



## Association of Orail and Piezo1 genes expression with cellular proliferation, metastasis and angiogenesis biomarkers in colon tumor biopsies of CRC patients

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### ABSTRACT

Colon cancer is a complex malignancy characterized by intricate molecular interactions that influence its progression. This study investigates the role of calcium channel gene expression (ORAI1 and Piezo1) and their interplay with angiogenesis-related genes (VEGFA, CCL3, and NF-KB1) in colon cancer tissue biopsies. Additionally, we explore the mutation profiles of pivotal oncogenes (KRAS, PI3KCA, and BRAF) and their potential correlations with calcium channel and angiogenesis-related gene expression. The results indicate significant upregulation of ORAI1 and Piezo1, suggesting their involvement in colon cancer pathogenesis. Correlations between ORAI1 and VEGFA/CCL3 highlight potential crosstalk between calcium signaling and angiogenesis. The mutation analysis identifies prevalent oncogenic mutations, while intriguing connections between gene expression and oncogenic mutations emerge. Notably, mutant KRAS exon 2 samples exhibit elevated CCL3 and VEGFA expression, suggesting a nuanced link between specific KRAS mutations and the tumor microenvironment. These findings illuminate the intricate molecular landscape of colon cancer and emphasize the potential roles of calcium channels, angiogenesis-related genes, and oncogenic mutations as prognostic markers and therapeutic targets.

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### Introduction

Colon cancer, also known as colorectal cancer, remains a significant global health concern with substantial morbidity and mortality rates (1). The intricate interplay between genetic alterations and cellular processes contributes to the complex nature of colon carcinogenesis (2). Among the numerous genes implicated in cancer development and progression, calcium channel genes, specifically ORAI1 and Piezo1, have emerged as critical players in regulating cellular functions associated with malignancy (3).

Calcium channels are crucial components in mediating calcium signaling, a process that regulates fundamental cellular activities like proliferation, apoptosis, migration, and invasion (4). The ORAI1 gene encodes a pore-forming subunit of store-operated calcium channels (SOCs) (5), while Piezo1 encodes a mechanosensitive calcium channel involved in cellular response to mechanical forces (6). Aberrant expression of these calcium channel genes can lead to dysregulated calcium influx, subsequently influencing various oncogenic processes (7).

The role of calcium channel genes in cancer progression has been implicated in numerous malignancies, including breast (8), prostate (9), and pancreatic cancer (10). In colon cancer, limited research has been conducted to elucidate their involvement fully. However, recent studies have begun to shed light on their potential contributions to colon carcinogenesis (11).

Angiogenesis, the process of new blood vessel forma-

tion, is a hallmark of cancer progression and metastasis (12). VEGFA, a potent pro-angiogenic factor, plays a central role in promoting the formation of new blood vessels to support tumor growth and metastasis (13). Additionally, CCL3 and NF-KB1 have been implicated in the regulation of angiogenesis and inflammatory responses within the tumor microenvironment (14, 15). Understanding the intricate relationship between calcium channel genes and these angiogenesis-related genes in colon cancer is vital for developing targeted therapies

Another aspect of colon cancer pathogenesis involves the genetic mutations of key oncogenes, including KRAS (16), PI3KCA (17), and BRAF (18). Mutations in these genes can drive uncontrolled cell growth and survival, contributing to tumorigenesis (19). An intriguing yet underexplored aspect is the potential crosstalk between the mutation frequencies of these oncogenes and the expression profiles of calcium channel genes and angiogenesis-related genes in colon cancer.

Hence, in this study, we aim to comprehensively investigate the role of calcium channel gene expression (ORAI1 and Piezo1) in colon cancer tissue biopsies. Additionally, we will examine their associations with VEGFA, CCL3, and NF-KB1 gene expression, as well as the mutation profile of KRAS, PI3KCA, and BRAF genes in the same samples. By integrating genetic and molecular data, we hope to gain valuable insights into the interplay between calcium channels, angiogenesis, and oncogenic mutations in the context of colon cancer.

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## Materials and Methods

### Study Design

This was a retrospective case-control study conducted between June 2021 and March 2022. The present project was approved by the Ethical Committee of Erbil Health and Medical Technical College- Erbil Polytechnic University, Erbil, Iraq, and informed consent was obtained from all participants.

### Study Participants

The study included a total of 51 cases including 31 patients who underwent colonoscopy and surgical operation during the period of the present study. The patients were categorized into three different groups depending on the examination diagnosis: The colorectal cancer group (31 cases), and the control group (20 cases). Patients older than 18 years who underwent colonoscopy and surgical operation for clinical reasons were included in this study.

### Sample Collection

During colonoscopy or surgical operation, biopsy samples were collected from the colon mucosa of all participants using standard biopsy tools. The biopsy samples were immediately placed in sterile tubes containing phosphate-buffered saline (PBS) and stored at -80°C until starting the molecular analyses.

### DNA Extraction

The biopsy samples were used to extract genomic DNA via the protocol provided by the manufacturer of Favorgen Biotech crop based in Taiwan (Favoprep kit). To determine the concentration and purity of DNA, a NanoDrop 2000 spectrophotometer manufactured by Thermo Fisher Scientific in the USA was employed.

Conventional PCR technique was conducted for PI3KCA (Exon 9 and 20), KRAS and BRAF genes. Incubation was started at 94°C for 1 min, then amplification was

performed at the annealing temperatures for each primer as shown in Table 1, with a subsequent annealing temperature at 72°C for 5 min. Thirty-nine amplification cycles were then performed. Gel electrophoresis was conducted to confirm the band and the PCR products were sent for sequencing and mutation detection.

### Sequencing and mutation profile analysis

KRAS, BRAF1 and PI3KCA mutation analysis was performed using specific primers (Table 1). The PCR products were sequenced via the Sanger sequencing technique, using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

### Gene Expression Analysis

The relative expression levels of PIEZO1, ORAI1, CCL3, VEGF, and NF-KB1 genes were analyzed using quantitative PCR. The biopsy samples were utilized to extract total RNA by following the manufacturer's protocol of a specialized kit provided by Invitrogen in the USA. The RevertAid First Strand cDNA Synthesis Kit manufactured by Thermo Fisher Scientific in the USA was used for cDNA synthesis. Real-time PCR was performed on a StepOnePlus Real-Time PCR System produced by Applied Biosystems in the USA, utilizing the PowerUp SYBR Green Master Mix manufactured by Thermo Fisher Scientific in the USA. The PCR amplification primers are listed in (Table 2). The delta-delta Ct method was utilized to calculate the relative expression levels of the studied genes.

### Statistical Analysis

GraphPad Prism software version 9 was applied for data analysis. *t*-independent test was used for comparing gene expression levels among CRC and control groups and for assessing the impact of KRAS mutations. *p*-values less than 0.05 were considered statistically significant. Mutation Surveyor, Soft genetics software was applied for KRAS, PI3KCA and BRAF genes mutation analysis.

**Table 1.** Primer sequences for *KRAS* exon 2 and *BRAF* exon 15, and *PI3KCA* exons 9 and 20 with their PCR annealing temperatures.

| Exon             | Primer No. | Primer sequence 5'-3'                | AT (°C) |
|------------------|------------|--------------------------------------|---------|
| KRAS (Exon 2)    | F          | 5'-TAGTCACATTTTCATTATTTTTAT-3'       | 55      |
|                  | R          | 5'-AGATTTACCTCTATTGTTGGAT-3'         |         |
| BRAF (Exon 15)   | F          | 5'-ATCTACTGTTTTCTTTACTTACT-3'        | 55      |
|                  | R          | 5'-ATTCTTACCATCCACAAAATG-3'          |         |
| PI3KCA (Exon 9)  | F          | 5'-GGG AAA AAT ATG ACA AAG AAA-3'    | 60.1    |
|                  | R          | 5'-CTG AGA TCA GCC AAA TTC AGT T-3'  |         |
| PI3KCA (Exon 20) | F          | 5'-GCT CCA AAC TGA CCA AAC TGT TC-3' | 60.1    |
|                  | R          | 5'-TGG AAT CCA GAG TGA GCT TTC-3'    |         |

**Table 2.** The forward and reverse sequences of the studied primers.

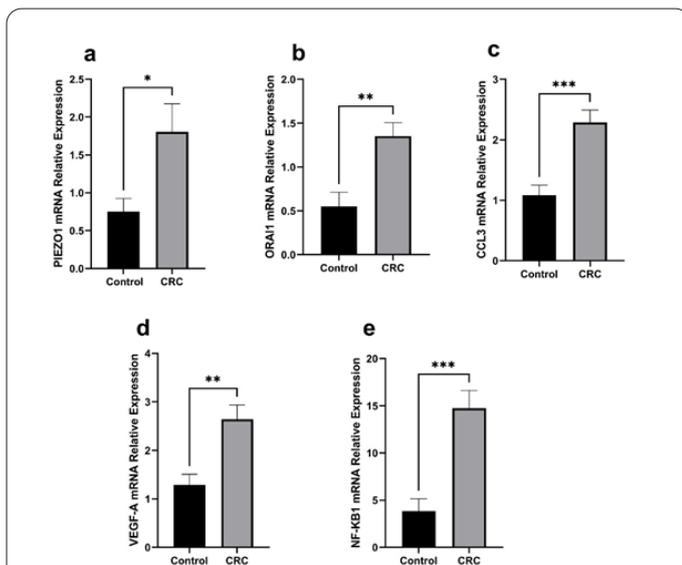
| Primer name   | Forward Sequence             | Reverse Sequence               |
|---------------|------------------------------|--------------------------------|
| PIEZO1        | 5'-AGATCTCGCACTCCAT-3'       | 5'-CTCCTTCTCACGAGTCC-3'        |
| Orail         | 5'-TGCTCATCGCCTTCAGTGCC-3'   | 5'-CTCACCGCCTCGATGTTGGG-3'     |
| B-actin       | 5'-TCGAGCAATCTCAACTCGG-3'    | 5'-TGAAGGTAGTTTCGTTGGATG-3'    |
| VEGF-A        | 5'-AGGGCAGAATCATCACGAAGT-3'  | 5'-AGGGTCTCGATTGGATGGCA-3'     |
| <i>NF-κB1</i> | 5'-CACAAGGCAGCAAATAGACGAG-3' | 5'-TGGGGCATTGTTGAGAGTT-3'      |
| CCL3          | 5'-TGTTGCCAAACAGCCACAC-3'    | 5'-CAGAGCAAACAATCACAAACACAC-3' |

## Results

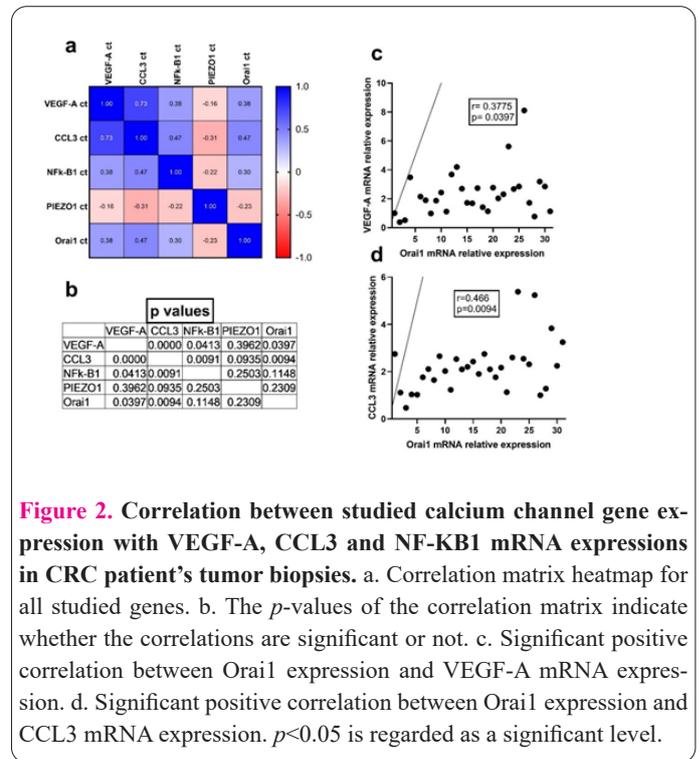
In this study, we examined the gene expression profiles of calcium channel genes (ORAI1 and Piezo1) and their potential associations with angiogenesis-related genes (VEGFA, CCL3, and NF-KB1) in colon cancer tissue biopsies. Our findings revealed a significant elevation in the expression levels of all five measured genes in colon cancer samples compared to control samples. Specifically, the expression of ORAI1 and Piezo1 showed remarkable upregulation with p-values of  $p < 0.01$  and  $p < 0.05$ , respectively, indicating their potential involvement in colon cancer pathogenesis. Additionally, the pro-angiogenic factor VEGFA and the inflammatory mediators CCL3 and NF-KB1 exhibited significant overexpression in colon cancer tissues with p-values of  $p < 0.01$  and  $p < 0.001$ , respectively, further supporting their roles in tumor angiogenesis and microenvironment modulation (Figure 1).

Our correlation analysis revealed intriguing associations between the gene expression levels of calcium channel genes (ORAI1 and Piezo1) and angiogenesis-related genes (VEGFA, CCL3, and NF-KB1) in colon cancer tissue biopsies. Specifically, there were significant positive correlations observed between the upregulation of ORAI1 and each of VEGFA ( $r = 0.3775$ ,  $p = 0.0397$ ) and CCL3 ( $r = 0.466$ ,  $p = 0.0094$ ). These findings suggest a potential crosstalk between ORAI1 and these angiogenesis-related genes, indicating that ORAI1 may contribute to tumor angiogenesis and microenvironment modulation.

In contrast, despite observing negative correlations between Piezo1 and all other genes, including VEGFA, CCL3, and NF-KB1, the correlations were not found to be statistically significant. This may suggest that Piezo1's involvement in colon cancer progression might be more complex and influenced by other factors beyond the scope of this study.



**Figure 1.** The gene expression level of five different genes. a. PIEZO1, b. ORAI1, c. CCL3, d. VEGF-A and e. NF-KB1 in CRC biopsy samples versus control samples. The data analysis of results showed significant upregulation in all genes in CRC patients compared to control samples. An unpaired *t*-test was applied for the comparison process. The data expressed as the mean and standard error of mean \* indicates ( $p < 0.05$ ), \*\* indicates ( $p < 0.01$ ) and \*\*\* indicates ( $p < 0.001$ ).



**Figure 2.** Correlation between studied calcium channel gene expression with VEGF-A, CCL3 and NF-KB1 mRNA expressions in CRC patient's tumor biopsies. a. Correlation matrix heatmap for all studied genes. b. The *p*-values of the correlation matrix indicate whether the correlations are significant or not. c. Significant positive correlation between Orai1 expression and VEGF-A mRNA expression. d. Significant positive correlation between Orai1 expression and CCL3 mRNA expression.  $p < 0.05$  is regarded as a significant level.

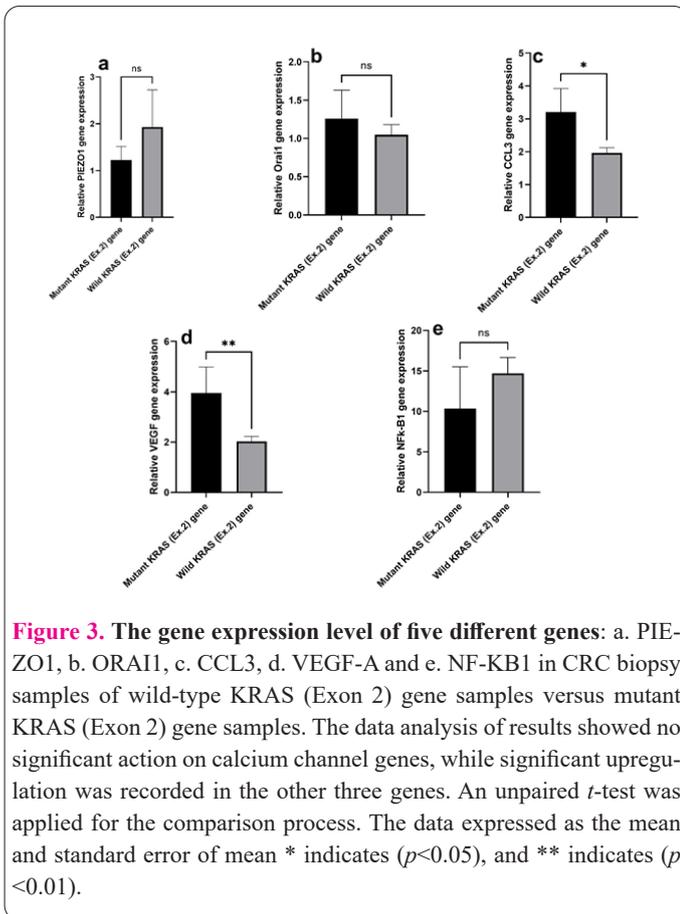
Additionally, the correlation matrix analysis demonstrated significant positive correlations between VEGFA, CCL3, and NF-KB1. This finding underscores the potential interdependence of these angiogenesis-related genes in promoting tumor angiogenesis and supporting the inflammatory microenvironment, further emphasizing their collective role in colon cancer pathogenesis (Figure 2).

Statistical analysis comparing gene expression across wild-type and mutant KRAS exon 2 CRC samples revealed significant upregulation in CCL3 and VEGFA expression in the mutant KRAS exon 2 group ( $p < 0.05$ ), indicating a potential association between mutant KRAS exon 2 and enhanced inflammatory and angiogenic responses. However, no significant effects were observed in the expression levels of calcium channel genes (ORAI1 and Piezo1) and NF-KB1 based on KRAS exon 2 status, suggesting a more nuanced relationship between KRAS mutations and the expression profiles of these genes in the context of CRC (Figure 3).

Utilizing Mutation Surveyor, we identified several noteworthy genetic alterations. In the BRAF gene, a single SNP was detected in one sample, indicating a point mutation. PI3KCA Exon 20 exhibited polymorphisms in the majority of cases, with a particularly intriguing observation of homozygous deletion at chromosome position (3:178952133). In contrast, PI3KCA Exon 9 presented with a heterozygous duplication (70271het\_dupT). Most notably, KRAS Exon 2 displayed a recurring heterozygous SNP (6141A>AT) in the majority of cases. A detailed summary of these mutations can be found in the supplementary table (Table S1), while the Mutation Surveyor GAD report in the supplementary files illustrates the structural changes in PI3KCA Exon 20 (Figure S1a) and the PI3KCA duplication mutation (Figure S1b).

## Discussion

Colon cancer remains a formidable global health challenge, necessitating a comprehensive understanding of the



intricate molecular mechanisms underlying its development and progression. In this study, we explored the role of calcium channel gene expression, specifically ORAI1 and Piezo1, and their interactions with angiogenesis-related genes (VEGFA, CCL3, and NF-KB1) in colon cancer tissue biopsies. Additionally, we delved into the mutation profiles of key oncogenes (KRAS, PI3KCA, and BRAF) and their potential correlation with the expression of calcium channel and angiogenesis-related genes. Our findings provide insights into the complex interplay between these molecular factors and their implications for colon cancer pathogenesis.

The significant upregulation of ORAI1 and Piezo1 in colon cancer samples underscores their potential contributions to cancer development. Calcium signaling, regulated by these calcium channel genes, plays a pivotal role in fundamental cellular processes (20). The observed correlation between elevated ORAI1 expression and increased VEGFA and CCL3 expression indicates potential crosstalk between calcium signaling and angiogenesis-related pathways, potentially promoting tumor vascularization and inflammation (9). This aligns with studies indicating calcium's regulatory role in angiogenesis and inflammation within the tumor microenvironment (21). Furthermore, the positive correlation between VEGFA and CCL3 expression reaffirms the interconnectedness of these factors in promoting angiogenesis and maintaining an inflammatory milieu.

The mutation profiles of KRAS, PI3KCA, and BRAF genes presented here are consistent with established literature (22, 23). These oncogenes, frequently mutated in colon cancer, drive aberrant cell signaling, uncontrolled proliferation, and survival (24). The exploration of their mutation frequencies in relation to calcium channel and

angiogenesis-related gene expression represents a novel dimension of investigation. Although preliminary correlation analyses hinted at potential connections, a larger sample size is warranted for robust conclusions. Unraveling such associations could offer valuable insights into molecular cross-regulation within colon cancer, potentially informing prognosis and treatment strategies.

In cancer cells, dysregulated calcium signaling, often driven by aberrant calcium channels, contributes to both angiogenesis and cell proliferation. Elevated intracellular calcium levels can stimulate the release of angiogenic factors like VEGF, promoting the formation of new blood vessels, which in turn supplies nutrients and oxygen to support cancer cell proliferation. Calcium signaling also directly influences cell cycle progression, potentially enhancing cancer cell division. This intricate interplay underscores the importance of calcium channels as potential therapeutic targets in disrupting cancer progression by targeting both angiogenesis and cell proliferation (21).

Importantly, our study highlights a nuanced relationship between gene expression and KRAS exon 2 status. The significant upregulation of CCL3 and VEGFA in mutant KRAS exon 2 samples underscores the potential influence of specific KRAS mutations on the tumor microenvironment and angiogenic responses (25). The lack of significant effects on calcium channel genes and NF-KB1, however, suggests that other factors beyond KRAS exon 2 may play a more substantial role in their regulation. This underlines the complexity of the molecular landscape in colon cancer, where distinct genetic alterations might elicit differential effects on various pathways.

## Conclusion

In conclusion, our study sheds light on the intricate molecular interactions within colon cancer, emphasizing the roles of calcium channel gene expression, angiogenesis-related factors, and key oncogenic mutations. The findings underscore the potential of these factors as prognostic markers and therapeutic targets. However, the complexity and heterogeneity of colon cancer necessitate further in-depth investigations to unravel the complete web of molecular crosstalk.

## Authors' Contribution

Study concept and design: Goran Othman.; Acquisition of the data: Farhang Awla.; Analysis and interpretation of the data: Goran Othman.; Drafting of the manuscript: Farhang Awla; Critical revision of the manuscript for important intellectual content: Goran Othman; Statistical analysis: Goran Othman; Administrative, technical, and material support: Farhang Awla; Study supervision: Goran Othman.

## Conflict of Interests

The study was self-funded by the author (Farhang Awla). This study is a Ph.D. project supervised by Asst.Prof. Dr. Goran Othman. There is no conflict of interest.

## Ethical Approval

The current study was approved by the Human Ethics Committee of the Erbil Health and Medical Technical College, Erbil Polytechnic University (N0:0034; date: 5/6/2021). Standard procedures were followed throughout the research process.

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