


## RESEARCH ARTICLE

# The RAS-PI3K-AKT-NF- $\kappa$ B pathway transcriptionally regulates the expression of BCL2 family and IAP family genes and inhibits apoptosis in fibrous epulis

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## Abstract

**Background:** Epulis has a tumor-like appearance but is considered to be a massive reactive lesion rather than a true neoplasia. Limited information about the pathogenesis of epulis is available. The purpose of our study was to identify potential signaling pathways in fibrous epulis through transcriptome profiling.

**Methods:** Differentially expressed genes (DEGs) between fibrous epulis lesions and normal gingival tissues were detected using RNA sequencing (RNAseq). The expression levels of eighteen genes were validated using quantitative real-time PCR (qRT-PCR).

**Results:** RNAseq identified 533 upregulated genes and 732 downregulated genes. The top 10 upregulated genes were *IL11*, *OSM*, *MMP3*, *KRT75*, *MMP1*, *IL6*, *IL1B*, *IL24*, *SP7*, and *ADGRG3*. The top 10 downregulated genes were *BCHE*, *TYR*, *DCT*, *KRT222*, *RP11-507K12.1*, *COL6A5*, *PMP2*, *GFRA1*, *SCN7A*, and *CDH19*. KEGG pathway analysis further indicated that the DEGs were enriched in "Pathways in cancer" and the "Ras signaling pathway". Quantitative real-time PCR verified that the expression levels of *SOS1*, *HRAS*, *PIK3CA*, *AKT3*, *IKBKA*, *IKBKB*, *NFKB1*, *BCL2*, *BCL2L1*, *XIAP*, *BIRC2*, and *BIRC3* were increased significantly.

**Conclusions:** The current transcriptomic profiling study reveals that in fibrous epulis, RAS-PI3K-AKT-NF- $\kappa$ B pathway transcriptionally regulates the expression of BCL2 family and IAP family genes, leading to increased proliferation and apoptosis inhibition.

## KEYWORDS

apoptosis, BCL2 family, fibrous epulis, RAS-PI3K-AKT-NF- $\kappa$ B pathway, RNA sequencing

## 1 | INTRODUCTION

Epulis, which exhibits a tumor-like appearance, is a kind of hyperplasia predominantly located on the gingival or alveolar mucosa.<sup>1</sup> Previous studies have indicated that this disease could be caused by low-grade local irritation, traumatic injury, hormonal factors, or certain drugs.<sup>2,3</sup>

Although epulis is classically categorized into many different subtypes, the main types are fibrous epulis, granulomatous epulis, and giant cell epulis.<sup>4,5</sup> The diagnosis of epulis is aided by its clinical and radiographic features, but histopathology is the key for final diagnosis.<sup>1,6</sup>

Limited studies have been carried out to delineate the pathogenesis of epulis and gingival hyperplasia. Several studies have found

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that apoptosis inhibition through overexpression of bcl-2 might play an important role in the development of epulis.<sup>7-9</sup> Extracellular matrix degradation and remodeling are also important aspects, and matrix metalloproteinases (MMPs) play an important role in periodontal tissue destruction.<sup>10</sup>

Systems biology approaches such as whole-genome expression profiling may provide new insights into the molecular mechanisms of fibrous epulis. In the present study, we used genome-wide RNA sequencing (RNAseq) to identify alterations in gene expression in the transcriptome. The results may provide relevant information for the further development of mechanism-based diagnostics and therapies.

## 2 | MATERIALS AND METHODS

### 2.1 | Participants

The participants were recruited from the Ningbo NO. 2 Hospital and the Ninth People's Hospital affiliated with the Shanghai Jiao Tong University School of Medicine from Jan 2018 to June 2018 with informed written consent and ethics review board approval. The epulides were diagnosed clinically and confirmed by histopathological examination. The inclusive criteria diagnosis with fibrous epulis; tooth mobility less than degree I; and a lack of a history of familial gingival hyperplasia. The exclusion criteria were moderate and severe periodontitis; a history of pregnancy; taking immunosuppressive agents or calcium channel antagonists; congenital epulis; and a history of familial gingival hyperplasia. Surgical treatment was performed on outpatients, and all lesions were removed using a scalpel (No. 11) without extraction of any of the affected teeth. Each removed lesion was divided into two parts. One part was fixed in formalin and processed conventionally by histopathological examination at the Department of Pathology; the other part was stored in RNAlater for further RNA extraction. At the same time, normal gingival tissues near the lesion were collected as the control specimens. The normal gingival tissues were also confirmed by histopathological examination.

Finally, a total of 35 lesions and 35 normal gingival tissues were recruited for the current study, among which 5 pairs were used in microarray analysis, and 30 pairs were used in validation stage.

### 2.2 | RNA preparation, library preparation, and sequencing

Total RNA was isolated from gingival tissues by using a TaKaRa MiniBEST Universal RNA Extraction Kit according to the manufacturer's instructions. The RNA quality was determined using a Qubit<sup>®</sup> 3.0 Fluorometer (Life Technologies). RNA integrity was measured using an Agilent 4200 system (Agilent Technologies).

A total of 1  $\mu$ g of RNA per sample was used as the initial material for RNA sample preparation. Ribosomal RNA was removed using Ribo-Zero<sup>™</sup> Gold Kits (Epicentre). Subsequently, the sequencing

libraries were generated following the manufacturer's recommendations with different index labels by using the NEBNext<sup>®</sup> Ultra<sup>™</sup> Directional RNA Library Prep Kit for Illumina (NEB). The libraries were clustered and sequenced on the Illumina NovaSeq 6000 platform, and 150 bp paired-end reads were generated. Raw reads (fastq files) were aligned to the reference genome GRCh38 using the High-performance Integrated Virtual Environment (HIVE).

### 2.3 | Statistical analysis of the RNAseq data

The RNAseq data were normalized using the R/Bioconductor Limma package.<sup>11</sup> An empirical Bayes model was used to compare the differentially expressed genes (DEGs) between the groups, and the set criteria were an FDR < 0.05 and fold change  $\geq 2$  or  $\leq 0.5$ .

To investigate the potential role of the DEGs, the functional classification and biological pathway interpretation of these genes were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.<sup>12</sup> Biological functions, represented by Gene Ontology (GO) terms (<http://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (<http://genome.jp/kegg/>), were deemed significant based on exhibiting at least three DEGs and a Benjamini-Hochberg corrected  $P < .05$ .

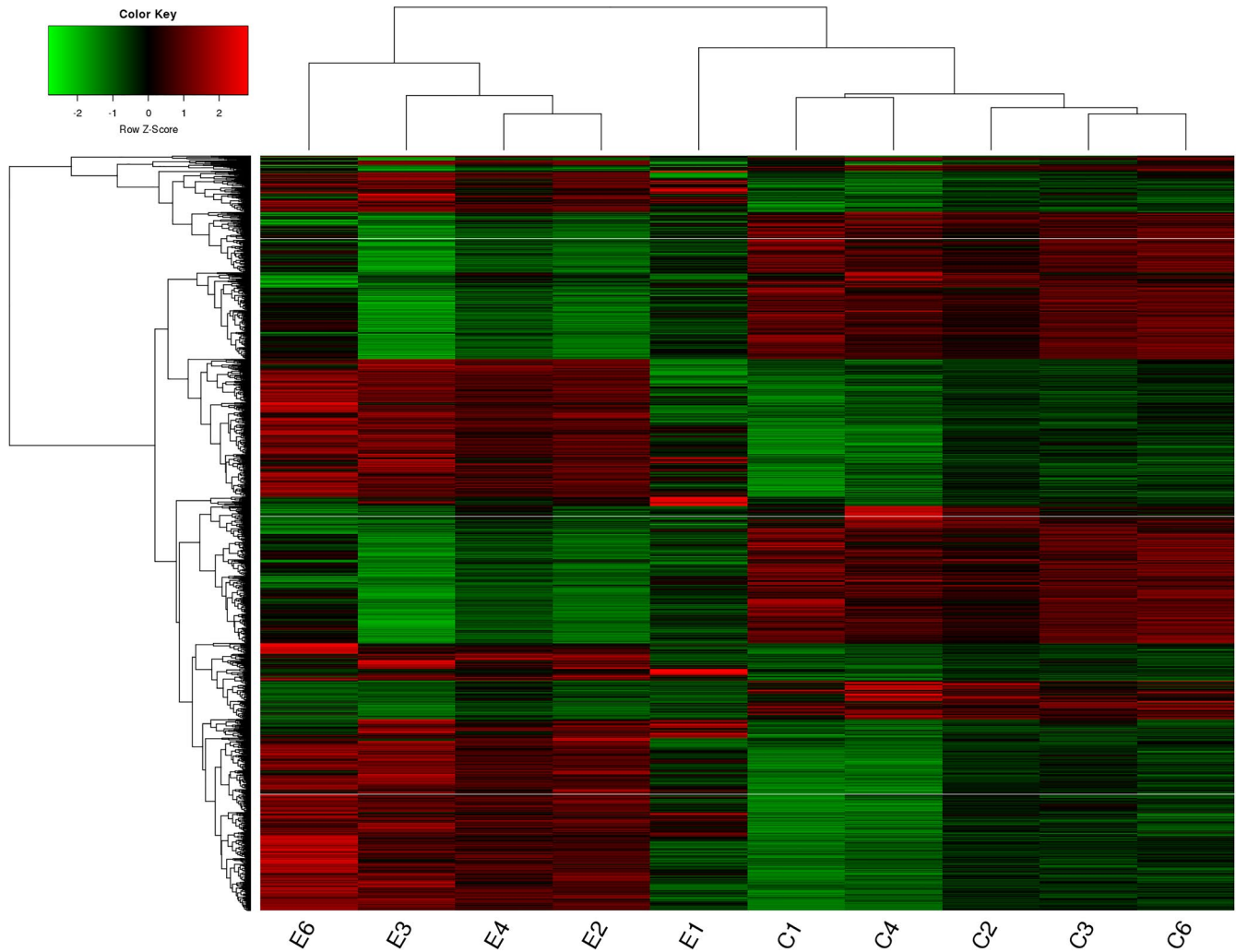
### 2.4 | Quantitative real-time PCR (qRT-PCR) validation

Eighteen representative genes involved in "Pathways in cancer" and the "Ras signaling pathway" were validated by qRT-PCR in another 30 pairs of gingival tissues. Total RNA was extracted as described above, and double-stranded cDNA was synthesized using the TaKaRa PrimeScript RT Reagent Kit according to the manufacturer's instructions. Subsequently, qRT-PCR was performed using Roche LightCycler 480 SYBR Green I Master mix. The  $2^{-\Delta\Delta CT}$  method was used to quantify the relative expression of each mRNA, using *GAPDH* as an internal control. All experiments were conducted in triplicate and were repeated three times. Differences in mRNA expression between the groups were evaluated with a paired-samples *t* test using SPSS 18.0 software. Significance was considered to be  $P < .05$ .

## 3 | RESULTS

### 3.1 | Identification of differentially expressed DEGs between fibrous epulis lesions and normal gingival tissues

Global gene expression profiling was performed using RNAseq. Compared with the normal gingival tissues, the fibrous epulis lesions exhibited 533 upregulated genes, whereas 732 genes were expressed at a lower level. The top 10 upregulated genes were *IL11*,



**FIGURE 1** Gene cluster analysis for all identified mRNAs from the control and epulis groups. The red intensity represents an increase in expression, whereas green represents a decrease in the expression relative to the average of the mean centered data

*OSM*, *MMP3*, *KRT75*, *MMP1*, *IL6*, *IL1B*, *IL24*, *SP7*, and *ADGRG3*. The top 10 downregulated genes were *BCHE*, *TYR*, *DCT*, *KRT222*, *RP11-507K12.1*, *COL6A5*, *PMP2*, *GFRA1*, *SCN7A*, and *CDH19*. The cluster assessment of the DEGs indicated that the fibrous epulis lesions exhibited a distinct expression pattern (Figure 1).

### 3.2 | Functional annotation and enrichment analysis of the DEGs

According to the GO analysis (Table 1), the biological process (BP) of the DEGs was enriched in cell-cell adhesion, and actin cytoskeleton reorganization, epidermis development. The cellular component (CC) of the DEGs was enriched in cytoplasm, cell-cell adherens junction, extracellular exosome, cytosol, cytoskeleton, apical part of cell, cell-cell junction, dendritic spine, actin cytoskeleton, ruffle membrane, brush border, and membrane. The molecular function (MF) of the DEGs was enriched in cadherin binding involved in cell-cell adhesion, protein binding, ATP binding, protein serine/threonine kinase activity, and growth factor binding.

Kyoto Encyclopedia of Genes and Genomes pathway analysis identified ten pathways that were significantly enriched with a Benjamini-Hochberg corrected  $P < .05$  and contained at least 3 DEGs (Table 2): Pathways in cancer (hsa05200), Ras signaling pathway (hsa04014), vascular smooth muscle contraction (hsa04270), axon guidance (hsa04360), Rap1 signaling pathway (hsa04015), tight junction (hsa04530), cGMP-PKG signaling pathway (hsa04022), proteoglycans in cancer (hsa05205), renin secretion (hsa04924), and platelet activation (hsa04611).

### 3.3 | Validation of DEGs in the “Pathways in cancer” and “Ras signaling pathway”

Eighteen representative genes involved in the “Pathways in cancer” and “Ras signaling pathway” categories were further validated in an additional 30 pairs of gingival tissues by qRT-PCR. These genes were *SOS1* (SOS Ras/Rac guanine nucleotide exchange factor 1), *KRAS* (*KRAS* proto-oncogene, GTPase), *NRAS* (*NRAS* proto-oncogene, GTPase), *HRAS* (*HRAS* proto-oncogene, GTPase), *ARAF* (*A-Raf*

**TABLE 1** The significant GO terms for the DEGs of fibrous epulis

Category	Term	Count	Benjamini
BP	GO:0098609~cell-cell adhesion	65	5.64E-05
BP	GO:0031532~actin cytoskeleton reorganization	18	2.23E-02
BP	GO:0008544~epidermis development	25	3.24E-02
CC	GO:0005737~cytoplasm	704	3.26E-07
CC	GO:0005913~cell-cell adhesion junction	75	4.04E-07
CC	GO:0070062~extracellular exosome	406	1.21E-06
CC	GO:0005829~cytosol	448	4.80E-04
CC	GO:0005856~cytoskeleton	71	1.14E-03
CC	GO:0045177~apical part of cell	23	1.96E-03
CC	GO:0005911~cell-cell junction	39	3.12E-03
CC	GO:0043197~dendritic spine	26	7.82E-03
CC	GO:0015629~actin cytoskeleton	43	2.52E-02
CC	GO:0032587~ruffle membrane	21	4.37E-02
CC	GO:0005903~brush border	17	4.88E-02
CC	GO:0016020~membrane	292	4.68E-02
MF	GO:0098641~cadherin binding involved in cell-cell adhesion	67	4.38E-05
MF	GO:0005515~protein binding	1113	9.28E-05
MF	GO:0005524~ATP binding	226	2.75E-03
MF	GO:0004674~protein serine/threonine kinase activity	73	2.81E-03
MF	GO:0019838~growth factor binding	12	3.07E-02

proto-oncogene, serine/threonine kinase), *BRAF* (B-Raf proto-oncogene, serine/threonine kinase), *RAF1* (Raf-1 proto-oncogene, serine/threonine kinase), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, also known as PI3K), *RASSF1* (Ras association domain family member 1), *AKT3* (AKT serine/threonine kinase 3), *IKBKA* (inhibitor of nuclear factor kappa B kinase subunit alpha, also known as IKK $\alpha$ ), *IKBKB* (inhibitor of nuclear factor kappa B kinase subunit beta, also known as IKK $\beta$ ), *NFKB1* (nuclear factor kappa B subunit 1), *BCL2* (BCL2 apoptosis regulator), *BCL2L1* (BCL2-like 1, also known as BCL-XL), *BIRC2* (baculoviral IAP repeat-containing 2, also known as CIAP1), *BIRC3* (baculoviral IAP repeat-containing 3, also known as CIAP2), and *XIAP* (X-linked inhibitor of apoptosis). Among these genes, 13 genes were DEGs identified from RNAseq, and the other 5 genes were important members of the Ras

**TABLE 2** The significant KEGG pathways for the DEGs of fibrous epulis

Term	Count	Fold	Benjamini
hsa05200:Pathways in cancer	71	1.65	0.005
hsa04014:Ras signaling pathway	46	1.86	0.005
hsa04270:Vascular smooth muscle contraction	29	2.27	0.004
hsa04360:Axon guidance	30	2.16	0.005
hsa04015:Rap1 signaling pathway	42	1.83	0.008
hsa04530:Tight junction	22	2.31	0.015
hsa04022:cGMP-PKG signaling pathway	33	1.91	0.015
hsa05205:Proteoglycans in cancer	39	1.78	0.014
hsa04924:Renin secretion	17	2.43	0.034
hsa04611:Platelet activation	27	1.90	0.042

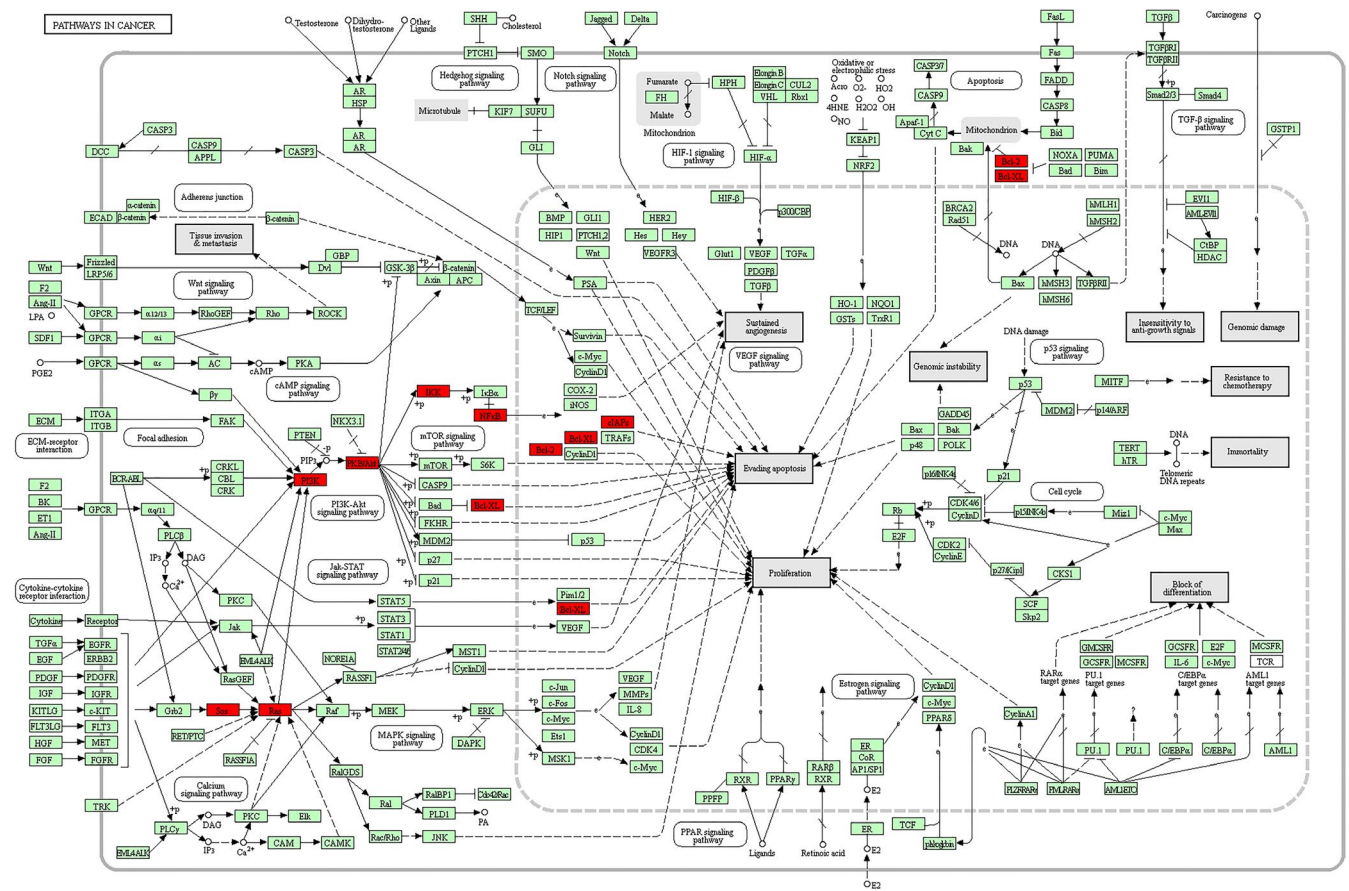
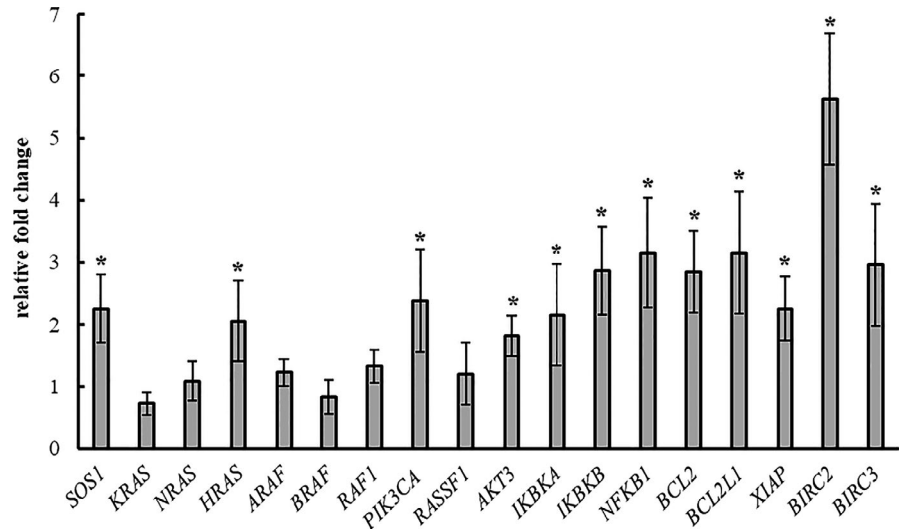
family (*KRAS* and *NRAS*) and the Raf family (*ARAF*, *BRAF*, and *RAF1*). The results confirmed the initial RNAseq findings for all of the genes (Figure 2). The KEGG Pathview analysis of these 18 genes further showed that noncanonical ras-mediated cancer pathways were the major pathogenetic pathways in fibrous epulis (Figure 3).

## 4 | DISCUSSION

Epulis is considered to be a reactive massive lesion rather than a true neoplasia and is usually asymptomatic with a variable growth rate.<sup>5</sup> Studies have confirmed that gingival hyperplasia is the result of reduced gingival epithelial cell death and/or increased proliferation, and it is speculated that apoptosis inhibition plays an important role in the development of fibrous epulis.<sup>13,14</sup> Although "Pathways in cancer" was listed as the first KEGG pathway in the current study, when all 71 DEGs categorized under this pathway were reanalyzed, the majority of the genes were also categorized under "Ras signaling" and "Regulation of programmed cell death." Therefore, the term "Pathways in cancer" identified in this study does not mean that gingival cells undergo the canonical cancer pathway in fibrous epulis but that they instead evade apoptosis and hyperproliferation.

Apoptosis plays an important role in cell metabolism and maintaining a relatively constant number of cells in a certain tissue.<sup>15</sup> In the normal gingival epithelium, the Bcl-2 protein is often restricted to the basal cell layer, while in gingival hyperplasia, the distribution of Bcl-2 expands from the basal cell layer to the upper layer.<sup>16</sup> In drug-induced gingival hyperplasia, it has been confirmed that the major pathogenesis involves the Bcl-2 family-regulated inhibition of apoptosis, leading to gingival fibroblast proliferation and accumulation.<sup>17-19</sup> In our previous study, we identified the expression of 84

**FIGURE 2** Microarray data were confirmed by qRT-PCR. The results are expressed as the mean  $\pm$  SD from three independent experiments. The asterisks represent statistical significance ( $P < .05$ ) compared with the control group



**FIGURE 3** Activation of the RAS-PI3K-AKT-NF- $\kappa$ B in epulis

apoptotic genes in fibrous epulis using the Qiagen RT<sup>2</sup> Profiler PCR Array. The results indicated that compared with normal gingival tissues, the expression levels of 12 genes were significantly increased in fibrous epulis tissues, including BCL2, BCL2L1, and BCL2L2. Interestingly, both BCL2 and BCL2L1 were identified as DEGs in the current study, and their expression levels were also increased significantly. These results strongly indicated that activation of anti-apoptotic BCL2 family members might be an important mechanism of

fibrous epulis, and these genes might be used as biomarkers in future diagnosis.

In addition to the BCL2 family, the current study also identified significant increases in the expression levels of three important members of the inhibitor of apoptosis protein (IAP) family. *BIRC2* encodes the cIAP1 protein; *BIRC3* encodes the cIAP2 protein; and *XIAP* (also known as *BIRC4*) encodes the XIAP protein. The IAPs are a family of functionally and structurally related proteins that serve

as endogenous inhibitors of apoptosis. A common feature of all IAPs is the presence of a baculovirus IAP repeat (BIR, an ~70 amino acid domain) in one to three copies.<sup>20</sup> The human IAP family consists of 8 members, including cIAP1, cIAP2, XIAP, NAIP, Livin, Survivin, Cp-IAP, and Op-IAP.<sup>21</sup> Among these members, XIAP is the best known, since it binds to caspase-9, caspase-3, and caspase-7, thereby inhibiting their activation and preventing apoptosis.<sup>22</sup> Additionally, cIAP1 and cIAP2 have been shown to bind caspases; however, their protein structure does not have specific caspase-inhibitory interaction sites.<sup>23</sup> According to the original RNAseq data, none of the caspase genes reached the threshold for a DEG (fold change  $\geq 2$  or  $\leq 0.5$ ) in fibrous epulis. However, the expression levels of CASP3 and CASP9 decreased to 0.6. Therefore, similar to BCL2 family genes, it was speculated that overexpression of IAPs inhibits the occurrence of gingival apoptosis and promotes fibrous epulis.

The current study also identified Ras signaling as one of the major mechanisms of fibrous epulis. Ras proteins are key signaling switches that are essential for the control of a variety of cellular processes, including the proliferation, differentiation, and survival of eukaryotic cells.<sup>24</sup> The Ras proteins and their downstream signaling proteins, such as the Raf kinases (BRAF and CRAF), mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK), together constitute the canonical RAS-RAF-MAPK pathway.<sup>25</sup> In the human genome, three genes encode HRAS, KRAS, and NRAS. The N-terminal halves of the Ras isoforms share complete amino acid identity, including the effector-binding domain and the majority of residues responsible for coordinating GDP/GTP binding, GTPase activity, and regulators of Ras activity.<sup>26</sup> Despite their similarity, Ras proteins are not functionally redundant. The mechanistic basis for isoform-specific signaling is thought to involve differences in the presentation of the effector-binding domain. Raf binding is favored by the KRAS orientation, whereas PI3K binding favors the orientation adopted by HRAS.<sup>27</sup> Interestingly, among these three members, only the expression of HRAS increased significantly in the current study. Moreover, elevated PI3K and AKT expression was identified, while the expression levels of ARAF, BRAF, and RAF1 remained unchanged. The fact that the DEGs identified in fibrous epulis were involved not in the canonical RAS-RAF pathway but in the RAS-PI3K-AKT pathway instead strongly indicated that although fibrous epulis has a tumor-like appearance, it is not a true neoplasia because the significant signaling pathways all led to proliferation and anti-apoptosis effects but not to other cancer pathways.

Downstream of the RAS-PI3K-AKT pathway, IKBKA (encoding IKK $\alpha$ ), IKBKB (encoding IKK $\beta$ ), and NFKB1 (encoding NF- $\kappa$ B) were also activated. Two kinase subunits (IKK $\alpha$  and IKK $\beta$ ) together with the regulatory subunit (NEMO/IKK $\gamma$ ) constitute the I $\kappa$ B kinase complex (IKK).<sup>28</sup> It has been confirmed that IKK $\alpha$  and IKK $\beta$  are necessary for I $\kappa$ B phosphorylation and that stimulation of the IKK complex is the key step in activating the NF- $\kappa$ B pathway.<sup>29,30</sup> NF- $\kappa$ B activation further induces a wide variety of target genes, such as pro-proliferative and anti-apoptotic genes that are involved in the regulation of cellular proliferation and survival.<sup>31</sup> It has been reported that Bcl-2 is directly transcriptionally targeted by NF- $\kappa$ B, leading to suppression

of apoptosis.<sup>32,33</sup> Moreover, it has been found that the activation of NF- $\kappa$ B suppresses caspase-8 activation through the induction of cIAP1 and cIAP2.<sup>34,35</sup> At this point, the Ras signaling pathway is connected to the anti-apoptosis pathway, which might be the major mechanism of fibrous epulis.

In conclusion, the current transcriptomic profiling study reveals that in fibrous epulis, the RAS-PI3K-AKT-NF- $\kappa$ B pathway transcriptionally regulates the expression of BCL2 family and IAP family genes, leading to increased proliferation and apoptosis inhibition.

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## CONFLICT OF INTERESTS

None.

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