Gene expression profile of pulpitis

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Abstract

The cost, prevalence and pain associated with endodontic disease necessitate an understanding of the fundamental molecular aspects of its pathogenesis. This study was aimed to identify the genetic contributors to pulpal pain and inflammation. Inflamed pulps were collected from patients diagnosed with irreversible pulpitis (n=20). Normal pulps from teeth extracted for various reasons served as controls (n=20). Pain level was assessed using a visual analog scale (VAS). Genome-wide microarray analysis was performed using Affymetrix GeneTitan Multichannel Instrument. The difference in gene expression levels were determined by the Significance Analysis of Microarray program using a false discovery rate (q-value) of 5%. Genes involved in immune response, cytokine-cytokine receptor interaction and signaling, integrin cell surface interactions, and others were expressed at relatively higher levels in the in the pulpitis group. Moreover, several genes known to modulate pain and inflammation showed differential expression in asymptomatic and mild pain patients (30mm on VAS) compared to those with moderate to severe pain. This exploratory study provides a molecular basis for the clinical diagnosis of pulpitis. With an enhanced understanding of pulpal inflammation, future studies on treatment and management of pulpitis and on pain associated with it can have a biological reference to bridge treatment strategies with pulpal biology.

Introduction

Inflammation of the dental pulp (pulpitis) can be a progressive and devastating pain experience characterized by spontaneous or provoked pain, hyperalgesia, allodynia and difficulty in achieving adequate local anesthesia [1]. The activation of dental pulp or peridental

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nociceptors during endodontic inflammation elicits a pain response that contributes to approximately 90% of dental emergency visits in both private dental clinics and in hospitals [2-5]. The economic implication of these emergency visits is reported to cost almost US$1 billion per year [4]. However, despite the cost and the prevalence of endodontic disease and the great discomfort associated with it, the fundamental molecular aspects of its pathogenesis are still not fully understood. The current literature on pulpal immune response to microbial infection continues to expand but very little is known on the regulatory mechanisms behind pulpal disease.

Cells comprising the human dental pulp trigger immune responses to a complex array of microorganisms that invade dental tissues [6-8]. These immunocompetent cells also form mechanical barriers (i.e. odontoblasts), detect and transmit sensations (nerve fibers) or differentiate (i.e. dental pulp stem cells) to limit infection, signal injury and promote repair, respectively. These cascades of events resulting from dental pulp stimulation by microorganisms result in the release of a plethora of immune mediators that trigger pulpal or odontogenic pain, inflammation or in advanced stages, pulpal necrosis. In addition, several studies have suggested that pulpal events can be reflected in the gingival crevicular fluid (GCF) through measurable levels of protein markers that correlated with pulpal symptoms [9, 10]. This shows that the dental pulp is not an isolated environment but rather a vital, reactive tissue that communicates with the outside environment.

Several studies have identified the biological differences between healthy and inflamed dental pulp. Cytokines, cell surface receptors and other protein markers are shown to be either highly increased or decreased in inflamed dental pulp.[7, 11-21]. A limited number of studies have examined gene expression in inflamed human pulps [22-24]. These studies, however, did not explore the differences in gene expression between normal and inflamed dental pulp and the clinical presentation of donor patients (i.e. pain and swelling), which may provide biologic explanation on the variability of clinical signs and symptoms of pulpal inflammation that confound diagnosis.

Although the clinical value of a molecular diagnostic marker may at first appear limited in scope, the emerging correlation of gene expression with clinical signs and symptoms will enhance our understanding on the development of pulpal inflammation - this will add not only to the knowledge base but it will also provide a biological basis for the varying clinical presentations of pulpitis. Prior studies that focused on histological findings have shown a wide variation – from poor to strong - in correlating clinical signs and symptoms with histological findings [25-27]. In this study, data from the full genome scan will be utilized to determine if an association exists between gene expression and clinical presentation (i.e. pain) of pulpitis patients.

Results and Discussion

Normal and pulpitis samples exhibited differentially expressed genes

The SAM software generated GSEA data which showed a significantly higher expression of various gene sets that are associated with immune response activation, maintaining cellular function and cell-to-cell interaction, among others (Figure 1) in pulpitis samples. This

underscores the utility of both the subjective (patient-derived history) and objective (endodontist-performed testing) diagnostic techniques that clinically delineate a normal from an inflamed dental pulp. Furthermore, the results above re-establish the immunocompetency of the dental pulp that has been shown to carry Toll-like receptor (TLR) -2/4+ cells [7, 28].

Differences in gene expression between mild and moderate to severe pain

Among the patients diagnosed with irreversible pulpitis, eight patients reported experiencing zero to mild pain ((30mm on VAS) and twelve patient reported moderate to severe pain. The mean VAS scores for patients who reported zero to mild pain and moderate to severe pain was 6.63 (SD = 9.29) and 81.93 (SD = 11.21), respectively ($P<0.0001$). There were differentially expressed genes between the two groups (Figure 2). Mild pain samples showed a significantly higher expression of genes involved in adaptive immune system, cytokine to cytokine interaction and cytokine signaling, among others. However, when looking at specific genes, several of them that have key roles in inflammation are significantly under-expressed, unchanged or over-expressed in asymptomatic or mild pain patients compared to those with moderate to severe pain (Table 1).

Relief from pain is a very important part in the practice of endodontics. Patients often judge the success of treatment and the efficiency of the dentist based on their pain experience. The decision whether to perform root canal therapy (RCT) to relieve pain or manage infection relies heavily on clinical diagnostic tests which dichotomize the pulpal diagnosis to reversible and irreversible pulpitis. To arrive at a diagnosis, dentists depend mainly on pain history and responses to sensibility tests (namely thermal tests and electric pulp testing,) that have been used for decades; but whether the responses to these clinical tests correlate well with pulpal histopathology or not remains unclear [25-27, 29-31].

This study was aimed to identify the differences in gene expression between normal pulps and pulpitis samples and between mild and moderate to severe pulpitis pain, based on the diagnostic guidelines set by the AAE and on the patient-reported pain experience at the time of endodontic treatment, respectively. Gene Set Enrichment Analysis (GSEA) was employed for the analysis of the thousands of genes included in the microarray screening [32, 33]. The Broad Institute defines GSEA as a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states.

Several applications are envisioned for the data that were gathered in this study. First, a biological basis at the molecular level can now be correlated not only with the current diagnostic testing procedures but also with the diagnosis of irreversible pulpitis itself. Molecular alterations precede histopathological changes and in the case of the dental pulp, there can be a wide array of histological presentations depending on the timing of pulp extirpation; thus, there are conflicting reports concerning the clinical presentation and actual histopathology of the pulp. Studies investigating inflammation at the molecular level may therefore provide a more accurate representation of the inflammatory changes in the dental pulp.

Next, the results may be used to cluster genes that are overexpressed or underexpressed in inflamed pulps and to use this information as biological basis for future studies on acute pain and on oral biology, especially those that focus on odontogenic pain and regeneration as these studies require a broad understanding of molecular changes that dictate clinical and biological outcomes. The difference in gene expression between normal and inflamed dental pulps (Figure 1) may have been the expected result considering that cytokines, chemokines and their receptors are known to be overexpressed in inflammatory conditions; however, this study is still an important addition to our current understanding of pulp biology as it provides a broad picture or a "snap-shot" of the molecular changes occurring in the dental pulp during the very moment that the patient is seeking endodontic treatment on the dental chair.

Another remarkable finding in the study is the overexpressed genes related to inflammation and immune response in pulps from patients who reported zero to mild pain (30mm on VAS) during their endodontic appointment compared to those who had moderate to severe pain (Figure 2). Moreover, looking at individual genes separately from the a priori gene sets in the GSEA software revealed that genes involved in key inflammatory response like IL8, TNFA, IL1A, ILIB and several immunoglobulins (i.e. IGHM, IGHV4-31, IGKV2D-40), among others (Table 1) are expressed at relatively lower levels in pulp samples from asymptomatic to mild pain patient compared to those with moderate to severe pain. IL8, TNFA and IL1B have been shown to be potent mediators of pain [34, 35].

IL8 has been recently associated with complex regional pain syndrome (CRPS) [36] and IL1B has been shown to contribute to the upregulation of nerve growth factor (NGF) during inflammation that consequently induces inflammatory hyperalgesia [37]. Both IL8 and IL1B are expressed at relatively lower levelsin zero to mild pain pulpitis patients. Several genes that are upstream on the inflammatory cascade of both IL1B and IL8 (e.g. TLR2 and TLR44) are significantly increased in pulpitis samples (Table 1). Furthermore, most genes associated with structural formation and integrity (e.g. COL1-4 and MMPs) are expressed at relatively higher levels in pulpitis samples. In contrast however, several genes that are known to be pain mediators or associated with increased pain experience did not have significant discordance between mild and moderate to severe pain (Table 1). For example, Prostaglandin-Endoperoxide Synthase 2 or Cyclooxygenase-2 (PGS2 or COX2), an important inflammatory enzyme that has been shown to be significantly elevated in pulpitis tissues[38] did not have expression difference in mild pain but is significantly elevated in pulpitis compared with normal controls (Table 1). IL6 showed the same results.

Pathogen–associated molecular pattern receptors, including TLRs, are key molecules in the response to pathological stimuli. Pulpitis is a disease that is mainly due to microbial insults. TLRs are expressed by both immune and non-immune cells in the pulp including neurons, fibroblasts, endothelial cells, epithelial cells and others. They recognize viral and microbial structures as well as self molecules (such as single stranded RNAs) that may accumulate in non-physiologic amounts or sites [39-43]. TLR ligands stimulate the production of pro-pain molecules and, in this way, may contribute to the pathogenesis of pain [42-47]. In this study, the expression of several TLRs (1,2,4,6, 8 and 9) were upregulated and TLR 3 was down regulated in inflamed pulps as compared to normal pulps. Our data also show that expression

of TLR8 was higher in pulpits patients experiencing severe pain as compared to those who reported no pain or mild pain. This is of particular interest given the recent finding that murine TLR7 (which is phylogenetically and structurally related to human TLR8) interacts with TRPA1 and that specific miRNAs activate nociceptors via TLR7 and TRPA1 [47].

Arriving at a reliable diagnosis that matches the histopathology of the dental pulp, especially during a pain episode is arguably the most challenging part of endodontics. The dental pulp is a dynamic tissue that is capable of responding to insults and of healing so that diagnosis may change over time [48]. Furthermore, individual variations in immunologic and pain response further complicate not only the clinical but also the biological aspect of endodontic diagnosis. This may be the one of the main reasons for the observational differences among studies correlating clinical and histopathological diagnosis as pointed out in the earlier part of this paper. Understanding the molecular changes in pulpitis may help develop better diagnostic tools. For example, molecular markers collected from dentinal fluids may be used to determine the pulpal status. This would be a non-invasive and easy diagnostic tool.

The study of tissue molecular profiles to better understand and diagnose disease pathophysiology has been examined in another common oral disease- periodontitis. Using biopsies from patients diagnosed with either chronic periodontitis or aggressive periodontitis, Kebschull et al., explored whether molecular profiling can form the basis for a pathobiology based classification that correlates with the phenotypic features of the disease [49]. They identified two *de novo* clusters with high similarity in transcriptional profiles. The clusters had distinct molecular signatures which did not align with the current classifications of periodontitis. However, they did translate into distinct phenotypic differences and can provide a basis for novel classification. This study along with current study supports the use of molecular profiling to classify disease.

While microarray analysis is a powerful tool to study the simultaneous expression of several genes it has some inherent limitations. These include technical factors such as limited dynamic range, signal saturations and cross-hybridization. It is important to also remember that the cellular profile of inflamed pulps differs from that of normal pulps. The residents cells in normal pulps include fibroblasts, odontoblasts and some immune cells such as macrophages, dendritic cells and mast cells. Inflamed pulps are characterized by an influx of immune cells and as such the results reported here may be in part, due to differences in the cellular makeup as well.

The present study shows that there are differences in gene expression between normal and inflamed pulps and; between zero to mild and moderate to severe pulpitis pain. Among the genes reported to have a strong association with pain and inflammation, IL8 showed the highest fold change (35.9, q-value = 0) in pulpitis samples and it is significantly under-expressed in mild pain compared with moderate to severe pain (0.14, q-value = 0). IL8 is increased in gingival crevicular fluid from patients with acute pulpitis and might be a useful measurement for staging patients with acute pulpitis [9]. The same trend was also noted in IL1A, IL1B and IL10RA.

This study provides a molecular basis for the clinical diagnosis of pulpitis and for the differences or similarities between asymptomatic to mild pulpal pain vis-à-vis moderate to severe pain. With an enhanced understanding of pulpal inflammation, future studies on treatment and management of pulpitis and on pain associated with it can have a biological reference to bridge treatment strategies with pulpal biology.

Materials and Methods

The study was approved by the University of North Carolina Office of Human Ethics. Eligible patients seeking treatment at the School of Dentistry were recruited to participate in the study. Informed consent was obtained from each participant. Demographic details, history of odontogenic pain, vital signs (blood pressure, pulse rate), medical history (including full details of any medication) and patient's smoking and oral habits were recorded.

Patient selection and pulpal diagnosis

The inclusion criteria were adults (18 years or older) presenting for endodontic treatment with no evidence of periapical pathoses (i.e radiolucency, swelling, pressure sensitivity) and no previous pulp therapy (i.e., pulp capping, etc). Exclusion criteria were conditions requiring antibiotic prophylaxis or additional treatment procedures, debilitating disease, chronic pain, diabetes mellitus, hematological disorders or a history of taking centrally acting drugs (e.g. tricyclic antidepressants) known to interfere with the release of various pain mediators and/or modify pain experience within the previous 6 months or over-the-counter pain medicine within the last six hours, chronic use of medication known to affect the immune response and patients who were immunocompromised. Teeth with incompletely developed roots were also excluded.

Diagnosis of normal pulp (NP) and irreversible pulpitis (IP) was based on subjective and objective findings and was in accordance with the American Association of Endodontists (AAE) guidelines. In brief, NP is a clinical diagnostic category in which the pulp is symptom-free and normally responsive to diagnostic testing On the other hand, IP is clinical diagnosis indicating that the vital, inflamed pulp is incapable of healing. The symptoms include lingering and exacerbated pain in response to thermal stimuli, spontaneous pain, referred pain or no clinical symptoms but inflammation is evident after caries excavation or trauma. Pain history, cold test (Endo Ice® Refrigerant Spray, Coltene Inc, Cuyahoga Falls, OH) and electric pulp testing (Vitality Scanner 2006, Sybron Endodontics, Orange, CA) were used to determine pulpal diagnosis. Percussion, palpation, probing, mobility and radiographs were used to rule out periapical pathosis.

Pain levels were assessed using a visual analogue scale (VAS). If lingering pain is present or significantly evoked by a stimulus compared with a control tooth, the patient was asked to quantify the intensity of pain by placing a mark on a 10-cm VAS with anchors of "no pain" and "worst pain imaginable". Using a modified version of the VAS recommended by Jensen et al [50], a VAS mark of 30mm was classified under mild, 31-74 mm under moderate and 75-100 under severe pain.

A total of 40 pulp samples from 28 patients with IP and from seven patients with NP were included in the study. Twelve of the IP samples presented with moderate to severe pain at the time of pulp extirpation. The total number of samples was based on a recently published paper by our group on the differences of micro-RNA expression between normal and inflamed pulps [51] and on a power analysis. With 20 samples in each group, we had 80% power to detect an effect size of 0.9 using a threshold of $p<0.05$ for significance. If we required a more stringent threshold of $p<0.001$, then we would have 80% power to detect an effect size of 1.4. Given that large effect sizes are common in microarray experiments [52], we had adequate power to detect the genes that exhibited the largest expression differences between the two groups. Moreover, we increased the power as much as needed by increasing the acceptable false discovery rate. Given that this initial analysis is intended to be exploratory, a higher false discovery rate is acceptable in this study. Thus, this study had adequate power to detect the genes with the largest group differences.

Sample collection

The inflamed pulps were extirpated during root canal treatment and the normal pulps were collected right after tooth extraction for orthodontic or restorative purposes or for third molar removal. Pulp collection methods were adapted from Awawdeh *et al* [53] with modifications. Briefly, local anesthesia (2% lidocaine with 1:100,000 epinephrine) was administered and an access cavity was prepared under rubber dam isolation. Pulp tissue was extirpated using a sterile barbed broach or Hedström-file, placed into a pre-weighed Eppendorf tube, immediately frozen in liquid nitrogen and stored at −70°C. The root canal treatment was then completed. For the controls and for some patients who chose to have extraction instead of root canal treatment, all extractions were performed under local anesthesia. Immediately after extraction, each tooth were split in a vice fitted with a cutting edge and the pulp tissue was removed using a barbed broach or Hedström-file. The pulp was then placed in an Eppendorf tube, immediately frozen in liquid nitrogen and stored at −70°C.

RNA Extraction and Microarray analysis

Total RNA was isolated using a commercial kit (Qiagen RNEasy Kit, Valencia, CA) following the manufacturer's specifications. Quantity and purity of the total RNA was analyzed using Nano-Drop™ (Thermo Scientific, Wilmington, DE) and Bioanalyzer™ (Agilent Technologies, Sta. Clara, CA). Microarray experiments were conducted at the Microarray Core Facility of UNC-Chapel Hill Lineberger Cancer Center. The Nugen Ovation Pico WTA system V2 and the Nugen Encore Biotin module were used to prepare RNA for hybridization onto Affymetrix GeneChip Human Gene 2.0 arrays. Briefly, 25 ng of total RNA was converted into amplified SPIA cDNA using the Nugen Ovation Pico WTA system V2 and accompanying user guide. The amplified SPIA cDNA was purified using the Qiagen QIAquick PCR purification kit. 2.5 ug of SPIA cDNA was fragmented and labeled using the Nugen Encore Biotin module and accompanying user guide. The cocktail for hybridization onto an Affymetrix Human Gene 2.1 ST peg plate was prepared using the Nugen Ovation PicoSL WT System V2 user guide's cocktail assembly table. The hybridization cocktails were denatured and hybridized on the Human Gene 2.1 ST peg plate in the Affymetrix GeneTitan MC Instrument. Washing and scanning was also carried out on the Affymetrix GeneTitan MC Instrument. Individual sample RNAs were hybridized

individually to microarrays. Basic data analysis of the arrays was carried out using the Affymetrix Expression Console software. Gene expression levels between the control and diseased pulps were compared. Changes in gene expressions were analyzed by Gene Set Enrichment analysis (GSEA) (Broad Institute, Cambridge, MA) and its leading edge analysis software that uses the permutation method.

The difference in gene expression levels between groups were determined by the Significance Analysis of Microarray (SAM) program using a false discovery rate (q-value) of 5%. Since this study analyzed over 53,600 genes, only those that were previously reported to play key roles in pain and inflammation will be discussed in this paper. In addition, only the key genes with a SAM fold change of over 1.5 (overexpressed) and under 0.5 (underexpressed) to signify a potentially relevant clinical effect are reported [36].

Acknowledgments

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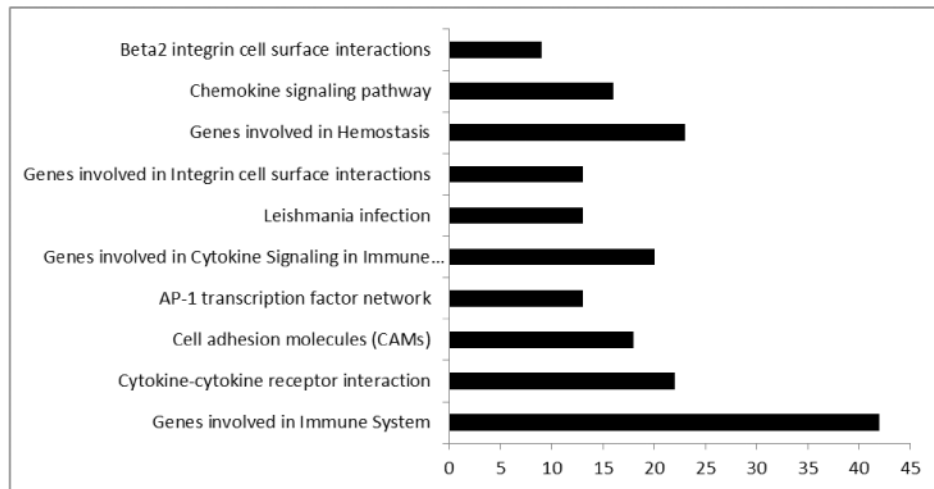**Figure 1.**
Gene Set Enrichment Analysis (GSEA) results between pulpitis and normal samples. Each bar represents the functional categories and the number of significantly regulated genes between pulpitis and normal groups (q<0.05).
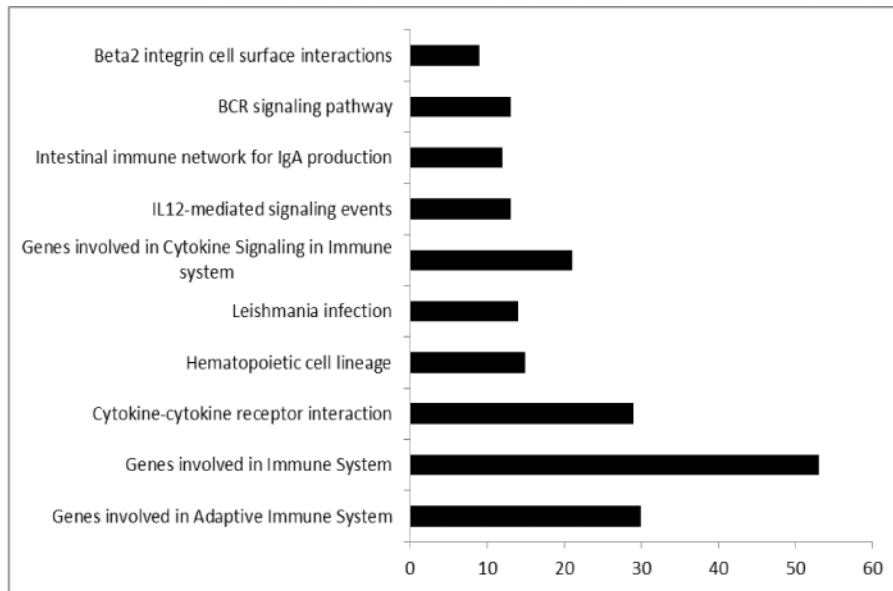
Figure 2.
GSEA results between groups that reported none to mild pain and those that reported moderate to severe pain on VAS. Each bar represents the functional categories and the number of significantly regulated genes between none to mild and moderate to severe pain groups (q<0.05).

Table 1
Selected up-regulated or down-regulated genes in pulpitis patients

Gene Symbol	Pulpitis vs Normal	None to Mild vs Moderate to Severe Pain	Gene Function [genecards.org]
AMELX	green	red	Enamel biomineralization
CALCRL	red	green	Bone metabolism
CCL20	red	black	Chemotaxis
CD14	green	red	Innate immune response
CD163	red	green	Acute phase receptor
CD79A	red	black	B-cell receptor function
COL10A1	red	green	Collagen formation
COL11A2	red	green	Collagen formation
COL12A1	red	black	Collagen formation
COL14A1	red	green	Collagen formation
COL15A1	red	green	Collagen formation
COL15A1	red	green	Collagen formation
COL18A1	red	green	Collagen formation
COL1A1	green	black	Collagen formation
COL1A2	green	black	Collagen formation
COL21A1	green	black	Collagen formation
COL4A1	red	green	Collagen formation
COL4A2	red	green	Collagen formation
CXCL3	red	green	Chemotaxis
DEFA1B/1A	green	black	Antimicrobial activity
DEFA3	green	black	Antimicrobial activity
DSPP	green	red	Dentin formation
IL10RA	red	black	IL-10 signaling
IL1A	red	black	Inflammatory response
IL1B	red	black	Inflammatory response
IL6	red	green	Inflammation, B-cell maturation
IL8	red	black	Chemotaxis
LBP	red	black	Innate immune response
MMP13	green	red	Collagen degradation
MMP20	black	green	Amelogenin degradation
MMP9	red	green	Collagen degradation
NOD2	red	black	Innate immune response
NR5A2	red	green	Antiviral activity
PTGS2	red	green	Prostaglandin formation
SCN8A	black	green	Sodium ion permeability
TLR1	red	green	Innate immune response
TLR2	red	green	Innate immune response
TLR3	black	green	Innate immune response
TLR4	red	green	Innate immune response

Gene Symbol	Pulpitis vs Normal	None to Mild vs Moderate to Severe Pain	Gene Function (genecards.org)
TLR6	🟥	🟩	Innate immune response
TLR8	🟥	⬛	Innate immune response
TLR9	🟥	🟩	Innate immune response
TNFA	🟥	⬛	Inflammatory response

⬛ Downregulated 🟥 Upregulated 🟩 No difference

Fold change: Downregulated 0.5; Upregulated: 1.5; q<0.05