

MicroRNAs: New Insights Into the Pathogenesis of Endodontic Periapical Disease

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Endodontics, School of Dentistry

Chapel Hill
2013

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ABSTRACT

LINDA CHAN: MicroRNAs: New Insights Into the Pathogenesis of
Endodontic Periapical Disease

(Under the direction of Asma Khan, Salvador Nares, Ricardo Padilla and Eric Rivera)

Apical periodontitis is an inflammatory disease of the periradicular tissues caused by the host's immune response to infection of the root canal system. MicroRNAs (miRNA) have been shown to play an important role in the regulation of inflammation and the immune response; however, their role in the pathogenesis of endodontic periapical disease has not been explored. The purpose of this study was to examine the differential expression of miRNAs in diseased periapical tissues as compared to healthy periodontal ligament and pulp tissues. miRNA profiles were assessed using microarray technology and expression levels of selected miRNAs linked to inflammation and the immune response were confirmed by quantitative RT-PCR. Of the 381 miRNAs identified using microarray, 24 miRNAs were down-regulated in diseased periapical tissues compared to controls (n=13) ($P<0.003$). Down-regulation of 7 of 9 selected miRNAs was confirmed by qRT-PCR in a separate set of diseased and healthy tissues (n=19) ($P<0.05$). Target genes of these miRNAs include key mediators in the immune and inflammatory response such as of IL-6, MMP-9 and TGF- β . These findings offer new insight into the pathogenesis of endodontic disease and have the potential to impact the development of new methods for prevention, diagnosis, and treatment of apical periodontitis.

Acknowledgements

This thesis would not have been possible without the guidance and limitless help of my thesis committee. Special thanks to Dr. Asma Khan for leading the research project and her tremendous support throughout the study. I truly appreciate the guidance that Dr. Nares has provided throughout this process as well as his generosity in sharing his lab.

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CHAPTER 1: INTRODUCTION

1.1 Pathogenesis of endodontic disease

Endodontic disease is characterized by inflammation of the pulp and the periapical tissues. In endodontic disease, the tooth pulp first becomes infected with oral microflora either via caries, fractures, cracks, coronal microleakage or operative dental procedures, which then leads to necrosis of the pulp tissues. This involves a mixed, predominantly gram-negative, anaerobic bacterial flora that elicits an initial immune response in the dental pulp (1-3).

These microorganisms accumulate and become organized in biofilms where the cells are embedded in a polysaccharide complex that the host defenses are incapable of eliminating (4). The microbial invaders progress towards the apex and eventually their byproducts such as lipopolysaccharides (LPS) are able to move through the apical foramen, reaching the periapex (5). Their egress from the apical foramen stimulates a secondary immune response in the periapical region that involves an initial innate immune response followed by the activation of the adaptive immune response. This process can be viewed as the body's defense response to the infected root canal space in its attempt to localize the infection and prevent its spreading and systemization (6, 7). First, the innate immune response is activated, which involves phagocytic leukocyte migration and proinflammatory cytokine production. Next the adaptive immune response becomes activated, which involves T and B cells. Host derived factors such as cytokines, arachidonic acid metabolites and neuropeptides are expressed to modulate this inflammatory process.

1.2 Pathogenesis of apical periodontitis

The inflammatory disease of the periradicular tissues, apical periodontitis, has great structural variation, especially in chronic lesions (8). However, initial tissue changes are characterized by hyperemia, vascular congestion, edema of the periodontal ligament and extravasation of neutrophils (9). High concentrations of neutrophils and some macrophages are found in the acute phase of apical periodontitis while lymphocytes, macrophages and plasma cells rise in number during the chronic phase of the disease process (10, 11). Neutrophils are drawn to the area of tissue injury by chemotaxis induced by LPS and complement factor C5a. Neutrophils then release prostaglandins and leukotrienes such as leukotriene B₄ (LTB₄) which attracts more neutrophils as well as macrophages to the periapex. Prostaglandins are derived from arachidonic acid when it is metabolized through the cyclooxygenase pathway. The prostaglandins, prostaglandin E₂ (PGE₂) and prostaglandin I₂ (PGI₂) activate osteoclasts resulting in bone resorption (12). Neutrophils, which are the predominant cell type in the acute phase of inflammation, are an important source of PGE₂. Leukotrienes are formed from the oxidization of arachidonic acid via the lipoxygenase pathway. LTB₄ is a strong chemotactic agent for neutrophils and facilitates the adhesion of PMNs to endothelial cell walls. PMNs contain cytoplasmic granules with many enzymes capable of degrading cell structures and extracellular matrices, including several matrix metalloproteinases. Although the primary role of PMNs is to destroy microorganisms, host tissues inevitably become damaged in the process as neutrophils die in large numbers at the site of inflammation, releasing their degradative enzymes, which do not discriminate between microbes and host tissues.

Macrophages enter the periapex later in the acute stage of the inflammatory response and produce several key mediators such as proinflammatory cytokines, IL-1, IL-6, and TNF, as well as chemotactic cytokine, IL-8. These cytokines increase the local vascular response, bone resorption and degradation of the extracellular matrices. They also work with IL-6 to up-regulate hematopoietic colony stimulating factor production, which mobilizes neutrophils and promacrophages from the bone marrow. Antigen-antibody complexes are formed in the later stages of the acute response as well (13). Proinflammatory and chemotactic cytokines involved in the pathogenesis of apical periodontitis include IL-1, IL-6, IL-8, and TNF (14). IL-1 β is the main form expressed in human periapical lesions and their exudates (15, 16). This cytokine enhances the adhesion of leukocytes to endothelial walls, stimulates lymphocytes, potentiates neutrophils, activates prostaglandin and proteolytic enzyme synthesis, increases bone resorption and inhibits bone formation (12, 17). IL-6 down-regulates the production of IL-1. IL-8 is a chemotactic cytokine produced by monocytes and fibroblasts under the influence of IL-1 β and TNF- α and is involved in regulating PMN and monocyte infiltration (18). TNF- α from macrophages and TNF- β from T-lymphocytes have many local effects similar to IL-1 (12).

In the chronic phase of apical periodontitis, the cellular composition shifts from a neutrophil dominated lesion to a macrophage, lymphocyte and plasma cell dominated lesion enclosed in a collagenous connective tissue periphery. Macrophage derived proinflammatory cytokines stimulate lymphocytes differentiation. T-cells outnumber B-cells and there are more CD4⁺ cells than CD8⁺ (11, 19-21). T-cells produce many cytokines that down-regulate the production of proinflammatory cytokines, thus suppressing osteoclastic activity, while also up-regulating the production of growth factors such as TGF- β . TGF- β is involved in the

activation of macrophages, proliferation of fibroblasts, synthesis of connective tissue, angiogenesis and down-regulation of many T-lymphocyte functions, thus helping to counter some of the damaging effects of the inflammatory response (22, 23).

A periapical lesion is formed as a result of this dynamic interaction between the host defense response and the bacteria contained in the root canal system. This inflammatory disease of the periradicular tissues, referred to as apical periodontitis, is of central importance in the practice of endodontics. In fact, the ultimate biological aim of root canal treatment itself is to prevent or cure apical periodontitis (24).

The apical inflammatory response to infection of the root canal system involves the induction of several hundreds of genes. It is a process that must be carefully regulated in order to achieve pathogen clearance and at the same time prevent the consequence of unregulated tissue damage. Despite advancements in the understanding of the pathogenesis of endodontic disease, there is still much to discover about the genetic regulation of this process.

1.3 microRNAs in inflammation and the immune response

The discovery of microRNAs (miRNAs) is one of the most significant scientific breakthroughs in recent years and has dramatically changed the previous view of a linear relationship between gene and protein expression (25). The first miRNAs were characterized in 1993; however, they were not recognized as a distinct class of regulators until 2001 when the term “microRNA” was introduced (26, 27). miRNAs are short, non-coding, single stranded RNA molecules (18-25 nucleotides long) that mediate RNA-interference through post-transcriptional modulation of gene expression. miRNAs silence genes by binding to

complementary sequences of their respective target messenger RNAs (mRNAs) and either inhibiting their translation into proteins or initiating cleavage of the mRNA leading to its degradation (28). Each miRNA can target many genes and it is thought that each gene is regulated by multiple miRNAs.

miRNAs play a fundamental role in mediating biological events and are involved in virtually all physiologic processes such as proliferation, differentiation, apoptosis, cell fate determination, signal transduction and organ development (25). They have also been implicated in a multitude of pathologic states such as cancer, developmental abnormalities, cardiovascular diseases, neurodegenerative disorders and inflammatory diseases (29-33). They are emerging as novel biomarkers of disease, prognostic indicators, and targets for drug therapy. The high sequence conservation of across species and tissue specificity are just some of the properties that make miRNAs ideal biomarkers (34). Stable miRNAs have recently been identified in many body fluids including saliva and plasma, which allows for a non-invasive means to easily measure miRNA profiles (35, 36). Functional miRNAs have also been discovered in exosomes, which presents a novel strategy to deliver RNA therapeutic agents (37). miRNA-based technology is currently being implemented in a wide range of applications such as cancer diagnosis and prognosis, predicting risk of transplant rejection, determining the quality of stored blood, and prenatal diagnostics (38).

miRNAs have been demonstrated to play an important role in the regulation of inflammation and the immune response and their role in dentistry is just beginning to be explored. Altered miRNA expression levels have been demonstrated in periodontal disease by comparing healthy and inflamed gingival tissues (39-41). The first miRNA study in the field of endodontics found significant differential expression of several miRNAs between

healthy and diseased human dental pulps (42). miRNAs from the miR-181 family were among those identified, whose targets include IL-6 – one of the most important mediators of the acute phase of the inflammatory response and a key player in the stimulation of osteoclastic activity. Although some insight has been gained on the role of miRNAs in endodontic pulpal disease, its role in endodontic periapical pathogenesis has not been explored. The purpose of this study is to determine the differential expression of miRNAs in diseased periapical tissues by comparing the miRNA profiles of diseased periapical tissues and healthy control tissues.

CHAPTER 2

MicroRNAs: New Insights Into the Pathogenesis of Endodontic Periapical Disease

Introduction:

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Materials and Methods:

Study participants and sample collection: This study was approved by the Institutional Review Board, at the University of North Carolina at Chapel Hill. The inclusion criteria were patients of age ≥ 12 years old and American Society of Anesthesiologists class I or II.

Patients who were immune compromised or currently taking antibiotics or other medications known to influence the immune response were excluded from the study. Written informed consent was obtained from all study participants.

Diseased periapical tissues were collected from teeth undergoing surgical endodontic treatment (apicoectomy). These teeth had previous non-surgical endodontic treatment and were associated with a non-healing periapical lesion. During the apicoectomy procedure, granulation tissue from the periapical lesion was curetted from the bony cavity prior to root end resection. Two different types of tissues were used as controls: normal periodontal ligament and pulp tissues. These were collected from extracted non-carious third molars or premolars. Healthy periodontal ligament was collected immediately following extraction using a scaling instrument to separate the tissues from the surface of the root. Healthy pulp tissue was extirpated using sterilized barbed broaches immediately after extraction. Tissues samples were placed in a sterile eppendorf tube with 0.5ml RNAsafer Stabilizer Reagent (VWR, Bridgeport, NJ) and stored at -80° C until processing. 13 samples (eight diseased periapical tissues and five healthy pulps) were used for the microarray experiment and 19 samples (eight diseased periapical tissues, eight periodontal ligaments and three healthy pulps) were used for qRT- PCR.

RNA isolation and miRNA microarray: Samples were thawed on ice and centrifuged at 4°C for 2 minutes at 12,000 rpm to remove the stabilizer reagent. Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA was quantitated using the NanoDrop (Thermo Scientific, Wilmington, DE) and RNA integrity assessed using the 2100 Bioanalyzer (Agilent, Foster

City, CA). The miRNA expression profiles were interrogated using Human miRNA Microarrays (V3) and the miRNA Complete Labeling and Hyb Kit (both from Agilent Technologies, Santa Clara, CA). The microarrays consist of glass slides containing 8 identical 15K oligonucleotide microarrays incorporating probes for 866 human miRNAs represented from the Sanger miRBase 12.0. The procedure was performed as described previously (42). Slides were scanned using the Agilent Microarray Scanner and the Agilent Feature Extraction Software version 10.5.1.1 (both from Agilent, Foster City, CA).

Bioinformatics miRNA analysis and target selection: Potential mRNA target genes for differentially expressed miRNAs in diseased periapical tissues were identified using miRWalk (<http://www.rna.uni-heidelberg.de/apps/zmf/mirwalk/index.html>). miRWalk is a comprehensive database that provides information on human and murine miRNAs on their predicted and validated targets associated with genes, pathways, diseases, organs, cell lines and transcription factors. It is based on a comparison of computed mRNA 3' UTRs miRNA binding sites with 8 miRNA-target prediction programs. Candidate mRNAs were selected if they were identified as validated miRNA targets in at least 5 out of 8 databases and were linked to immunity, inflammation and pain by GO Biological Process (www.geneontology.org). Results from miRWalk and PUBMED search were integrated to reach our final results.

Quantitative RT-PCR: 9 miRNAs that demonstrated significant differential expression in the microarray analysis and were known to be linked to inflammation and the immune response were selected for further validation using qRT-PCR. miScript primers and miScript II RT

Kit were purchased from Qiagen (Germantown, MD, USA). 450 ng total RNA was reverse transcribed using cDNA synthesis kit according to manufacturer's instructions. The PCR reactions were run using miRNA specific primers and the miScript universal primer (Qiagen, Catalogue #: MS00006692, MS00006699, MS00008841, MS00031500, MS00007588, MS00009744, MS00031878, MS00010906, MS00003570, MS00007518). 20 µl reaction mixes were prepared using 2X EvaGreen Master Mix (Biotium, Hayward, CA, USA), 2 µl of 1:10 diluted cDNA, and 10 pmoles of each forward and reverse primer. The real-time PCR was carried out in the StepOne 7500 thermocycler (Applied Biosystems, Carlsbad CA, USA). SNORD 44 served as an internal control and all reactions were run in triplicates.

Statistical analysis: For microarray data analysis, any expression value that was lower than the reported error for that particular gene (which includes negative expression values) was set to be equal to the estimated error rate. Quantile normalization was applied to the expression data. To identify genes that were differentially expressed in each group, we applied a permutation test to test the null hypothesis that the mean expression of each gene was the same in both groups. An exact hypothesis test was used since the sample size was small. We used the resulting p-values to estimate the false discovery rate q-value when the differential expression of each miRNA is called "significant". For each resulting p-value, we computed the q-value, which is defined to be the false discovery rate when all tests with a p-value less than or equal to the given p-value are called "significant."

For qRT-PCR analysis, the relative expression of miRNA as compared SNORD44 was computed using the $2^{(-\Delta\Delta Ct)}$ method (43). Significance was determined by applying Welch's t-test to the relative fold changes of periapical tissues and control tissues.

Differences were considered significant when the probability value was less than 5% ($P < 0.05$).

Results:

No significant differences were noted in gender distribution between experimental and control groups. However, there was a significant difference in age between subjects from which periapical tissue were collected and subjects from which healthy PDL and pulp were collected ($P < 0.05$). The mean age of the periapical group was 53 yrs. (± 15), while that of the PDL controls was 28 yrs. (± 16), and that of the pulp group was 18 yrs. (± 3). Two subjects from which periapical lesion tissue was collected for qRT-PCR analysis experienced pain symptoms associated with the tooth prior to sample collection, while the remaining were asymptomatic.

Microarray results

Of the 381 human miRNAs identified in periapical tissues, 24 miRNAs were significantly down-regulated ($P < .003$, $q < .08$) in periapical tissues compared to healthy pulp control tissues. Of these 24 down-regulated miRNAs, 15 showed a 2-5 fold change and six had more than a five-fold change (Fig. 1). Of the 24 down-regulated miRNAs identified, nine miRNAs that are linked to inflammation and immunity were selected for further analysis with qRT-PCR. The fold change in expression of these nine miRNAs ranged from 2.4 to 10.8.

qRT-PCR results

The comparison between healthy periodontal ligament samples and diseased periapical tissue samples showed a significant down-regulation of seven out of nine miRNAs tested ($P < .05$) (Fig. 2). The fold change for this comparison varied from 1.2-fold to 21.2-fold. miR-95 was down-regulated 1.2 fold while the remaining six miRNAs were down-regulated more than 4-fold. These same seven miRNAs were also significantly down-regulated when comparing healthy pulp tissues to periapical lesion tissues ($P \leq .001$) (Fig. 3). The fold regulation for this comparison varied from 16-fold to 45-fold. These results were similar to those obtained with microarray as both showed significant down-regulation of the seven miRNAs, however the magnitude of the fold change was greater with qRT-PCR (16 to 45 fold change) compared to microarray (2.4 to 10.8 fold change). The remaining two miRNAs, miR-455-5p and miR-181d, were not detected in the samples using qRT-PCR.

Bioinformatics data

The potential targets of the differentially expressed miRNAs identified include key cytokines involved in inflammation (IL-6, IL-10), chemokines (CCL8), pathogen recognition receptors (TLR-4), growth factors (TGF- β 1, VEGF- α) and proteins involved in macrophage differentiation and the immune response to LPS (MMP-9) (Table 1).

Discussion:

In this study, multiple miRNAs from the miR-181 family (miR-181a*, miR-181b and miR-181c) were demonstrated to be significantly down-regulated in diseased periapical

tissues compared to healthy control tissues. Down-regulation of miRNAs, which are negative regulators themselves, results in an increase in their respective target messenger RNAs (mRNA). The targets of miR-181a* include toll-like receptor-4 (TLR-4), which plays a key role in pathogen recognition and activation of the innate immune response, and IL-6, which stimulates neutrophil production and supports B-cell maturation. miR-181b also targets IL-6 as well as CCL-8, MMP-9 and TGF- β 1, which are involved in a wide range of inflammatory pathways. For example, CCL-8 is chemotactic for and activates several immune cells including monocytes, T cells and NK cells. MMP-9 is closely associated with macrophage differentiation and TGF- β 1 increases collagen biosynthesis and fibroblast proliferation. The targets of miR-181c include SOCS1, which is involved in the LPS response, and IL-2, which plays an essential role in the immune response to antigenic stimuli and is important for the proliferation of T and B lymphocytes. The miR-181 family is also notable for altering T-cell receptor signaling and increasing IFN- γ /IL-17 production by Th1/17 cells.

Increasing evidence supports the role of the miR-181 family in inflammatory pathologies. Circulating levels of miR-181a, miR-181a-2* and miR181c in whole blood were significantly lower in patients diagnosed with complex regional pain syndrome, a disorder in which neurogenic inflammation plays a key role (44). Blood plasma levels of miR-181b was found to be lower in patients with sepsis (a whole body inflammatory condition due to infection) and in animal models of sepsis (45). Data from our previous study show that members of the miR-181 family are down-regulated in inflamed human pulps as compared to normal pulps ($p \leq 0.001$) (42). Conversely, miR-181c is upregulated in inflamed gingival tissues (40). These differences in expression of miR-181c in distinct inflammatory

pathologies could possibly be due to differences in the type of infection or the host tissue or the sampling point after initiation of inflammation.

In addition to the miR-181 family, miR-24-1*, miR-95, miR-149 and miR-455-3p were significantly down-regulated in diseased periapical tissues. These miRNAs also have a variety of targets that are implicated in the immune and inflammatory response. For example, miR-149 targets VEGF- α , which acts on endothelial cells to mediate increased vascular permeability and promote cell migration to the site of inflammation. miR-455-3p targets TLR-4 as well as IL-10, a cytokine produced primarily from monocytes that serve to enhance B cell survival, proliferation and antibody production.

A limited number of studies have examined miRNA expression in inflammation related to endodontic infection. Our previous study examined the differential expression of miRNAs in inflamed and healthy pulps using microarray techniques (42). When comparing our microarray data to that of our previous study, we see that there is significant differential expression of 13 of the same miRNAs in both periapical and pulp tissues. These include: miR-29a*, miR-30b*, miR-181a-2*, miR-181d, miR-455-5p, miR199-5p and miR-664. All seven of the significantly down-regulated miRNAs identified in diseased periapical tissues were also shown to be down-regulated in inflamed pulp tissue compared to healthy pulp tissue. The six of which showed significant down-regulation in both tissues include: miR-24-1*, miR-95, miR-181a*, miR-181b, miR-181c and miR-455-3p. The extent of this cross-over suggests some common miRNA regulatory network in pulpal and periapical disease pathogenesis. This could be due to similar inflammatory processes occurring in both tissues. However, the tissue specificity of miRNAs is apparent in the difference between the types of miRNAs identified in pulp and periapical tissues using microarray. Although some overlap

exists, it is possible that the other miRNAs identified in this study could be unique to the periapical disease process.

The limitations of this study include the small sample size and age matched controls were not used. Both PDL and pulp were used as control tissues. In the absence of periapical disease the tissues present in the periapex consist of PDL, pulp and bone tissue. Bone tissue was not included as a control since its removal is not recommended in apicoectomy procedures as this could delay healing. Bone tissue is also relatively acellular and its cellular components are difficult to isolate from the hard tissue. After the microarray experiments, an additional cohort of tissue samples were collected for qRT-PCR analysis. Despite using new tissue samples for the qRT-PCR, the results confirmed the down-regulation of the same miRNAs identified using microarray. Also, the same miRNAs were consistently down-regulated in diseased periapical tissues when comparing to both pulp and PDL control tissues. This correlation strengthens the findings and supports the inclusion of the selected control tissues. The magnitude of down-regulation of the seven miRNAs was greater when comparing diseased periapical tissues to pulp controls than when comparing to PDL controls. This may be due to higher baseline expression levels of the miRNAs in the pulp since it is more cellular in nature than PDL.

A significant difference in mean age between groups from which tissue samples were collected was observed. Participants in the PDL and pulp control groups consisted primarily of younger individuals undergoing extraction of third molars or premolars for orthodontic purposes. Participants in the periapical group were older as these teeth had previous endodontic treatment and were planned for apicoectomy surgery.

Future studies that include a larger number of subjects are necessary to identify any miRNAs that might correlate with pain symptoms. Studies that implement in situ hybridization techniques could be used to determine the cellular sources of the identified miRNAs. Confirmation of the altered target gene expression could then be investigated with miRNA overexpression or knockdown studies.

This study explores the role of miRNAs in endodontic disease and provides new insight into the genetic regulation of endodontic periapical pathogenesis. Seven specific miRNAs that target key mediators in the inflammatory and immune response were determined to be significantly down-regulated in diseased periapical tissues. This study offers potential candidates for further investigation of miRNAs in endodontic disease. These findings could facilitate the development of potential biomarkers and possible therapeutic targets for the treatment of endodontic disease.

Acknowledgements:

This work was supported by the School of Dentistry, University of North Carolina-Chapel Hill and NIH, NIDCR DE021052.

Figures & Tables:

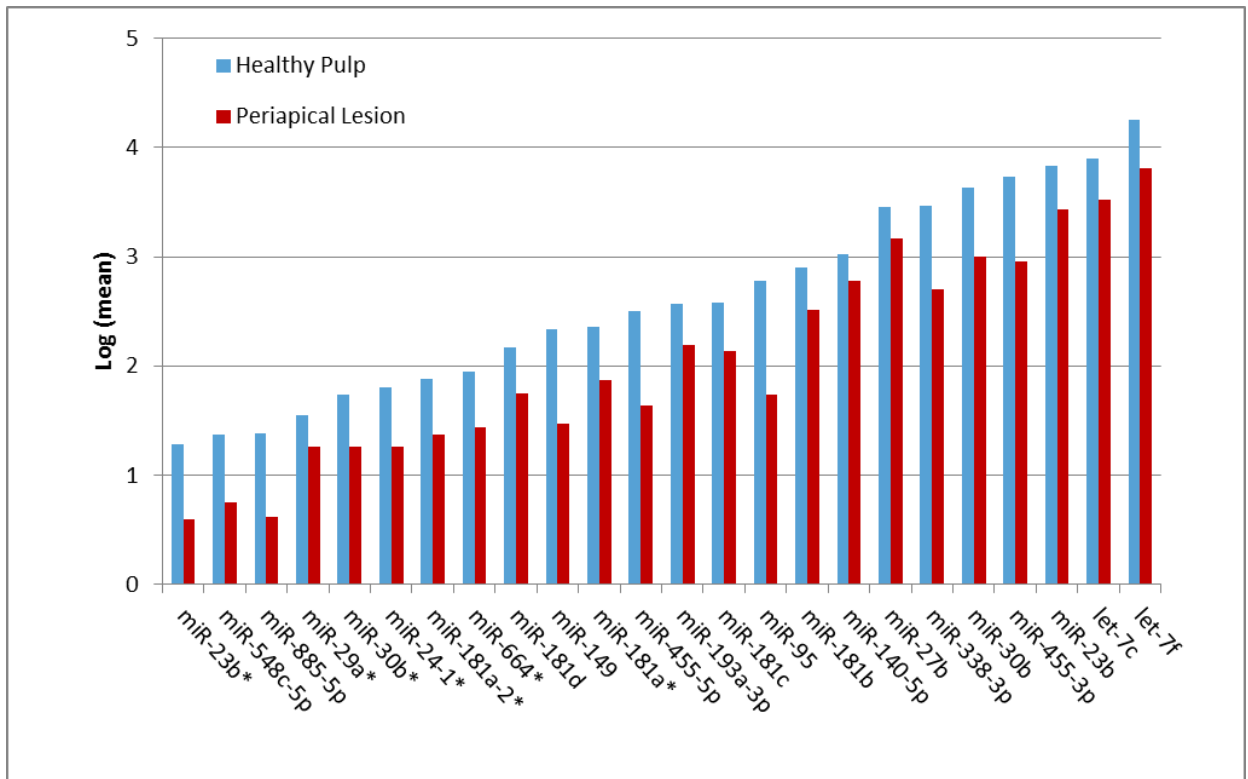


Figure 1. Differential expression of miRNAs in healthy pulp (n = 5) and diseased periapical tissues (n = 8) evaluated by microarray. Twenty-four miRNAs were significantly down-regulated in diseased periapical tissues ($P < .003$, $q < .08$). Data was analyzed using an exact hypothesis test and expressed as log values.

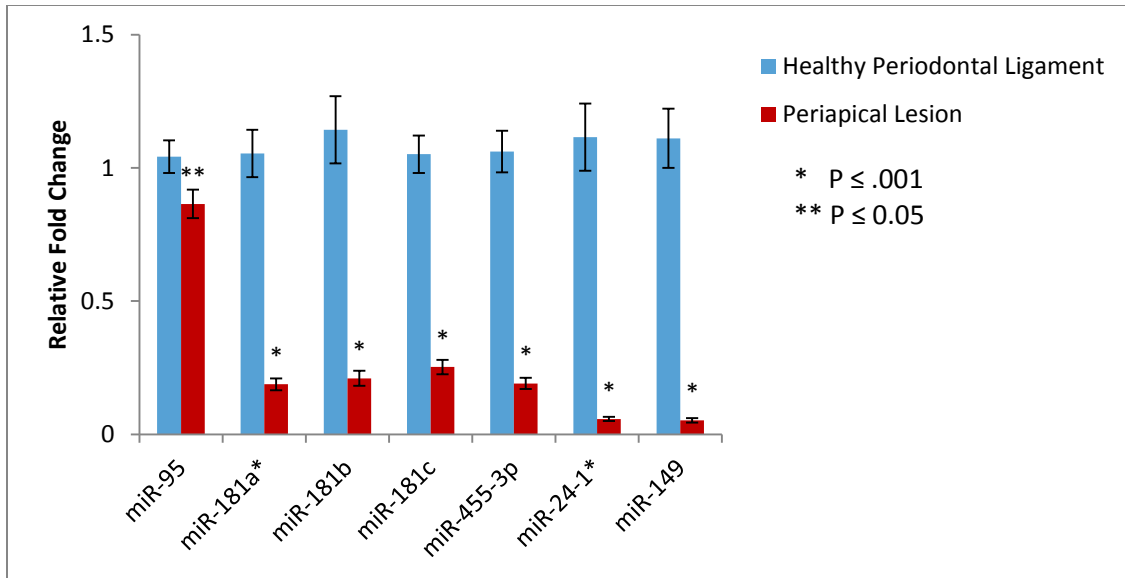


Figure 2. Relative fold changes of seven miRNAs in diseased periapical and healthy periodontal ligament tissues analyzed by qRT-PCR. Data was normalized to expression of endogenous control, SNORD44. Relative expression computed using the $2^{(-\Delta\Delta Ct)}$ method. * $P \leq .001$, ** $P \leq 0.05$

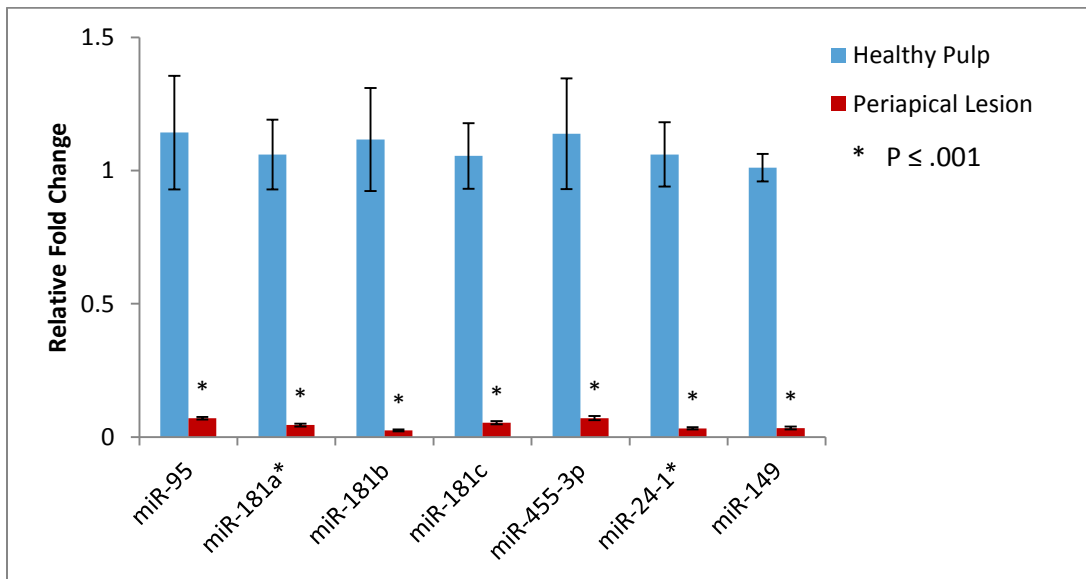


Figure 3. Relative fold changes of seven miRNAs in diseased periapical and healthy pulp tissues analyzed by qRT-PCR. Data was normalized to expression of endogenous control, SNORD44. Relative expression computed using the $2^{(-\Delta\Delta Ct)}$ method. * $P \leq .001$

Table 1. Potential miRNA target genes were identified using PUBMED and miRWalk (http://www.rna.uni-heidelberg.de/apps/zmf/mirwalk/index_html).

miRNA	Target gene	Gene product function	GO Term (Accession, Ontology)
miR-181a	TLR-4	Toll-like receptor-4 (TLR) plays a fundamental role in pathogen recognition and activation of innate immunity	GO:0035662, Molecular Function
	IL-6	Acute and chronic inflammation and the maturation of B cells	GO:0019981, Molecular Function
miR-181b	IL-6	Negative regulation of cytokine secretion; negative regulation of collagen biosynthetic process; positive regulation of acute inflammatory response; response to cold, heat and mechanical stimuli	GO:0005138, Molecular Function
	CCL8	Immune response, inflammatory response, chemokine activity, phospholipase activator	
	MMP9	Cell response to IL-1, LPS; macrophage differentiation; response to heat and mechanical stimuli; positive regulation of apoptosis and angiogenesis	GO:0004229, Molecular Function
	TGFB1	Adaptive immune response; positive regulation of collagen biosynthesis, chemotaxis, fibroblasts migration, and odontogenesis	GO:0034713, Molecular Function
miR-181c	SOCS1	Cytokine mediated signaling pathway; negative regulator of JAK-STAT pathway; LPS response	
	IL-2	Cytokine produced by T-cells in response to antigen or mitogen stimulation	GO:0005134, Molecular Function
miR-24	MAPK14	MAP kinase activated by various environmental stresses and proinflammatory cytokines, act as an integration point for multiple biochemical signals	GO:0051403, Biological Process
miR-95	EIF2C2	Eukaryotic translation initiation factor interacted with dicer1 in short-interfering-RNA-mediated gene silencing	
miR-149	VEGFA	Increased vascular permeability, angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis	GO:0035924, Biological Process
miR-455	IL-10	Pleiotropic effects in immunoregulation and inflammation, B cell survival, proliferation, and antibody production, block NF-kappa B activity	GO:0019969, Molecular Function
	TLR-4	Toll-like receptor-4 (TLR) plays a fundamental role in pathogen recognition and activation of innate immunity	GO:0035662, Molecular Function

CHAPTER 3: CONCLUSION

In this study, 24 miRNAs were found to be significantly down-regulated in periapical tissues compared to healthy control tissues using microarray techniques. Of these 24 miRNAs, 9 which were linked to immunity and inflammation were then subjected to further validation using qRT-PCR. Additional tissue samples were collected for qRT-PCR and results showed significant down-regulation of 7 of the 9 miRNAs tested when comparing diseased periapical tissue to both healthy pulp and periodontal ligament tissues.

Multiple miRNAs from the miR-181 family (miR-181a*, miR-181b and miR-181c) were among those identified to be significantly down-regulated in diseased periapical tissues. Down-regulation of miRNAs, which are negative regulators themselves, results in an increase in their respective target messenger RNAs (mRNA). The targets of miR-181a* include toll-like receptor-4 (TLR-4), which plays a key role in pathogen recognition and activation of the innate immune response, and IL-6, which stimulates neutrophil production and supports B-cell maturation. miR-181b also targets IL-6 as well as CCL-8, MMP-9 and TGF- β 1, which are involved in a wide range of inflammatory pathways. For example, CCL-8 is chemotactic for and activates several immune cells including monocytes, T cells and NK cells. MMP-9 is closely associated with macrophage differentiation and TGF- β 1 increases collagen biosynthesis and fibroblast proliferation. Studies using animal models have also implicated miR-181b in the regulation of NF- κ B-mediated endothelial cell activation and vascular inflammation and suggests that rescue of miR-181b expression could be

implemented in anti-inflammatory therapy (45). The targets of miR-181c include SOCS1, which is involved in the LPS response, and IL-2, which plays an essential role in the immune response to antigenic stimuli and is important for the proliferation of T and B lymphocytes.

Many miRNAs from the miR-181 family have been implicated in various inflammatory pathologies. For example, circulating levels of miR-181a, miR-181a-2* and miR181c are significantly lower in patients diagnosed with complex regional pain syndrome, a disorder in which neurogenic inflammation plays a key role (44). In addition, circulating levels of miR-181b are lower in patients with sepsis (a whole body inflammatory condition due to infection) and in animal models of sepsis (45). Data from our previous study show that members of the miR-181 family are down-regulated in inflamed human pulps as compared to normal pulps ($p \leq 0.001$) (42). Conversely, miR-181c is upregulated in inflamed gingival tissues (40). These differences in expression of miR-181c in distinct inflammatory pathologies could possibly be due to differences in the type of infection or the host tissue or the sampling point after initiation of inflammation.

In addition to the miR-181 family, miR-24-1*, miR-95, miR-149 and miR-455-3p were significantly down-regulated in diseased periapical tissues. These miRNAs also have a variety of targets that are implicated in the immune and inflammatory response. For example, miR-149 targets VEGF- α , which acts on endothelial cells to mediate increased vascular permeability and promote cell migration to the site of inflammation. miR-455-3p targets TLR-4 as well as IL-10, a cytokine produced primarily from monocytes that serve to enhance B cell survival, proliferation and antibody production.

A limited number of studies have examined miRNA expression in inflammation related to endodontic infection. Our previous study examined the differential expression of

miRNAs in inflamed and healthy pulps using microarray techniques (42). When comparing our microarray data to that of our previous study, we see that there is significant differential expression of 13 of the same miRNAs in both periapical and pulp tissues. These include: miR-29a*, miR-30b*, miR-181a-2*, miR-181d, miR-455-5p, miR199-5p and miR-664. Of the seven significantly down-regulated miRNAs identified in diseased periapical tissues, six miRNAs have also been shown to be down-regulated in inflamed pulp tissue compared to healthy pulp tissue. These include miR-24-1*, miR-95, miR-181a*, miR-181b, miR-181c and miR-455-3p. The extent of this cross-over suggests a common miRNA regulatory network in pulpal and periapical disease pathogenesis.

Future studies that include a larger number of subjects are necessary to identify any miRNAs that might correlate with pain symptoms. In this study, only two subjects presented with pain symptoms prior to periapical tissue collection. A larger scale study could include more symptomatic patients which would allow for statistical analysis to determine if any specific miRNAs show differential expression between patients experiencing pain and asymptomatic patients. Future studies could also investigate the cellular sources of the identified differentially expressed miRNAs. Formalin fixed periapical lesion tissues collected from apicoectomy procedures could be used with in situ hybridization techniques to determine which cell types each of the identified miRNAs is expressed in. In the present study, samples were determined to be periapical granulomas rather than cysts based on only clinical impression. However, in situ hybridization would allow for confirmation of the type of lesion while also providing a qualitative determination of differential expression of miRNAs. Identifying the cellular sources of the differentially

expressed miRNAs could also shed light on whether the observed changes in their expression levels are solely due to a change in the cell profile of the lesion or not.

In addition, the down-stream effects of altered miRNA expression could be explored. Gene-specific experimental validation of miRNA targets can be done to identify individual miRNA:mRNA interactions. Downstream effects of differential miRNA expression can be observed at the protein level by western blot and at the mRNA level by qRT-PCR. Confirmation of the altered target gene expression could then be investigated with miRNA overexpression or knockdown studies. Over-expression can be achieved using transient transfection of a synthetic miRNA precursor or by stable introduction of a miRNA expression construct such as a lentiviral vector (46). miRNAs can be inhibited by expressing modified antisense oligonucleotides that bind to mature miRNAs thus blocking their activity. Animal studies could also be used to sample lesions throughout the healing process in order to determine whether there are changes in the miRNA profiles at different stages of healing.

This study is the first to explore the role of miRNAs in periapical disease, providing new insight into the genetic regulation of endodontic disease. Seven specific miRNAs that target key mediators in the inflammatory and immune response were determined to be significantly down-regulated in diseased periapical tissues. This study begins to elucidate the complex miRNA regulatory network in endodontic disease and offers potential candidates for further investigation. The identified miRNAs have potential to serve as possible biomarkers or therapeutic targets for the treatment of endodontic disease. These findings could facilitate the development of improved methods of diagnosis and management of endodontic disease.

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