, Billerica, MA) supplemented with Complete Mini (Roche Diagnostics, Indianapolis, IN) and centrifuged at 12,000 rpm for 20 min at 4 °C. The lysates were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to Trans-Blot Turbo Transfer Pack 0.2  $\mu$ M polyvinylidene membranes (BioRad Laboratories, Hercules, CA), immunoblotted with specific antibodies, and visualized using ImageQuant LAS4000 (GE Healthcare, Buckinghamshire, UK) as described previously<sup>12</sup>. The concentration of antibodies was optimized to 1:1,500 for anti- $\beta$ -actin and anti-LAL antibodies and 1:3,000 for goat anti-rabbit IgG H&L (HRP) and sheep anti-mouse IgG HRP-linked antibodies.

## **2.6. Construction and infection of *LIPA* lentiviral vectors**

*LIPA* wild-type (*LIPA* WT) and *LIPA* SNP rs143793106 (*LIPA* mut) lentivirus vectors were generated by Vector Builder (Chicago, IL). An empty vector (EV) (Vector Builder) was used as a control. The virus vectors were transfected into HPDL cells. The number of viruses per cell, expressed as the multiplicity of infection (MOI) was adjusted so the same amount of LAL was expressed in the HPDL cells infected with all vectors (MOI = 1 for the EV and *LIPA* WT-transfected cells and MOI = 2 for the *LIPA* mut-transfected cells). After washing the cultured HPDL cells ( $1 \times 10^6$  cells per well) with 4 °C phosphate-buffered saline (PBS, Wako), the lentivirus vectors lysed in  $\alpha$ MEM containing 5  $\mu$ g/ml polybrene (Vector Builder) were added and incubated with HPDL cells for 12 h. The medium was then replaced with  $\alpha$ MEM containing 10% FBS and kanamycin. After 24 h, the infected cells were selected using puromycin, and then cultured for 48 h.

## **2.7. LAL activity assay**

LAL activity was determined using a LysoLive Lysosomal Acid Lipase Kit (Abcam) according to the manufacturer's instructions. Briefly, HPDL cells ( $1 \times 10^6$  cells per well) were incubated for 72 h after transfection. After washing the cells with PBS, 1 ml of  $\alpha$ MEM containing 1  $\mu$ l of LAL substrate was added to the cells and incubated for 4 h. Finally, HPDL cells were collected and suspended in Flow Holding and Sorting



Buffer (Abcam), and analyzed using a flow cytometer (BD FACS Calibur; BD Bioscience, Franklin Lakes, NJ).

## **2.8. Alkaline phosphatase (ALPase) activity assay**

ALPase activity was measured as previously described<sup>13</sup>. Briefly, after washing the cultured cells ( $1 \times 10^6$  cells per well) with PBS, 500  $\mu$ l of Tris-HCl (pH 7.4) was added to the cells. The cells were sonicated for 30 s on ice using a sonicator device (Handy-Sonic model UR-20P, TOMY, Tokyo, Japan). Then, 50  $\mu$ l of 1 M Tris-HCl (pH 9.0), 10  $\mu$ l of 5 mM magnesium chloride (Wako), and 10  $\mu$ l of 50 mM sodium para-nitrophenyl-2-phosphate (Wako) were added to the supernatant. Enzymatic activity was analyzed using Multiskan FC (Thermo Fisher Scientific). The DNA content in the sonicated cells was measured by staining with Hoechst 33258. Briefly, Hoechst 33258 solution was prepared by dissolution in 2M NaCl and 25 mM Tris-HCl (pH7.5) and 5  $\mu$ g/ml of the Hoechst 33258 solution was added to 100  $\mu$ l of the sonicated supernatants. The fluorescence was monitored at an emission wavelength of 450 nm after excitation at 356 nm using FLUOROSKAN ASCENT (Thermo Fisher Scientific). ALPase activity was normalized by the DNA content in each sample.

## **2.9. Alizarin red staining**

Calcified nodules were measured using a modified alizarin red staining protocol, as previously described<sup>14</sup>. Briefly, after removing the supernatant, the cell layer ( $1 \times 10^6$  cells per well) was washed with PBS and fixed with 100% cold ethanol (Wako) for 15 min. Then, the cell layer was washed with distilled water, stained with 1% alizarin red S (pH 6.4) (Wako) for 5 min, and then washed again with distilled water. To quantify the number of calcified nodules, the stained cell layer was scanned using a scanner (EPSON GT-X970, EPSON, Nagano, Japan). WINROOF (Mitani, Fukui, Japan) was used to measure the density of alizarin-stained nodules.

## **2.10. Statistical analyses**

The Student's *t*-test was used to compare two groups, and one-way analysis of variance (One way ANOVA) was used to compare more than two groups. A chi-square test was used for exome sequencing analysis. Statistical significance was set at  $p < 0.05$ .

## **3. Results**

### **3.1. Association of *LIPA* SNP rs143793106 with AgP in a Japanese population**

We performed a GWAS using the database of AgP patients (DNA Data Bank of Japan: JGAS00000000024 and JGAS00000000040) and the healthy individuals from the *iJGVD*, which is an expanded database of genetic variants in 3,550 healthy Japanese individuals. We identified top 11 AgP-associated variants that were located in 11 different genes (Table 1). Among them, we decided to focus on a lipid-metabolism-related variant, the SNP rs143793106 (c.1009 T<C, p.Thr 337 Ala), located in the *LIPA* gene. To analyze whether *LIPA* SNP rs143793106 was associated with AgP, we performed Sanger sequencing of the T/T, T/C, and C/C genotypes in 44 AgP patients and used the *iJGVD* data as a control. As shown in Table 2, 40 AgP patients had the T/T genotype and 4 AgP patients had the T/C genotype, whereas none had the C/C genotype. In the control group, 3,432 and 118 patients had the T/T and T/C genotypes, respectively. Similar to the AgP cases, no subjects had C/C. The minor allele frequency (MAF) of rs143793106 (T/C) was significantly higher in AgP subjects (4.55%) than in the control subjects (1.66%) ( $P = 3.7 \times 10^{-2}$ ). The odds ratio was 2.82, and the 95% confidence intervals for the MAF of rs143793106 (T/C) was 1.02–7.81 (Table 2).

### **3.2. *LIPA* expression levels in periodontium-composing cells**

We then analyzed the *LIPA* mRNA expression in HPDL, HGF, and HGE cells using real-time PCR. We found that *LIPA* mRNA expression was significantly higher in HPDL and HGF cells than that in HGE cells (Figure 1A). In addition, we confirmed higher *LIPA* mRNA expression in HPDL cells among the periodontium-composing cells derived from the other donors (Appendices Figure 1). We further analyzed LAL protein expression in HPDL and HGF cells by western blotting. LAL protein expression was higher in HPDL cells than that in HGF cells (Figure 1B). Result from conventional PCR confirmed that the *LIPA* mRNA expression was detected in HPDL cells derived from 3 different donors (Figure 1C).

### **3.3. *LIPA*/LAL is associated with the cytodifferentiation of HPDL cells**

Considering that the identified AgP-related genes by previous GWAS were lipid metabolism-related genes such as *SMPD3*<sup>2</sup> and *PONI*<sup>3</sup> and that they were involved in the pathogenesis of AgP by regulating the cytodifferentiation of HPDL cells, we hypothesized that *LIPA* might also be involved in the cytodifferentiation of HPDL cells. To further test this hypothesis, we induced the cytodifferentiation of HPDL cells by culturing them in the mineralization medium for 15 days. Subsequently, we assessed *LIPA* mRNA and calcification-related gene expression using real-time PCR. The mRNA expression of *LIPA* was also upregulated in HPDL cells, as the mRNA

expression of runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALPL*), collagen type 1 alpha 1 chain (*COL1A1*) and osteocalcin (*BGLAP*), which are the marker genes of the cytodifferentiation of PDL cells, was upregulated during the cytodifferentiation of HPDL cells (Figure 2).

### **3.4. *LIPA* SNP rs143793106 enhances LAL activity in HPDL cells**

To further analyze the effects of *LIPA* wild-type (WT) and the mutated rs143793106 (*LIPA* mut) on the cytodifferentiation of HPDL cells, the lentiviral vectors encoding *LIPA* WT and *LIPA* mut were transfected into HPDL cells. An empty vector (EV) was used as a control. The result of western blotting showed that the expression levels of LAL were significantly higher in the *LIPA* WT- and *LIPA* mut-transfected HPDL cells than those in the cells transfected with EV, indicating that the *LIPA* vectors were transfected in HPDL cells (Figure 3A and B). No significant differences in LAL expression were found between *LIPA* WT-transfected HPDL cells and *LIPA* mut-transfected HPDL cells (Figure 3A and B).

Next, to examine the specific effects of the mutated genotype on LAL activity, we measured the activity of LAL enzyme. As expected, we observed significantly higher LAL activity in *LIPA* WT- and *LIPA* mut-transfected HPDL cells than that in EV-transfected HPDL cells. Additionally, *LIPA* mut-transfected HPDL cells presented significantly lower LAL activity than *LIPA* WT-transfected HPDL cells (Figure 3C).

### **3.5. The negative effect of *LIPA* SNP rs143793106 on the cytodifferentiation of HPDL cells**

To analyze the effects of *LIPA* WT and *LIPA* mut on the cytodifferentiation of HPDL cells, we performed osteogenic induction of HPDL cells. Subsequently, we measured ALPase activity and stained the calcified nodules using alizarin red. On day 15 of the cytodifferentiation of HPDL cells, the ALPase activity in the *LIPA* mut-transfected HPDL cells was significantly lower than that in the *LIPA* WT-transfected HPDL cells (Figure 4A). On day 27 of the cytodifferentiation, alizarin red staining illustrated that the *LIPA* mut-transfected HPDL cells showed significantly lower density of calcified nodules than the *LIPA* WT-transfected HPDL cells, whereas the density of calcified nodules in *LIPA* WT-transfected HPDL cells was similar to that in EV-transfected HPDL cells (Figure 4B). Additionally, real-time PCR results revealed that there was no significant difference in the *LIPA* mRNA expression levels between *LIPA* WT-transfected and *LIPA* mut-transfected HPDL cells throughout the cytodifferentiation of HPDL cells (Figure 5A). The mRNA expression of *RUNX2*,

*ALPL* and *COL1A1* was significantly lower in *LIPA* mut-transfected HPDL cells than that in *LIPA* WT-transfected HPDL cells at least one time point during the cytodifferentiation of HPDL cells (Figure 5B to D). The *BGLAP* mRNA expression showed a lower tendency in the *LIPA* mut-transfected HPDL cells than that in the *LIPA* WT-transfected HPDL cells at the late stage of cytodifferentiation, although there was no statistically significant difference (Figure 5E). In addition, similar tendency was observed in HPDL cells derived from two different donors (Appendice Figure 2 and 3).

#### 4. Discussion

In this study, we identified 11 AgP-related genes in a Japanese population using a GWAS. Among these genes, we focused on the SNP rs143793106, located on the *LIPA* gene. We then investigated the effects of this SNP on the function of HPDL cells and their relationship with AgP. Using *in vitro*-experiments, we found that the mutated genotype of rs143793106 negatively regulated the cytodifferentiation of HPDL cells by reducing LAL activity.

The presence of the alternative allele in the rs143793106 induces a missense change of threonine to alanine at the 337th amino acid position of the LAL enzyme, which presumably has a small effect on the enzymatic activities. It is still unknown how *LIPA* SNP rs143793106 affects the protein structure of LAL. It has been reported that *LIPA* SNP rs143793106 reduces intracellular and extracellular LAL enzyme activities by approximately 33% and 16%, respectively<sup>15</sup>. A severe reduction in LAL activity causes LAL deficiency, also termed Wolman's disease, a rare disorder characterized by severe clinical symptoms, such as hepatomegaly, splenomegaly, and atherosclerosis due to the accumulation of triglycerides and cholesteryl esters. It has been reported that one of the causing pathogenic mutations, consisting on a guanine to alanine change in the 894th base of the gene, results in the disruption of the LAL active site, thereby leading to a striking reduction of its activity<sup>16,17</sup>. Other SNPs located in this gene, including rs1412444 and rs2246833, have been identified as risk factors for coronary artery disease<sup>18</sup>.

A new classification of periodontal disease was introduced in 2017<sup>19</sup>. By the definition of the new classification system, stages indicate severity and treatment complexity, whereas grades indicate disease progression. We used the nomenclature, aggressive periodontitis, because the samples used in this study were collected before the introduction of the new classification system. In our study, all patients were diagnosed as Stage IV Grade C periodontitis by the new classification. It is considered that most AgP-patients are classified as Stage III/IV and Grade C periodontitis because

one of the characteristics of AgP is rapid destruction of periodontal tissue in comparison with age<sup>18</sup>.

Previous studies support an association between dyslipidemia and chronic periodontitis. For example, the amount of calcification in atherosclerotic lesions increases the destruction of the periodontal tissue<sup>20</sup>, and the same genes were identified as disease-related genes for both coronary artery disease and periodontal disease<sup>21</sup>. The 44 AgP patients included in this study were generally healthy and had no LAL-D-like symptoms at their first visit to our dental hospital, suggesting that the small decrease in the enzyme activities of LAL caused by *LIPA* SNP rs143793106 did not have serious systemic effects. However, the average age of the AgP subjects in this study was 32.6 years, younger than the age of the onset of atherosclerosis (35 years old). Hence, we cannot exclude the possibility that these subjects will suffer from atherosclerosis in the future. Thus, it may be interesting to follow-up longitudinally the subpopulation of AgP patients carrying the *LIPA* SNP rs143793106 mutation.

Interestingly, combining with the previous GWAS studies, 3 lipid metabolism-related genes were selected as AgP associated genes in the Japanese population<sup>2,3</sup>. Several studies have reported that the lipid metabolism plays an essential role in homeostasis and osteoblast differentiation<sup>22,23,24</sup>. In addition, in periodontal fibroblasts, oleic acids, one of the fatty acids, enhance the cytodifferentiation<sup>25</sup>. It is more apparent that lipids, including fatty acids, regulate ATP production through oxidative phosphorylation in mitochondria and fuel energy which is necessary for cytodifferentiation and calcification, although the studies in this regard are still insufficient and inconclusive<sup>26</sup>. *LIPA*-encoded LAL has a function to hydrolyze triglycerides and cholesteryl esters to fatty acids, cholesterols and glycerols, resulting in regulating the intracellular lipids<sup>4</sup>. It will be necessary to address how *LIPA* controls lipid consumption in HPDL cells and elucidate the mechanisms by which *LIPA*-induced lipids regulate the cytodifferentiation of HPDL cells.

In general, identifying disease-related genes using a GWAS requires approximately 10,000 subjects<sup>27</sup>. In this study, we could not identify the variants meeting genome-wide significance ( $p < 5 \times 10^{-8}$ ) mainly because the sample size of the AgP patients was small (n=44) compared to the standard sample size of GWAS. The prevalence of AgP in the Japanese population is approximately 0.03%<sup>28</sup>; therefore, it is difficult to recruit a large number of individuals from a single institute. This is the limitation of this study. In the future, we expect to validate our results with expanded data and identify novel AgP-related genes in the Japanese population.

In conclusion, *LIPA* SNP rs143793106 reduces ALPase activity, calcification formation, and mRNA expression of calcification-related genes during the cytodifferentiation of HPDL cells by reducing LAL activity. Based on these results, we suggest that *LIPA* SNP rs143793106 negatively controls the cytodifferentiation of HPDL cells, which disturbs the homeostasis of periodontal tissue and leads to the onset and progression of AgP. In the future, *LIPA* SNP rs143793106 may be used as a risk marker to predict AgP and provide preventive interventions for pre-AgP patients.

#### **AUTHOR CONTRIBUTIONS**

MM and CF contributed to acquisition of data, analysis and interpretation of data. drafting the article and revised the manuscript. TN, JK and YY contributed to acquisition of data and drafting the article. SY, KM and SM contributed to critical revision of the manuscript. All authors approved the current and any revised version to be submitted.

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#### **CONFLICT OF INTEREST**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

#### **SUPPORTING MATERIAL**

A supporting material to this article is available online.

#### **APPENDICES**

Appendices Figure 1, 2 and 3 to this article are available online.

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## Figure Legends

**Figure 1.** Expression levels of *LIPA* mRNA and LAL protein in periodontium-composing cells. (A) The mRNA expression of *LIPA* in human periodontal ligament cells (HPDL), human gingival fibroblasts (HGF), and human gingival epithelial (HGE) cells was assessed using real-time PCR. The *LIPA* expression in HPDL cells and HGF cells was calculated as relative ratios to that in HGE. Data represent the average and standard deviation (SD) of three independent experiments. \*\*\*  $p < 0.001$  (B) The protein expression of LAL and  $\beta$ -actin in HPDL cells and HGF cells was assessed using western blotting. The left panels show representative images of three independent experiments. The right panel indicates the densitometry of immunoblots showing the ratio of LAL relative to  $\beta$ -actin. Data represent the average and standard deviation (SD) of three independent experiments. \* $p < 0.05$  (C) The mRNA expression of *LIPA* and *HPRT* in HPDL cells derived from three different donors (Donor #1 to #3) was analyzed by conventional PCR. Data are representative of three independent experiments. *HPRT*: hypoxanthine phosphoribosyltransferase (an internal control gene).

**Figure 2.** Alteration in mRNA expression of *LIPA* during the course of the cytodifferentiation of human periodontal ligament (HPDL) cells. HPDL cells were cultured for 15 days. The mRNA expression levels of *LIPA*, *RUNX2*, *ALPL*, *COL1A1* and *BGLAP* were assessed using real-time PCR. The expression levels of each gene were calculated as relative ratios to the expression level of *HPRT* (an internal control gene). Data represent the average and standard deviation (SD) of three independent experiments. \*  $p < 0.05$  compared with day 0.

**Figure 3.** Transfection of *LIPA* wild-type (WT) and *LIPA* SNP rs143793106 (mut)-expressing lentivirus vectors in HPDL cells. EV, WT, and mut-expressing lentivirus vectors were transfected into HPDL cells. (A) The protein expression levels of LAL and  $\beta$ -actin in the transfected HPDL cells were assessed using western blotting. Data are representative of three independent experiments. (B) The graph shows densitometry analysis of (A). The ratios of LAL relative to  $\beta$ -actin in WT and mut were normalized by that in EV. Data represent the average and standard deviation (SD) of three independent experiments. (C) The LAL activity in EV, WT and mut-expressing HPDL cells was measured. The graph shows the relative ratios of LAL activity to that in EV. Data represent the average and standard deviation (SD) of three independent experiments. EV: empty vector-HPDL cells, WT: *LIPA* wild-type HPDL cells, mut: *LIPA* SNP rs143793106-HPDL cells. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

**Figure 4.** Effects of *LIPA* WT (WT) and *LIPA* mut (mut) on the cytodifferentiation of HPDL cells. HPDL cells were cultured in mineralization medium for 27 days. (A) ALPase activity was measured on day 0 and 15 of the cytodifferentiation of HPDL cells. Data represent the average and standard deviation (SD) of three independent experiments. (B) The formation of calcified nodules in EV, WT, and mut-HPDL cells was examined using alizarin red staining on day 27 of the cytodifferentiation. Alizarin red-stained calcified nodules were quantified using the densitometric analysis. *Left*: the image of alizarin red staining; *right*: the densitometry of *left*. The density of the calcified nodules in EV was set as 100%, and the graph indicates a relative density to EV. Data represent the average and standard deviation (SD) of three independent experiments. EV: empty vector-HPDL cells, WT: *LIPA* wild-type HPDL cells, mut: *LIPA* SNP rs143793106-HPDL cells. \*:  $p < 0.05$ , \*\*:  $p < 0.01$

**Figure 5.** Effects of *LIPA* WT (WT) and *LIPA* mut (mut) on mRNA expression of the calcification-related genes during the cytodifferentiation of HPDL cells derived from donor #1. HPDL cells were cultured in the mineralization medium for 15 days. The mRNA expression levels of *LIPA* (A), *RUNX2* (B), *ALPL* (C), *COL1A1* (D), and *BGLAP* (E) were assessed using real-time PCR every 5 days. The expression level of each gene was calculated as relative ratios to the expression level of *HPRT* (an internal control gene). Data represent the average and standard deviation (SD) of three independent experiments. EV: empty vector-HPDL, WT: *LIPA* wild-type HPDL, mut: *LIPA* SNP rs143793106-HPDL. \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$