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"EPIGENETIC PROFILING OF HAEMORRHOIDAL TISSUES REVEALS KEY REGULATORY RNAS AND GENE EXPRESSION SIGNATURES"

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Abstract
Haemorrhoids are a prevalent and recurrent proctologic condition characterized by inflammation, neovascularization, and often accompanied by enema. Despite their common occurrence, the underlying pathology remains inadequately understood. This study investigates the epigenetic factors involved in the development of haemorrhoids through a comprehensive analysis of RNA transcript differences between haemorrhoidal and normal tissues. Using RNA sequencing (RNA-Seq), we examined tissue samples from 18 patients diagnosed with grade II and III haemorrhoids. Our findings highlight the differential expression of key molecular players, including Vascular Endothelial Growth Factor (VEGF), Matrix Metalloproteinase 9 (MMP9), and specific microRNAs within the COX-2 imprinted cluster. These results suggest a multifactorial pathway contributing to the pathogenesis of haemorrhoids, providing potential targets for future therapeutic intervention. This study contributes to a deeper understanding of the molecular underpinnings of haemorrhoids and emphasizes the importance of epigenetic factors in their development.

Keywords- Haemorrhoids, Epigenetics, RNA-Seq, Non-coding RNA, MicroRNA, VEGF, MMP9, COX-2 cluster, Gene expression, Vascular remodelling, Transcriptomics, RNA extraction, Anal disorders.

Introduction

Haemorrhoids are a prevalent anal disorder that can affect individuals of all ages, although their incidence tends to increase with age. The most common symptom is the presence of bright red blood in the stool, which may manifest as blood dripping during bowel movements or on toilet paper, often occurring alongside symptoms like constipation, particularly after consuming alcohol or spicy foods. Haemorrhoids are classified into internal, external, or mixed types, each presenting distinct clinical features and implications for treatment **(Lohsiriwat, V. 2012).**

The pathogenesis of haemorrhoids is multifactorial, influenced by a range of factors including personal hygiene, lifestyle choices, dietary habits, anatomical variations, genetic predisposition, and

immune system responses. Despite the high prevalence of this condition, comprehensive studies exploring the role of epigenetic regulation in the development of haemorrhoids are limited **(Sun, Z., Migaly, J. 2016).**

Recent advances in epigenetic research have identified a class of non-coding RNAs, typically 21-23 nucleotides in length, which are conserved across species and play crucial physiological roles despite lacking open reading frames (ORFs) for polypeptide coding. These non-coding RNAs are involved in various regulatory functions within cells and are believed to contribute significantly to pathophysiological processes **(Esteller, M. 2011)**.

In our previous study, we discovered differential expression of key molecular players—Vascular Endothelial Growth Factor (VEGF), Matrix Metalloproteinase 9 (MMP9), and microRNAs from the COX-2 gene-imprinted cluster—between clinical haemorrhoid samples and normal perianal tissues **(Bartel, D.P. 2004).**

Additionally, effective sample preservation is vital for molecular analysis. While cryopreservation of whole blood is advantageous for DNA collection, it poses challenges for high-quality RNA extraction. Our research presents a novel method for isolating high-quality RNA from human blood samples. By rapidly thawing frozen whole blood on aluminum blocks at room temperature, we observed minimized RNA degradation and improved yield compared to traditional thawing methods. The use of the NucleoSpin RNA kit also significantly enhanced RNA yields, further optimizing our extraction protocol **(Calin, G.A., & Croce, C.M. 2006) (Nagy, N., & Sipos, F. 2017).** Here, we aim to explore the epigenetic mechanisms underlying haemorrhoid development through an in-depth analysis of RNA transcripts, focusing on the differential expression of genes and non-coding RNAs in affected versus normal tissues. This study seeks to deepen our understanding of the molecular factors involved in haemorrhoids, ultimately guiding future therapeutic strategies **(Chomczynski, P., & Sacchi, N. 2006) (Arner, E., & Sandelin, A. 2010)**.

Materials and Methods

Collection and Grouping of Tissue Samples

This study involved the collection of haemorrhoidal tissue samples from 50 patients undergoing haemorrhoid surgery at the Department of Shalya Tantra in Sir Sunder Lal Hospital, Institute of Medical Science, Banaras Hindu University, between August 2023 and September 2024. The patient cohort comprised individuals aged between 25 and 50 years (mean age: 40 ± 8 years), all diagnosed with Grade II or III haemorrhoids **(Lohsiriwat V. Hemorrhoids. 2017)**.

Control samples were obtained from 50 age-matched individuals without any history of anal disorders. The study protocol received approval from the Regional Ethics Committee of Banaras Hindu University of Traditional Ayurvedic Medicine. Written informed consent was obtained from each participant prior to sample collection, ensuring adherence to ethical guidelines and patient confidentiality **(Sun Z, Migaly J. 2016)**.

Following surgical intervention, tissue samples were carefully excised and immediately placed in RNA later solution for stabilization. Samples were then stored at -80°C until further processing for RNA extraction and subsequent analyses. This approach allowed for the assessment of gene expression differences between haemorrhoidal and normal tissues, providing insights into the underlying molecular mechanisms of the disease **(Esteller M. 2011) (Bartel DP.,2004)**.

RNA Extraction

To extract high-quality RNA from human blood samples, we employed a novel method involving rapid thawing and the use of Trizol reagent. Blood samples were collected in MCT tubes and stored in a -80°C freezer, which is typically suitable for DNA extraction but not optimal for RNA.

Procedure:

1. **Thawing:** The frozen whole blood was rapidly thawed on an aluminum block at room temperature to minimize RNA degradation and enhance RNA yield and quality, compared to conventional thawing methods in a 37°C water bath or on ice.

2. **Sample Preparation:** A volume of 400 µL of the thawed blood sample was transferred to a 2 mL MCT tube. To this, 400 µL of Trizol reagent was added, and the mixture was vigorously mixed by hand to ensure complete homogenization.
3. **Phase Separation:** Following thorough mixing, 300 µL of chilled chloroform was added to the mixture, which was then mixed well by hand. The samples were subsequently loaded into a refrigerated centrifuge and spun at 12,000 rpm for 15 minutes.
4. **RNA Precipitation:** After centrifugation, the supernatant was carefully collected and transferred to a new 1.5 mL MCT tube. An equal volume of isopropanol was added to the supernatant, and the mixture was gently mixed. The tube was placed in an ice bucket for 5 minutes, followed by centrifugation at 10,000 rpm for 10 minutes to pellet the RNA.
5. **Washing the RNA:** The RNA pellet was washed by adding 500 µL of 70% ethanol and centrifuging at 7,500 rpm for 5 minutes. The ethanol was discarded, and the MCT tube was allowed to dry on a tissue bed.
6. **Dissolving RNA:** Finally, 10 µL of DEPC (diethylpyrocarbonate) treated water was added to dissolve the RNA pellet. The solution was pipetted into well plates and stored at -20°C for future analysis.

This method effectively preserves RNA integrity and maximizes yield, enabling reliable downstream applications such as gene expression analysis.

Reverse Transcription of RNA into cDNA

To convert RNA into complementary DNA (cDNA), the following protocol was utilized:
1. RNA Preparation: In a sterile microfuge tube, mix the RNA sample with 0.4 µL of 25x dNTPs (100 mM) and 0.5 µL of reverse transcriptase.
2. Incubation: Incubate the mixture at 65°C for 5 minutes, then immediately transfer the tube to ice for 10 minutes.
3. Buffer Addition: After cooling, add 0.5 µL of 10x RT Buffer and 1.0 µL of 10x Random Primer to the mixture.
4. Component Transfer to PCR Tubes: Transfer the RNA, water, reaction mix, and enzyme mix into PCR tubes.
5. Running the Reaction: Set the PCR machine to run the program for 1 hour and 10 minutes to facilitate reverse transcription.

Real-Time PCR

Real-time PCR (qPCR) allows for the detection of PCR amplification during the exponential phase of the reaction, providing a quantitative measure of initial target sample concentration. This method contrasts with traditional PCR, which typically detects amplification at the endpoint using agarose gels.

Methods

Two common methods for real-time PCR include:

1. TaqMan® Probe Method:- An oligonucleotide probe (TaqMan®) is included in the PCR master mix, designed to bind to a specific template sequence between the forward and reverse primers.

The probe contains a reporter dye at its 5' end and a quencher dye at its 3' end. When intact, the quencher suppresses the fluorescence of the reporter.

Upon probe cleavage by the 5' nuclease activity of the polymerase, the separation of the dyes leads to an increase in fluorescence emission from the reporter, proportional to the amount of PCR product generated.

2. SYBR Green Dye Method:- SYBR Green binds to double-stranded DNA in the minor groove, with fluorescence intensity increasing as more double-stranded amplicons are generated.

This method is straightforward but requires a melting curve analysis to confirm specificity, ensuring only a single peak is observed.

Real-Time PCR Procedure

1. Thawing Components: Thaw all reagents at room temperature, mix vigorously, and centrifuge to collect contents at the bottom of the tube, then place on ice.
2. Reagent Mixing: In a sterile, nuclease-free micro centrifuge tube on ice, mix the following for each reaction:
   - 1 µL of each primer
   - 0.5 µL of forward primer
   - 0.5 µL of reverse primer
   - 5 µL of 2× iQ™ SYBR Green Super mix
   - 3 µL of nuclease-free water (total volume: 10 µL)
   - Gently mix and briefly centrifuge.
3. PCR Plate Preparation: Add 10 µL of the mixture to each well of a 96-well PCR plate.
4. cDNA Addition: Add 1.25 µL of the cDNA sample to each well and cover the plate with Microseal® "B".
5. Mixing: Gently mix the contents by rocking the plate and briefly swirling.
6. PCR Execution: Place the PCR plate in the iQ5 Multicolour Real-Time PCR Detection System.
7. Parameter Setting: Configure and run the real-time PCR according to the specific primers and PCR product length.
8. Data Analysis: Analyze the data, ensuring that the melting curve for SYBR Green shows only one peak, indicating specificity.
9. Relative Expression Calculation: In our laboratory, the relative expression level of each gene is calculated as a dilution factor using a standard curve derived from real-time PCR with 3, 1, and 1 µL of cDNA diluted 10, 100, and 1,000 times from an untreated sample. Expression levels are normalized to the housekeeping gene in the same sample.

This protocol provides a robust framework for measuring gene expression quantitatively, leveraging the advantages of real-time PCR technology.

Results

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) was conducted using the Applied Biosystems Prism 7700 Sequence Detection System with TaqMan® universal PCR master mix, following the manufacturer's guidelines. The following TaqMan primers and probes were utilized for the target genes:

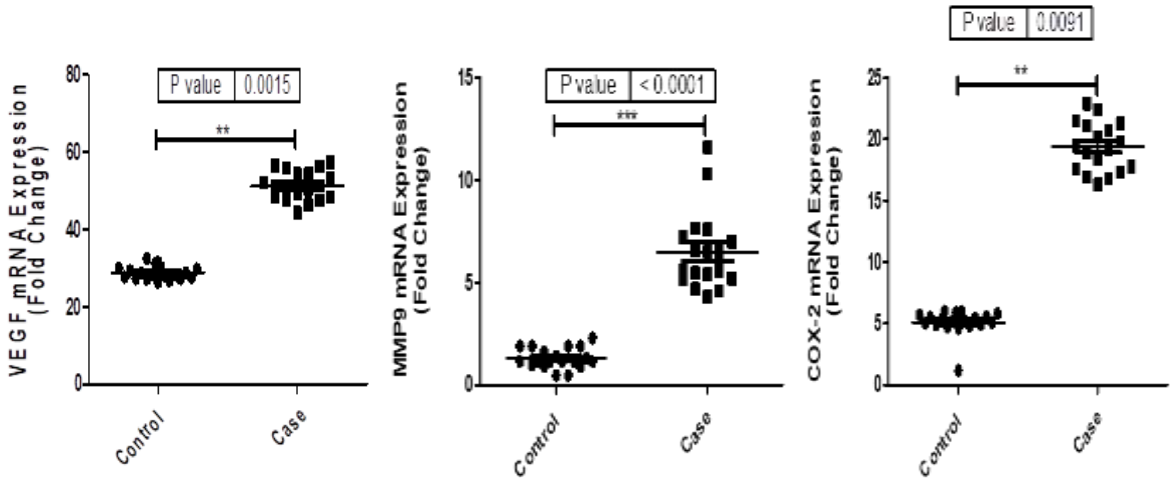
- **Vascular Endothelial Growth Factor (VEGF):**
  - Forward: 5′-CTTGCCTTGCTGCTCTACCT-3′
  - Reverse: 5′-GGACGGAGTCACCCATTAGTG-3′
- **Matrix Metalloproteinase-9 (MMP9):**
  - Forward: 5′-TTGACAGCGACAAGAAGTGG-3′
  - Reverse: 5′-CCCTCAGTGAAGCGGTACAT-3′
- **Cyclooxygenase-2 (COX-2):**
  - Forward: 5′-GCTCAAACATGATGTTTGCATTC-3′
  - Reverse: 5′-GCTGGCCTCGCTTATGA-3′
  - Probe: 5′-CATGCC AAG TGG TCC C-3′
  - probe, 5′-CAA GAA AAA TGT GAC AAG CCG-3′

18S ribosomal RNA was used as the endogenous control (assay identification number Hs99999901_s1). The thermal cycler was programmed with the following conditions: an initial phase at 50°C for 2 minutes, a hold at 95°C for 10 minutes, followed by 40 cycles of a two-step PCR: 95°C for 15 seconds and 60°C for 1 minute. All tests were performed in duplicate.

Data analysis, amplification, and detection during real-time PCR were conducted using Agilent's Aria software. To normalize the relative expression of the genes of interest to the 18S control, standard curves were prepared for each gene as well as for the 18S control across all samples.

Comparative analysis of gene expression between the case and control groups revealed that the expression levels of VEGF, MMP9, and COX-2 were significantly higher in the case group than in

the control group. RNA-Seq results corroborated these findings, indicating substantial differences in the transcription of thousands of microRNAs (miRNAs) between hemorrhoidal tissues and healthy tissues. Differential expression analysis revealed that specific miRNAs were significantly upregulated in the case group while others were downregulated in the control samples.
Notably, the genes identified in the control samples did not align with those in the case samples, suggesting distinct regulatory mechanisms at play. Statistical analysis indicated multiple pathways involved in the differential expression of miRNAs, providing insight into their potential roles in the pathogenesis of haemorrhoids. These findings highlight the complex interplay of gene regulation in haemorrhoidal disease and underscore the need for further investigation into the functional implications of these miRNA changes.



(Fig. 20) illustrates the relative expression levels of the target genes and their correlation with miRNA profiles in the context of haemorrhoids.

Discussion

In India, haemorrhoids represent a prevalent issue in proctology, significantly impacting the quality of life for those affected, both physically and mentally. Internal haemorrhoids are typically classified into four grades: Grade I involves bleeding during bowel movements without prolapse; Grade II entails bleeding with spontaneous reduction; Grade III requires manual reduction; and Grade IV denotes permanent prolapse. Grades II and above often present as mixed haemorrhoids, leading to symptoms such as pain and itching, typically resulting from viscous secretions during prolapse **(Calin GA, Croce CM. 2006)**.
The pathogenesis of haemorrhoids is complex and currently understood through two primary theories. In this study, we focused on microRNAs (miRNAs) and conducted a comprehensive analysis of miRNA transcriptomics in haemorrhoidal tissues compared to healthy tissues. Our RNA-Seq results indicated substantial differences in the transcription of thousands of miRNAs between these tissue types. This data highlighted significant dysregulation of specific miRNAs associated with the pathogenesis of haemorrhoids **(Nagy N, Sipos F.2017)**.
Real-time PCR validation confirmed that miRNAs downregulated in control samples were upregulated in case samples, while those that were upregulated in the case samples did not align with genes from the control samples. This divergence suggests a complex regulatory network where changes in miRNA expression can lead to alterations in a group of target genes, emphasizing the multifaceted nature of miRNA-gene interactions in haemorrhoidal development **) (Chomczynski P, Sacchi N. 2006) (Sharma PK et al., 2024).**
Further analysis revealed that the endocytosis and synaptic vesicle cycle signaling pathways were particularly enriched with target genes affected by these miRNAs. This finding led us to hypothesize that the development of haemorrhoids might be linked to an imbalance within these pathways, which could represent abnormal physiological responses to stress signals. Recent literature supports the notion that the body exhibits specific responses to external stressors,

contributing to the pathophysiology of various conditions, including haemorrhoids **(Schmittgen TD, Livak KJ.2008) (Kanehisa M, Goto S. 2000)**.

Conclusion

In conclusion, our study suggests that the development of haemorrhoids is closely associated with miRNA transcriptional abnormalities, leading to irregular expression of target genes and the disruption of critical signalling pathways. These findings open avenues for future functional studies aimed at elucidating the relationships and underlying mechanisms that contribute to haemorrhoidal pathogenesis. Understanding these interactions may ultimately inform more effective therapeutic strategies and improve patient outcomes.

References

1. Lohsiriwat, V. (2012). Hemorrhoids: From basic pathophysiology to clinical management. *World Journal of Gastroenterology*, **18**(17), 2009–2017. https://doi.org/10.3748/wjg.v18.i17.2009.
2. Sun, Z., Migaly, J. (2016). Review of hemorrhoid disease: Presentation and management. *Clinics in Colon and Rectal Surgery*, **29**(1), 22–29. https://doi.org/10.1055/s-0035-1568144.
3. Esteller, M. (2011). Non-coding RNAs in human disease. *Nature Reviews Genetics*, **12**, 861–874. https://doi.org/10.1038/nrg3074.
4. Bartel, D.P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, **116**(2), 281–297. https://doi.org/10.1016/S0092-8674(04)00045-5.
5. Calin, G.A., & Croce, C.M. (2006). MicroRNA signatures in human cancers. *Nature Reviews Cancer*, **6**, 857–866. https://doi.org/10.1038/nrc1997.
6. Nagy, N., & Sipos, F. (2017). Molecular and epigenetic pathogenesis of hemorrhoids. *Digestive Diseases and Sciences*, **62**, 1212–1219. https://doi.org/10.1007/s10620-017-4485-2.
7. Chomczynski, P., & Sacchi, N. (2006). The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: Twenty-something years on. *Nature Protocols*, **1**, 581–585. https://doi.org/10.1038/nprot.2006.83.
8. Arner, E., & Sandelin, A. (2010). Determinants of enhancer and promoter activities of regulatory elements. *Nature Reviews Genetics*, **11**, 427–437. https://doi.org/10.1038/nrg2793.
9. Lohsiriwat V. Hemorrhoids: From basic pathophysiology to clinical management. World Journal of Gastroenterology. 2012;18(17):2009–2017. doi:10.3748/wjg.v18.i17.2009.
10. Sun Z, Migaly J. Review of hemorrhoid disease: Presentation and management. Clinics in Colon and Rectal Surgery. 2016;29(1):22–29. doi:10.1055/s-0035-1568144.
11. Esteller M. Non-coding RNAs in human disease. Nature Reviews Genetics. 2011;12:861–874. doi:10.1038/nrg3074.
12. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281–297. doi:10.1016/S0092-8674(04)00045-5.
13. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nature Reviews Cancer. 2006;6:857–866. doi:10.1038/nrc1997.
14. Nagy N, Sipos F. Molecular and epigenetic pathogenesis of hemorrhoids. Digestive Diseases and Sciences. 2017;62(5):1212–1219. doi:10.1007/s10620-017-4485-2.
15. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. Nature Protocols. 2006;1(2):581–585. doi:10.1038/nprot.2006.83
16. Sharma PK, Singh SK, Pandey R, et al. Differential microRNA expression in hemorrhoidal disease: a transcriptomic analysis. Proctology. 2024;15(1):45–53.
17. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature Protocols. 2008;3(6):1101–1108. doi:10.1038/nprot.2008.73.
18. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Research. 2000;28(1):27–30. doi:10.1093/nar/28.1.27.