Transcriptional expression in human periodontal ligament cell driven by orthodontic force : RNA-sequencing study

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감사의 글

논문이 완성되기까지 많은 격려와 가르침을 주신 최윤정 교수님께 깊은 감사를 드립니다. 더 나은 방향으로 논문이 마무리 될 수 있도록 길을 제시해 주시고 많은 조언을 해주신 유형석 교수님, 차정열 교수님, 육종인 교수님, 김의성 교수님께도 진심으로 감사를 드립니다.

수련 과정 동안 교정과 의사로 성장할 수 있도록 기반을 만들어 주시고 이끌어 주신 박영철 교수님, 백형선 교수님, 황충주 교수님, 김경호 교수님, 이기준 교수님, 정주령 교수님, 최성환 교수님께도 다시 한번 감사의 말씀을 드리고 싶습니다. 또한 수련 기간 큰 힘이 되어 주었던 동기들 및 지금 제 옆을 든든하게 지켜주고 있는 가족에게도 감사의 마음을 전합니다.

2019년 6월

저자 씀

TABLE OF CONTENTS

LEGEND OF FIGURES

LEGEND OF TABLES

ABSTRACT

Transcriptional expression in human periodontal ligament cell driven by orthodontic force : RNA-sequencing study

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(Directed by professor Yoon Jeong Choi, D.D.S., Ph.D)

The aims of this study were to investigate changes in gene expression of periodontal ligament (PDL) cells after mechanical stimulus and to establish mechanism (signaling pathway) through RNA sequencing. We selected ten patients who required tooth extraction for orthodontic treatment. To stimulate the PDL, orthodontic force was applied to the first premolar for three weeks (the experimental group; n=10), while the first premolar on the other side were left untreated (the control group; n=10). After the PDL cells were isolated from the extracted teeth, gene set enrichment analysis (GSEA), differential expressed gene (DEG)

analysis, and real time PCR were performed between the experimental and control groups.

GSEA demonstrated that most of the gene sets were related to the cell cycle pathway. In the experimental group, 13 up-regulated genes and 20 down-regulated genes were found compared to the control group through DEG analysis. Real time PCR results confirmed that five up-regulation genes and six down-regulated genes were consistent with RNA sequencing results. We finally ruled out three genes (CPNE3, OPHN1, and PPM1F) which is related periodontal ligament regeneration by these analyses.

After mechanical stimulus, CPNE3 and OPHN1 genes are up-regulated and PPM1F gene is down-regulated in periodontal ligament cells. We found the three genes can directly affect periodontal ligament, and further studies are needed to clarify the relationship between mechanical stimulus and periodontal ligament cells.

Keywords: RNA-sequencing, mechanical stimulus, periodontal ligament, GSEA, DEG

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I. INTRODUCTION

Mechanical stimulus is reported to contribute to periodontal ligament (PDL) regeneration (Kraft et al. 2010). In particular, low-magnitude, high frequency (LMHF) mechanical vibration promotes human PDL stem cells (PDLSC) differentiation (Zhang et al. 2012). Also, mechanical shear stress promotes osteogenic differentiation of the dental stem cells derived from pulp, alveolar bone, and PDL (Pavlin, Gluhak-Heinrich 2001). An animal study showed that occlusal stimulus promoted regeneration of the PDL and prevented dentoalveolar ankylosis (Mine et al. 2005), which was based on histologic observations. There have been few studies investigating relation between the mechanical stimulus and PDL regeneration in human.

The signaling pathway for changes in cytokine and bone remodeling under orthodontic force is already known, although changes in PDL and its signaling pathways have barely been

reported. Teeth without occlusal contact showed decrease of PDL volume and mechanoreceptors, and reduced ability to recover from root resorption. On the other hand, periodontal ligaments with mechanical stimulus show an increase in length, and remodeling occurs vigorously (Howard et al. 1998).

RNA sequencing (RNA-seq) is a useful technique for analyzing dynamic transcriptomes rather than static genomes (Kukurba, Montgomery 2015). Comparing with microarray-based methods, RNA-seq can analyze a wide range of transcriptomes and it is possible to discover novel transcripts from obtained data as well as quantitative analysis of gene expression (Qian et al. 2014). In dentistry, not only studies about physiology of cariogenic pathogen through RNA-seq (Zeng, Burne 2016), but also studies about the correlation of specific gene (IGFBP2) in cleidocranial dysplasia have been reported (Greene et al. 2018).

In the present study, we simulated the mechanical stimulus to the PDL by applying orthodontic force before tooth extraction. If the mechanical stimulus can be related to changes in gene expression of PDL cells in human, finding signaling pathway of PDL regeneration would be valuable to understand PDL changes after mechanical stimulus such as orthodontic treatment and trauma to the teeth. Therefore, the aims of this study were to investigate changes in gene expression of PDL cells after mechanical stimulus and to establish mechanism (signaling pathway) through RNA sequencing.

II. MATERIALS AND METHOD

1. Patient selection

Ten healthy patients (7 men and 3 women) free from any underlying medical conditions were selected from orthodontic patients who visited Yonsei University Dental Hospital between June 2017 and May 2018. All participants gave written informed consent. The study protocol was approved by the human ethics committee of Yonsei University Dental Hospital

(IRB No. 2-2017-0028). The patients were planned for extractions of the first premolars as part of orthodontic treatment. The inclusion criteria were that patients had a sound first premolar in each quadrant, without caries or restorations, and were not taking any steroidal or non-steroidal anti-inflammatory drugs during force application. The exclusion criteria were an asymmetrical arch form or any root malformations on either side, such as root dilaceration, which might result in a difficult extraction of the first premolars.

2. Mechanical stimulus (applying orthodontic force)

To stimulate the PDL tissues, orthodontic force was applied to the first premolars (the experimental group; n=10), while the other side first premolars were left untreated (the control group; n=10). The study was a split-mouth, randomized, controlled trial with a 1:1 allocation ratio (Park et al. 2019). A 0.016-inch nickel-titanium (NiTi) wire (Tomy International, Yokohama, Japan) were used for mechanical stimulus in the experimental group. (Figure 1) According to the manufacturer's instructions (Tomy International, Inc.), the wire has the shape memory effect that uses body temperature to generate light continuous force (100 g) with a deflection of 0.5–1.8 mm. The appliance was maintained for three weeks before extraction of the first premolars. PDL cells were fixed with PBS after extraction, and the tissues were analyzed for RNA sequencing (n=10).

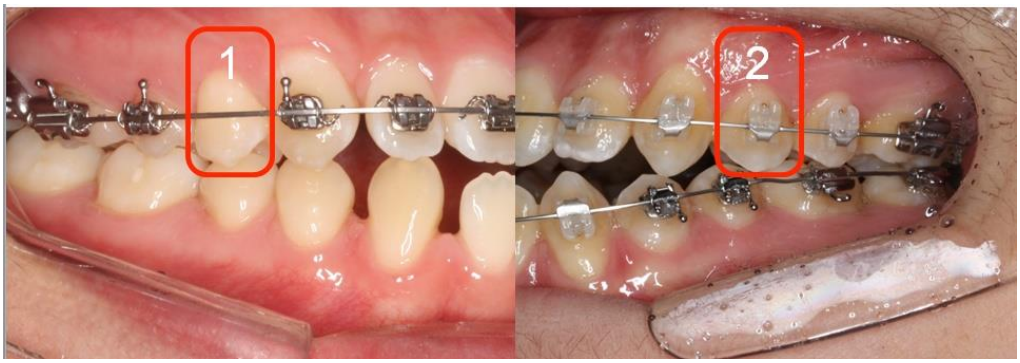

Figure 1. Split-mouth design (1) control group and (2) experimental group (applying orthodontic force)

3. Sample preparation for RNA sequencing

After periodontal ligament cells were isolated from the extracted teeth, the obtained cDNA was amplified by reverse transcription. After that, the library was constructed with the data obtained through RNA sequencing. Through gene set enrichment analysis (GSEA), a gene set was found underlying mechanical stimulus. Thereafter, differential expressed gene (DEG) analysis was performed between the experimental group and the control group.

4. Real time PCR

To evaluate the changes in gene expression after mechanical stimulus, PDL cells from each group were obtained, and total RNA was extracted using TRIzol Reagent (Ivitrogen, Waltham, Massachusetts, USA). The RNA was stored at −70℃ and was measured at an optical density of 260 nm. The mixtures of total RNA were incubated with Oligo dT (Gibco BRL), and a cDNA synthesis reaction was performed using a mixture of AccuPower PCR PreMix (Bioneer, Daejeon, Korean) and water. The cDNA was synthesized from the total RNA obtained from both groups using SuperScript First-Strand Synthesis System (Invitrogen) and was amplified with ABI-7300 (Applied biosystems, Mortlake, Waltham, Massachusetts, USA). The amplified cDNA was detected with the SYBR Green PCR Master Mix Reagent Kit (Takara, Seoul, Korean). PCR conditions were as follows: incubation for 10 min at 95℃, followed by 40 cycles of 10 s denaturation at 95℃, annealing for 60 s at 60℃. The reaction mixture lacking cDNA was used as a negative control in each run. Primer sequences are summarized in Table 1. Ratios of the intensities of the target genes and GAPDH signals were used as a relative measure of the expression level of the target genes. To ensure accuracy of the experiment, real-time PCR assays were performed in triplicate for each sample. The mean fold change of expression in the experimental group as compared to the control group was calculated from the △△Ct values, and the range of the fold changes was represented by the

standard deviations of the values (Fu et al. 2006).

Table 1. Primer sequences used for Real-time quantitative PCR

Genes	Forward	Reverse
CC2D1B-2	GAGTCGCAGCTAGCCTCTGT	TCTGTCTCAGGGCTCCTGTT
TMEM253-1	CTTGCTGAGCCAGAGGAAAC	CAAACCAGGAACCTCTTCCA
TENM4-2	CCGTCTTCCTTTCTGACAGC	ATCAGCCCAAACTTGTCCAC
CPNE3-1	TCGACCACTGGTGATGAAAA	CCGATGAACCATTAGCCAGT
MYOM2-1	CGGTGAATACAAGGCAACCT	TCACATATCTGCAGCCAAGC
PPM1F-2	GTACAGCAGGGACAGGTGGT	ACAGGCAAGCAGCAGGTAGT
PCDP1-2	TCAACAAGTAGCACGCAAGG	ATCCGCCTCAGGAAGAATTT
ATP2A1-1	TGGCTCTTCTTCCGCTACAT	GCCTCGAAGACCTCACAGTC
GPR171-1	CAACCGTTGTGTGGCTAATG	TATGATGTAGCCCGTGGTCA
OPHN1-2	GTCCCCAAGCAGGCCTAT	GTCCATTGGTGGCCTTTG
TANGO2-2	TCCCTGGAGGAAGCTGTG	GCTGCGCCTCTTCATTGT
UAP1-2	TCCAAAGCTGGGCAAGAG	GGTTCCATTCGTGCATCC
RP1-34H18.1-1	GCGGAGGAGGGAAGAAAG	AAAACCAACCGAGGCACA
GAPDH	TCCGCGGCTATATGAAAACAG	TCGTAGTGGGCTTGCTG AA

III. RESULT

1. GSEA

Most of the gene sets were related to the cell cycle (Figure 2).

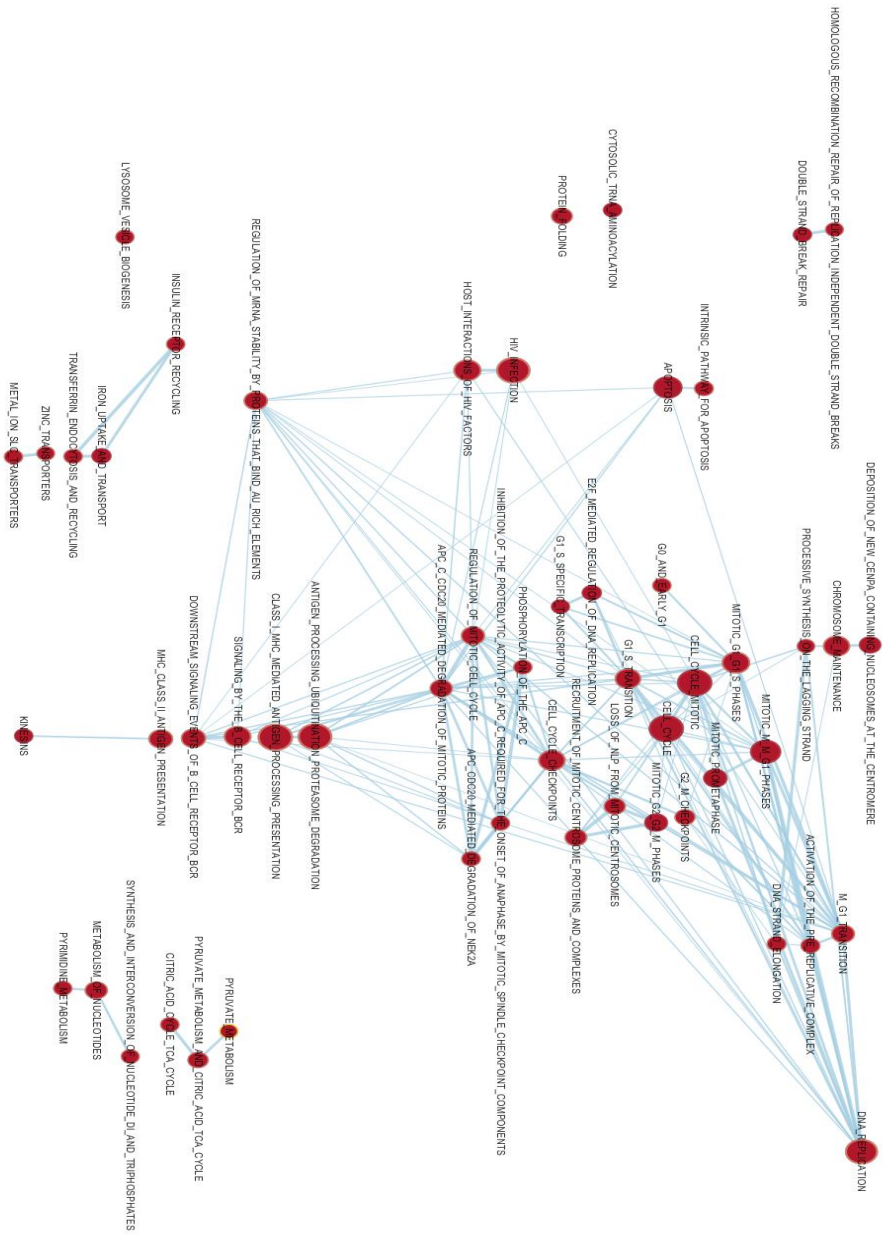

Figure 2 Results of GSEA. Through gene set enrichment analysis (GSEA), a gene set was found underlying mechanical stimulus and these genes were associated with the cell cycle.

2. DEG analysis

In the experimental group, thirteen up-regulated genes and twenty down-regulated genes were found compared with the control group (Tables 2 and 3)

Table 2. 13 up-regulated genes were found from differential expressed gene analysis. (fold ratio ≥ 1.5)

Gene Name	Description	Control	Experimental	log2fc	P value	q value
OPHN1	oligophrenin1	5.31	781	7.2	5.00E-05	0.0149
TANGO2	transport and golgi organization 2 homolog (Drosophila)	6.66	86.1	3.69	5.00E-05	0.0149
CC2D1B	coiled-coil and C2 domain containing 1B	12	150	3.58	5.00E-05	0.0149
UAP1	UDP-N-acteylglucosamine pyrophosphorylase1	43	468	3.45	5.00E-05	0.0149
TMEM253	transmembrane protein 253	0.836	7.66	3.2	5.00E-05	0.0149
TENM4	teneurin transmembrane protein 4	13.5	93.6	2.8	5.00E-05	0.014911
ABHD4	abhydrolase domain containing 4	20.9	137	2.71	5.00E-05	0.0149
CPNE3	copine III	23.7	134	2.5	5.00E-05	0.0149
RP11-820L6.1	-	1.76	9.19	2.38	5.00E-05	0.0149
SPATA22	spermatogenesis associated 22	0.379	1.89	2.31	5.00E-05	0.0149
SCUBE1	signalpeptide, CUB domain, EGF-like1	0.217	0.88	2.02	0.0001	0.0277
ARMC8	armadillo repeat containing 8	13.5	54	2	5.00E-05	0.0149
ERI2	ERI1 exoribonuclease family member 2	6.69	26.5	1.99	5.00E-05	0.0149

Table 3. 20 down-regulated genes were found from differential expressed gene analysis. (fold ratio ≤ -1.5)

Gene Name	Description	Control	Experimental	log2fc	P value	q value
ITCH	itchy E3 ubiquitin protein ligase	41.6	14.4	-1.53	5.00E-05	0.0149
ARL14EP	ADP-ribosylation factor-like 14 effector protein	71.8	22.8	-1.65	5.00E-05	0.0149
RFX1	regulatory factor X, 1 (influences HLA class II expression)	17.1	5.47	-1.65	5.00E-05	0.0149
PLEKHH1	pleckstrin homology domain containing, family H(with MyTH4 domain) member1	6.91	2.18	-1.66	5.00E-05	0.0149
EHBP1L1	EH domain binding protein1-like 1	162	46	-1.81	5.00E-05	0.0149
PIGQ	phosphatidylinositol glycan anchor biosynthesis, class Q	286	81.7	-1.81	5.00E-05	0.0149
ASPG	asparaginase homolog(S.cerevisiae)	56.8	15.2	-1.9	5.00E-05	0.0149
BOD1L1	biorientation of chromosomes in cell division 1-like 1	92.5	22.5	-2.04	5.00E-05	0.0149
PTPN12	protein tyrosine phosphatase, non-receptortype12	268	55.9	-2.26	5.00E-05	0.0149
USH1C	Usher syndrome 1C (autosomal recessive,severe)	4.9	0.927	-2.4	5.00E-05	0.0149
MYOM2	myomesin 2	18.2	3.03	-2.58	5.00E-05	0.0149

PRR11	proline rich11	16.7	2.72	-2.62	5.00E-05	0.0149
CCDC91	coiled-coil domain containing 91	166	26.8	-2.63	5.00E-05	0.0149
PPM1F	protein phosphatase, Mg2+/Mn2+ dependent, 1F	165	24.5	-2.75	5.00E-05	0.0149
PCDP1	Homo sapiens primary ciliary dyskinesia protein1(PCDP1), transcript variant1, mRNA.	2.35	0.254	-3.21	5.00E-05	0.0149
TSPAN8	tetraspanin 8	34.8	3.1	-3.49	5.00E-05	0.0149
RCCD1	RCC1 domain containing1	114	7.76	-3.87	5.00E-05	0.0149
ATP2A1	ATPase, Ca++ transporting, cardiac muscle,fast twitch 1	13.7	0.916	-3.91	5.00E-05	0.0149
GPR171	G protein-coupled receptor 171	49	0.782	-5.97	5.00E-05	0.0149
RP1-34H18.1	-	4.14E+03	0.774	-12.4	5.00E-05	0.0149

3. Real time PCR

We selected some target genes among up- and down-regulated genes from RNA-sequencing, and real time PCR was performed to confirm the DEG result. Real time PCR results confirmed that five up-regulation genes and six down-regulated genes were consistent with RNA sequencing results (Table 4). Among them, we found specific genes that affect the cell cycle through gene research.

Table 4. After qPCR, five up-regulated genes and six down-regulated genes were consistent with RNA sequencing results.

	Official full name	Description
Up-regulated genes		
CC2D1B	coiled-coil and C2 domain containing 1B	
CPNE3	copine III	CPNE3 is the same family with copine 1, 6, and 7
OPHN1	oligophrenin1	OPHN1 has a Rho-GAP domain shown to negatively regulate RhoA, Rac, and Cdc42
TANGO2	transport and golgi organization 2 homolog (Drosophila)	
UAP-1	UDP-N-acteylglucosaminepyrophosphorylase1	
Down-regulated genes		
MYOM2	myomesin 2	
PPM1F	protein phosphatase, Mg2+/Mn2+ dependent, 1F	PPM1F is a member of the serine / threonine protein phosphatase. (involved in caspase-dependent apoptosis)
PCDP1	Homo sapiens primary ciliary dyskinesia protein1(PCDP1), transcript variant1, mRNA.	
ATP2A1	ATPase, Ca++ transporting, cardiac muscle,fast twitch 1	
GPR171	G protein-coupled receptor 171	
RP1-34H18.1-1	-	

IV. DISCUSSION

In some samples, qPCR results were inconsistent with DEG analysis. Up and down regulated genes in the DEG analysis did not change or went into reverse in the qPCR. The cause of this error may be the presence of occlusal contact in the control group. Gene expression of the control group with occlusal force may be different from the control group without occlusal force (Kraft et al. 2010), which may affect the outcome. It would be better if we split the control group by presence of occlusion in the experimental design.

We examined the function and signaling pathway of the genes from the qPCR results, and CPNE3, OPHN1 and PPM1F were noticeable. CPNE3 is a calcium-dependent membrane-binding protein that encodes a protein that plays a major role in the synthesis of phospholipids and the immune system. CPNE3 is the same family with copine 1, 6, and 7, which can bind both calcium ions and phospholipids simultaneously. According to a previous report (Choung et al. 2019), CPNE7 (a family gene with CPNE3) involved in periodontal ligament regeneration. CPNE7 supports attachment of periodontal ligament cells to cementum and promotes physiological arrangement of PDL fibers. Therefore, CPNE3 may play a role in the regeneration of periodontal ligaments like CPNE7. Further studies are needed to determine whether CPNE3 affects PDL regeneration.

OPHN1 encodes the protein oligophrenin-1 that has a Rho-GAP domain shown to negatively regulate RhoA, Rac, and Cdc42 in vitro and in nonneuronal cells (Billuart et al. 1998). Rho GTPases have been related in important cell biological processes, including cell growth control, cell motility, cell development (Govek, Newey, Van Aelst 2005). As expression of OPHN1 gene increases, rhoA, Rac, and Cdc42 proteins will decrease. (RhoA, Rac, and Cdc42 proteins are known to activate caspase 3 and finally activate apoptosis.)

Decreasing of these proteins leads to a diminution in the apoptosis of periodontal ligament cells. Therefore, up-regulation of OPHN1 genes may contribute to the maintenance of periodontal ligament cells.

PPM1F is a member of the serine / threonine protein phosphatase. This gene is known to be involved in caspase-dependent apoptosis (Ishida et al. 1998). When the orthodontic force is applied, down-regulation of PPM1F means that apoptosis is inhibited in periodontal ligament cells. We hypothesize that down regulation of PPM1F will eventually have a positive effect on PDL regeneration. Further research is needed to prove this hypothesis.

V. CONCLUSION

When orthodontic force is applied, CPNE3, OPHN1 genes are up-regulated and PPM1F is down-regulated in periodontal ligament cells. We found three novel genes that can directly affect periodontal ligament, further studies are needed to clarify the relationship between orthodontic force and periodontal ligament cells.

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ABSTRACT (KOREAN)

교정력에 의한 사람 치주인대 세포의 유전자 발현 : RNA-sequencing 연구

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(지도 교수: 최 윤 정)

본 연구의 목적은 교정력을 적용했을 때, 치주인대 세포에서의 유전자 발현의 변화를 RNA sequencing을 통해 알아보는 것이다. 교정 진단 결과, 발치가 필요한 10명의 환자를 대상으로 연구를 진행하였다. 치주인대 세포가 물리적 자극을 받는 상황을 시뮬레이션 하기 위해 실험군에 교정력을 가하였고, 대조군은 힘을 가하지 않은 채로 두었다. 발치한 세포로부터 치주인대 세포를 분리한 후, gene set enrichment analysis (GSEA), Differential Expressed Gene (DEG) analysis 및 real time PCR 분석을 실시하였다.

GSEA 결과 대부분의 유전자 세트들은 세포 주기와 관련되어 있었고, DEG 분석을 통해 13개의 up-regulated genes 과 20개의 down-regulated gene을 찾았다. Real time PCR을 통해 5개의 up-regulated genes 과 6개의 down-

regulated genes 이 RNA sequencing 결과와 일치함을 발견하였다. 유전자 기능 탐색을 통해 최종적으로 치주인대 세포의 재생과 관련된 3개의 유전자(CPNE3, OPHN1 and PPM1F)를 찾았다. 교정력이 가해졌을 때, 치주인대 세포 내 CPNE3 및 OPHN1 유전자는 up-regulated 되며, PPM1F 유전자는 down-regulated 된다. 후속적인 연구(예를 들면, knock-out model을 이용한 functional study)를 통해 이러한 3개의 유전자가 직접적으로 치주인대 세포 재생에 영향을 미치는 것을 확인하는 것이 필요하다.

핵심이 되는 말: RNA-sequencing, 교정력, 치주인대 세포, 전향적 임상 연구