

Identifying Effective Pathways and Genes in Colorectal Cancer According to Stage by Analyzing RNA Sequencing Data

Morteza-Ali Rahmani¹, MSc;  Melika Aramfar¹, MSc; Zohreh Hojati^{1*}, PhD 

¹Division of Genetics, Department of Cell and Molecular Biology and Microbiology, Faculty of Biological Sciences and Technology, University of Isfahan, Isfahan, Iran

*Corresponding authors:

Zohreh Hojati, PhD; Division of Genetics, Department of Cell and Molecular Biology and Microbiology, Faculty of Biological Sciences and Technology, University of Isfahan, Isfahan, Iran. Tel: +98 313 7932455, Email: z.hojati@sci.ui.ac.ir

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Abstract

Introduction: Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide. Numerous studies have demonstrated dysregulated gene expression in CRC. However, comprehensive investigations are still needed to clarify the underlying biological pathways disrupted by these dysregulated genes. This study was designed to identify differentially expressed genes (DEGs) common across all CRC stages compared to normal samples, as well as to identify hub genes and their related pathways.

Methods: RNA sequencing data were downloaded from the TCGA database. Samples were classified into four stages, and DEGs between each stage and normal samples were identified. Genes present in all four groups were selected for further analysis. Gene enrichment analyses were performed using the DAVID database to validate the data. A protein-protein interaction (PPI) network was constructed, and hub genes were identified using the CytoHubba plugin. The UALCAN database was used to perform in silico validation of the potential genes of interest.

Results: A total of 2,899 genes were commonly expressed across all four groups. Biological pathway analysis showed that these genes are enriched in known CRC pathways. PPI network analysis and hub gene identification using the CytoHubba plugin highlighted key hub genes. Validation through the UALCAN database confirmed the relevance of these genes, and enrichment analysis demonstrated their association with G protein-coupled receptor (GPCR) signaling.

Conclusion: The hub genes are functionally associated with the GPCR signaling pathway. Given the well-documented involvement of the GPCR pathway in various cancers, especially CRC, further research on these genes and pathways is essential to enhance our understanding of this disease.

Keywords: Colorectal Neoplasms, RNA-Seq, Neoplasm Staging

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death worldwide. In 2020, more than 1.9

million new cases and over 900,000 deaths due to CRC were estimated, making it a major health problem (1). Although some countries with very high human development have seen a decline or stabilization in CRC incidence, attributed primarily to healthier

lifestyles and established screening programs, CRC continues to pose a substantial financial burden and public health challenge worldwide (2).

CRC affects approximately 4% to 5% of people worldwide. Age is the primary risk factor, with a sharp increase in risk after 50 years old, while cases occurring before 50 are rare, except for inherited forms (3). Other unavoidable risk factors include a personal history of CRC and inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, which cause chronic inflammation and abnormal cell growth (dysplasia) that may lead to cancer. A family history of CRC, particularly in relatives diagnosed before age 50, also increases risk due to genetic or environmental factors (4).

Lifestyle-related risks can be reduced with healthier habits. A sedentary lifestyle is associated with an increased risk of CRC, partly because it contributes to obesity. Visceral fat produces inflammatory substances that promote cancer development. Diet plays a significant role: unhealthy eating habits can increase CRC risk by up to 70% (5). Consumption of red meat, especially when cooked at high temperatures, releases carcinogens in the gut (6). Smoking and alcohol consumption also increase CRC risk. Smoking increases risk by approximately 10.8%, primarily among long-term smokers, due to carcinogens reaching the intestines. The role of alcohol is less clear; however, acetaldehyde, its metabolite, is carcinogenic, with effects varying depending on individual enzyme variations (7).

Early detection plays a key role in preventing metastasis, reducing mortality, and improving both prognosis and future quality of life. Extensive studies are needed to achieve this aim. Cancer stage significantly influences survival outcomes. The earliest stage of CRCs are called stage 0, which represents very early cancer, followed by stages I through IV. Generally, the lower the stage number, the less the cancer has spread; conversely, a higher number, such as stage IV, indicates more extensive cancer spread (8).

The average five-year relative survival rate for all stages of CRC is 65.1%. However, in stage IV CRC, where the cancer has metastasized to distant sites, the average five-year survival rate drops significantly to 15.5%. Evidence indicates that mutations and changes in the expression of multiple genes disrupt various cellular pathways, contributing to the onset and progression of CRC (9). A lack of comprehensive understanding of the underlying cellular mechanisms has limited the ability to identify the causes and develop effective prevention strategies for CRC. Recent advancements in technologies such as microarray analysis and RNA sequencing, as well as the high-throughput data they generate, have enabled the identification of differential gene expression associated with CRC. Recently, several studies have used bioinformatic analyses to identify gene signatures related to CRC (10, 11). Liang, B. et al.

identified key pathways and genes in CRC using bioinformatics analysis. They used microarray data for their analysis and identified hub genes along with their associated pathways (12). Additionally, similar studies have been conducted in other cancer types. For example, Xiaoyu Zeng et al. used a bioinformatic approach to predict potential biomarkers for breast cancer, using three different microarray datasets and online bioinformatic tools (13). Their work provides a valuable framework for applying bioinformatics techniques to shed light on the genes and pathways involved in CRC, as demonstrated in our study.

This study aimed to extend previous investigations on CRC by identifying common differentially expressed genes (DEGs) across the four stages of CRC compared to normal samples and by finding hub genes that exhibit strong interconnections. Additionally, identifying significant pathways related to these hub genes could reveal the potential molecular mechanisms underlying this cancer.

Materials and Methods

Download the Data and Preprocess It

RNA-seq data for 481 tumor samples and 41 adjacent normal tissue samples from the TCGA-COAD project were downloaded from the TCGA website (www.cancergenome.nih.gov). The data were normalized using the DESeq2 package with the R program (version 4.4.0) (14). Outlier samples were identified through heatmap and a principal component analysis (PCA) plots. Samples exhibiting aberrant gene expression patterns inconsistent with their designated normal or tumor status were identified as outliers and removed from the analysis; these plots were drawn using the ggplot2 package. Subsequently, clinical data for 459 colon cancer patients were downloaded. According to the American Joint Committee on Cancer staging system, we classified tumor samples were classified into four groups: stage I, stage II, stage III, and stage IV. After removing outliers and organizing samples by stage, the dataset comprised 376 tumor samples and 39 normal samples.

Identification of Differentially Expressed Genes and Their Subgrouping

DEGs were identified using the DESeq2 package by comparing each stage against normal samples. This resulted in four groups of DEGs (DEGs in stages I-IV (DEG I, II, III, and IV)). Genes in each group were filtered based on an adjusted P-value of less than 0.001 and $|\log_2 \text{fold change}|$ greater than 2. The genes in these groups exhibit certain similarities; for example, a gene may be exclusive to DEG I, while another may be present in both DEG I and DEG II. Since there are four groups, the total number of possible combinations is 16 (2^4), including one combination representing genes that do not belong to any group. Therefore, the genes were classified

into 15 distinct groups based on their presence or absence patterns across these four groups.

Gene Enrichment Analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

DAVID is an open database used to investigate the functional annotation of genes and pathways (<http://david.ncifcrf.gov>; version 2021) (15). KEGG is a database designed for analyzing relevant pathways generated by high-throughput experimental techniques (16). To identify the roles of DEGs, gene ontology analysis including biological process, cellular component, and molecular function, as well as KEGG pathway analysis were performed using the DAVID database.

PPI Network Analysis

The Search Tool for the Retrieval of Interacting Genes (STRING; <https://string-db.org/>) was used to construct a PPI network for protein-coding genes with a confidence score cutoff set at 0.4. Cytoscape software (version 3.10.1) was used to visualize the interaction network (17). CytoHubba, a powerful plugin for Cytoscape, was used to identify key hub genes by applying various topological analysis methods, including betweenness, degree, edge percolated component, maximal clique centrality, maximum neighborhood component, and stress (18). In this study, degree, maximum neighborhood component, and edge percolated component metrics were used.

UALCAN Validation

UALCAN is an interactive and comprehensive web resource that provides easy access to cancer OMICS data (TCGA, MET500, CPTAC, CBTTTC) and enables in silico validation of potential genes of interest. In this study, the UALCAN database was used to validate the expression of hub genes

based on transcripts per million, a measure of gene expression values across sample types and individual cancer stages (19).

Results

Screening DEGs

Before performing DEG analysis, normalization and outlier removal were conducted. Outliers were identified using boxplots, heatmaps, and PCA. After removing outliers, PCA and heatmap plots revealed distinct groups between normal and tumor samples. Ultimately, a total of 415 samples were selected for further analysis. According to the clinical data, the samples included 68 in Stage I, 150 in Stage II, 105 in Stage III, 53 in Stage IV, and 39 normal samples. DEG analysis was performed by comparing each stage against the normal samples (DEG I, DEG II, DEG III, and DEG IV). To filter DEGs and identify significant genes, genes with $|\log_2 \text{fold change}|$ greater than 2 and an adjusted p-value less than 0.001 were selected.

Genes can be differentially expressed in any combination of stages or not expressed in any stage at all. There are 16 (equal to 2^4) possible outcomes based on the presence or absence of gene expression in each group. DEGs were categorized into 15 subgroups. The Venn diagram illustrates the distribution of genes among the four groups of DEGs (Figure 1).

Among these subgroups, DEGs present in all four groups (highlighted in red in the Venn diagram) were used for subsequent analysis. These 2899 genes are significantly upregulated or downregulated across all stages compared to normal samples. Therefore, they may play important roles in the occurrence and progression of CRC. In this group, there were 1,551 protein-coding genes and 816 lncRNAs while pseudogenes and other other RNA types were excluded.

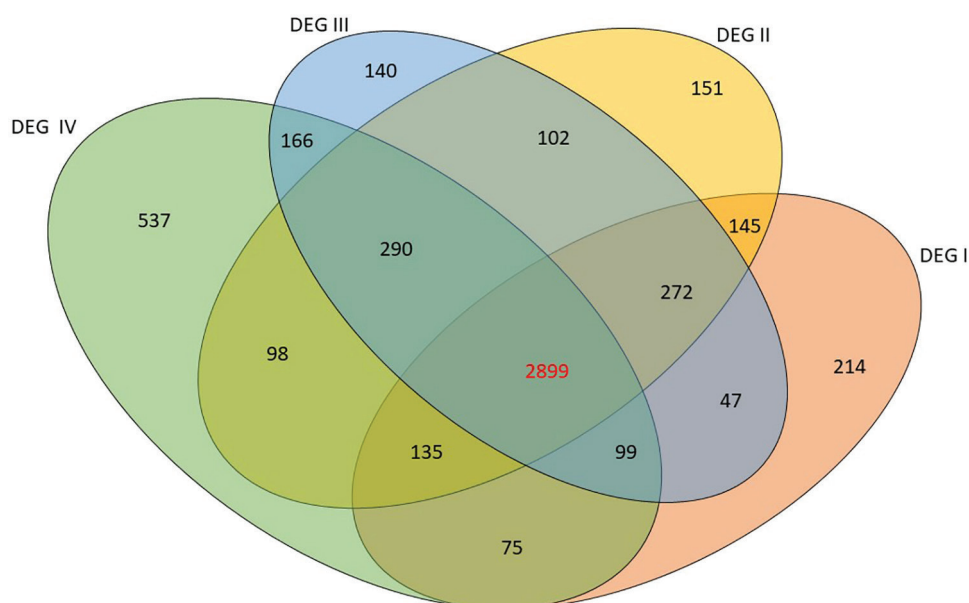


Figure 1: The Venn diagram shows the segregation of genes among four groups of differentially expressed genes. Different colored areas represented different differentially expressed genes. Some genes appear in more than one group, and this diagram indicates the number of these genes. A total of 2,899 genes (shown in red) are present in all four groups of differentially expressed genes.

Table 1: Names of hub genes, cytohubba methods, and expression values (indicating their upregulation or downregulation) are presented in this table.

	Hub genes	Cytohubba method	Expression value
1	Protein kinase cAMP-activated catalytic subunit beta (<i>PRKACB</i>)	Degree/MNC/EPC	down
2	C-X-C Motif Chemokine Ligand 8 (<i>CXCL8</i>)	Degree/MNC/EPC	up
3	Somatostatin (<i>SST</i>)	Degree/MNC/EPC	down
4	Neural Cell Adhesion Molecule 1 (<i>NCAM1</i>)	Degree/MNC/EPC	down
5	Angiotensinogen (<i>AGT</i>)	Degree/MNC/EPC	up
6	Sonic hedgehog signaling molecule (<i>SHH</i>)	Degree/MNC	up
7	Interleukin 2 (<i>IL2</i>)	Degree/MNC	down
8	Calmodulin Like 3 (<i>ALML3</i>)	Degree/MNC	up
9	Glucagon (<i>GCG</i>)	Degree/MNC	down
10	Angiotensin II Receptor Type 1 (<i>AGTR1</i>)	MNC/EPC	down
11	Collagen Type I Alpha 1 Chain (<i>COL1A1</i>)	Degree	up
12	Dopamine Receptor (<i>DRD2</i>)	EPC	up
13	Glutamate Decarboxylase 1 (<i>GADI</i>)	EPC	up
14	Fibroblast Growth Factor 8 (<i>FGF8</i>)	EPC	up
15	Motif Chemokine Ligand 12 (<i>CXCL12</i>)	EPC	down

PPI Network Analysis

To identify protein-protein interactions within the subgroup of interest (2,899 genes differentially expressed across all four groups), a PPI network was constructed specifically for the 1551 protein-coding genes in this subgroup using the STRING database. The cytoHubba plugin identified three different sets of 10 hub genes through degree, maximum neighborhood component, and edge percolated component methods. Table 1 presents the gene IDs along with their expression levels (upregulated or downregulated) identified by these three methods.

UALCAN Database Analysis

The TCGA analysis on the UALCAN platform validated the expression levels of these hub genes; however, the transcripts per million values for IL2 and FGF8 were extremely low. As mentioned, all of these genes are differentially expressed across all stages based on our RNA-seq analysis. Figure 3 demonstrates that the UALCAN analysis confirmed the differential expression of these hub genes in all four stages.

Gene Enrichment Analysis for hub Genes

Gene enrichment analysis was performed for the hub genes. In the Biological Process section, the G protein-coupled receptor (GPCR) signaling pathway was identified as a significant pathway. Among all the hub genes identified in this study, C-X-C Motif Chemokine Ligand 12 (*CXCL12*), C-X-C Motif Chemokine Ligand 8 (*CXCL8*), Angiotensin II Receptor Type 1 (*AGTR1*), Angiotensinogen (*AGT*), Glucagon (*GCG*), Interleukin 2 (*IL2*), Somatostatin (*SST*), and Dopamine Receptor (*DRD2*) were involved in this pathway.

Discussion

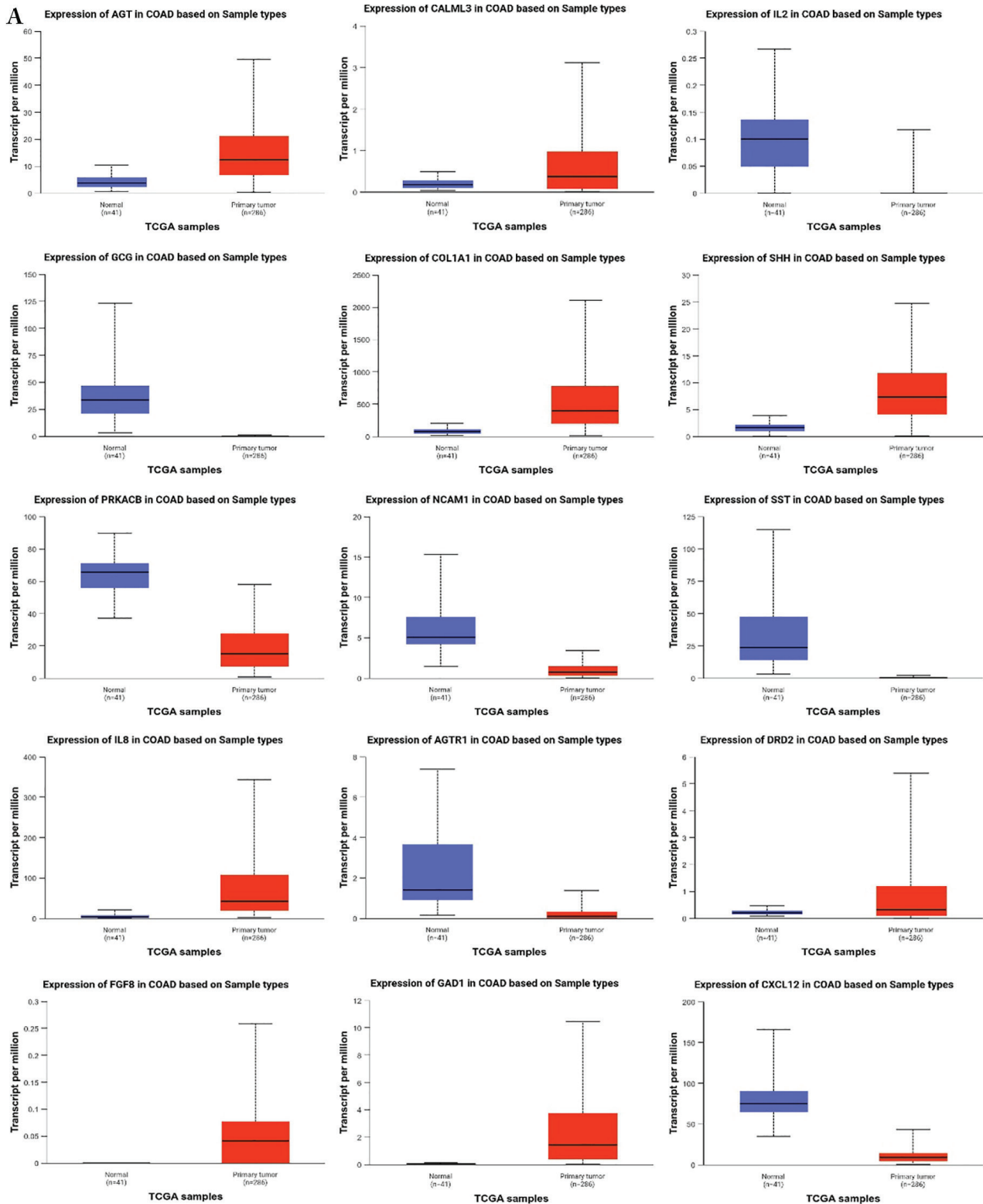
In this study, RNA sequencing data from TCGA were analyzed to identify hub genes that may play a crucial role in the initiation or progression of CRC. A

total of 2899 DEGs common across all four stages of CRC compared to the normal group were identified. Functional enrichment analyses demonstrated that these DEGs were significantly involved in biological processes such as keratinization, phagocytosis recognition, intermediate filament organization, and immune response. Previous studies have demonstrated that dysregulation of these biological processes plays a crucial role in cancer development and progression (24-26), thereby supporting the validity of this study. PPI network analysis identified three different sets of hub genes including *PRKACB*, *CXCL8*, *SST*, *IL2*, *COL1A1*, *SHH*, *NCAM1*, *CALML3*, *AGT*, *GCG*, *AGTR1*, *CXCL12*, *DRD2*, *FGF8*, and *GADI*. To validate the expression levels of these genes, the UALCAN database, based on TCGA data, was utilized, confirming that the dysregulation of these hub genes is consistent with CRC. Finally, enrichment analysis using the DAVID database identified biological pathways associated with the validated hub genes. The genes were classified into two main pathways: GPCR signaling and cell-cell signaling.

A significant number of the hub genes are involved in the GPCR pathway. The seven-transmembrane GPCRs, which belong to the largest superfamily of signal transduction proteins, regulate multiple biological functions by coupling to heterotrimeric G-protein associated with the inner surface of the plasma membrane. G proteins are classified into four main subgroups: $G_{\alpha s}$, $G_{\alpha q/11}$, $G_{\alpha i/o}$, and $G_{\alpha 12/13}$, which selectively associate upon ligand activation to initiate a potential downstream signaling pathway. These G proteins consist of three subunits, G_{α} , G_{β} , and G_{γ} , located on the inner part of the plasma membrane (27). GPCRs play a crucial role in numerous physiological functions as well as in tumor growth and metastasis. For instance, overexpression of various GPCRs has been observed in a range of primary and metastatic cancers, including head and neck squamous cell carcinoma, non-small cell lung cancer, breast, prostate, gastric tumors, melanoma, and diffuse large B-cell lymphoma (28, 29).

Various molecules such as hormones, lipids, peptides, and neurotransmitters exert their biological effects by binding to these seven-transmembrane receptors coupled to heterotrimeric G proteins, which are highly specialized transducers capable of modulating multiple signaling pathways. Furthermore, many GPCR-mediated responses do not rely on a single biochemical route but result from the integration of a complex network of transduction cascades involved in various physiological activities and

tumor development (30). The WNT pathway, a key signaling cascade associated with cancer, is initiated when WNT ligands bind to the G protein-coupled Frizzled receptors, which subsequently associate with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6). In the presence of WNTs, β -catenin translocates into the nucleus and activates TCF/LEF transcription factors, thereby regulating the expression of genes involved in cell differentiation and proliferation (31, 32).



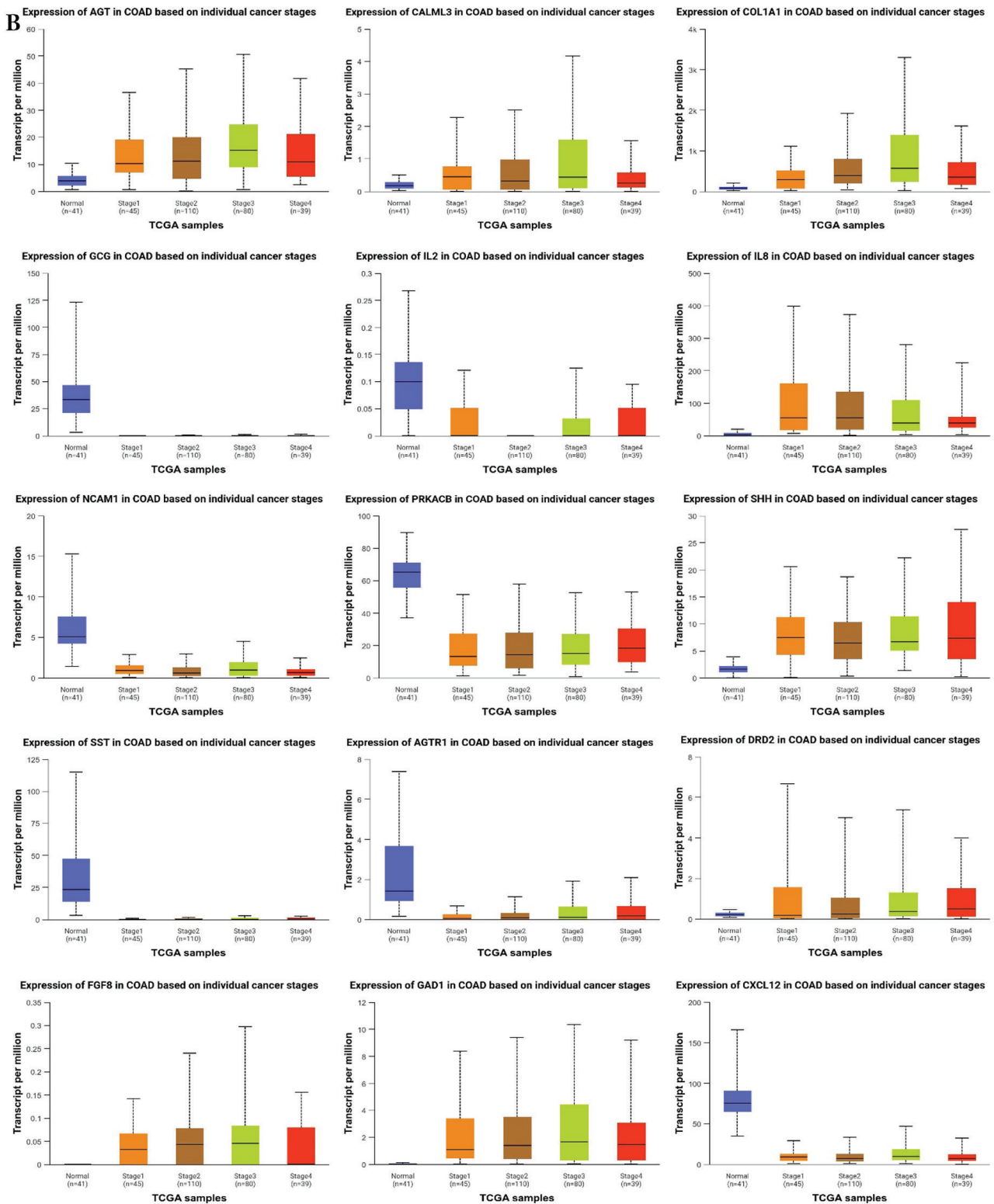


Figure 3: The UALCAN platform was used to validate the expression of the 15 identified hub genes based on sample types (A) and individual cancer stages (B). The transcription per million values for IL2 and FGF8 were extremely low (I).

The CXC family of chemokines and their receptors are crucial for inflammation and antitumor immunity, both of which are key factors in CRC progression. These small proteins are secreted not only by tumor cells but also by leukocytes, fibroblasts, endothelial cells, and epithelial cells. They influence tumor behavior by regulating angiogenesis, activating tumor-specific immune responses, and directly stimulating tumor proliferation through autocrine

or paracrine mechanisms. The CXC chemokines and their receptors has also been associated with metastasis and treatment resistance. Several studies have reported the expression of CXC chemokines and/or their receptors in tumors, whether in epithelial tumor cells, fibroblasts, or infiltrating leukocytes as well as in plasma or in plasma/serum samples from CRC patients, with this expression has been associated with patient outcomes (33).

Two chemokines, C-X-C Motif Chemokine Ligand 8 (*CXCL8*) and *CXCL12*, and their roles in cancer development have been well-studied. *CXCL8*, an inflammatory cytokine, binds to *CXCR1* and *CXCR2* on neutrophils, activating G-protein and β -arrestin-mediated signal transduction pathways, which ultimately promote neutrophil chemotaxis (34). Additionally, the binding of *CXCL8* to C-X-C Motif Chemokine Receptor 1 (*CXCR1*) and C-X-C Motif Chemokine Receptor 2 (*CXCR2*) induces receptor internalization, primarily mediated by β -arrestins, leading to desensitization of G protein-mediated signaling. This process results in receptor degradation, recycling back to the membrane, or initiation of additional MAPK or tyrosine kinase signaling pathways (35).

CXCL12 is a homeostatic chemokine that binds to C-X-C Motif Chemokine Ligand 4 (*CXCR4*), atypical chemokine receptor 1 (*ACKR1*), and atypical chemokine receptor 3 (*ACKR3*), promoting the migration and activation of hematopoietic progenitor cells, endothelial cells, and various leukocytes. Therefore, it plays an important role in regulating embryogenesis, hematopoiesis, and angiogenesis (36-38). Additionally, *CXCL12* has an inflammatory function and is associated with *CXCL8* in this context. Notably, *CXCR4* is a GPCR, indicating the significance of this pathway in cancer (36).

You-Chuan Xiao et al. demonstrate that a high abundance of *CXCL8* is strongly correlated with poor overall and disease-free survival in 186 patients with CRC.

Angiotensin II type I receptor (*AGTR1*) is a member of the G protein-coupled receptor superfamily. Upregulation of this gene has been observed in related tumor tissues (39). Activation of *AGTR1* by its ligand, angiotensin II, promotes a pathway that ultimately promotes cell proliferation and migration. Persistent triggers by angiotensin II can induce changes in downstream gene expression and facilitate the phenotypic transition from epithelial-to-mesenchymal transition (40).

The physiologically active enzyme angiotensin II is involved in maintaining blood pressure, body fluid balance, and electrolyte homeostasis, as well as in the pathogenesis of essential hypertension and preeclampsia. Dysregulated expression of its gene, *AGT*, has been validated in CRC; suggesting that *AGT* can be a potential biomarker for this tumor (41). Wei Chen et al. confirmed the overexpression of *AGT* in CRC tissues. Subsequently, a series of *in vitro* experiments were conducted to evaluate the potential role of *AGT* in the proliferation, migration, and invasion of CRC cells (41).

The neuroendocrine peptide glucagon (*GCG*) is implicated in CRC (42). Previous research has demonstrated that aberrant *GCG* gene expression distinguishes CRC tissue from hyperplastic polyps with 100% sensitivity (42). In human colon cancer cell lines, *GCG* activates its receptor, which leads to

cancer cell proliferation by affecting AMPK/MAPK signaling pathways (43).

Somatostatin, an endogenous peptide hormone, is a ligand for five types of somatostatin receptors (SSTRs), which belong to the transmembrane GPCRs superfamily. Decreased expression of somatostatin has been observed in CRC compared to normal tissues, suggesting an antitumor role for this peptide (44, 45).

DRD2, a dopamine receptor characterized by a seven-transmembrane structure, is another gene belonging to the GPCR family. *DRD2* couples with the $G\alpha i/o$ family of G proteins, leading to a decrease in cAMP levels (46-48).

Conclusion

The identified hub genes involved in the GPCR pathway highlight the significance of this pathway in CRC. This study suggests that further *in vitro* investigations of this pathway could validate the findings.

Authors' Contribution

Morteza-Ali Rahmani: Conception and design of the study, Data curation, Acquisition of data, Analysis and interpretation of data, Investigation, visualization, Drafting the article or revising it critically for important intellectual content, Final approval of the version to be submitted. Melika Aramfar: Conception and design of the study, Acquisition of data, Analysis and interpretation of data, Writing – review & editing, Drafting the article or revising it critically for important intellectual content, Final approval of the version to be submitted. Zohreh Hojati: Project administration, Conception and design of the study, Acquisition of data, Analysis and interpretation of data, writing – review & editing, Drafting the article or revising it critically for important intellectual content, Final approval of the version to be submitted.

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N/A

Conflict of Interest

The authors declare no conflict of interest. There aren't any of the authors of this manuscript who are current Editors or Editorial Board Members of the Iranian Journal of Colorectal Research

Ethics Statement

This article is an independent study and has been conducted under the institutional oversight of the University of Isfahan. All ethical guidelines were followed, and the study protocol was approved by the University's Ethics Committee (IR.UI.REC 1403.119)

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