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ORIGINAL ARTICLE

# Notch – a possible mediator between Epstein-Barr virus infection and bone resorption in apical periodontitis

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**ABSTRACT**

**Objectives:** This study aimed to investigate whether Epstein-Barr virus (EBV) positive periapical lesions exhibited higher mRNA levels of Notch signalling molecules (Notch2 and Jagged1), bone resorption regulators (receptor activator of nuclear factor kappa-β ligand (RANKL) and osteoprotegerin (OPG)), and proinflammatory cytokines (tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-6) compared to EBV negative lesions. Additionally, the potential correlation between investigated molecules in periapical lesions was analyzed.

**Materials and methods:** Sixty-four apical periodontitis lesions were obtained subsequent to standard apicoectomy procedure. The presence of EBV was determined using nested PCR. Based on the presence of EBV all periapical lesions were divided into two groups, 29 EBV positive and 35 EBV negative lesions. A reverse transcriptase real-time PCR was used to determine mRNA levels of Notch2, Jagged1, RANKL, OPG, TNF-α, IL-1β and IL-6.

**Results:** Significantly higher mRNA levels of Notch2, Jagged1, RANKL and IL-1β were observed in EBV positive compared to EBV negative lesions. Significant positive correlation was present between Notch2 and Jagged1, Jagged1 and RANKL, and IL-β and TNF-α in EBV positive periapical lesions.

**Conclusions:** Notch signalling pathway may be involved in alveolar bone resorption in apical periodontitis lesions infected by EBV.

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Apical periodontitis; Epstein-Barr virus; Notch signalling pathway

## Introduction

Epstein–Barr virus (EBV) belongs to the *Herpesviridae* family, the gamma subfamily, and the *Lymphocryptovirus* genus [1]. It is an ubiquitous virus that establishes a lifelong persistent infection and over 90% of adults worldwide show seropositivity [2]. EBV is an important human pathogen associated with several diseases, including infectious mononucleosis, lymphoproliferative diseases in immunocompromised and EBV-associated malignancies in nonimmunocompromised individuals (for instance Burkitt and Hodgkin lymphoma, nasopharyngeal and gastric carcinoma) [1]. Additionally, EBV is implicated in the pathogenesis of several inflammatory diseases of the oral cavity, including gingivitis, chronic and aggressive periodontitis [3], pericoronitis [4], apical periodontitis [5], peri-implantitis [6] etc.

EBV has been frequently detected in apical periodontitis lesions [7] and has also been related to symptomatic and large-sized lesions, and radicular cysts , both in immunocompetent and immunocompromised patients [7,8]. EBV may be implicated in the pathogenesis of apical periodontitis either by direct cytopathic action on infected cells or through virally induced impairment of host defense, which in turn helps bacterial growth [9]. Although EBV has been thoroughly investigated, its exact role in apical periodontitis development remains obscure.

Progressive bone loss in the periradicular area, a distinctive feature of apical periodontitis, is associated with inflammation which is the consequence of polymicrobial infections in the root canal [10,11]. A recent study reported that EBV may induce periapical bone resorption in apical periodontitis via increased levels of reactive oxygen species and disturbed balance between receptor activator of nuclear factor kappa-Β ligand (RANKL) and osteoprotegerin (OPG) [12]. It is well established that about 100 different antigens are produced during the EBV active phase, including immediate early (IE), early (E) and late (L) proteins, and nine antigens in the latent phase, including nuclear antigens and latent membrane proteins (LMPs) [1]. EBV LMPs are mimic receptors of the tumour necrosis factor (TNF) receptor superfamily and can activate several immune-signalling cascades, for instance nuclear factor kappa-Β, c-Rel, p38, c-Jun N-terminal kinase (JNK) and phosphatidylinositol 3-kinase (PI3K)/Akt, to produce numerous cytokines and chemokines at the site of inflammation [13,14].

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Recently, significant attention has been focused on the involvement of Notch signalling in bone homeostasis [15,16]. It is an evolutionarily conserved pathway essential for cell proliferation, differentiation and apoptosis [17]. It is also one of the most important regulators of inflammatory response via cells of the innate and adaptive immune response [18]. Cell communication is achieved through communication between ligands (Jagged 1 and -2, Delta 1, -3 and -4) and receptors (Notch 1 to 4) on opposing cells. The ligand-receptor interaction causes proteolytic break of the Notch receptor [17] followed by the translocation of Notch receptor intracellular domain (NICD) to the nucleus and transcription of its target genes [19].

Several studies have reported that Notch signalling pathway enhanced osteoclastogenesis of RANKL pre-stimulated osteoclast precursors and boosted osteoclastic resorption [20,21]. Previous studies, using immunohistochemical analyses, have proved the existence of Notch pathway molecules in the lining epithelium of periapical cysts [22,23]. A more recent studies hypothesized [24] and reported [25] that periapical lesions with increased RANKL expression exhibit significantly higher mRNA levels of Notch signalling molecules and proinflammatory cytokines compared to OPG predominant lesions, thus confirming the involvement of Notch cascade in alveolar bone resorption. Since EBV has the potential to induce alveolar bone resorption via stimulated production of RANKL, it would be important to examine the relationship between Notch signalling and EBV in apical periodontitis. Therefore, this study aimed to investigate whether EBV positive apical periodontitis lesions exhibited increased expression of Notch signalling pathway molecules (Notch2 and Jagged1), bone remodelling markers (RANKL and OPG) and cytokines (TNF-α, interleukin-1 beta (IL-1β) and IL-6) compared to EBV negative lesions. Additionally, the potential correlation between investigated molecules in EBV positive and EBV negative lesions was analyzed.

## Material and methods

### Participants and sample collection

This cross-sectional study was conducted in full accordance with the World Medical Association Declaration of Helsinki (version 2002). The study protocol was reviewed and approved by the Ethical Committee of the School of Dental Medicine, University of Belgrade (approval number: 36/12-2013). All the participants were fully informed and they signed a written informed consent prior to all procedures.

The apical periodontitis samples were collected from 64 consecutive patients (aged 18–68 years; mean age 35.7 ± 13.1 years; 27 males and 37 females) at the Department of Oral Surgery, School of Dental Medicine, University of Belgrade, during the period from October 2016 to March 2017. All patients included in the study were in good general health (American Society of Anesthesia level I or II), and required surgical apicoectomy due to the failure of conventional root canal treatment. Patients with periodontally involved teeth (probing depth > 4 mm, with periodontal bone loss), vertical root fracture, immunocompromised, or patients treated with antibiotics, antiviral or immunosuppressive therapy 3 months before the examination were excluded from the study. Clinical, radiographic and histopathological features of apical periodontitis lesions were gathered from all included patients based on previously reported criteria [26].

Collection of periapical samples was done upon standard periapical surgery as fully explained elsewhere [26]. After harvesting, two equal tissue specimens were subjected to further analysis. One part of each lesion was fixed in 10% formalin for histopathological evaluation. The other portion was used for DNA and RNA isolation. It was placed into a sterile 1.5 mL centrifugation tube (Eppendorf AG, Hamburg, Germany) containing solution for RNA stabilization (RNAlater® Stabilization Solution; Thermo Fisher Scientific, Waltham, MA), kept overnight at 2–8 °C and finally stored at −70 °C until DNA and RNA isolation.

### RNA/DNA co-extraction

Both RNA and DNA were extracted using TRIzol reagent (Thermo Fisher Scientific, Grand Island, NY) according to manufacturer's recommendations. RNA was precipitated from the aqueous phase utilizing isopropanol and DNA was precipitated from the interphase/organic phase with ethanol. Isolated RNA was stored at −70 °C and DNA at −20 °C pending further analysis.

### Nested polymerase chain reaction analysis – EBV detection

The prevalence of EBV in apical periodontitis samples was assessed using nested PCR. The detection of EBV was done using primers that correspond to EBV nuclear antigen 2 (EBNA-2) gene. PCR analysis was performed as described previously [26]. Based on the presence of EBV, all periapical lesions were further divided into two subgroups: 29 EBV positive and 35 EBV negative periapical lesions.

### Gene expression analysis

Complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Pittsburgh, PA), 1 μg of RNA and oligo (dT) primers. Real-time PCR amplification of the regions of Notch2, Jagged1, IL–1β, IL–6, TNF–α, RANKL and OPG genes was done by using SensiFAST™ SYBR® Hi-ROX Kit (Bioline, London, UK) as described previously [25]. Normalization of gene expression results was done using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control. The amplification specificity was confirmed by generating melting curve after each PCR run. Relative expression levels were calculated using the comparative 2-ΔΔCt method [27]. The values were calculated as the mean value of duplicates for each sample, and the relative gene expression levels for all samples were defined as the ratio of each gene of interest to GAPDH expression.

### Statistical analysis

Statistical calculations were done using IBM SPSS Statistics for Windows Software (Version 22.0; IBM Corp, Armonk, NY). Categorical data were compared by Pearson's chi-square test ($\chi 2$). Numerical data were analyzed using Student's *t*-test (mean values) and Mann–Whitney U test (median values), depending on data distribution. The relationship between mRNA levels for the investigated genes was assessed by calculating the Spearman's rank correlation coefficient ($\rho$). *p* values less than .05 were regarded as significant.

## Results

No significant differences were observed between the subgroups for the demographic parameters (Student's *t*-test, $p = .532$; Chi-square test, $p = .698$) (Table 1). The presence of EBV was more frequently detected in symptomatic, large-sized lesions and radicular cysts, but the difference between the subgroups was not significant (Table 2). Significantly higher Notch2, Jagged1, RANKL and IL-1β mRNA levels were found in EBV positive lesions compared to EBV negative lesions (Table 3). Significant positive correlation was detected in EBV positive between Notch2 and Jagged1 ($\rho = .452$, $p = .001$), Notch2 and RANKL ($\rho = .321$, $p = .003$), Notch2 and IL-6 ($\rho = .270$, $p = .003$) Jagged1 and RANKL ($\rho = .295$, $p = .014$), Jagged1 and IL-1β ($\rho = .329$, $p = .007$), TNF-α and IL-1β ($\rho = .290$, $p = .001$), and IL-6 and OPG ($\rho = .276$, $p = .015$). Moreover, a significant negative correlation between OPG and Jagged1 ($\rho = -.250$, $p = .013$) and IL-1β ($\rho = -.144$, $p = .032$) was found (Table 4). Similar trends were observed in EBV negative lesions and the corresponding correlation coefficients are presented in Table 5.

**Table 1.** Demographic characteristics of study patients.

| | Apical periodontitis lesions | | |
|---|---|---|---|
| | EBV positive ($n = 29$) | EBV negative ($n = 35$) | *p* Value |
| Age (years, Mn ± SD) | 34.6 ± 13.4 | 36.7 ± 12.8 | .532[a] |
| Gender (F/M) | 16/13 | 21/14 | .698[b] |

[a]Student's *t*-test;
[b]Pearson's Chi-square test;
F: female; M: male; Mn: mean; SD: standard deviation.

**Table 2.** Clinical, radiographic and histopathological features of apical periodontitis lesions regarding the presence of Epstein-Barr virus infection.

| | Epstein-Barr virus | | |
|---|---|---|---|
| | Positive ($n = 29$) | Negative ($n = 35$) | *p* Value* |
| Symptomatic lesions ($n = 39$, %) | 20 (52.6) | 19 (47.4) | .231 |
| Asymptomatic lesions ($n = 25$, %) | 9 (36) | 16 (64) | |
| Large-sized lesions ($n = 29$, %) | 22 (75.9) | 7 (24.1) | .418 |
| Small-sized lesions ($n = 35$, %) | 21 (60) | 14 (40) | |
| Periapical granulomas ($n = 24$, %) | 10 (41.7) | 14 (58.3) | .650 |
| Radicular cysts ($n = 40$, %) | 19 (47.5) | 21 (52.5) | |

*Pearson's Chi-square test.

**Table 3.** Relative mRNA levels of analysed genes in EBV positive and negative apical periodontitis lesions.

| | Relative gene expression, mean ± SD, (median) | | |
|---|---|---|---|
| Gene | EBV positive periapical lesions ($n = 29$) | EBV negative periapical lesions ($n = 35$) | *p* Value* |
| Notch2 | 0.0661 ± 0.1814 (0.0108) | 0.0250 ± 0.050 (0.0034) | .012 |
| Jagged1 | 0.1249 ± 0.2226 (0.0310) | 0.0404 ± 0.0624 (0.0132) | .009 |
| RANKL | 0.0012 ± 0.001 (0.0008) | 0.0007 ± 0.0007 (0.0005) | .011 |
| OPG | 0.0186 ± 0.0635 (0.0005) | 0.0050 ± 0.0170 (0.0011) | .235 |
| TNF-α | 0.3323 ± 0.3489 (0.1684) | 0.9073 ± 2.3631 (0.1518) | .914 |
| IL-1β | 0.0162 ± 0.0171 (0.0103) | 0.0093 ± 0.0112 (0.0058) | .043 |
| IL-6 | 0.0043 ± 0.0073 (0.0015) | 0.0058 ± 0.0070 (0.0018) | .608 |

*Mann–Whitney U-test.
EBV: Epstein-Barr virus; RANKL: Receptor activator of nuclear factor kappa-Β ligand; OPG: Osteoprotegerin; TNF-α: Tumour necrosis factor – alpha; IL-1β: Interleukin 1 beta; SD: standard deviation.

## Discussion

Apical periodontitis is considered to be a polymicrobial disease caused by agents of endodontic origin. This infection is characterized by complex synergistic, antagonistic and commensal interrelationships among resident microorganisms [28]. Different microbial species with a significant potential to participate in the development of apical periodontitis have been identified in necrotic pulp tissue [26,29–31]. At the beginning of the 21st century Slots et al. [9] reported the presence of herpesviruses in apical periodontitis samples and proposed a hypothesis by which combined bacterial-viral infections may exacerbate the severity of the inflammatory response within the periradicular area. In addition to their direct cytopathogenic effect on different cells, herpesviruses also utilize indirect mechanisms to impair local host defense allowing them to persist throughout the hosts' lifetime [3].

EBV has been the most frequently detected human herpesvirus in apical periodontitis lesions [7]. Several studies

**Table 4.** Correlations between mRNA levels of analysed genes in EBV positive periapical lesions.

| Spearman's rho correlation coefficients ($\rho$) in EBV + lesions | | | | | |
|---|---|---|---|---|---|
| Gene | RANKL | OPG | Notch2 | Jagged1 | TNF-α | IL-1β |
| OPG | −0.001 | | | | | |
| Notch2 | 0.321[b] | −0.107 | | | | |
| Jagged1 | 0.295[b] | −0.250[b] | 0.452[a] | | | |
| TNF-α | 0.004 | −0.16 | 0.048 | 0.232 | | |
| IL-1β | 0.126 | −0.144[b] | 0.225 | 0.329[a] | 0.290[b] | |
| IL-6 | −0.029 | 0.276[b] | 0.270[b] | 0.164 | −0.211 | −0.052 |

EBV: Epstein-Barr virus; RANKL: Receptor activator of nuclear factor kappa-Β ligand; OPG: Osteoprotegerin; TNF-α: Tumour necrosis factor – alpha; IL-1β: Interleukin 1 beta; SD: standard deviation.
[a]Correlation is significant at the 0.01 level.
[b]Correlation is significant at the 0.05 level.

**Table 5.** Correlations between mRNA levels of analysed genes in EBV negative periapical lesions.

| Spearman's rho correlation coefficients ($\rho$) in EBV − lesions | | | | | | |
|---|---|---|---|---|---|---|
| Gene | RANKL | OPG | Notch2 | Jagged1 | TNF-α | IL-1β |
| OPG | −0.001 | | | | | |
| Notch2 | 0.311[b] | −0.176 | | | | |
| Jagged1 | 0.270[b] | −0.190[b] | 0.350[a] | | | |
| TNF-α | 0.005 | −0.142 | 0.053 | 0.23 | | |
| IL-1β | 0.118 | −0.123[b] | 0.227 | 0.299[a] | 0.293[b] | |
| IL-6 | −0.049 | 0.250[b] | 0.292[b] | 0.158 | −0.209 | −0.052 |

EBV: Epstein-Barr virus; RANKL: Receptor activator of nuclear factor kappa-Β ligand; OPG: Osteoprotegerin; TNF-α: Tumour necrosis factor – alpha; IL-1β: Interleukin 1 beta; SD: standard deviation.
[a]Correlation is significant at the 0.01 level.
[b]Correlation is significant at the 0.05 level.

reported that EBV infection is related to acute exacerbation of periapical disease [32–34]. On the other side, pooled data in a recent meta-analysis suggest that there is no significant difference in the occurrence of EBV between symptomatic and asymptomatic periapical lesions [7], which is in accordance with the results of this study.

The majority of previous studies dealt with the association between EBV detection and clinical, radiographic or histopathological features of periapical lesions [32–34]. Only a few have investigated possible pathophysiological mechanisms by which EBV induces apical periodontitis. Notably, Jakovljevic et al. [12,35] hypothesized and reported that EBV may induce periapical bone resorption via excessive production of reactive oxygen species and imbalance between bone resorption regulators.

The present study reported a significantly higher mRNA expression of RANKL and IL-1β in EBV positive compared to EBV negative periapical lesions. The role of bone resorption regulators and pro-inflammatory cytokines in periapical bone resorption has been extensively investigated. Both animal and human studies confirmed that increased levels of pro-inflammatory cytokines and imbalance between RANKL and OPG are strongly related to the development of apical periodontitis [36,37]. EBV infection induces the production of pro-inflammatory cytokines [32,33,38] and RANKL [12], which consequently leads to osteoclast differentiation and bone resorption in the periapical area. The results of the present study are in agreement with previously reported levels of pro-inflammatory cytokines and RANKL in periapical lesions infected by EBV [12,32,33].

Notch signalling pathway represents a canonical mechanism of signal transduction based on the relationship between corresponding ligands and receptors. This evolutionary conserved signalling pathway is involved in a variety of processes, including bone homeostasis [15,16]. The present study has revealed a significant positive correlation between Notch2 and Jagged1, Notch2 and RANKL, and Jagged1 and RANKL, which is in line with the report of Fukushima et al. [21] who have showed that Notch2 and Jagged1 activation in macrophages derived from bone marrow led to a RANKL dose-dependent osteoclast differentiation.

Previous studies investigated the role of Notch signalling molecules in animal models related to normal and pathological states of dental pulp [39,40]. In 2011, Meliou et al. [22] reported strong and moderate staining of Notch2 and Hes1 in the lining epithelium of human periapical cysts and suggested that downstream activation of Notch pathway was related to cyst expansion. Furthermore, Nikolic et al. [25] showed that lesions with RANKL predominance exhibited significantly higher relative mRNA levels of Notch signalling molecules and proinflammatory cytokines compared to lesions with OPG predominance. Most recently, Lina et al. [41] reported a significantly higher expression of microRNA-146a and Hairy and enhancer-of-split related with YRPW motif 2 (Hey2) in periapical samples compared to healthy controls. The expression of Hey 2, a target gene of Notch signalling pathway, was also significantly increased in MC3T3-E1 cells stimulated with different concentrations of *Porphyromonas endodontalis* lipopolysaccharide. All these results support the involvement of Notch in the pathogenesis of apical periodontitis and consequent periapical bone resorption.

In the present study both Notch2 and Jagged1 mRNA levels were significantly higher in EBV positive compared to EBV negative periapical lesions ($p = .012$, $p = .009$, respectively). These results are in agreement with previous findings on the expression of Notch2 and Jagged1 in bone resorption observed during orthodontic tooth movements [42], as well as under *in vitro* conditions in the periodontal ligament cells model [43]. Although both studies [42,43] dealt with the role of Notch signalling pathway in bone resorption, none of them took into consideration the potential involvement of EBV.

The link between EBV nuclear antigen EBNA-2 and Notch signalling molecules has been presented in several previously reported *in vitro* investigations [44–46]. In the late '90s, Strobl et al. [44] put forward the hypothesis that EBNA2 and Notch receptors are able to activate different genes in the same way, by interacting with cellular repressor protein RBP-Jκ located in the nucleus. Their experiments revealed that activated Notch serves as a potential functional cellular equivalent to EBNA-2, which is used for B cells immortalization and which enables lifelong latent EBV infection [45]. Gordadze et al. [46], by using the transcomplementation system in EREB2.5 cell lines, confirmed previous findings and revealed that both Notch and EBNA-2 activate an important subset of cellular genes associated with type III latency and B-cell growth and proliferation.

Although we have demonstrated that mRNA expression of Notch signalling molecules is significantly higher in EBV positive compared to EBV negative periapical lesions, all aspects of the relationship between EBV infection and the Notch signalling activation in apical periodontitis are still poorly investigated. However, the excessive production of reactive oxygen species, proinflammatory cytokines and bone resorption regulators at the site of inflammation could be perceived as the potential link between them. Based on previous studies, active EBV infection may induce excessive production of reactive oxygen species [47] and proinflammatory cytokines [32,33] in the periapical area which leads to overproduction of RANKL and consequent osteoclastogenesis [48]. The activation of Notch signalling pathway has been related to excessive oxidative stress [49], while the increased production of proinflammatory cytokines induced the expression of Notch ligands and receptors, and promoted NICD nuclear translocation [18]. Fukushima et al. [21] reported that RANKL induced Notch2 expression and activation; they also established a bi-directional relationship between RANKL and Notch signalling in osteoclastogenesis.

Based on the presented results, the possibility of controlling Notch signalling in the therapy of periapical bone resorption should also be mentioned. In this context, the proven efficacy of a RANKL-specific antibody for postmenopausal osteoporosis and rheumatoid arthritis in clinical trials must be stressed [50,51]. Moreover, Kuritani et al. [52], in a mice periodontitis model, reported that intraperitoneal

administration of anti-mouse monoclonal RANKL antibodies significantly inhibited alveolar bone destruction and tooth root exposure. Down-regulation of Notch signalling includes the use of biochemical inhibitors of Notch activation and antibodies to Notch receptors or their ligands. Several studies reported that the generalized inhibition of Notch signalling is associated with the development of serious unwanted events (e.g. skin cancer, vascular tumours and hepatotoxicity) [53,54]. On the other hand, the application of selective antibodies blocking Notch1 or Notch2 avoids severe cell metaplasia associated with pan-Notch inhibition [55]. This therapeutic approach could be used in future experimental animal models to evaluate the potential efficacy of Notch signalling blocking in periapical bone resorption control.

Although additional experiment is necessary to confirm our findings, there is a possibility that active EBV infection indirectly induces bone resorption in apical periodontitis via excessive oxidative stress and proinflammatory cytokines production and activation of RANKL-Notch bi-directional signalling cascade.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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