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Original Article

The differential regulation of tumor suppressor genes (SAMD9, SPRED1, TGFBI, DUSP6, CDX2, TP53) and MAPK/ERK signaling pathway in colorectal cancer

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Abstract

Despite considerable advancements in research, particularly in oncology, colorectal cancer (CRC) remains a formidable and deadly disease. It is crucial to delve deeper into the effects of targeted therapies, signaling pathways, and genetic regulation to ensure the effectiveness of cancer treatments. In this study, we obtained microarray data for four patient-derived CRC organoids from the Gene Expression Omnibus (GEO) database (accession number: GSE114060). We then used several next-generation knowledge discovery (NGKD) tools, such as GEO2R, Metascape, WebGestalt, and Ingenuity Pathway Analysis (IPA) software, to investigate the underlying molecular mechanisms in colorectal cancer-derived organoids treated with trametinib compared to those treated with DMSO. Our NGKD analysis revealed upregulation of SAMD, TP53, and SPTLC3 and downregulation of SPRED1, TGFBI, and DUSP6. The MAPK/ERK signaling pathway was significantly downregulated and was associated with reduced expression of CDX2 in CRC organoids treated with trametinib. We concluded that SAMD9, SPRED1, TGFBI, TP53, and DUSP6 are differentially regulated and can play a pivotal role in the downregulation of the MAPK/ERK signaling pathway to suppress CRC. The use of targeted therapies to regulate the specific gene signatures identified in the current study may be beneficial in the CRC associated tumor suppression . On the other hand, upregulation of SPTLC3 may be induced by MEK inhibitors and may cause hepatotoxicity alongside nonalcoholic steatohepatitis.

Keywords: Colorectal cancer, Organoids, Tumor suppression, MAPK/ERK signaling pathway, MEK inhibitor, Hepatotoxicity

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths globally, according to the Global Cancer Observatory (GLOBOCAN) in 2020, resulting in 10 million deaths worldwide (Sung et al., 2021). Several studies have been pursuing various approaches to cure and treat cancer, and with promising advancements in new therapeutics and the continuous evolution of treatments, such as targeted therapy (Debela et al., 2021; Price et al., 2024), to develop molecular inhibitors that target signaling pathways in cancer therapy (Yip and Papa, 2021; Lee et al., 2023). These signaling pathways regulate cell growth and apoptosis; however, if a gene is mutated, it can disrupt the regulation of cells and lead to cancer (Sever and Brugge, 2015). Several genes, including SAMD9, SPRED1, TGFBI, DUSP6, CDX2, and TP53, differentially regulate CRC. However, SPRED1 can be found in the Tumor Suppressor Gene database (TSGene) (Zhao et al., 2013; Zhao et al., 2016). The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, which regulates cell proliferation, differentiation, and apoptosis, is involved in the progression of colon cancer (Guo et al., 2020). MAPK/ERK is crucial for cell proliferation, differentiation, and apoptosis, and plays a significant role in the progression of colon cancer (Guo et al., 2020).

Our study focused on the influence of MEK inhibitor on the up/downregulation of the MAPK/ERK pathway and its associated genes in CRC using the gene expression data derived from microarray experiment using organoids derived from CRC patient and normal colonic epithelium derived from healthy individual. Here, we studied specific gene signatures, pathways, gene ontologies that significantly affect the MAPK/ERK pathway in CRC using high-throughput microarray data of four patient-derived CRC organoids and normal organoids and employed several next-generation knowledge discovery (NGKD) tools, such as GEO2R, Metascape, WebGestalt, and Ingenuity Pathway Analysis (IPA) software to dissect the underlying molecular mechanisms involving tumor suppressor genes and the MAPK/ERK pathway in CRC.

Material and Methods

In the present study, we obtained the gene expression microarray data derived from four patient-derived colorectal cancer organoid (PDOs) samples from the Gene Expression Omnibus (GEO) database (accession number GSE114060) (Zhan et al., 2019).

GEO2R analysis

GEO2R analysis was performed to identify the differentially expressed genes (DEGs) in the CRC organoids compared to normal organoids. The CRC organoids were treated with trametinib (100 nM) and the normal organoids were treated with dimethyl sulfoxide (DMSO) as described before (Zhan et al., 2019). Together with the use of Log2FC cutoff ± 2, the significance level cut-off was set as ≤ 0.05 with Benjamini & Hochberg method (false discovery rate), to generate with the Volcano and Mean-Average (MA) Plots.

Metascape analysis

Utilizing pathway and process enrichment analysis across various ontology databases, including KEGG Pathway (Kyoto Encyclopedia of Genes and Genomes), Gene Ontology (GO) Biological Processes, Reactome Gene Sets, Canonical Pathways, CORUM, WiKiPathways, and PANTHER Pathway (Zhou et al., 2019), we conducted a comprehensive analysis of the gene list. The parameters for input and analysis were set as species: H. sapiens (244), and the input of the gene symbol from the gene list, with a log-fold change (LogFC) cut-off between -2 and 2. After the filtering process, we identified a total of 306 DEGs.

WebGestalt analysis

We utilized two methods, over-representation analysis (ORA) and gene set enrichment analysis (GSEA), in the WebGestalt (WEB-based GEne Set Analysis Toolkit) (Liao et al., 2019) for our study. For ORA, we employed the organism of interest Homo sapiens, and the functional database was the Gene Ontology biological process with a reference set for the gene list as affy hg u133 plus 2. In GSEA, we utilized the organism of interest Homo sapiens, the method of interest was GSEA, and the functional database was a non-redundant Gene Ontology biological process. We analyzed other functional database pathways, networks, and drugs in GSEA, including the Reactome pathway and KEGG network as kinase targets, and the drug used was GLAD4U. The advanced parameters in both ORA and GSEA were set to their default values, with ORA utilizing

the input of gene symbols from the gene list, and GSEA using both the gene symbol and the logFC value from the gene list, with the logFC cut-off set at (-2 to 2). After applying the cut, the total number of genes was 306.

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) (Qiagen, USA), a web-based software application, was used to analyze, integrate, and understand gene expression data (442 differentially expressed genes (DEGs) significantly passing logFC > ±1.5 with p values <0.05). Based on the identified DEGs and their observed fold changes, IPA predicted canonical pathways, networks, and upstream/downstream effects and discovered potential biomarkers/drug targets. Up/downstream effect analysis indicated the most affected/associated diseases and functions with activation/suppression states.

Results

Our analysis of differentially expressed genes using GEO2R revealed that SAMD9 and SPTLC3 were upregulated, with LogFC values of 3.3 and 3.13, respectively, while SPRED1, TGFBI, and DUSP6 were downregulated. SPRED1 had logFC values of -3.98, TGFBI -4.44, and DUSP6 -6.9, all significant in the GEO2R analysis (Figure 1). However, DUSP6 was not significant in WebGestalt analysis.

The Metascape analysis

Metascape analysis revealed the top 20 clusters of enriched terms from the gene list, with "vasculature development" (GO:0001944) having the highest Log10(P) of -10.10. Other related clusters included "positive regulation of cell death" (GO:0010942) with Log10(P) of -7.32, "negative regulation of cell adhesion" (GO:0007162) with Log10(P) of -6.49, "response to xenobiotic stimulus" (GO:0009410) with Log10(P) of -6.31, "positive regulation of cell adhesion" (GO:0045785) with Log10(P) of -6.27, and "regulation of MAPK cascade" (GO:0043408) with Log10(P) of -5.89. These clusters were all part of the Gene Ontology Biological Process category (Figure 2).

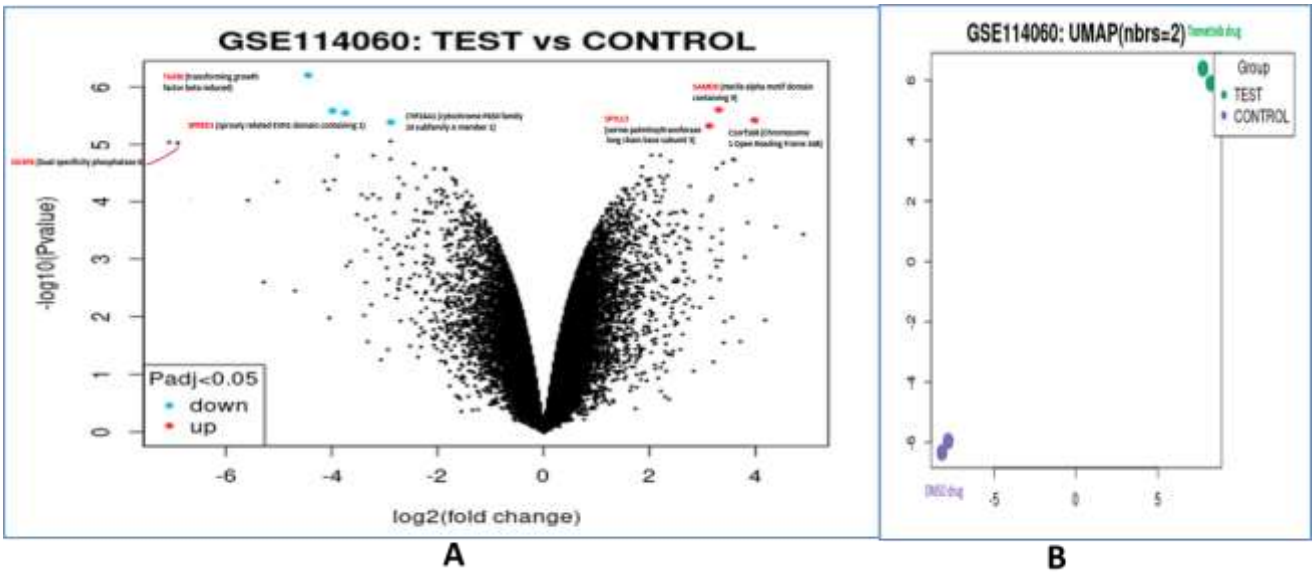

Figure-1. A: The volcano plot derived from the GEO2R analysis presents a comparison of two groups (Test and Control) and highlights the top differentially expressed genes, five of which are pertinent to our study's findings. Of these five genes, four (SAMD9, SPRED1, TGFBI, and DUSP6) are associated with the MAPK/ERK pathway, and four (SAMD9, SPRED1, TGFBI, and SPTLC3) are deemed significant. SAMD9 and SPTLC3, denoted by red circles, exhibit upregulated expression, while SPRED1 and TGFBI, indicated by blue circles, show downregulated expression. The DUSP6 gene is located in the upper left corner of the graph, with a black dot connected to it by a red line, indicating downregulated expression and non-significance. The UMAP graph from the GEO2R analysis features four samples from patient-derived colorectal cancer organoids. The top right two green circles represent the test group that received MEKi inhibitor treatment, while the bottom left two purple circles correspond to the control group that was administered dimethyl sulfoxide (DMSO) as a placebo drug.

WebGestalt analysis

We performed an ORA with the functional database as a gene ontology biological process and obtained statistically significant findings with (FDR ≤ 0.05). The top enriched gene set was "vasculature development" (GO:0001944) with (FDR 0.0014876) and it had *SPRED1* and *TGFBI* (Table 1). The "regulation of apoptotic process" (GO:0042981) with (FDR 0.0011518), "regulation of programmed cell death" (GO:0043067) with (FDR 0.0011518), "regulation of cell death" (GO:0010941) with (FDR 0.0011518), "apoptotic process" (GO:0006915) with (FDR 0.00053731), "programmed cell death" (GO:0012501) with (FDR 0.00073905) and all of them, except "vasculature development" have *DUSP6* gene (Figure 3).

The GSEA using WebGestalt

In the pathway reactome, we found three statistically significant downregulated gene sets (FDR ≤ 0.05) (Figure 4A). At the bottom of the enriched gene set "MAPK family signaling cascades" (R-HSA-5683057) with (FDR 0.0037960), "MAPK1/MAPK3 signaling" (R-HSA-5684996) with (FDR 0.0067484), "RAF/MAP kinase cascade" (R-HSA-5673001) with (FDR 0.0067484). All significant gene sets had DUSP6 with a score of -6.5133 and SPRED1 with a score of -3.8733 (Table 2).

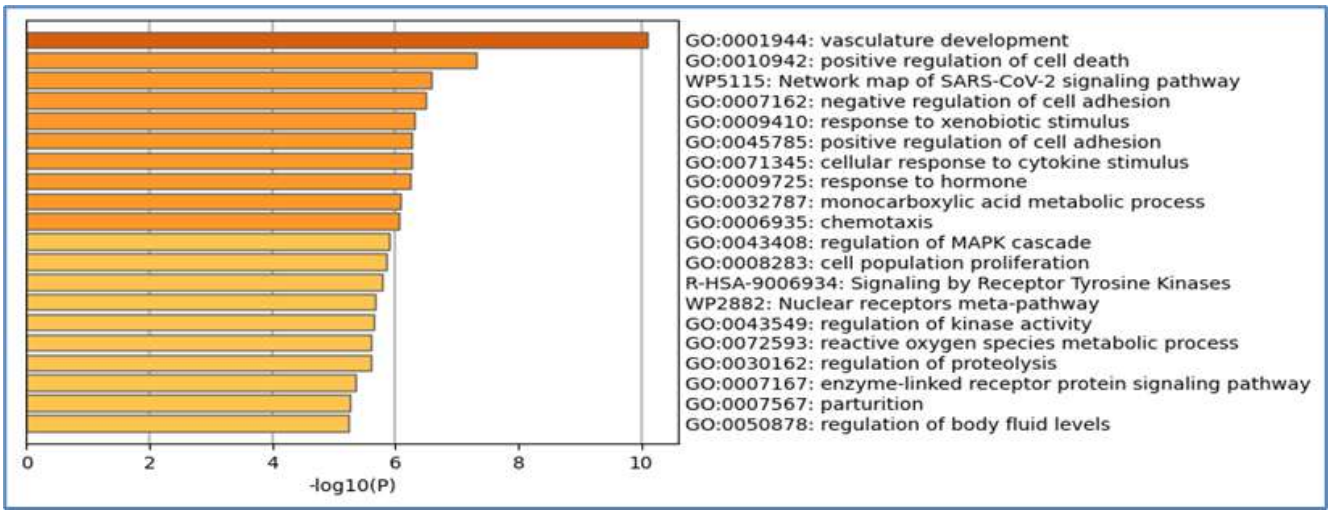

Figure-2. The bar graph from the Metascape analysis depicts the top 20 clusters, and according to the results, "vasculature development" was found to be the most significant Gene Ontology Biological Process modulated in the test group.

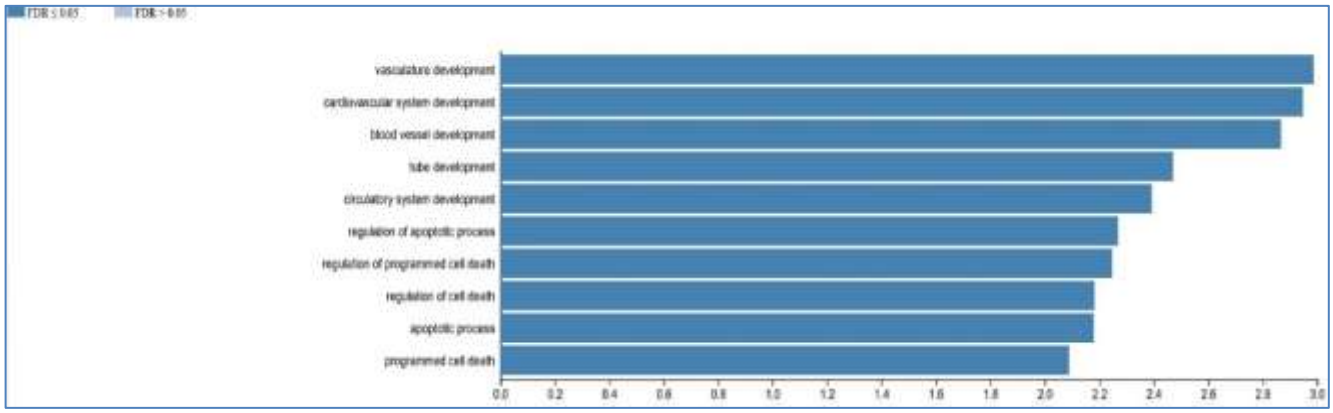

Figure-3. A bar chart showcasing the results of the over-representation analysis (ORA) of gene ontology biological processes revealed a statistically significant outcome. The enriched gene set comprised of related genes, namely SPRED1, TGFBI, and DUSP6, was derived from WebGestalt.

Table-1. The SPRED1 and TGFBI genes have been identified as part of the same gene set (GO:0001944) through the use of the WebGestalt analysis tool, and this identification has been found to have a significant value (FDR ≤ 0.05).

Analysis Tools	Gene Symbol	Gene set	FDR	Enrichment Ratio	P Value
ORA based on GO Biological Process	SPRED1 TGFBI	GO:0001944 vasculature development	0.0014876	2.9880	9.8606e-7

Table-2. The WebGestalt analysis indicated the downregulation of three genes—MAPK/ERK pathway, DUSP6, and SPRED1—with a significance value (FDR ≤ 0.05)

Analysis Tools	Gene Symbol	Gene set	Score	FDR	Enrichment Score	P Value	up/down-regulation
GSEA based on Reactome Pathway analysis	DUSP6 SPRED1	R-HSA-5683057 MAPK family signaling cascades	-6.5133	0.014036	-0.69195	<2.2e-16	Down
		R-HSA-5684996 MAPK1/MAPK3 signaling		0.017438	-0.68112	0.0020243	
		R-HSA-5673001 RAF/MAP kinase cascade	-3.8733	0.017438	-0.68112	0.0020243	

GSEA with KEGG pathway analysis revealed significant downregulation of the MAPK/ERK and *DUSP6* genes. "MAPK signaling pathway" (hsa04010) with (FDR 0.0012314), *DUSP6* with a score of -6.513 (Figure 4B). In the network kinase target analysis using GSEA, we found two significant results related to the MAPK/ERK–ERK pathway. A downregulated "mitogen-activated protein kinase 1" (Kinase_MAPK1) with (FDR 0.030747) and "mitogen-activated protein kinase 3" (Kinase_MAPK3) with (FDR 0.037335), both have *DUSP6* gene with a score of -6.5133 (Figure 4C). GSEA with GLAD4U showed two other significant results; a downregulated "protein kinase inhibitors" (PA164713204) with (FDR <2.2e-16) and "l-tyrosine" (PA451822) with (FDR 0.044717). Both had *DUSP6* with a score of -6.5133 and *SPRED1* with a score of -3.8733 (Figure 4D). The GSEA with gene ontology biological process was not redundant, and we obtained (FDR > 0.05) without any statistical significance, but we found a downregulated gene set that is related to our genes. A "negative regulation of intracellular signal transduction" (GO:1902532) with (FDR 0.057461), "negative regulation of transferase activity" (GO:0051348) with (FDR 0.22874), "negative regulation of phosphorylation" (GO:0042326) with (FDR 0.18841), "dephosphorylation" (GO:0016311) with (FDR 0.22432), all the previous gene set have *DUSP6* with a score of -6.5133 and *SPRED1* with a score of -3.8733 (Figure 4E). In addition, in the GSEA with gene ontology, the molecular function was not redundant and (FDR > 0.05) was not statistically significant, but we obtained upregulated and downregulated genes. The upregulated gene set "cofactor binding" (GO:0048037) with (FDR 0.74439) *SPTLC3* with a score of 2.805, and the downregulated gene set "cell adhesion molecule binding" (GO:0050839) with (FDR 0.94589) *TGFBI* with a score of -4.44 (Figure 4F).

Ingenuity Pathway Analysis

IPA predicted 490 canonical pathways, including the most significant p53 Signaling, FXR/RXR Activation, β-alanine Degradation I, LPS/IL-1 Mediated Inhibition of RXR function, and histamine degradation (Table 3, Figure 5, 6). Activation of the TP53 signaling pathway induces cell cycle progression and reduces apoptosis

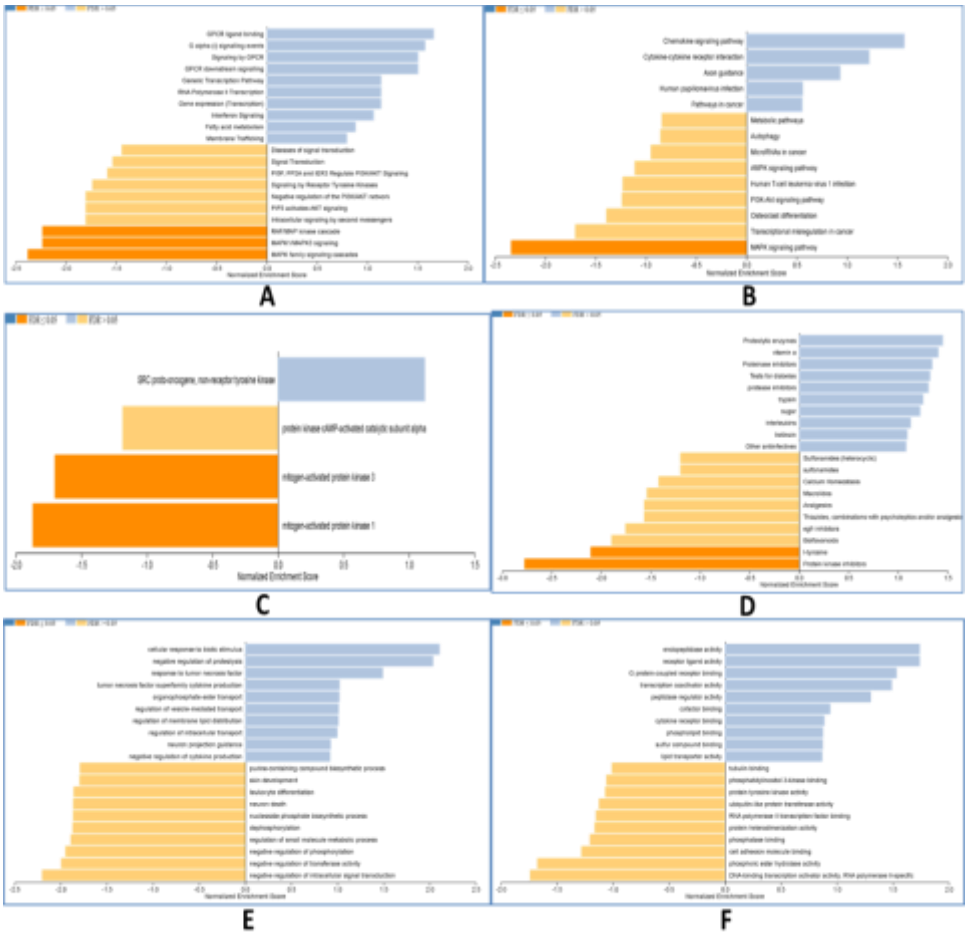

Figure-4. Different bar graphs depicting gene set enrichment analysis (GSEA) from WebGestalt are presented below. A: This graph utilizes the Reactome pathway and demonstrates a significant downregulation of the MAPK/ERK pathway, which is highlighted in orange and includes the genes DUSP6 and SPRED1. B: Another graph using the KEGG pathway also reveals a significant downregulation of the MAPK/ERK pathway, with the gene DUSP6 highlighted in orange. C: The network kinase target graph displays two significant downregulations of the MAPK/ERK pathway, highlighted in orange with the DUSP6 gene. D: The drug GLAD4U is associated with two significant downregulated enriched gene sets, colored in orange and including the related genes DUSP6 and SPRED1. E: The gene ontology biological process graph shows downregulation, highlighted in yellow for DUSP6 and SPRED1. F: Finally, the gene ontology molecular function graph shows upregulation in light blue for SPTLC3 and downregulation in yellow for TGFBI, with no redundancy present.

Table-3: Top 10 Ingenuity Canonical Pathways

Ingenuity Canonical Pathways	-log (p-value)	Molecules
p53 Signaling	3.64	KAT2B,PIK3R1,PIK3R3,PMAIP1,THBS1,TNFRSF10A,TP53I3,TP53INP1
FXR/RXR Activation	3.59	CLU,FABP6,FOXA2,IL33,NR5A2,PON1,SLCO1B3,SULT2A1,VLDLR
β-alanine Degradation I	3.56	ABAT,ALDH6A1
LPS/IL-1 Mediated Inhibition of RXR Function	3.46	ACSL6,ALDH1A1,ALDH1A3,ALDH1B1,ALDH1L2,ALDH6A1,FABP6,IL33,NR5A2,PAPSS2,SLCO1B3,SULT2A1,UST
Histamine Degradation	3.45	ALDH1A1,ALDH1A3,ALDH1B1,AOC1
Putrescine Degradation III	3.37	ALDH1A1,ALDH1A3,ALDH1B1,SAT1
Senescence Pathway	3.3	BHLHE40,BMPR2,CAPN6,CCNB2,CDKN2B,CXCL8,EIF4EBP1,ELF3,KAT2B,PDK3,PIK3R1,PIK3R3,PPP2R2C,TLR2
Role of Hypercytokinemia / hyperchemokinemia in the Pathogenesis of Influenza	3.25	AREG,CASP1,CXCL8,IFIT2,IFIT3,IL33,TLR3
CDX Gastrointestinal Cancer Signaling Pathway	3.24	CDHR5,CXCL8,FOS,FZD7,IL33,NOX1,PIK3R1,PIK3R3,TNFSF10,WNT10A,WNT6
Xenobiotic Metabolism AHR Signaling Pathway	3.22	ABCG2,ALDH1A1,ALDH1A3,ALDH1B1,ALDH1L2,ALDH6A1,PON1

We obtained results for the *CDX2* and *TP53* genes as follows: 1- Negative expression of *CDX2* in CRC via activation of *BRAF* mutations in the MAPK/ERK signaling pathway. 2- The activation of *TP53*, which induces cell cycle arrest, is triggered by ultraviolet (UV) radiation, which targets the MAPK/ERK signaling pathway

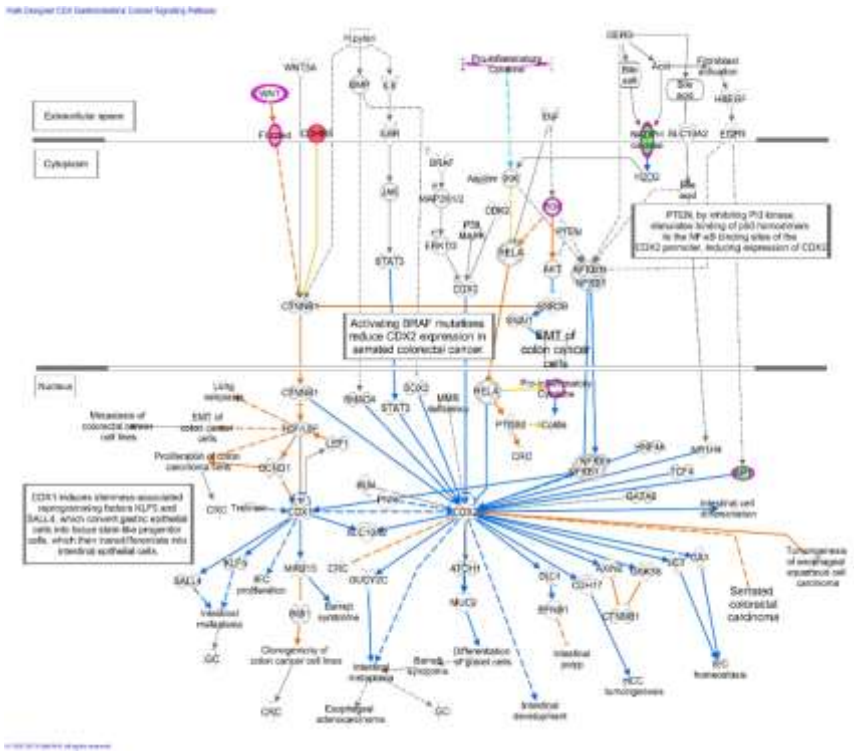

Figure-5. A network of *CDX* gastrointestinal cancer signaling pathways obtained from IPA analysis showed that the MAPK/ERK signaling pathway was associated with reduced expression of the *CDX2* gene in colorectal cancer.

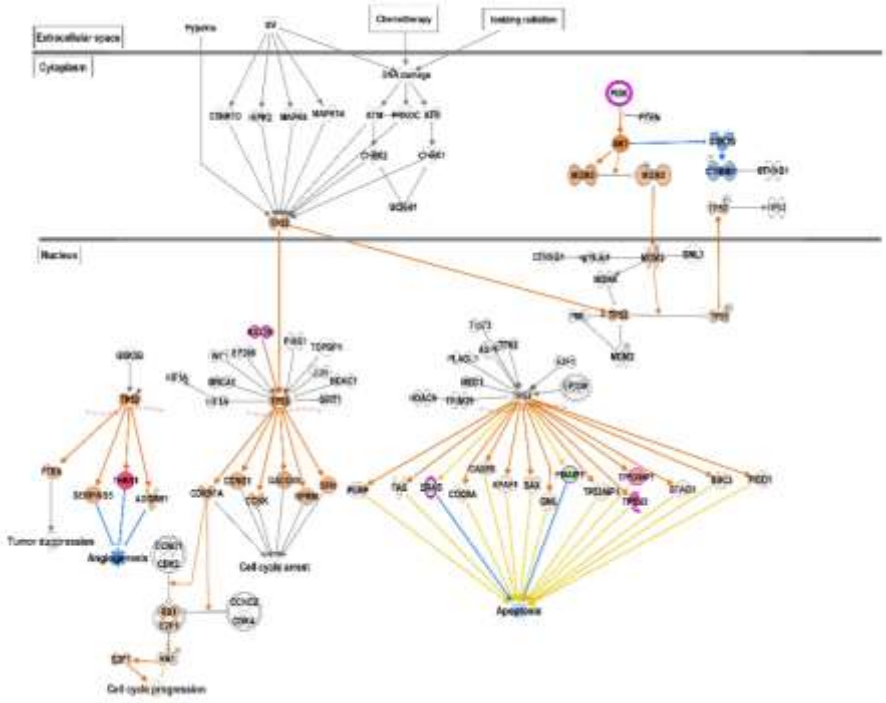

Figure-6. A network of the *TP53* pathway and activation of *TP53* via UV light targeted two members of the MAP kinase family 8 and 14 as indicated by the IPA analysis.
As shown in (Figure 6), the TP53 upregulation leading to an increase in apoptosis and reduced angiogenesis in cancer.

Discussion

From these results, we can infer the differential regulation of SAMD9, SPRED1, TGFBI, DUSP6, CDX2, and TP53 genes and the role of MEKi in its downregulation CRC. Overexpression of SAMD9 can suppress MAPK/ERK signaling and decrease the growth of colon cancer cells (Wong et al., 2018). Although the function of SAMD9/9L is not fully understood, it is believed to act as a tumor suppressor (Peng et al., 2022). SPRED1 and TGFBI are also involved in regulating the MAPK/ERK pathway, but their overexpression can lead to apoptosis (Zhang et al., 2020; Pajares et al., 2014). Conversely, the TGFBI gene is downregulated in most cancers owing to the effect of MEKi, but it is upregulated in lung cancer (Pajares et al., 2014). SPRED1 and TGFBI are known to act as tumor suppressors (Ablain et al., 2021; Yeh et al., 2022). DUSP6 is another tumor suppressor that regulates the MAPK/ERK pathway (Ma et al., 2013; Kanda et al., 2021).

We observed that the MEK inhibitor downregulated both the SPRED1 and TGFBI genes while upregulating SAMD9. Further investigation is needed, particularly for the TGFBI protein, which is associated with tumor growth when it is upregulated (Chen et al., 2021). Although some studies have explored the relationship between TSGs, a meta-analysis found that they were highly correlated with other genes (Cho, 2022). Additionally, upregulation of SPTLC3 may be associated with hepatotoxicity induced by MEKi and has been linked to nonalcoholic steatohepatitis in mouse models. If NAFLD progresses, SPTLC3 gene expression will be upregulated (Ijuin et al., 2022). BRAF and MEK inhibitors such as vemurafenib, dabrafenib (BRAFi), and trametinib (MEKi), can cause liver toxicity and deranged liver function (Welsh and Corrie, 2015).

CDX2 and TP53 are both tumor suppressors that have been extensively studied in CRC (Bode and Dong, 2003; Balbinot et al., 2018; Aubrey et al., 2016). While the downregulation of CDX2 is influenced by the activation of the MAPK/ERK pathway, MEKi treatment may upregulate CDX2 expression, as demonstrated in CRC cell lines (Krueger et al., 2009). Additionally, CDX2 can serve as a prognostic biomarker for patients with stage 1 (Dalerba et al., 2016) (Aasebø et al., 2020). The TP53 and MAPK/ERK pathways play crucial roles in the regulation of cell cycle arrest (De et al., 2020).

However, it is important to note that this study focused only on the MAPK/ERK pathway and did not investigate other pathways or TSGs associated with CRC.

Conclusion

Based on the results of this study, it can be concluded that there is a regulatory relationship between SAMD9, SPRED1, TGFBI, DUSP6, CDX2, and TP53 genes and the MAPK/ERK signaling pathway. These genes are TSGs and may affect the downregulation of the MAPK/ERK pathway or its upregulation if they are mutated, as observed in the data analysis of MEKi in the test group and DMSO in the control group. Therefore, it may play a role in reducing the number of tumors in CRC and other cancers. As research techniques improve, it may be possible to understand and control TSGs in the future to treat cancer cells.

In addition, the upregulation of SPTLC3 in the CRC Organoids may have been induced by trametinib treatment linked to hepatotoxicity, as shown in the bar graph with a high level of expression in the test group. SPTLC3 upregulation is associated with NASH and hepatotoxicity and can be a predictive biomarker for liver cancer. These findings may aid in the understanding of TSGs and their relationship to CRC, the MAPK/ERK pathway, and cancer treatment.

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Contribution of Authors

Rasool M & Pushparaj PN: Planned the study, secured funds, edited the manuscript, and approved the final manuscript.

Alhassan KI, Karim S & Haque A: Performed the experiments and wrote the first draft.

Mutwakil MHZ, Alharthi M & Chaudhary AG: Helped in statistical analysis, read and approved the final manuscript, supervised study.

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