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Original Article

The roles of FGFR, EGFR and AMP-activated protein kinase pathway in colorectal cancer stem cells derived spheroids: Implications in colorectal cancer treatment

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Abstract

The aim of our research was to identify molecular targets that can be targeted by drugs and patient-specific models for personalized medicine for colorectal cancer (CRC). Here, we obtained high-throughput RNA sequencing data from Gene Expression Omnibus (GEO) with accession number GSE205787 and analyzed it using next-generation knowledge discovery tools such as BioJupies and Ingenuity Pathway Analysis (IPA) software. Differentially expressed genes (DEGs) were identified by comparing the raw counts from 47 CRC patient-derived spheroids (CRC-CSCs) with those from normal spheroids from the epithelium of the colon and rectum of healthy individuals, using BioJupies tools. IPA was used to identify differentially regulated canonical pathways, upstream regulators of CRC, non-directional networks, diseases, and biofunctions, as well as to conduct subsequent perturbation analysis using the Molecular Prediction Analysis (MAP) tool. Our study demonstrates that several KEGG pathways, including the AMPK, Phospholipase D, MAPK, and PI3-AKT signaling pathways, were significantly downregulated in the CRC-CSC group. Additionally, Wnt signaling and FGFR pathways were significantly upregulated. Moreover, according to Wikipathways, the EGF/EGFR signaling pathway, MAPK signaling pathway, G-protein signaling pathway, and Focal Adhesion-PI3-AKT pathway were downregulated in the CRC-CSC group. Furthermore, based on the Reactome, the Metabolism, Vesicle-mediated transport, RAF signaling, and G-alpha (12/13) signaling pathways were also downregulated in the CRC-CSC group. Utilizing innovative drug combination approaches and innovative drug delivery techniques, CRC treatments can be enhanced by modulating the FGFR, EGFR, and AMPK signaling pathways, which may ultimately lead to improved patient outcomes.

Keywords: Colorectal cancer, Cancer stem cell spheroids, AMP-activated protein kinase signaling, Fibroblast Growth Factor Receptor, Epidermal Growth Factor Receptor, BioJupies, Ingenuity Pathway Analysis

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Introduction

Colorectal cancer (CRC), which primarily affects the colon and rectum and plays a crucial role in the absorption of water and nutrients from food, as well as in the storage of stool, is a prevalent form of cancer worldwide. According to the World Health Organization (WHO), it is responsible for 1.9 million new cases and 935,000 deaths in 2020 (Sung et al., 2021). CRC is the second leading cause of cancer-related deaths in women and the third in men, but when detected early, it can be treated effectively, resulting in a five-year survival rate of up to 90% (Siegel et al., 2022). In Saudi Arabia, CRC rates are increasing, making it a significant public health concern. The Saudi Cancer Registry reports that men have an age-standardized incidence rate (ASR) of 14.2/100,000 and women 11.5/100,000. The Eastern Region and Riyadh had the highest ASR, whereas Tabuk and Najran had the lowest. The median age at diagnosis of CRC is 60 years for men and 58 years for women (Almatroudi, 2020). The incidence rates have been increasing in Saudi Arabia for several decades, and lifestyle changes, such as consuming more processed foods, red meat, and sugary drinks, and engaging in less physical activity, have contributed to this trend. The prevalence of obesity, a major risk factor for CRC, is increasing in Saudi Arabia. Better diagnostic techniques have also led to the identification of more CRC patients (Almatroudi, 2020). CRC develops from localized to metastatic due to mutations in APC, KRAS, and TP53. As cancer progresses, asymptomatic individuals may experience rectal bleeding, changes in bowel movement, and abdominal pain. After surgery, adjuvant or palliative chemotherapy and radiation therapy can be used to treat CRC. Immunotherapy is a potential therapeutic option (Wan et al., 2020; Siegel et al., 2022).

Colorectal cancer stem cells (CSCs) are believed to play a crucial role in the initiation, progression, and metastasis of CRC (Vermeulen et al., 2008). Unfortunately, some patients experience disease recurrence following chemotherapy and radiation treatment due to CSC resistance. Three-dimensional (3D) models of CRC tumors, known as colorectal cancer-derived stem cell spheroids (CRC-SCSs), can be created by cultivating CSCs in a specific medium, and have emerged as valuable tools for researching CRC biology and developing new CSC-targeted therapies (Kiwaki and Kataoka, 2022). CRC-SCSs offer several advantages over two-dimensional (2D) cell cultures, including a more accurate representation of CRC tumors, the ability to study cell-cell interactions between CSCs and the tumor microenvironment, and the potential for CSC-targeted drug testing. By utilizing CRC-SCSs, we can gain a better understanding of CRC biology and develop more effective treatments. In the present study, we employed next-generation knowledge discovery (NGKD) technologies to analyze a high-throughput RNA-seq dataset of CRC-CSCs to identify differentially regulated pathogenic pathways in CRC-SCSs compared with healthy colonic epithelial spheroids. We aimed to identify druggable molecular targets, prognostic and therapeutic response biomarkers, and patient-specific models for personalized medicine. Ultimately, our goal is to improve our understanding of CRC and develop more effective therapies utilizing CRC-SCSs.

Material and Methods

Data source

High throughput RNA sequencing data generated by Kitano et al. (2022) were obtained from the Gene Expression Omnibus (Accession Number: GSE205787). These data were derived from 47 CRC-CSCs and three normal colorectal epithelial spheroid lines. The raw data were subjected to BioJupies analysis as previously described (Torre et al., 2018; Pushparaj et al., 2021). This study did not require ethical approval from the Institutional Review Board (IRB) ethical approval because it did not involve animal models or human subjects and was conducted using RNA-seq datasets from the Gene Expression Omnibus (GEO) (Barrett et al., 2013; Pushparaj et al., 2021).

Data normalization and differentially expressed genes

Using BioJupies, the raw counts were normalized to log10-Counts Per Million (logCPM) by dividing each column by the total sum of its counts, multiplying it by 10^6, and then applying a log10-transform (Torre et al., 2018). The signature of differentially expressed genes (DEGs) was generated by comparing gene expression levels between the control group and the experimental group using the limma R package available on Bioconductor (Ritchie et al., 2015). (<http://bioconductor.org/packages/release/bioc/html/limma.html>)

Principal component analysis, heatmap and

volcano plot

The Python module sklearn's principal component analysis (PCA) function was utilized. Initially, the raw gene counts were normalized using the logCPM method, followed by the selection of 2500 genes exhibiting the most variable expression. Next, a Z-score transformation was applied before the PCA was conducted. An interactive heatmap was then generated using Clustergrammer (Fernandez et al., 2017), which is accessible at <http://amp.pharm.mssm.edu/clustergrammer/>. For Library Size Analysis, the raw gene counts were normalized using logCPM, and 2500 genes exhibiting the most variable expression were selected. Z-score transformation was applied, and the resulting data were used to create a heatmap. Read counts were obtained by summing each column of the raw gene count matrix. The total count was divided by 10^6 to display the values in millions of reads. The x-axis of the heatmap displays the log2 transformed gene fold changes, and the y-axis displays the -log10 transformed P-values after applying the Benjamini-Hochberg correction method (Torre et al., 2018).

Pathway enrichment analysis

Enrichr is a web-based tool that employs an extensive gene set library and interactive methods to present enrichment outcomes. By examining upregulated and downregulated gene sets, Enrichr generated enrichment results. In this study, the gene set libraries KEGG, Reactome and WiKiPathways (2016) were utilized. The significant terms were determined using a p-value less than 0.1, following the application of the Benjamini-Hochberg adjustment. Enrichment analysis, a statistical method that integrates gene function knowledge from multiple sources, identifies over-represented biological terms such as signaling pathways, molecular functions, and diseases in a gene set (Xie et al., 2021).

Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA) software (Qiagen, USA) was used to analyze the significantly regulated genes (DEGs) with logFC > ±2 and p values < 0.05, as previously described (Shaath et al., 2023). The analysis included identification of canonical pathways, gene networks, upstream/downstream effectors, and biomarkers/drug targets. Additionally, the Molecular Activity Prediction (MAP) tool was used to investigate the influence of DEGs in colorectal cancer (CRC) on cancer metastasis and to elucidate the molecular mechanisms underlying cancer pathways.

Results

BioJupies analysis of high throughput RNA sequencing data

Principal Component Analysis (PCA) is a widely used method for the analysis of high-dimensional datasets, enabling the identification of global patterns. In this study, a 3D scatter plot illustrates the PCs of normal spheroids and CRC-CSC RNA-seq (Figure 1A). Additionally, a volcano plot with significance versus fold-change estimates from differential expression analysis was displayed on the axes of the scatter plot for each gene (Figure 1B). Clustergrammer tool was used to display and analyze high-dimensional data as dynamic hierarchically clustered heatmaps online. This is particularly useful for comparing RNA-seq samples and identifying genes that cluster samples (Figure 1C). To assess gene expression, sequencing reads from the GSE205787 RNA-seq dataset were mapped to the reference genome and consolidated into gene counts. CRC-CSCs exhibit altered gene expression profiles compared to normal spheroids. By comparing gene expression between CRC-CSCs and normal spheroids, we identified differentially expressed genes (DEGs) to gain a deeper understanding of the underlying pathological mechanisms associated with CRC.

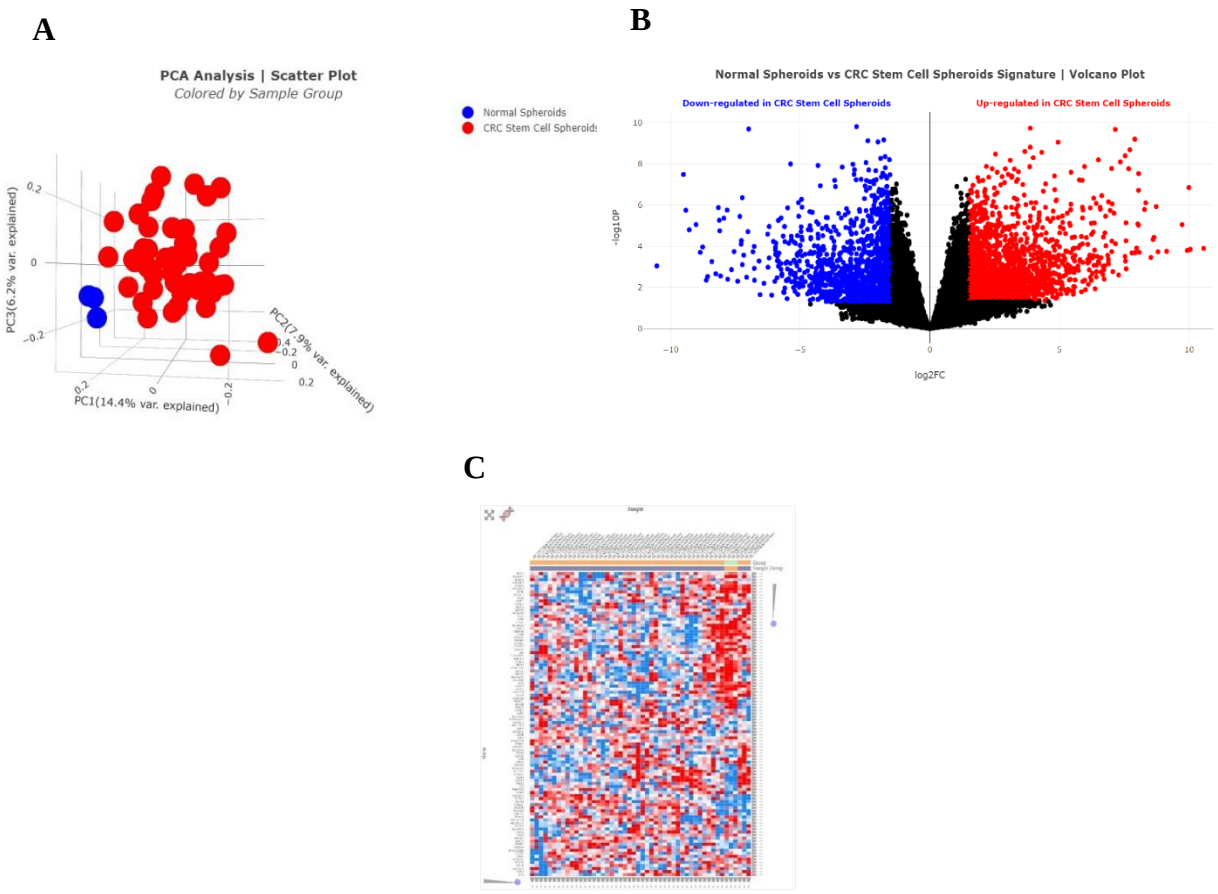

Figure-1. (A) The three-dimensional scatter plot displaying the results of the Principal Component Analysis (PCA) of the RNA-seq samples. The red circles represent the 47-colon cancer stem cell derived spheroid samples, while the blue denotes the 3 spheroids derived from healthy normal colon epithelium. (B) Volcano plot of differential gene expression analysis, illustrating the upregulated and downregulated genes. The blue and red points indicate statistically significant genes. (C) Heatmap depicting gene expression across different samples in the RNA-sequencing dataset. Each row in the heatmap corresponds to a gene and each column represents a sample. The color intensity of each cell reflected the normalized gene expression values, with darker shades indicating higher expression levels. The heatmap also includes color bars alongside each column, which provides contextual information about the samples, such as their tissue origin or experimental treatment.

Pathway enrichment analysis

Biological pathways are a series of biochemical interactions that play a crucial role in determining cellular behavior. These pathways have been extensively documented in databases such as KEGG, Reactome, and WiKi Pathways. By utilizing Enrichr, these databases were used to identify the biological pathways that are over-represented in the upregulated and downregulated genes identified by comparing CRC-CSCs with normal spheroid samples. According to BioJupies analysis, several KEGG pathways, including the AMPK signaling pathway, Phospholipase D signaling pathway, MAPK signaling pathway, and PI3-AKT signaling pathway, were significantly downregulated in the CRC-CSC group (Figure 2A). In addition, the Wnt signaling pathway was significantly upregulated in the CRC-CSC group. Furthermore, according to Wikipathways, the EGF/EGFR signaling pathway, MAPK signaling pathway, G-protein signaling pathway, and Focal Adhesion-PI3-AKT pathway were downregulated in the CRC-CSC group (Figure 2B). Additionally, based on Reactome, the Metabolism, Vesicle-mediated transport, RAF signaling, and G-alpha (12/13) signaling pathways were downregulated in the CRC-CSC group (Figure 2C).

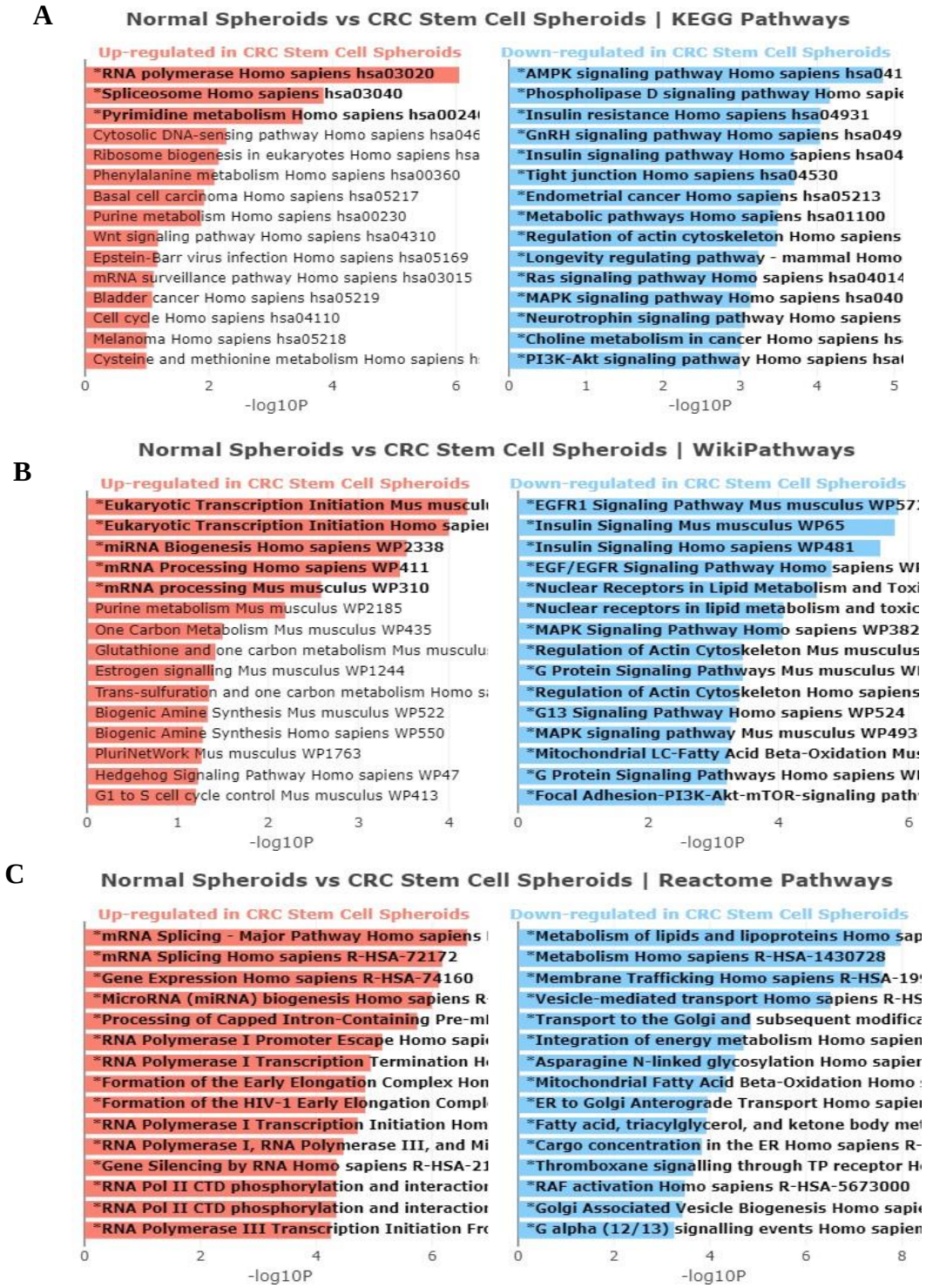

Figure-2. Illustration of differentially regulated pathways in CRC stem cell spheroids compared to normal colon epithelium derived spheroids, based on (A) KEGG, (B) Wikipathways, and (C) Reactome pathways, as demonstrated by BioJupies web tool.

Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA) revealed a notable enrichment of the CRC metastasis pathway among the DEGs (Figure 3). The overlay of DEGs from the CRC-CSC group and the Molecular Activity Prediction analysis using IPA showed downregulation of TP53 and apoptosis signaling, as well as upregulation of cellular functions such as cell growth, metastasis, cell survival, angiogenesis, and tumor progression (Figure 3). Moreover, the analysis revealed the upregulation of a variety of signaling molecules involved in cancer cell migration, survival, metastasis, and cell cycle progression, as well as the prediction of downregulated Tp53 signaling and apoptosis signaling mechanisms (Figure 4).

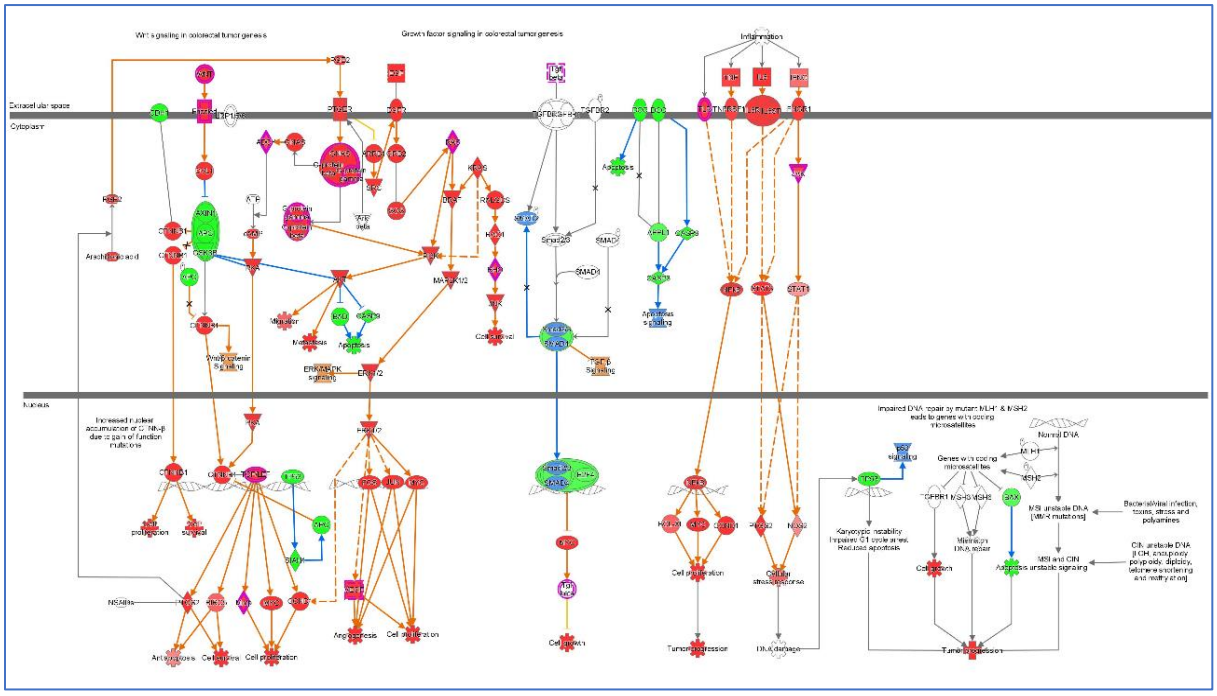

Figure-3. Molecular Mechanisms of Colorectal Cancer. The results of the analysis of differentially expressed genes (DEGs) from the colorectal cancer (CRC) group, in conjunction with the use of the Molecular Activity Prediction tool in IPA, indicated downregulation of TP53 and apoptotic signaling. The IPA analysis revealed the upregulation of a variety of signaling molecules that are involved in cancer cell migration, cell survival, metastasis, and cell cycle progression.

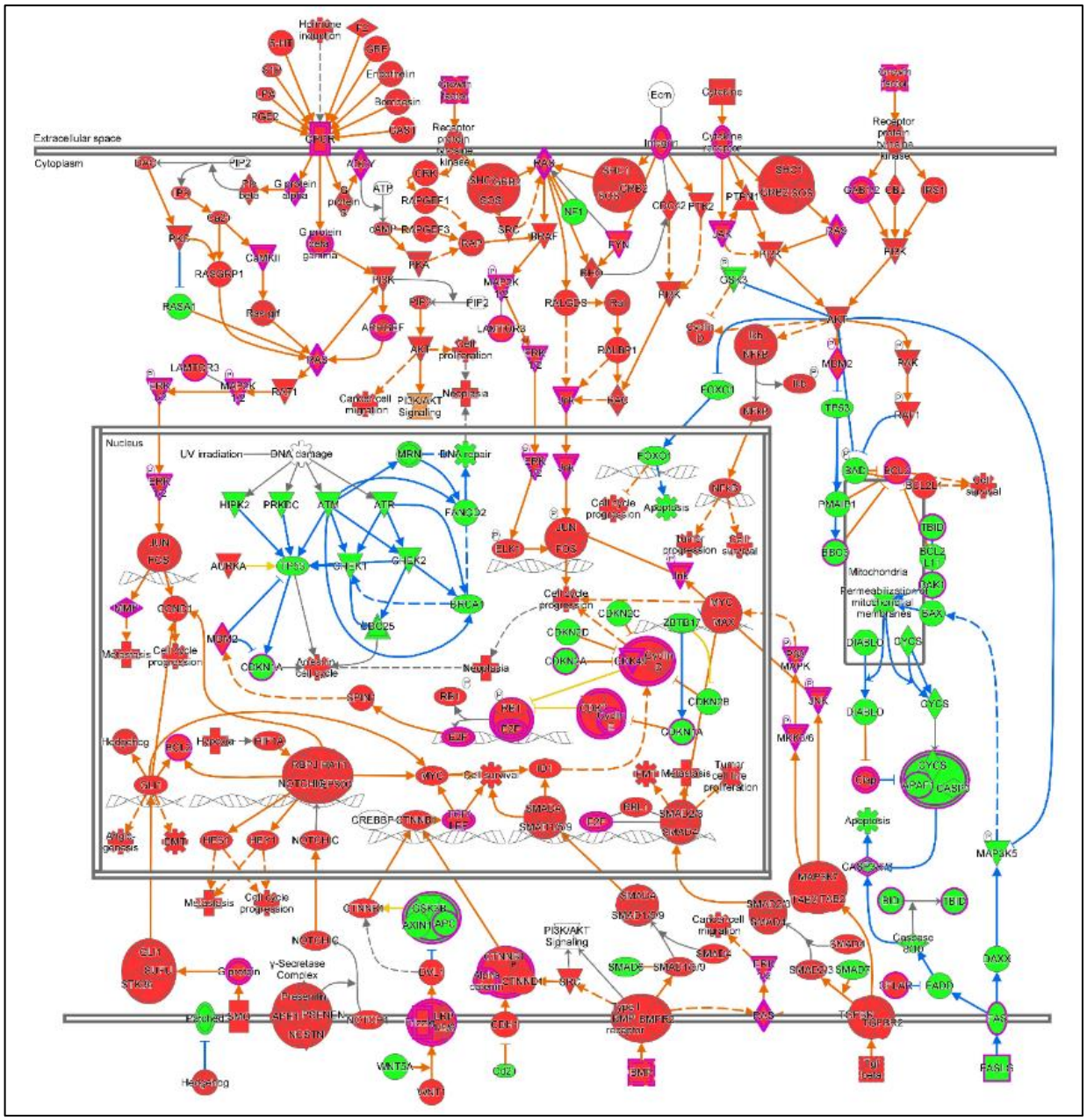

Figure-4. The IPA analysis revealed the upregulation of a variety of signaling molecules that are involved in cancer cell migration, cell survival, metastasis, and cell cycle progression.

Discussion

CRC, the third most prevalent cancer globally, is a major public health concern. Despite advances in therapy, recurrence and metastasis have persisted. Understanding the intricate relationship between CSCs and their environment may provide insight into more effective treatments. Alqahtani et al. (2020) reported that CRC was the second most common cancer in Saudi Arabia from 2010 to 2019. In the United States, CRC is projected to be one of the deadliest diseases by 2023, with an estimated 153,020 new diagnoses and 52,550 deaths (Siegel et al., 2023). Pathway analysis of gene expression data can yield statistically significant results and enhance our understanding of cancer pathophysiology and gene mutations. Genetic analysis can improve CRC treatment by refining molecular cancer treatments and correlating genetic routes (Karagiannakos et al., 2022). The metabolic regulator AMPK suppresses cell

growth and proliferation.

NGKD studies have shown downregulation of the AMPK, EGF/EGFR, and FGF/FGFR signaling pathways and upregulation of the FGF/FGFR and Wnt signaling pathways in CRC-CSCs. AMPK activation may represent a potential therapeutic approach for CRC, as suggested by our findings. AMPK, a master regulator of energy metabolism, is essential for the maintenance of cellular homeostasis. In CRC, the AMPK signaling pathway can either suppress or promote tumor growth, depending on the context (Guo et al., 2018). The antitumor effects of AMPK include phosphorylation and inhibition of mTOR, a crucial protein involved in cell growth and proliferation (Hsu et al., 2022). AMPK activation also promotes apoptosis by activating pro-apoptotic proteins and inhibiting anti-apoptotic pathways (Hsu et al., 2022).

AMPK activation has been demonstrated to limit cell motility and invasion, which in turn reduces cancer cell invasion and metastasis (Hsu et al., 2022). Certain chemotherapeutic drugs have been reported to be more effective when AMPK activation promotes cell death and inhibits pro-survival pathways (Hsu et al., 2022). Notably, the pro-tumorigenic actions of AMPK are stronger in advanced CRC, in which tumor cells are subjected to metabolic stress and require alternative energy sources (Saxena et al., 2018). AMPK activation induces cancer cell epithelial-to-mesenchymal transition (EMT), which enhances cell motility, invasion, and metastasis (Saxena et al., 2018). Understanding the dual role of AMPK in colorectal cancer (CRC) is essential for developing effective treatments. Targeting AMPK with specific activators may slow tumor growth in early CRC. Further research is needed to develop AMPK-activating drugs that decrease tumor growth and minimize side effects in the advanced stages. By understanding AMPK's complex role of AMPK in CRC, customized and effective cancer treatments can be designed to improve patient outcomes. The FGFR, EGFR, and AMPK signaling pathways are crucial to CRC proliferation, survival, invasion, angiogenesis, and metastasis, as well as affecting CSCs, which causes tumor recurrence and resistance to traditional treatments (Ye et al., 2020).

FGFRs are overexpressed and activated in several types of cancer, including CRC (Babina and Turner, 2017). Research has shown that FGFR signaling promotes the growth, survival, and migration of CRC cells. Furthermore, studies have indicated that FGFRs play a role in maintaining CSCs and making them resistant to therapy. Preclinical and clinical trials of FGFR inhibitors for CRC treatment have shown promising results. In addition to FGFRs, EGFR is a receptor tyrosine kinase that is important for the development and progression of CRC. The Wnt signaling pathway is upregulated in CRC-CSCs and is influenced by the microenvironment (Vermeulen et al., 2010). EGFR mutations are found in various types of cancers. EGFR signaling stimulates the growth, survival, and invasion of CRC cells. Cetuximab and panitumumab are approved for the treatment of advanced CRC. However, these medications often lead to resistance, highlighting the need for further studies on EGFR signaling in CRC and CSCs (Uribe et al., 2021; Mizukami et al., 2019).

Further investigation into the FGFR, EGFR, and AMPK signaling pathways in CRC-SCSs is essential for the development of more effective and targeted therapies for patients with CRC. This research is crucial for overcoming resistance to current therapies, improving patient outcomes, and understanding the complex relationship between these signaling pathways and cancer stem cell biology. FGFR, EGFR, and AMPK signaling pathways are promising therapeutic targets in CRC, particularly when combined with cancer stem cell-targeted therapies. The use of CRC-SCSs in research can accelerate therapeutic development and enhance patient survival. However, it is important to note that the participation of each pathway depends on the genetic and molecular characteristics of each tumor; therefore, these pathways must be studied alongside other CRC signaling networks to understand tumor biology. New drug combinations targeting cancer stem cells could improve FGFR, EGFR, and AMPK signaling therapeutics (Kitano et al., 2022). Addressing these challenges and conducting further research can unlock the full potential of targeting these signaling pathways for CRC treatment. Cancer stem cell-SCSs have significant potential for cancer research and drug discovery; however, they must overcome several obstacles. Standardizing culture methodologies ensures repeatability and comparability, whereas high-throughput screening platforms accelerate drug discovery. Overcoming these challenges and researching cancer stem cell-SCSs will lead to the development of novel and more effective CRC treatments, a better understanding of cancer stem cell biology, tumor development and progression, and personalized medicine approaches for CRC treatment. Overall, cancer stem cell-SCS and differentially

expressed pathogenic pathway research have great potential to improve the outcomes of CRC patients.

Conclusion

Our research, based on NGKD methodology, has demonstrated the critical role that CRC-SCSs play in understanding CRC and developing effective treatments. By targeting CSCs with new drug combinations and novel drug delivery strategies, we may be able to improve treatments for CRC by modulating the FGFR, EGFR, and AMPK signaling pathways. However, significant challenges must be overcome, and further studies should be conducted before such treatments can be implemented. It is essential to examine these signaling pathways along with other CRC signaling networks to gain a comprehensive understanding of tumor biology. Future research directions include manipulating CSCs using CRISPR-Cas9, integrating CRC-SCSs with organoids, utilizing genetic and molecular profiling for personalized treatment, and developing novel drugs targeting CSC signaling pathways. These advances hold great promise for individualized therapy and improvement of patient outcomes in CRC.

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Contribution of Authors

Rasool M: Planned the study, secured funds, reviewed and edited manuscript and approved final draft
Alhassan KI: Performed the experiments, reviewed literature and wrote the first draft.
Karim S: Reviewed & edited manuscript, performed software analysis and data interpretation
Haque A: Reviewed & edited manuscript, performed software analysis and data interpretation
Mutwakil MNZ: Helped in statistical analysis, edited and approved the final manuscript and supervised study
Alharthi M: Helped in statistical analysis, edited and approved the final manuscript and supervised study
Chaudhary AG: Helped in statistical analysis, edited and approved the final manuscript and supervised study
Pushparaj PN: Planned the study, secured funds, reviewed and edited manuscript and approved final draft

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