Bioinformatics Analysis Reveals miR-98-5p as a potential Inhibitor of Tumor cell proliferation and metastasis in Colorectal Cancer by targeting the FZD3 receptor of the Wnt signaling pathway

Submitted by:

Mutebi John Kenneth

Student ID: 20376006

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the Master of Science in Biotechnology

Master of Science in Biotechnology

Department of Mathematics and Natural Sciences

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Dedicated to my late grandfather, my roommate and my best friend for their motivational sentiments during my MS Biotechnology studies

Declaration of Authenticity

I hereby declare that the research work embodying the results reported in this thesis entitled **"Bioinformatics Analysis Reveals miR-98-5p as a potential Inhibitor of Tumor cell proliferation and metastasis in Colorectal Cancer by targeting the FZD3 receptor of the Wnt signaling pathway"** has been written and submitted by me, Mutebi John Kenneth under the supervision of Dr. Fahim Kabir Monjurul Haque, Assistant Professor, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

I further declare that this thesis presented here has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma except where stated otherwise by reference or acknowledgement.

Student's Full Name & Signature:

_____

Mutebi John Kenneth

ID: 20376006

Approval

This thesis titled — "Bioinformatics Analysis Reveals miR-98-5p as a potential Inhibitor of Tumor cell proliferation and metastasis in Colorectal Cancer by targeting the FZD3 receptor of the Wnt signaling pathway" submitted by Mutebi John Kenneth (ID: 20376006) of Fall, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 13th January, 2022.

Examining Committee:

Supervisor: _____

(Member) **Dr. Fahim Kabir Monjurul Haque**

Assistant Professor, Biotechnology Program

Department of MNS

BRAC University

Departmental Head: _____

(Chairperson) **A F M Yusuf Haider, PhD**

Professor and Chairperson, Department of MNS

BRAC University

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Firstly, I am thankful to the Omnipotent God for my good health and wellbeing especially during this **COVID-19** pandemic that were necessary for me to sail through my entire Masters studies in this pandemic era, where I had to move from one country with less morbidity to another country with a high morbidity, all in good and sound health.

I am grateful to my family for always having faith in me, loving and supporting me in every aspect of my life and above all is their daily prayers for me to stay safe from **COVID-19**. They have been a constant source of inspiration.

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Mutebi John Kenneth

List of Abbreviations

The following abbreviations have been used throughout the text

CRC	Colorectal cancer
D.A.V.I.D	Database for Annotation, Visualization and Integrated Discovery
FDA	Food and Drug Administration
FZD	Frizzled Receptors
FZD3	Frizzled Class Receptor 3
DEGs	Differentially Expressed Genes
GEO	Gene Expression Omnibus
GEDS	Gene Expression Display Server
HDI	Human Development Index
miRNA	MicroRNA
KEGG	Kyoto Encyclopedia of Genes and Genomes
UTR	Untranslated Region
PPI	Protein-Protein interaction
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
Wnt	Wingless-related integration site
TCGA-COAD	The Cancer Genome Atlas – Colon adenocarcinoma
TCGA –READ	The cancer Genome Atlas – Rectal adenocarcinoma

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Abstract

According to the World Health Organization (WHO) report of 2020, Colorectal Cancer (CRC) is the third most common type of cancer and the second cause of cancer-related deaths in the world. However, the existing treatment, as well as prognosis strategies, need to be improved to increase the survival of CRC patients. Targeted therapies of CRC as opposed to ordinary therapies; target key biological features and pathways of cancerous cells hence minimizing the subsequent damage to normal cells. MicroRNAs have been reported to play a crucial role in inhibiting and/or suppressing major pathways in various cancer types by targeting transcripts of key genes in such pathways. This study aimed at *in silico* inhibiting cancer cell proliferation and metastasis by targeting a key gene – Frizzled receptor 3 (FZD3) in the Wnt signaling pathway, one of the major pathways in CRC; using microRNAs. The *in silico* analysis revealed that miR-98-5p is a direct target of FZD3, using 5 microarray datasets containing tumorous and control samples. Further analysis indicated that miR-98-5p inhibits the expression of this receptor by directly binding to the 3'UTR of its mRNA hence exerting a tumor-suppressor role in CRC through the Wnt signaling pathway. However, these results need to be validated in the future through basic research experiments using CRC cells *in vivo* and *in vitro*. The study reveals miR-98-5p as a novel target of FZD3 and an inhibitor of the Wnt signaling pathway hence being a potential candidate for developing targeted therapies against CRC.

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Chapter – 1: Introduction

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INTRODUCTION

Cancer is a major public health concern worldwide, accounting for nearly 10 million deaths in 2020 according to the World Health Organization (WHO) and it is believed to be the second leading cause of death in various countries such as the United States (Siegel et al., 2021). Colorectal Cancer (CRC) is the third most diagnosed type of cancer among men and women in the United States and according to the American Cancer Society's estimations, new colorectal cancer cases in the United States are expected to rise up to 104,270 and 45,230 new cases in colon cancer and rectal cancer respectively (American Cancer Society, n.d). According to the data of 2020 by WHO, it is indicated that colon and rectal cancer counted for 935,000 deaths, making CRC the second deadliest cancer type in 2020 ahead of liver, stomach, and breast cancers.

Colorectal cancer remains among the top three common malignancies in the world, and its risk factors are associated with one's lifestyle such as smoking and alcohol abuse, dietary behavior such as processed meat foods like hot dogs and red meat, old age, type 2 diabetes and obesity, genetics and environmental factors.

The present treatment strategies for CRC include surgical resection, chemotherapy, immunotherapy, or a combination of any two of these. However, the effectiveness may vary from patient to patient especially those with locally invasive or metastatic CRC. If we're to improve the prognosis and treatment of CRC patients, it is very essential to understand the mechanisms that drive the progression of this disease in order to identify biomarkers that can be potential therapeutic targets.

Targeted therapeutic agents of CRC can help to improve treatment by targeting unique biological features and pathways that are responsible for tumor progression (Piawah & Venook, 2019a). Such therapeutic agents are advantageous in cancer treatment in that, they target biological features of cancerous cells as opposed to other therapeutics that kill both tumorous and normal cells. Targeted therapy can be administered to turn off dominant pathways and processes in cancerous cells such as angiogenesis, proliferation, apoptosis inhibition, cell differentiation, RAS pathway, the Wnt signaling pathway, PI3K pathway, cell cycle pathway, among others (Sanchez-Vega et al., 2018).

A number of studies have proved that aberrant activation of the Wnt signaling pathway is a major culprit in the tumorigenesis of most human cancers, with a strong emphasis on CRC (Schatoff et al., 2017). Wnt signaling is an exceedingly conserved pathway that serves a crucial purpose in tissue homeostasis and developmental processes (L et al., 2015). This pathway controls a key modulator in signal transduction called β-catenin, through phosphorylation and ubiquitin-mediated degradation processes. This regulation involves key scaffold proteins such as AXIN and Dishevelled (DVL) which disrupt the beta-catenin destruction complex that is composed of core proteins adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 beta (GSK3β) (L et al., 2015).

In absence of the β-catenin destruction complex, β-catenin is no longer ubiquitinated or degraded, leading to the accumulation of free β-catenin in the cytoplasm (Piawah & Venook,

2019b) which is a hallmark of CRC progression (Cheng et al., 2019) and then its translocation into the nucleus (Schatoff et al., 2017). In the nucleus, β-catenin associates with T cell factor (TCF) and lymphoid enhancer-binding factor (LEF) transcription factors and displaces their repressor molecule Groucho. The formed β-catenin/TCF/LEF complex together with other co-activators form an active transcriptional complex that leads to the expression of Wnt target genes (Nie et al., 2020), which include MYC, CCND1, AXIN2, Cyclin D1, among others (Cheng et al., 2019). These target genes are mostly oncogenes which when aberrantly overexpressed, promote anti-apoptosis abilities, proliferation, survival, migration, polarity, all of which are very crucial for tumorigenesis, progression, and metastasis in benign cells (X. Li et al., 2020).

Wnt signaling pathway is aberrantly up-regulated in both classes of CRC i.e., non-hyper-mutated microsatellite stable (MSS) and hyper-mutated microsatellite instability (MSI) CRCs (L et al., 2015). In MSS CRCs, aberrant activation of the Wnt pathway is facilitated by inactivating mutations in APC, which is a negative regulator of this pathway. Whereas in MSI cancers, the DNA mismatch repair (MMR) defects result in high mutation rates that see-off frequent mutations in multiple oncogenes and suppressor genes in the Wnt pathway as well as Wnt pathway inhibitors by DNA hyper-methylation (Nie et al., 2020). These mutations mainly lead to the loss-of-function of APC hence up-regulating the Wnt signaling pathway and facilitating CRC cell proliferation and enhanced anti-apoptosis abilities through overexpression of the target genes of this pathway (X. Li et al., 2020).

Wnt signaling pathways are classified as either canonical (β-catenin dependent) or non-canonical (β-catenin independent); however the initiation of signaling events in both pathways requires a Wnt molecule to bind to a frizzled receptor and/or other co-receptors such as low-density lipoprotein receptor-related protein 5/6 (LRP5/6)/ROR2/RYK for signal transduction initiation (X. Li et al., 2020). Frizzled (FZD) is a family of trans-membrane ligand-activated receptors that serve as receptors of the Wnt pathway. This family of receptor proteins consists of 10 members, with each FZD member having a preferred Wnt ligand. Studies have indicated that loss-of-function mutations of E3 ubiquitin ligases ring-finger protein 43 (RNF43), which lead to excessive activation of the Wnt signaling pathway through ubiquitin-mediated degradation blockage of FZD receptors and LRP5/6 co-receptors are frequently detected in CRC (Cheng et al., 2019). Therefore signal transduction by the Wnt pathway may be disrupted or highly activated when the expression levels of crucial components of the pathway change (Schatoff et al., 2017).

A number of cancer-promoting functions including invasion, angiogenesis, cancer cell proliferation, migration, chemo-resistance upon recurrence are all mediated by FZD receptors (Zeng et al., 2018). Since it is the most implicated pathway in CRC, disrupting the Wnt pathway signal transduction through down-regulating the expression of crucial pathway components such as FZD receptors can be a therapeutic strategy for CRC. Studies have indicated that targeting FZD receptors can down-regulate Wnt signaling hence suppressing malignant cell proliferation, tumor growth, angiogenesis, and metastasis (Ji et al., 2022; C. Li et al., 2019).

Human Frizzled Homolog 3 protein (FZD3) was mapped to chromosome 8p21 (Sala et al., 2000) and its mRNA is expressed in a number of normal tissues, including skeletal muscles, pancreas, kidney, cerebellum, stomach, among others. Studies have shown that FZD3 is up-regulated in various cancers such as lung squamous cell carcinoma tissues, myeloma,

lymphoma, Ewing sarcoma, among others (He et al., 2011; Smith et al., 2021; Wong et al., 2013). A study by Wong et al, 2013 indicated that FZD3 was 100 % expressed in CRC spacemen, 89% in colorectal adenomas, and 75% in colorectal polyp spacemen (Wong et al., 2013). This indicated that FZD3 is so significant in CRC tumorigenesis and progression hence making it a potential candidate for chemo-preventive interventions (Sompel et al., 2021).

Recent studies have indicated that microRNAs (miRNAs) are potential suppressors of growth, proliferation, and metastasis in CRC cells by targeting FZD receptors and other oncogenes. (Smith et al., 2021; Ueno et al., 2013c; Q. Wang et al., 2017; Zeng et al., 2018).

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs that play an important role in negatively regulating gene expression by binding to the 3' untranslated region (3' UTR) of the target gene mRNA and then inducing its cleavage or repressing its translation thereby inhibiting the target protein (Ueno et al., 2013a).

MicroRNAs interact with FZD mRNAs hence influencing the expression of FZD proteins and the Wnt signaling pathway as a result (Smith et al., 2021). Identifying miRNAs that inhibit the expression of FZD genes in different cancers has been reported as a therapeutic strategy for human cancer (Zeng et al., 2018). Although other FZDs have been extensively studied, the FZD3 receptor is poorly studied among FZD family receptors in human cancers, especially CRC. The present study aims to identify a suitable miRNA target for FZD3 receptor mRNA and demonstrate through bioinformatics analysis that hsa-miR-98-5p is a suitable target for FZD3 in human CRC. This would allow the clinical evaluation of the potential of miR-98-5p in inhibiting CRC progression and its consideration as a therapeutic strategy in CRC treatment.

Chapter – 2: Methods and Materials

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METHODS AND MATERIALS

2.1 Gene Expression Datasets

Microarray datasets of five projects with gene expression profiles of colon and rectum normal and adenocarcinoma samples were downloaded from the public functional genomics data repository;- Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo), against query words such as CRC and colorectal cancer, on 27th September 2021. The selection criteria were based on the fact that samples were of *Homo sapiens* origin, excluding cell-line-based experiments. All the selected datasets contained enough samples (50 or more samples) to obtain statistically significant results.

Table 1 Characteristics of the datasets

Dataset	Total Samples	Selected Samples	Platform	Reference
GSE25071	50	Normal = 4 Carcinoma = 46	GPL2986	(Danielsen et al., 2011)
GSE62321	57	Normal = 18 Carcinoma = 30	GPL97 [HG-U133B]	(Del Rio et al., 2007)
GSE8671	64	Normal = 32 Carcinoma = 32	GPL570 [HG-U133_Plus_2]	(Sabates-Bellver et al., 2007)
GSE41657	88	Normal = 12 Tumor = 35	GPL6480	Zhang et al (2012)
GSE39582	585	Normal = 19 Carcinoma = 566	GPL570 [HG-U133_Plus_2]	(Marisa et al., 2013)

2.2 Identification of Differentially Expressed Genes (DEGs)

Normalization and identification of differentially expressed genes (DEGs) from each dataset were done by using both the limma R package (Ritchie et al., 2015) as well as the GEO2R tool. GEO2R is an interactive online tool for differential gene expression analysis in a GEO series, (GEO2R; https://www.ncbi.nlm.nih.gov/geo/geo2r/). The cut-off conditions were set to log fold change (log FC) > 1/ Log FC < -1 and P-value of < 0.05 as the threshold for obtaining DEGs in all the 5 datasets. To visualize the DEGs, volcano plots were plotted for DEGs from each dataset using *bioinfokit v2.0.1* tool in python (Bedre, 2020).

2.3 GO and KEGG Functional Enrichment Analysis

After obtaining DEGs, they were arranged in descending order with respect to the magnitude of their Log FC value. The top 20 DEGs from each dataset were obtained for enrichment analysis in order to understand the in-depth biological significance of these genes. A list of top 20 DEGs from each dataset was annotated using an open-source web server, Enrichr (https://maayanlab.cloud/Enrichr/) (Chen et al., 2013). This web-based gene enrichment analysis tool integrates results from multiple libraries of gene enrichment analysis. For example, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways option was used to obtain pathways against each gene list, with statistically significant pathways having a p-value < 0.05. Pathway enrichment for the top 20 DEGs was also done by using an online tool Database for Annotation, Visualization, and Integrated Discovery (D.A.V.I.D; https://david.ncifcrf.gov/). The threshold P-value of < 0.05 was set for statistically significant DEG pathways.

2.4 Protein-Protein Interaction Network Analysis

The protein-protein interactions (PPI) network of the DEGs was constructed by using the Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/) tool, taking the highest confidence score to be 0.900. The constructed networks were visualized in Cytoscape version 3.6.1 (http://www.cytoscape.org/).

2.5 Pathways in Cancer Analysis

The enriched pathways were further analyzed for their involvement as well as their respective genes in CRC by using KEGG (https://www.kegg.jp/), an online curated resource that integrates eighteen databases which are categorized into systems, genomic, chemical and health information (Kanehisa et al., 2021). Pathways in cancer were queried, with specific emphasis on highly enriched pathways among the DEGs of the five datasets.

2.6 Visualization of gene expression between Tumor and Normal Tissues

After choosing Frizzled receptors (FZD) as potential targets, gene expression analysis was performed to ascertain the expression levels of each of the 10 family member receptors in both tumor and normal tissues. This was done by using a freely available online tool Gene Expression Display Server (GEDS; http://bioinfo.life.hust.edu.cn/web/GEDS/), which is a comprehensive resource for searching, visualizing, and analyzing expression data of miRNAs, proteins, and genes (Xia et al., 2019). DEGs from each dataset were analyzed to identify all Frizzled receptors and their expression levels in both TCGA and Microarray datasets. A frizzled receptor whose expression levels were Log FC > 1 and P-value < 0.05 was selected as a potential target Frizzled receptor gene. The expression levels of potential target FZD receptors were visualized on Volcano plots in comparison with the top 5 DEGs from each dataset, using *bioinfokit v2.0.1* tool in python (Bedre, 2020).

2.7 Validation of gene expression between CRC and Normal colorectal tissues

To validate and improve the reliability of the results of this study, FZD gene expression data from the TCGA database were analyzed. The Cancer Genome Atlas (TCGA; https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) is a coordinated and comprehensive effort to improve our understanding of the molecular basis of cancer through the application of genome analysis technologies, including large-scale genome sequencing. TCGA is now a collection of more than 20 characterized tumor types. Searching, downloading, and preparing data for validation were all performed using a Bioconductor R package *TCGAbiolinks* (A et al., 2016). To identify DEGs, a Bioconductor R package edgeR was utilized (Robinson et al., 2010). DEGs were filtered with cut-off points being Log2 FC < -1 or Log2FC > 1 and P-value < 0.05 to indicate statistical significance.

2.8 Prediction and Enrichment of the target microRNAs

To predict the target miRNAs for the identified Frizzled receptor, an online tool DIANA-microT-CDS (http://www.microrna.gr/webServer) was used. This web server has been widely used among the scientific community since its initial launch in 2009 and is dedicated to predicting miRNA targets as well as their functional analysis (Paraskevopoulou et al., 2013). From the web server, all possible targets for the identified Frizzled receptor were identified and downloaded, with a threshold of 0.7.

To identify the potential target of the identified Frizzled receptor, miRNA-target enrichment analysis of the possible targets was performed by MicroRNA ENrichment TURned NETwork (MIENTURNET; http://userver.bio.uniroma1.it/apps/mienturnet/), an interactive web tool for microRNA-target enrichment and network-based analysis (Licursi et al., 2019).

Chapter – 3: Results

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RESULTS

Microarray datasets were separately analyzed to obtain DEGs between normal and tumor tissue cells. Volcano plot diagrams were plotted to visualize the expression levels of different genes between the two conditions. A total of 14227 DEGs were obtained from 5 datasets (Figure 2**).** The general trend indicated that differential gene expression was more of down-regulated genes than up-regulated ones. A list of overexpressed genes was generated and out of which the top 20 genes from each dataset were selected for further analysis.

Table 2: Number of DEGs from each dataset in this study

Dataset	No. of Up-regulated Genes	No. of Down-regulated Genes
GSE62321	427	999
GSE41657	1706	2150
GSE39582	1284	1204
GSE25071	1749	1323
GSE8671	1012	2419

Table 3: Top 200 DEGs detected from the 5 datasets of this study

GEGs	Genes Symbols
Up-regulated	TCN1,CDH3,CEMIP,MMP7,DEFA6,KLK10,C2CD4A,SLCO1B3,DPEP1,FOXQ1,MMP3,TACSTD2,CLDN2,S100A2,MSX2,ASCL2,CHI3L1,CRNDE,SERPINB5,FABP6,REG1A,SPP1,INHBA,UBD,KRT23,S100A8,CXCL8,SFRP4,S100A9,CXCL10,LY6G6D,SAA1,LY6E,CLDN1,COL11A1,CTHRC1,MMP1,CST1,COL1A1,AJUBA,CEL,COL12A1,KRT80,PPP6R1,IRX5,CABP7,LRRC2,RPA4,CD86,ATXN3L,RBM22,CA9,CGB1,FAM193B,ZFAS1,PABPC1L,CDCA5,ADAMTS2,BLACAT1,PSAT1,SLC6A6,EPHX4,SFTA2,DACH1,FAM3B,GOLT1A,ECT2,TNS4,STC2,LINC01120,DMD,COL1A2,REG3A,TOP1MT,ACSL6,IER5L,NKD1,MIR34A,AXIN2,ADAMTS12,TMPRSS3,ATP11A,ADAM12,C12orf66,SLC39A10,APCDD1,NFE2L3,WDR33,SLCO4A1,TNFRSF6B,DEUP1,LOC105376351,MSX1,LRP8,ESM1,GRAMD1C,CCDC150,KLK8,CXCL2,CXCL3
Down-regulated	DCD,TNS1,IFIT2,TCF4,CELF4,GTSF1,GNA11,HNAK,DST,MIXL1,KIAA0513,MAGEB6,GRIK1AS2,TEF,PLXNA2,MADCAM1,TNFRSF13C,RNU6501P,NPIPA5,MICALCL,RSRP1,PAN2,PDPR,DICER1,CYCS,ZNF773,KLK15,PTPRN,COLEC12,C4orf33,ARHGAP27,NAALADL2,TMC4,CHKB,AVPI1, HKDC1,FTCD,IGF1,SLC36A1,RTN4,ZDHHC21,ABCG1,MFSD9,SLC20A1,HMGN2P46,CP,SGMS2,PPARD,PPP1CB,MPP5,BDKRB1,SIPA1L2,PER3,STON2,NOVA1,GHITM,RNASE4,BICDL2,CEACAM1,ADGRL3,BMP6,BORCS6,SELT,NDUFB5,COL13A1,SMAD2,NSG1,YPEL2,ATPAF1,TLR6,HLA- DMA,RSU1,GLIS3,FCGR2A,SPRYD7,ID2,PIGK,ANKRD13A,GOLM1,ATG10,NMNAT1,TOMM40L,NT5DC3,LINC00473,PHLDB2,ELAVL4,EGLN1,CDC42SE2,SNORD11621,SLC16A7,PRICKLE2,PYGO1,STAM2,TMEM178B,CAMK2N1,DNALI1,CCDC159,GLOD5,LARP1B,LPP

3.1 Wnt Signaling Pathway is highly enriched in CRC patients

The enrichment analysis of the top 20 DEGs from each dataset was performed using Enrichr in which it was determined that these genes were involved in 16 different pathways, taking a P-value of < 0.05 as the cut-off point for statistically significant pathways. Although a number of cancer-related pathways such as PI3k-Akt signaling pathway, HIF-1 signaling pathway, and others were all enriched among the top DEGs in these datasets, Wnt signaling pathways were significantly enriched compared to other pathways, with genes such as SFRP4, MMP7, among others being involved.

The PPI which was performed by using STRING indicated the network of top 20 DEGs from each dataset in this study when combined together. When visualized in Cytoscape, the network indicated that genes involved in the Wnt signaling pathway were among those with the highest number of interactions i.e. MMP7 with 7 interactions and only second to MMP1 and MMP3 (9 interactions) which are involved in the IL-17 signaling pathway.

Figure 1: Results of the PPI network analysis of the top up-regulated DEGs

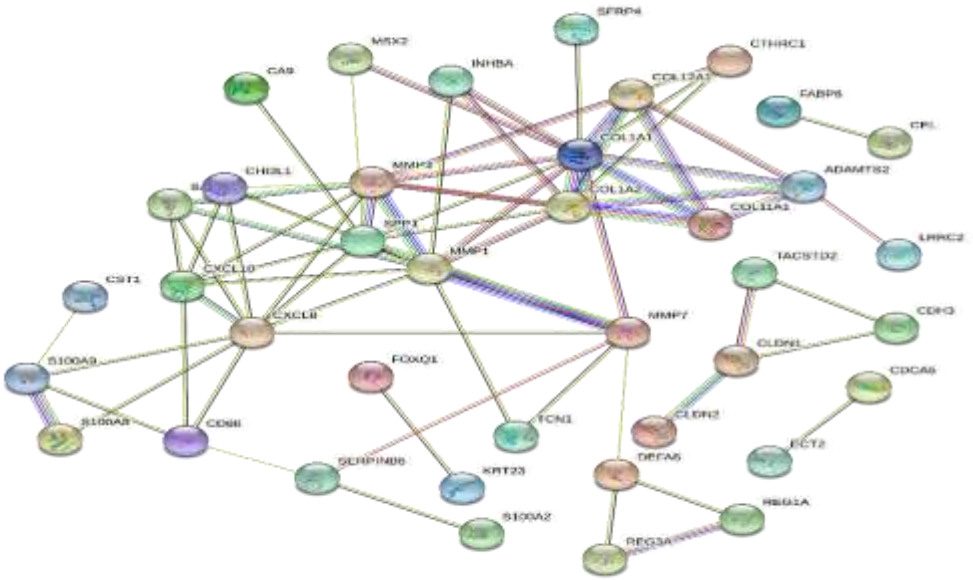
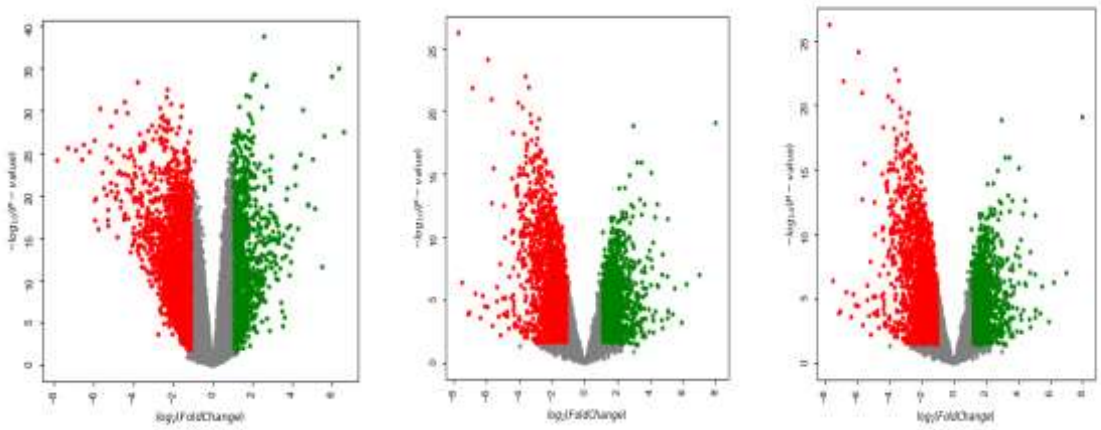
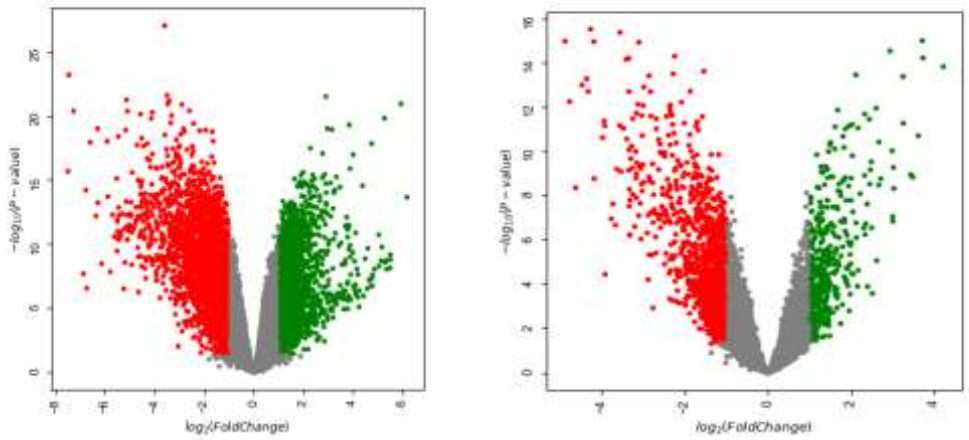


Figure 2: Volcano plots depicting differential expression and distribution in 5 datasets

GSE8671 **GSE25071** **GSE39582**



GSE41657 **GSE62321**



3.2 Wnt signaling is a Key pathway in Colorectal Carcinogenesis

Wnt signaling pathway was enriched in the top 20 DEGs of all five datasets which indicated that the pathway could have a key role in colorectal cancer. Based on these results, it was hypothesized that inhibiting the pathway can suppress CRC pathogenesis. To test the hypothesis, Wnt signaling pathway enrichment analysis in CRC was further analyzed in KEGG Pathways in the Cancer database. The analysis indicated that (Figure 2) the pathway is a gateway to a number of key genes and pathways in CRC such as (Table 3); some of which were identified among DEGs as overexpressed in all the 5 datasets. This pathway proceeds when the Wnt ligand binds to receptors of either Frizzled family or ROR1/ROR2 and RYK family, stimulating the downstream signaling cascade in a canonical or non-canonical pathway hence leading to the transcription of Wnt target genes (C. Li et al., 2019), (Figure 3). For this fact, therefore, it was hypothesized that inhibiting the expression of FZD receptors could suppress the binding of Wnt ligands hence inhibiting this highly implicated pathway in CRC.

Figure 3: The Wnt Signaling pathway (KEGG pathway database)

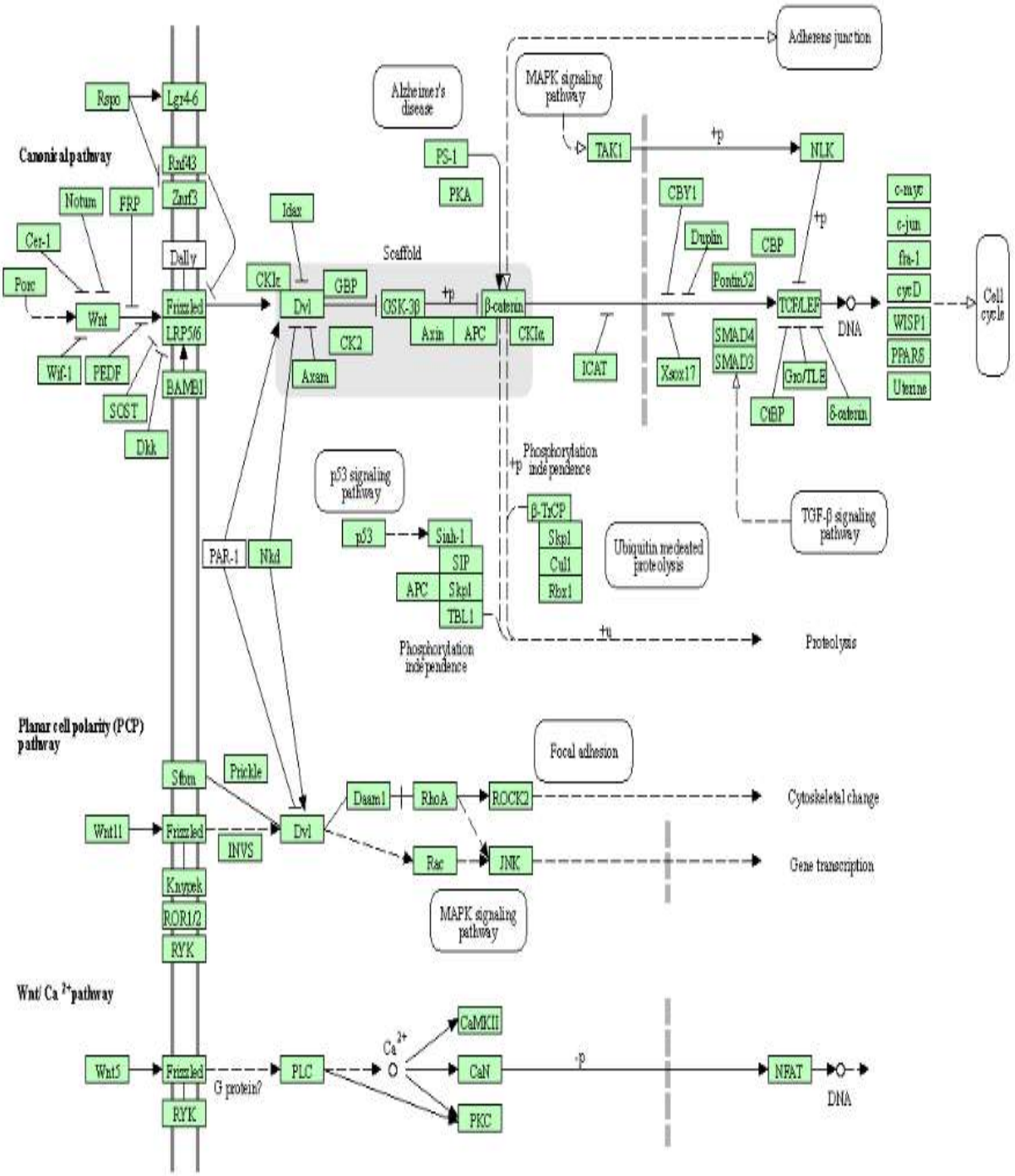
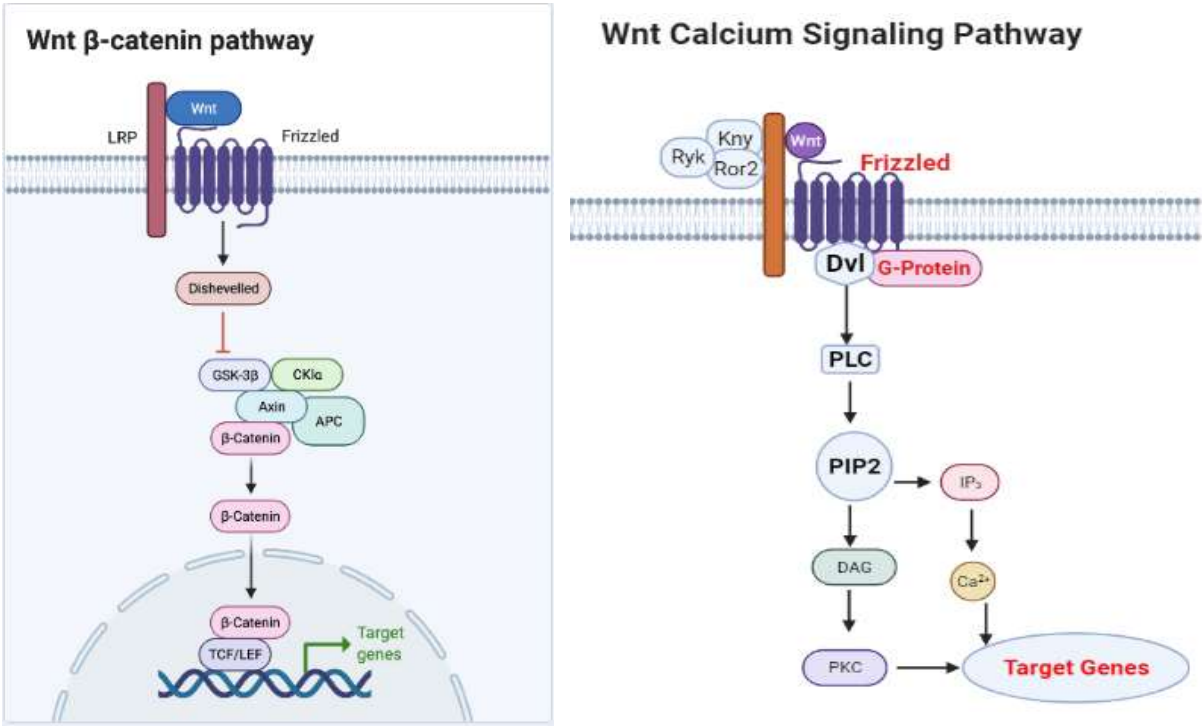


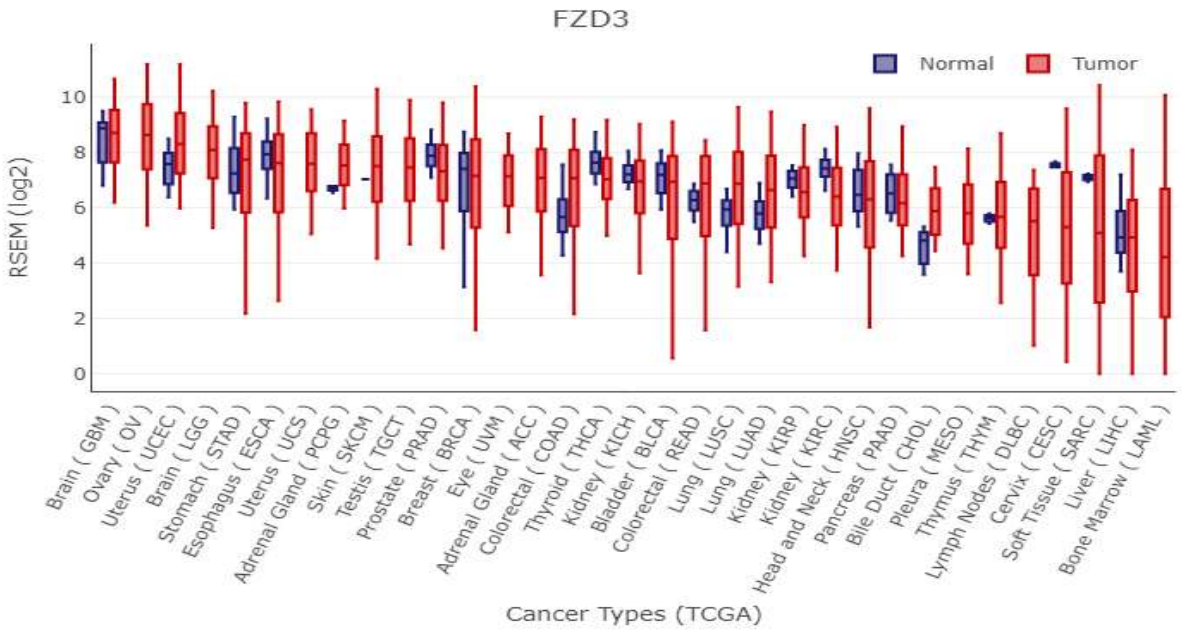
Figure 4: The Wnt Signaling pathway (Designed in BioRender)



3.3 FZD3 is a potential Candidate to inhibit the Wnt signaling pathway

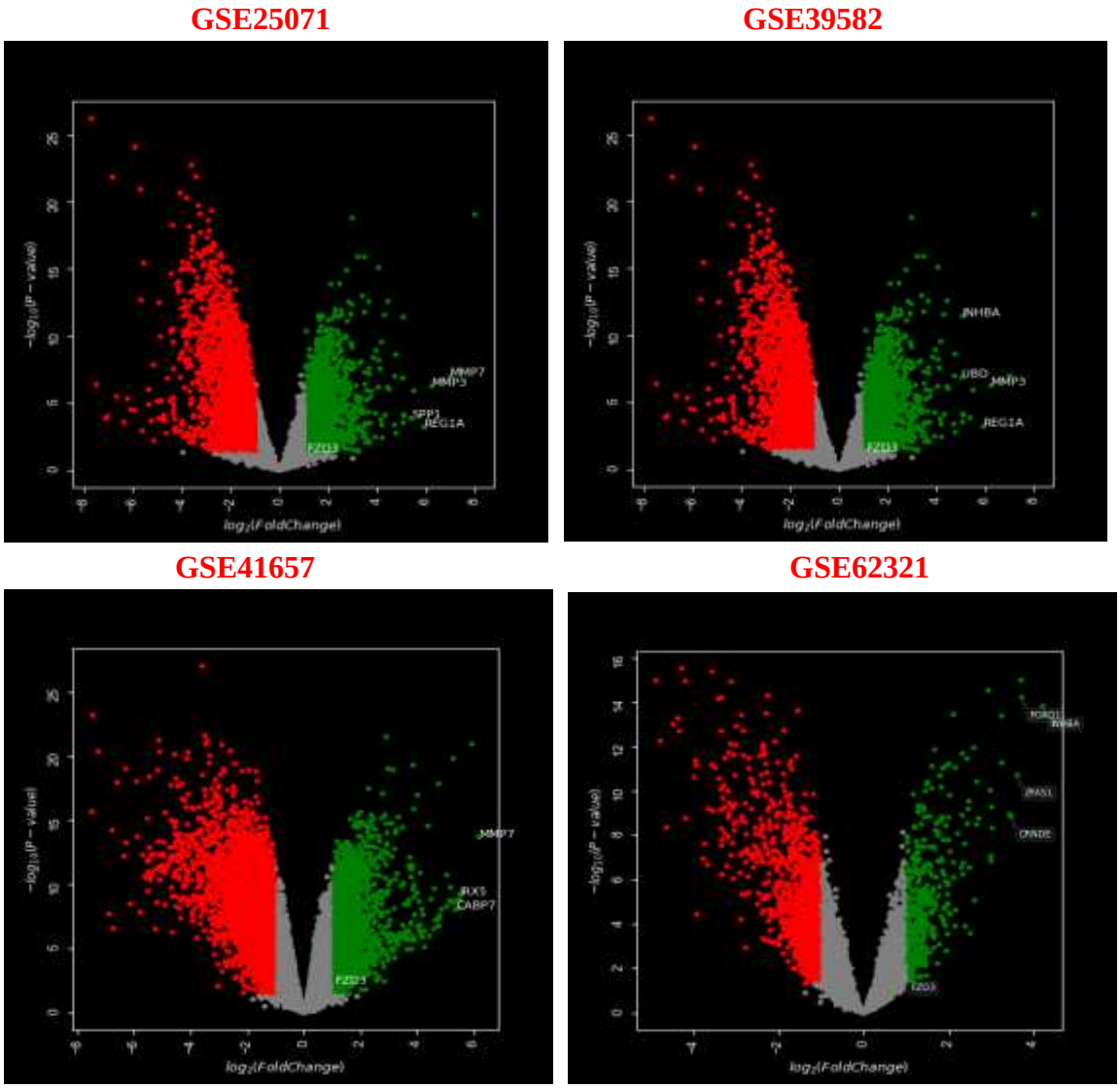
To test the hypothesis of inhibiting FZD receptors, the expression levels of each of the 10 FZD receptor family members were compared between normal and cancerous colorectal tissues, by using the Gene Expression Display Server (GEDS) (Figure 5).

Figure 5: Expression Levels of FZD3 receptor in different cancer types



The expression trend indicated high expression levels of FZD receptors in tumors as compared to normal tissues, with some receptors having a higher expression level in CRC than others among which is FZD3. The expression levels of all FZD family receptors were further analyzed in the 5 datasets. The analysis indicated that the FZD3 receptor was up-regulated across all 5 datasets, unlike other receptors. To validate this expression, TCGA analysis was performed on TCGA-COAD and TCGA-READ datasets using Bioconductor R packages *TCGAbiolinks* and edgeR for Colon Adenocarcinoma and Rectal Adenocarcinoma respectively, between normal and tumor tissues. Using the selection threshold for DEGs from GEO data, a total of 2096 genes in the TCGA-COAD dataset and 2885 genes in the TCGA-READ dataset were identified as differentially expressed between normal and tumor tissues. It was also found that FZD3 was up-regulated in tumor tissues compared to normal tissues, with a Log FC that correlates with that from the GEO datasets. The results indicated that FZD3 is up-regulated in CRC. Down-regulating this receptor, the Wnt signaling pathway can be inhibited and as a result, tumor proliferation and progression can be suppressed in human CRC.

Figure 6: Volcano plots depicting differential expression and distribution of FZD3 in 5 datasets



GSE8671

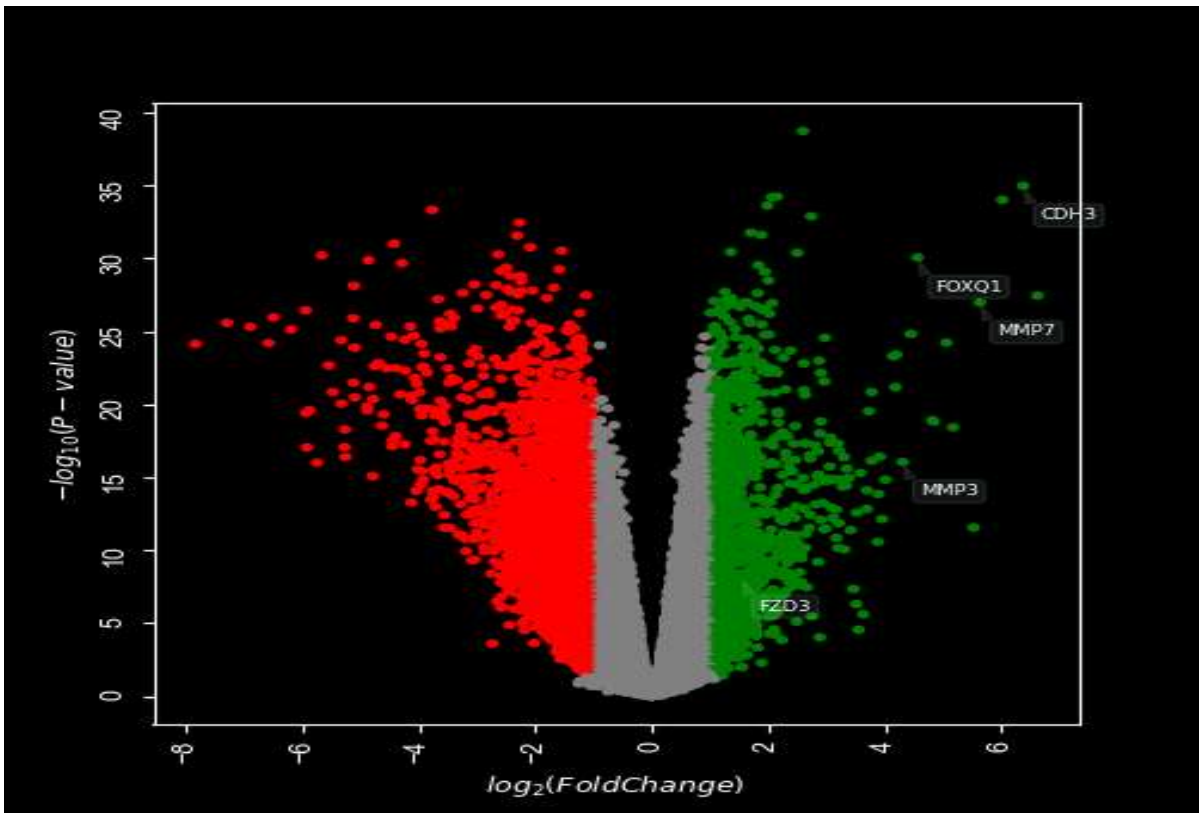
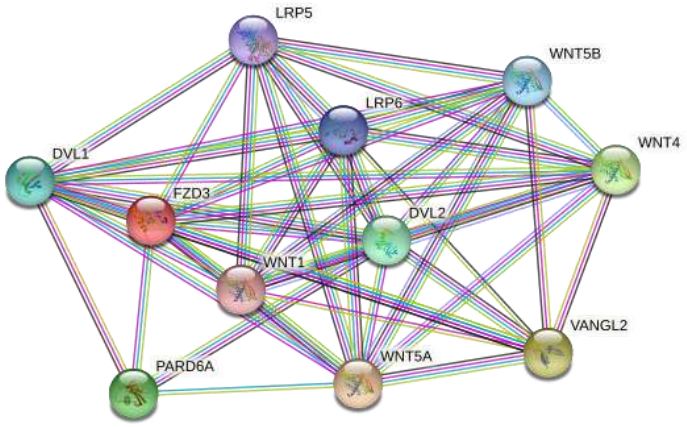


Figure 7: Results of the PPI network analysis of FZD3 receptor gene



3.4 MiR-98-5p is a suitable miRNA target for FZD3 receptor

A number of studies have identified and reported various microRNAs (miRNAs) as potential targets for Frizzled receptors in CRC as well as other cancer types, hence inhibiting the Wnt signaling pathway. This is done when miRNAs post-transcriptionally inhibit target FZD mRNAs expression by binding to their 3' untranslated regions (3'UTRs) (Kim et al., 2015; Moazzendizaji et al., n.d.; Tian et al., 2020; Ueno et al., 2013a). Having identified FZD3 as a

candidate receptor to inhibit Wnt signaling in addition to recent studies (C. Li et al., 2019), further investigations were made to identify potential miRNAs that targeted the 3'UTR of FZD3 mRNA and inhibit its expression using computer-based algorithms such as TargetScan (release 7.1), miRBase Targets, DIANA-microT-CDS as well as MIENTURNET web tools (Agarwal et al., 2015). The search for potential targets of FZD3 using DIANA-microT-CDS identified 606 *Homo sapiens* microRNAs (hsa-miRNAs) with a threshold of 0.7. Further analysis of the identified potential targets with the miRTarBase option of the MIENTURNET web tool indicated five miRNAs as candidate targets for the FZD3 receptor. These include hsa-miR-7856-5p, hsa-miR-3658, hsa-miR-31-5p, hsa-miR-98-5p, and hsa-miR-3653-3p. Functional enrichment analysis in the MIENTURNE web tool showed that only two of the five miRNAs were enriched in the Wnt signaling pathway i.e. hsa-miR-31-5p and hsa-miR-98-5p. These two miRNAs were further analyzed through Kaplan Meier survival analysis (Nagy et al., 2021) as well as recently published literature. Hsa-miR-98-5p was chosen as the best target for the FZD3 receptor on grounds that recently published literature has indicated that hsa-miR-31-5p has oncogenic properties in CRC (Mi et al., 2020) despite having a better Kaplan Meier median survival compared to hsa-miR-98-5p. Further analysis in the computational prediction program called TargetScan (http://www.targetscan.org/), (Agarwal et al., 2015) indicated that hsa-miR-98-5p binds to the 3' UTR of FZD3 mRNA at three positions i.e. 1873, 3523, 4957.

Figure 8: The putative binding sequence of miR-98-5p and miR-31-5p in FZD3 3'-UTR

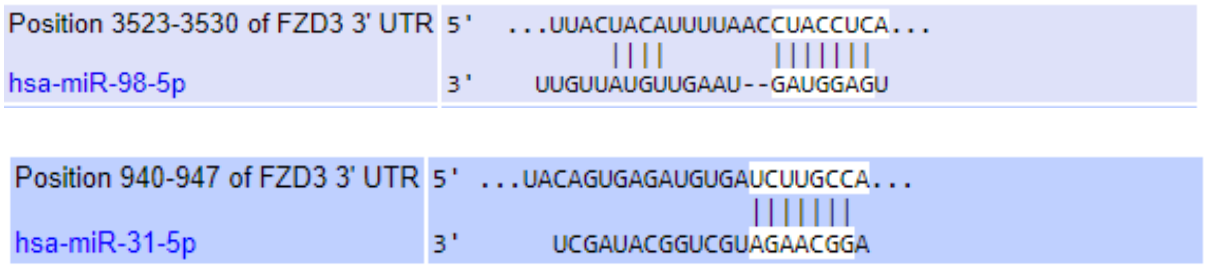


Figure 9: Kaplan-Meier survival curves associated with the two candidate miRNAs expression in colorectal cancer

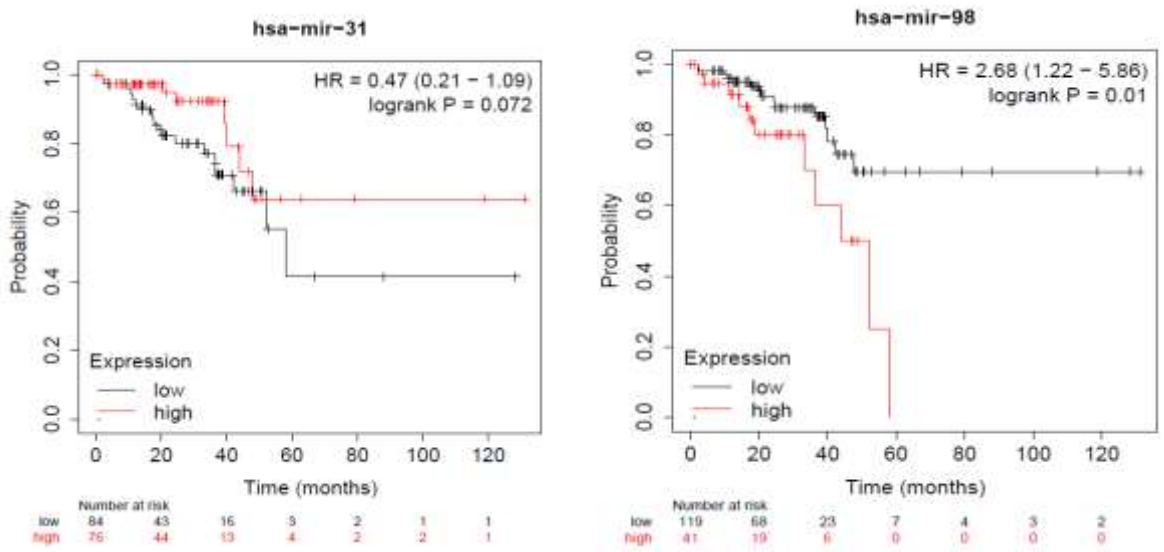
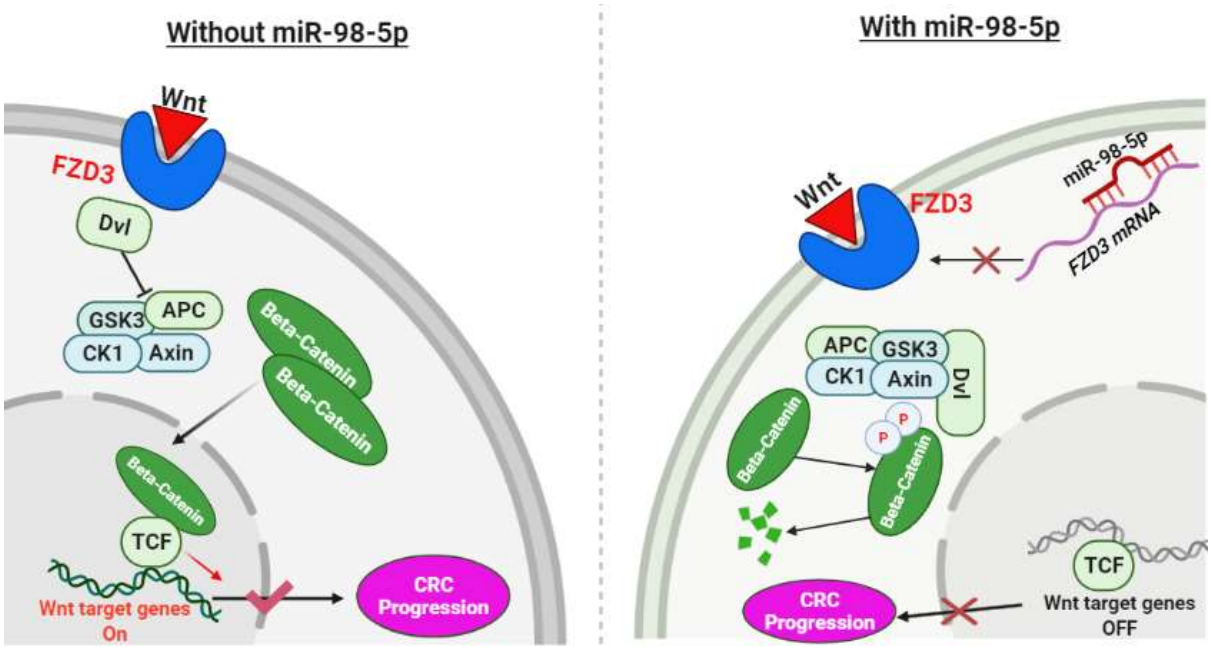


Figure 10: The putative progression of Wnt signaling pathway with/out miR-98-5p in CRC cells



Chapter – 4: Discussion

CHAPTER – 4

DISCUSSION

Although the trends in CRC incidences and mortalities have dropped since the mid-1980s worldwide, this malignancy accounted for more than 1.5 million new cancer cases and close to 1 million cancer-related deaths in 2020. The burden distribution of CRC varies globally but increasing incidences are reported in countries with high Human Development Index (HDI) such as Nordic countries, Western Europe, North America, Australia, New Zealand etcetera (Høydahl et al., 2020) due to the aging population in such countries. However, CRC is still a big challenge in developing countries whose health care system is still poor yet the lifestyle among natives exposes them to preventable CRC risk factors such as smoking, alcohol, unfavorable diet, lack of physical exercise, among others. Affordable high standard healthcare systems ensure that screening and early diagnosis increase survival rates whereas in countries where high-quality healthcare systems are unaffordable, treatment strategies need to be improved to increase survival. And surgical treatment. Routine CRC treatment strategies such as chemotherapy, immunotherapy, radiotherapy, and surgery have saved so many lives however, targeted therapy due to advancement in health science brings more specificity and increases survival among CRC patients.

Targeted therapy in CRC aims to block different critical pathways that are responsible for cell growth and proliferation, angiogenesis, migration, differentiation, and anti-apoptosis by using small molecules such as monoclonal antibodies. These molecules with molecular weight <900Da penetrate into cells thereby inhibiting target enzymes hence interfering with tumor growth and; in some cases inducing apoptosis (To et al., 2018; Xie et al., 2020). The United States Food and Drug Administration (FDA) has to date approved a number of targeted therapies for CRC such as Cetuximab; an anti-EGFR agent (Vascular Endothelial Growth Factor), anti-angiogenesis agent bevacizumab, etcetera, and the trend is promising (Di Nicolantonio et al., 2021). Pathways that offer potential sites for targeted therapy in CRC include among others; Wnt/β-catenin, HGF/c-MET pathway, notch, hedgehog, and EGFR-related pathways (Xie et al., 2020).

The Wnt/FZD signaling pathway is classified into canonical and non-canonical pathways. During the process, the nineteen members of the Wnt family bind to ten members of the Frizzled family hence activating downstream processes such as Wnt/beta-catenin; - a canonical pathway, Wnt/planar cell polarity, and Wnt/Ca2+; the non-canonical pathways (L et al., 2015; Schatoff et al., 2017).

Accumulating evidence from published studies indicates the biological importance of microRNAs (miRNAs) in the progression and metastasis in CRC hence attracting the attention of researchers. Despite them being referred to as non-coding RNAs, studies have shown that miRNAs are involved in post-transcriptional regulation of more than 60% of human genes (X. Wang et al., 2021). Studies have gone ahead to show that dysregulated expression of miRNAs is associated with the progression of CRC (Ye et al., 2019). This study aimed to target one of the critical pathways in CRC - Wnt signaling pathway with miR-98-5p, a rarely reported

miRNA in CRC - to inhibit FZD3, a regularly up-regulated frizzled receptor of this pathway in CRC.

To determine the expression pattern of the FZD3 receptor in CRC, differential gene expression analysis was done on five datasets from GEO. The statistical analysis by Bioconductor R package limma (Ritchie et al., 2015) showed that the quantitative changes in the expression levels between normal and tumor samples were significant for FZD3 to be a DEG alongside other genes. The gene ontology and KEGG enrichment analyses that were used to explore the functions of the identified DEGs indicated that FZD3 was significantly enriched in the Wnt signaling pathway. Like FZD3, a number of other top DEGs were enriched in Wnt signaling, which correlated with recent studies that implicate Wnt signaling as a key pathway in CRC (C. Li et al., 2019; X. Li et al., 2020; Patel et al., 2019; Ueno et al., 2013c; Wong et al., 2013). The molecular mechanisms, as well as the role of various FZD family members in the development of CRC, have been widely studied. However, an explicit method or antibody that targets the FZD3 receptor for colorectal cancer therapy is yet to be identified (Zeng et al., 2018).

The GEDS web server and Kaplan-Meier plotter were utilized to validate the expression of FZD3 mRNA in CRC. The expression in tumor colorectal tissues was significantly higher than in normal tissues and this correlated with poor overall survival. The protein-protein interaction analysis of FZD3 by STRING web server showed that this receptor gene interacts with a number of key Wnt signaling pathway genes such as DVL, WNT1, WNT5, LRP6, VANGL2 (Figure 7), which were found up-regulated too in all the five datasets of this study. More to this, studies have indicated that sFPR1 (secreted Frizzled Receptor Protein 1), a Wnt antagonist, is significantly decreased by up-regulation of FZD3 (Sompel et al., 2021; Ueno et al., 2013a) and it was found down-regulated in all the five datasets. High FZD3 expression levels have been reported by various studies to correlate with Wnt target genes such as Cyclin D1 and c-Myc (Ueno et al., 2013b). The expression of these genes correlated with that of FZD3 in all the 5 datasets. Based on these results as well as literature citations, it seemed reasonable to suggest that FZD3 is a key player in the Wnt signaling pathway and if knocked down, the pathway would be inhibited (He et al., 2011).

Recent studies have indicated that microRNA miR-98-5p inhibits tumor cell proliferation, migration and invasion in various cancers including ovarian cancer (Z. Wang et al., 2021), glioblastoma (Xu et al., 2017), gastric cancer (Zhan et al., 2021), Non-small cell lung cancer (Jiang et al., 2019), pancreatic ductal adenocarcinoma (Fu et al., 2018), and etcetera. Moreover, studies have shown that this microRNA suppresses tumor progression in various cancers by targeting the Wnt signaling pathway related genes (W. Li et al., 2019; Zheng et al., 2019). This makes it a potential target to FZD3, one of this pathway's key receptors.

Target prediction algorithms such as Targetscan showed that the FZD3 mRNA contained the binding sites for miR-98-5p in its 3'-UTR, which is a key feature in the miRNA post-translational gene regulation mechanism. However, the miRNA prediction algorithms can barely confirm that FZD3 is a direct target of miR-98-5p in CRC samples. To validate the results of this study, a luciferase reporter assay should be performed to compare the wild-type (WT) as well as the mutated (MUT) FZD3 in the 3'UTR binding site. A significant fluorescence from FZD3-WT than FZD3-MUT will confirm FZD3 gene as a direct target of miR-98-5p. (Kim et al., 2015; X. Wang et al., 2021). FZD3 and miR-98-5p could be forming an axis that inhibits Wnt signaling and CRC in general; however, the involvement of other

target genes in the process cannot be ruled out. To validate the mechanism by which miR-98-5p inhibits Wnt signaling pathway, all the predicted target genes by at least two miRNA prediction algorithms should be enriched in Wnt pathways by gene ontology and KEGG. The mRNA expression levels of such genes can then be measured with miR-98-5p mimic and inhibitor respectively (Kim et al., 2015).

In conclusion, the current study indicated that FZD3 is up-regulated in CRC and miR-98-5p inhibits the expression of this receptor by directly binding to the 3'UTR of its mRNA hence exerting a tumor-suppressor role in CRC through Wnt signaling pathway. However these results need to be validated by basic research in future to verify the regulatory mechanisms of miR-98-5p in CRC cells *in vitro* and *in vivo*. The study provides evidence for a new target of FZD3 that can potentially inhibit the proliferation and metastasis of colorectal tumor cells, and this may help in developing target-based therapies for CRC patients.

Chapter – 5: References

CHAPTER -5

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