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Research Article

MicroRNA Regulation of RAS/MAPK Pathway: Unveiling Interactions in Colorectal Cancer Tumorigenesis and Beyond

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ABSTRACT

The Rat Sarcoma virus/Mitogen Activated Protein Kinase (RAS/MAPK) pathway, pivotal in cancer development, intertwines with microRNAs (miRNAs), shaping Colorectal Cancers (CRCs) dynamic cellular landscape. This study utilizes a systems biology approach, employing advanced bioinformatics tools to unravel miRNA-mediated regulatory networks in CRC. Investigating miRNA dysregulation, identifying hub genes and exploring interplay between signalling pathways aim to provide nuanced insights into CRCs molecular landscape. Protein-protein network analysis uncovers the interconnected web of miRNA-targeted genes within the RAS/MAPK signaling pathway. Utilizing STRING and Cytoscape, we pinpoint 13 hub proteins orchestrating network dynamics. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses reveal complex regulatory mechanisms. Cluster analysis unveils 817 clusters, emphasizing CRC significance. Hub gene promoter motif analysis delves into transcriptional regulatory elements governing CRC pathogenesis. A comprehensive list of miRNAs targeting the Phosphatidylinositol 3-Kinase (PI3K) pathway in CRC is presented. Protein-protein interaction networks highlight 13 hub proteins, predominantly linked to transcriptional regulation. Enriched pathways, such as the MAPK cascade, underscore their pivotal roles. Cluster analysis reveals CRCs significance, emphasizing involvement in glucose metabolism. Promoter motif analysis uncovers significant motifs targeted by dysregulated miRNAs. Transcription factor motifs reveal biological roles, with implications in signal transduction and cell proliferation. Enrichment in nucleus-based terms and disruption of cell polarity underscore potential implications in cancer development. This integrative study resolves the regulatory complex of miRNAs in the RAS/MAPK signaling pathway, explaining on CRCs molecular landscape. Identification of hub genes, enriched pathways and regulatory motifs provides valuable insights for diagnostics and therapeutics, offering potential targets for simultaneous inhibition in cancer cell growth.

Keywords: Colorectal cancer; Network analysis; Promoter motif analysis; Signaling pathway

INTRODUCTION

Colorectal Cancer (CRC) remains a formidable global health challenge, prompting a fervent search for a deeper understanding of its molecular underpinnings [1]. At the cellular level, complex pathways complexly guide the development and progression of colorectal cancer, underscoring the multifaceted nature of this malignancy. The dynamic interplay of signaling pathways within cells plays a pivotal role in steering the trajectory of colorectal cancer, shaping its initiation, progression and response to therapeutic interventions [2].

Among the myriad cellular pathways implicated in colorectal cancer, the RAS/MAP kinase pathway emerges as a linchpin, wielding substantial influence over cellular processes important to cancer development. This signaling cascade, encompassing the RAS family of proteins and the Mitogen Activated Protein Kinase (MAPK) pathway, is renowned for its regulatory prowess in cell proliferation, survival and differentiation [3].

MicroRNAs (miRNAs), small non-coding Ribose Nucleic Acid (RNA) molecules, have emerged as significant orchestrators in the complex of cellular pathways, particularly impacting the RAS/MAP kinase pathway in colorectal cancer. These miRNAs, through their regulatory role, exert influence over key elements of the RAS/MAPK pathway, potentially tipping the balance in favor of cancer development. Understanding the nuanced interactions between miRNAs and the RAS/MAP kinase pathway unveils a layer of complexity that holds potential for solve novel therapeutic avenues in the management of colorectal cancer [4]. The pivotal role of microRNAs (miRNAs) in the regulation of important signaling

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pathways, particularly the RAS/MAPK pathway, has emerged as a focal point for investigations seeking to decipher the molecular symphony that underlies CRC [5].

This study begins on a systems biology approach, leveraging advanced bioinformatics tools, cluster analysis and promoter motif exploration to unravel the complexities of miRNA-mediated regulatory networks in colorectal cancer. Our exploration delves into the dysregulation of miRNA expression, the identification of hub genes and the complex crosstalk between signaling pathways. Through cluster analysis, we dissect the functional modules within these networks, providing a holistic view of the interplay among key molecular players. Furthermore, a detailed examination of hub gene promoter motifs sheds light on the transcriptional regulatory elements driving colorectal cancer pathogenesis. By integrating these diverse analyses, our study aims to contribute nuanced insights into the molecular landscape of colorectal cancer, with potential implications for diagnostic strategies and the identification of therapeutic targets. As we navigate the complexities of miRNA regulatory networks, this exploration marks a significant stride towards solving the molecular mosaic that defines colorectal cancer.

MATERIALS AND METHODS

Protein-protein network analysis

First of all, the list of miRNAs targeting the RAS/MAPK signaling pathway was obtained from review article. In pursuit of solving the complex web of Protein-Protein Interactions (PPIs), our study harnessed the predictive power of the STRING database version 11.5. This database not only encapsulates direct (physical) associations but also embraces indirect (functional) links. These interactions are derived from a synthesis of computational predictions, knowledge transfer between organisms and insights aggregated from primary databases.

To visually navigate the complexities of the PPI network and discern central players, we employed the Cytoscape software (version 3.9.1). This open-source platform seamlessly integrates network visualization with diverse attribute data. The identification of hub proteins, beneficial in orchestrating network dynamics, was facilitated by the CytoHubba plugin (version 0.1). CytoHubba offers an arsenal of 11 topological analysis methods, including degree, edge percolated component, maximum neighborhood component, density of maximum neighborhood component, maximal clique centrality and six centralities (bottleneck, eccentricity, closeness, radiality, betweenness and stress) based on shortest paths.

Our meticulous selection of hub proteins was anchored in the Maximum Clique Centrality (MCC), Maximum Neighborhood Component (MNC), Density of Maximum Neighborhood Component (DMNC) and Degree (D) algorithms. These algorithms collectively empowered us to pinpoint proteins playing pivotal roles in the complex of protein interactions, providing a deeper understanding of the network dynamics underlying the molecular landscape we aimed to dissect.

GO and KEGG enrichment analysis

To comprehensively scrutinize gene functions, we utilized STRING software version 11.5 for Gene Ontology (GO) analysis. GO analysis allows users to complexly describe a gene or gene product, considering three main aspects which are to delineate its Molecular Function (MF), to understand the Biological Processes (BP) in which it participates and to define its Cellular Component (CC). This multifaceted approach aims to solving the soft roles of genes within the cellular context, explains on their molecular functions, their participation in biological processes and their localization within cellular structures. Simultaneously, our investigation seeks to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, executed with precision using the STRING software. This analysis aims to afford a panoramic view of the pathways implicated in our study. By scrutinizing the KEGG pathways, we aim to gain insights into the interconnected molecular networks and pathways that shape the landscape of our investigated genes.

Analysis of the network's clusters

In the pursuit of deciphering the organizational principles within our network, we deployed CytoCluster (version 2.1.0) as our analytical compass. This algorithmic tool served as a discerning guide, orchestrating the grouping of network nodes. The utilization of the protein complex identification technique bestowed a stringent threshold of 2, akin to a selective gatekeeper, ensuring only nodes of significant influence gained entry [6].

Proceeding with meticulous precision, we enlisted the computational prowess of STRING software (version 11.5) to scrutinize the genetic composition of each cluster. This analysis aimed to expose the complex associations between genes within these clusters and specific Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Building like a complex molecular audit, dissecting the functional nuances and modular organization embedded within each cluster. Our study, armed with these computational revelations, delves into the profound realm of functional complex, explains on the orchestrated dance of molecular entities within the clusters of our network.

Hub gene promoter motif analysis

In the activity of simplifying the regulatory dynamics governing hub genes, we meticulously extracted Upstream Flanking Regions (UFRs) from Ensembl Services (https://grch37.ensembl.org/Homo_sapiens/Info/Index). These contiguous regions, precisely 1 kbp in length, serve as windows into the complex of gene regulation.

Employing MEME Suite (version 5.5.2) (meme.nbcr.net/meme/intro.html) with rigorously defined parameters, we embarked on the identification of conserved motifs within these genomic sequences [7]. The criteria for motif discovery were set with exacting standards, imposing thresholds for P and E values at 0.01, ensuring a robust selection process. To distill essential regulatory motifs and annotate them against established knowledge, the Tomtom tool (version 5.5.2) (http://meme-suite.org/tools/tomtom) took center stage. Employing thresholds of 0.01 and 0.1 for P and E values respectively, Tomtom meticulously sifted through motifs, eliminating redundancy and cross-referenced against the JASPAR CORE 2022 database to unveil known cis-regulatory elements [8].

Adding another layer to our analysis, the GoMo tool (http://meme-suite.org/tools/gomo) was invoked to elucidate potential functional roles associated with the identified motifs [9]. This computational tool acted as a lens through which we sought to interpret the probable biological implications encoded within these regulatory elements. In this multifaceted analysis, our endeavor is to unravel the nuanced regulatory syntax embedded within the UFRs of hub genes, contributing to the broader understanding of the complex molecular mechanisms governing gene expression.

RESULTS

Obtaining miRNAs targeting the PI3K pathway in colorectal cancer patients

In this section, we provide compelling evidence demonstrating the dysregulation of miRNA expression in human cancer, attributed to various mechanisms such as amplification or deletion of miRNA genes, abnormal transcriptional control, dysregulated epigenetic changes and defects in miRNA biogenesis [10]. MiRNAs exhibit dual roles as oncogenes or tumor suppressors, impacting cancer hallmarks, including proliferative signaling, growth suppression evasion, resistance to cell death, invasion and metastasis activation and angiogenesis induction.

Numerous studies highlight miRNAs as potential biomarkers for human cancer diagnosis, prognosis and therapeutic targets, emphasizing the need for further investigation and validation. Employing bioinformatics tools, our study explores the complex relationship between miRNAs targeting the RAS/MAPK signaling pathway and their involvement in colorectal cancer development and progression. By activating and cross-talking with other vital cellular pathways, these miRNAs play a pivotal role.

The miRNAs and their targeted genes, sourced from our group's review study and the miRDB database, reveal a comprehensive list of miRNAs targeting the PTK pathway in colorectal cancer patients. Upon acquisition of dysregulated miRNAs targeting the PI3K pathway from previous group studies, we identified the genes targeted by these miRNAs, emphasizing those with a score exceeding 90 in the mirDB database (Table 1).

Initially, we gathered 1064 genes targeted by both up- and down-regulated miRNAs affecting the Wnt pathway, all possessing a score exceeding 90 according to the miRDB database (http://www.mirdb.org). All high-scoring genes regulated by miRNAs were included in subsequent analyses. To visually represent the complex protein network among these genes, we utilized databases String and Cytoscape. The interactions revealed by Cytoscape encompass both direct (physical) and indirect (functional) associations, incorporating computational predictions, knowledge transfer between organisms and aggregated interactions from primary databases.

Table 1: Oncogenic or tumor-suppressor regulatory microRNAs (miRNAs) for the Rat Sarcoma/Mitogen-Activated Protein Kinase (RAS/MAPK) signaling pathway in colorectal cancer pathology.

MicroRNA (miR)	Target	Function
miR-21	RAS P21 Protein Activator 1 (RASA1)	Oncogene
miR-21-5p	Rat Sarcoma/Mitogen Activated Protein Kinase (RAS/MAPK) signaling	Oncogene
miR-335	RAS P21 Protein Activator 1 (RASA1)	Oncogene
miR-223	RAS P21 Protein Activator 1 (RASA1)	Oncogene
miR-31	RAS P21 Protein Activator 1 (RASA1)	Oncogene
miR-3148	Rat Sarcoma/Mitogen Activated Protein Kinase (RAS/MAPK) signaling	Oncogene
miR-1260b	Programmed Cell Death 4/Insulin-like Growth Factor 1 (PDCD4/*IGF1*)	Oncogene
miR-650	Inhibitor of Growth 4 (ING4)	Oncogene
miR-625-3p	Mitogen-Activated Protein Kinase 6 (MAP2K6)	Oncogene
miR-487b	Kirsten Rat Sarcoma (*KRAS*), Lipoprotein Receptor-related Protein 6 (LRP6)	Tumor-suppressor
miR-30b	Kirsten Rat Sarcoma (*KRAS*), Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit Delta (PIK3CD), B-Cell Lymphoma 2 (BCL2)	Tumor-suppressor
miR-622	Kirsten Rat Sarcoma (*KRAS*)	Tumor-suppressor
miR-543	Kirsten Rat Sarcoma (*KRAS*), Metastasis-Associated protein-1 (MTA1), High Mobility Group A protein-2 (HMGA2)	Tumor-suppressor
miR-337	Kirsten Rat Sarcoma (*KRAS*)	Tumor-suppressor
miR-139-5p	Ras-related protein (Rap1b)	Tumor-suppressor
miR-143	Kirsten Rat Sarcoma (*KRAS*)	Tumor-suppressor
miR-145	Neuroblastoma Ras viral oncogene (NRAS), Insulin Receptor Substrate 1 (IRS1), P21-Activated Kinase 4 (PAK4)	Tumor-suppressor
miR-143, miR-145	Kirsten Rat Sarcoma (*KRAS*), Ras Responsive Element Binding protein 1 (RREB1)	Tumor-suppressor
miR-126	Insulin Receptor Substrate 1 (IRS1), CXC Chemokine Receptor 4 (CXCR4)	Tumor-suppressor
miR-139	Insulin-like Growth Factor 1 Receptor (IGF-IR)	Tumor-suppressor
miR-331-3p	Human Epidermal Growth Factor Receptor 2 (HER2)	Tumor-suppressor

Constructing protein-protein interaction networks and analyzing hub proteins

Biological networks, serving as representations of complex biological systems, frequently involve various entity types. Cytoscape, a versatile software tool supported by numerous apps, aids in the analysis and visualization of such networks. The latest stringApp 2.0, a Cytoscape app, significantly enhances support for heterogeneous networks (Figure 1).

Protein-protein Interaction Networks (PPIN) mathematically depict the physical contacts between proteins within the cell. These contacts are specific, occurring between defined binding regions in proteins and hold particular biological significance, serving specific functions. In recent years, researchers have focused on protein substructures, utilizing protein interaction algorithms to predict interactions and understand protein functions. This approach enables the examination of key proteins important for optimal targeting.

Figure 1, displays proteins directly linked to dysregulated miRNAs, many of which play pivotal roles in cellular pathways essential for growth and proliferation. In subsequent sections, we aim to provide a more refined classification and understanding of these proteins, identifying those with the most interactions.

Leveraging four algorithms were outlined, we identified 13 hub proteins with the most interactions, signifying significant roles in the network (Figure 2).

The majority of proteins listed exhibit roles as transcription factors or direct involvement in the regulation of transcription factors. Molecular function analysis unveils their direct connection to Deoxyribose Nucleic Acid and their regulation of transcription, supported by an adjusted value of less than 0.02%. Specifically, Suppressor of Mothers Against Decapentaplegic 4 (*SMAD4*), Estrogen Receptor 1 (*ESR1*), Zeste Homolog 2 (*EZH2*), cAMP Responsive Element Binding 1 (*CREB1*) and Histone Deacetylase 2 (*HDAC2*) are proteins that bind to the DNA strand. Among them, Table 2, highlights that *SMAD4*, *HDAC2*, *CREB1* and *ESR1* possess the capability to identify and bind to specific DNA sequences (Tables 2 and 3).

In terms of cellular location, hub proteins with an adjusted value of less than 0.05 are predominantly present in the nucleus structure. Notably, *HDAC2* and *EZH2* are components of the ESC/E(Z) complex, known for its ability to methylate lysine-27 and lysine-9 amino acids of histone H3. This complex interacts with various noncoding RNAs and plays an important role in gene silencing. On the other hand, *CREB1* and *ESR1* are primarily found in euchromatin. Moreover, Table 4, reveals the presence of *SMAD4*, *HDAC2*, *CREB1*, Cysteine-Aspartic Acid Protease 3 (*CASP3*), Exportin 7 (*XPO7*), *MAPK1*, Karyopherin subunit alpha 4 (*KPNA4*), *ESR1*, YY1-Associated Factor 2 (*YAF2*) and *EZH2* in intracellular membrane bounded organelles (Table 4 and Figure 3).

Examining molecular function further, these hub proteins significantly contribute to the transcription cycle and cell proliferation. Specifically, *MAPK1* and *ESR1* play roles in the Steroid Hormone Mediated (SHM) signaling pathway, while *SMAD4*, *CREB1*, Insulin-like Growth Factor 1 (*IGF1*) and *EZH2* are involved in Positive Regulation of Cell Differentiation (*PRCD*). Additionally, *MAPK1*, *KRAS* and *IGF1* are components of the MAPK cascade and *SMAD4*, *MAPK1*, *KRAS* and *IGF1* are categorized within the cluster of Positive Regulation of Protein Phosphorylation (PRPP) are highlighted (Table 5).

Numerous studies have delved into the alterations in expression or sequence of the identified hub proteins and their association with the risk of colorectal cancer development [11,12]. In line with our findings, Wei et al., utilizing a subnetwork extraction algorithm (Limited K-walks algorithm), unearthed colorectal cancer-related genes within the protein-protein interaction network [13]. Notably, their computational predictions identified Ubiquitin C (UBC) and *SMAD4* as putative key genes associated with CRC [13]. A recent report has further substantiated the significance of *SMAD4*, documenting mutations in patients with juvenile polyposis. Remarkably, *SMAD4* is among the genes included in the hereditary colorectal cancer panel [14].

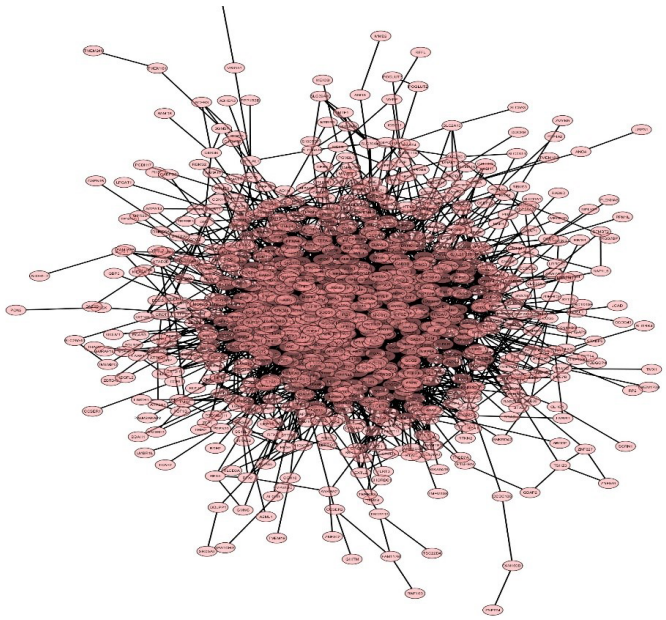

Figure 1: Gene network for all targeted genes.

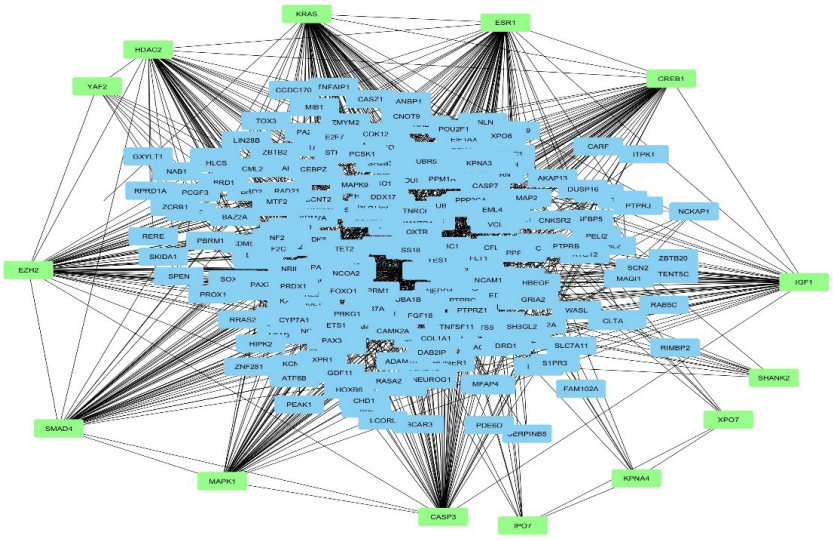

Figure 2: Subnetwork formed by hubs.

Table 2: Gene ontology (molecular function) of hub proteins.

Term	p-value	Adjusted p-value	Genes
Transcription coregulator binding (GO:0001221)	4.12E-05	0.00267539	*SMAD4, ESR1, EZH2*
Transcription coactivator binding (GO:0001223)	2.52E-04	0.008190405	*SMAD4, ESR1*
Transcription corepressor binding (GO:0001222)	3.86E-04	0.008353458	*ESR1, EZH2*
Sequence-specific DNA binding (GO:0043565)	0.00123	0.01785911	*SMAD4, HDAC2, CREB1, ESR1*
Chromatin DNA binding (GO:0031490)	0.001499	0.01785911	*HDAC2, EZH2*
DNA-binding transcription activator activity, RNA polymerase II-specific (GO:0001228)	0.001649	0.01785911	*SMAD4, CREB1, ESR1*

Note: GO: Gene Ontology; DNA: Deoxyribose Nucleic Acid; RNA: Ribose Nucleic Acid; *SMAD4*: Suppressor of Mothers Against Decapentaplegic 4; *ESR1*: Estrogen Receptor 1; *EZH2*: Zeste Homolog 2; *HDAC2*: Histone Deacetylase 2; *CREB1*: cAMP Responsive Element Binding 1.

Table 3: Hob proteins in genes targeted by deferentially expressed microRNA (miRNA) targeting the Rat Sarcoma/Mitogen-Activated Protein Kinase (RAS/MAPK) signaling pathway.

Hob proteins	Method	Rank	Gene description
ESR1	Degree/MNC/ MCC	1,1,3	Estrogen receptor; Nuclear hormone receptor. The steroid hormones and their receptors are involved in the regulation of eukaryotic gene expression and affect cellular proliferation and differentiation in target tissues
KRAS	Degree/MNC/ MCC	2,2,4	TPase *KRas*, N-terminally processed; Ras proteins bind GDP/GTP and possess intrinsic GTPase activity. Plays an important role in the regulation of cell proliferation. Plays a role in promoting oncogenic events by inducing transcriptional silencing of Tumor Suppressor Genes (TSGs) in Colorectal Cancer (CRC) cells in a ZNF304-dependent manner
EZH2	Degree/MNC	3,3	Histone-lysine N-methyltransferase *EZH2*; Polycomb Group (PcG) protein. Catalytic subunit of the PRC2/EED-*EZH2* complex, which methylates Lys-9 (H3K9me) and Lys-27 (H3K27me) of histone H3, leading to transcriptional repression of the affected target gene
CREB1	Degree/MNC	4,4	Cyclic AMP-responsive element-binding protein 1; Phosphorylation-dependent transcription factor that stimulates transcription upon binding to the DNA cAMP Response Element (CRE), a sequence present in many viral and cellular promoters

SMAD4	MNC	5	In muscle physiology, plays a central role in the balance between atrophy and hypertrophy. When recruited by MSTN, promotes atrophy response *via* phosphorylated SMAD2/4. Acts synergistically with SMAD1 and YY1 in Bone Morphogenetic Protein (BMP)-mediated cardiac- specific gene expression
CASP3	MCC	1	Caspase-3 subunit p12; Involved in the activation cascade of caspases responsible for apoptosis execution. At the onset of apoptosis it proteolytically cleaves Poly (ADP-Ribose) Polymerase (PARP) at a '216-Asp-/-Gly-217' bond
IGF1	MCC	2	Insulin-like growth factor I; The insulin-like growth factors, isolated from plasma, are structurally and functionally related to insulin but have a much higher growth-promoting activity. May be a physiological regulator of [1-14C]- 2-Deoxy-D-Glucose (2DG) transport and glycogen synthesis in osteoblasts
MAPK1	MCC	5	Mitogen-activated protein kinase 1; Serine/threonine kinase which acts as an essential component of the MAP kinase signal transduction pathway. *MAPK1*/ERK2 and MAPK3/ERK1 are the 2 MAPKs which play an important role in the MAPK/ERK cascade
HDAC2	Degree	5	Histone deacetylase 2; Responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Histone deacetylation gives a tag for epigenetic repression and plays an important role in transcriptional regulation, cell cycle progression and developmental events
YAF2	DMNC	1	YY1-associated factor 2; Binds to MYC and inhibits MYC-mediated transactivation. Also binds to MYCN and enhances MYCN-dependent transcriptional activation. Increases calpain 2-mediated proteolysis of YY1 *in vitro*
SHANK2	DMNC	2	H3 and multiple ankyrin repeat domains protein 2; Seems to be an adapter protein in the Postsynaptic Density (PSD) of excitatory synapses that interconnects receptors of the postsynaptic membrane including NMDA-type and metabotropic glutamate receptors and the actin-based cytoskeleton
IPO7	DMNC	3	Importin-7; Functions in nuclear protein import, either by acting as autonomous nuclear transport receptor or as an adapter-like protein in association with the importin-beta subunit KPNB1
XPO7	DMNC	4	Exportin-7; Mediates the nuclear export of proteins (cargos) with broad substrate specificity. In the nucleus binds cooperatively to its cargo and to the GTPase Ran in its active GTP-bound form
KPNA4	DMNC	5	Importin subunit alpha-3; Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1. Binds specifically and directly to substrates containing either a simple or bipartite NLS motif

Note: *ESR1*: Estrogen Receptor 1; MNC: Maximum Neighborhood Component; MCC: Maximum Clique Centrality; *KRAS*: Kirsten Rat Sarcoma; TPase: Triphosphatase; GDP: Guanosine Diphosphate; GTP: Guanosine Triphosphate; ZNF304: Zinc-Finger protein 304; *EZH2*: Zeste Homolog 2; PRC2: Polycomb Repressive Complex 2; EED: Embryonic Ectoderm Development; *CREB1*: cAMP Responsive Element Binding 1; AMP: Adenosine Monophosphate; DNA: Deoxyribose Nucleic Acid; *SMAD4*: Suppressor of Mothers Against Decapentaplegic 4; MSTN: Myostatin; YY1: Yin-Yang1; *CASP3*: Cysteine-Aspartic acid Protease; ADP: Adenosine Diphosphate; *IGF1*: Insulin-like Growth Factor 1; MAPK: Mitogen-Activated Protein Kinase; ERK2: Extracellular signal-Regulated Kinase 2; *HDAC2*: Histone Deacetylase 2; *YAF2*: YY1-Associated Factor 2; DMNC: Density of Maximum Neighborhood Component; MYC: Myelocytomatosis oncogene; NMDA: N-methyl-D-aspartate; IPO7: Importin 7; *XPO7*: Exportin 7; *KPNA4*: Karyopherin subunit Alpha 4; KPNB1: Karyopherin subunit Beta 1; NLS: Nuclear Localization Signal.

Table 4: Cellular component of hub proteins.

Term	P-value	Adjusted P-value	Genes
ESC/E(Z) Complex (GO:0035098)	9.54E-06	4.20E-04	*HDAC2*, *EZH2*
Nucleus (GO:0005634)	1.29E-04	0.002849	*SMAD4*, *HDAC2*, *CREB1*, *CASP3*, *XPO7*, *MAPK1*, *KPNA4*, *ESR1*, *YAF2*, *EZH2*
Euchromatin (GO:0000791)	4.23E-04	0.005067	*CREB1*, *ESR1*
Intracellular membrane-bounded organelle (GO:0043231)	4.61E-04	0.005067	*SMAD4*, *HDAC2*, *CREB1*, *CASP3*, *XPO7*, *MAPK1*, *KPNA4*, *ESR1*, *YAF2*, *EZH2*

Note: GO: Gene Ontology; ESC: Embryonic Stem Cells; *HDAC2*: Histone Deacetylase 2; *EZH2*: Zeste Homolog 2; *SMAD4*: Suppressor of Mothers Against Decapentaplegic 4; *CREB1*: cAMP Responsive Element Binding 1; *CASP3*: Cysteine-Aspartic Acid Protease 3; *XPO7*: Exportin 7; *MAPK1*: Mitogen-Activated Protein Kinase 1; *KPNA4*: Karyopherin subunit Alpha 4; *ESR1*: Estrogen Receptor 1; *YAF2*: YY1-Associated Factor 2; *EZH2*: Zeste Homolog 2.

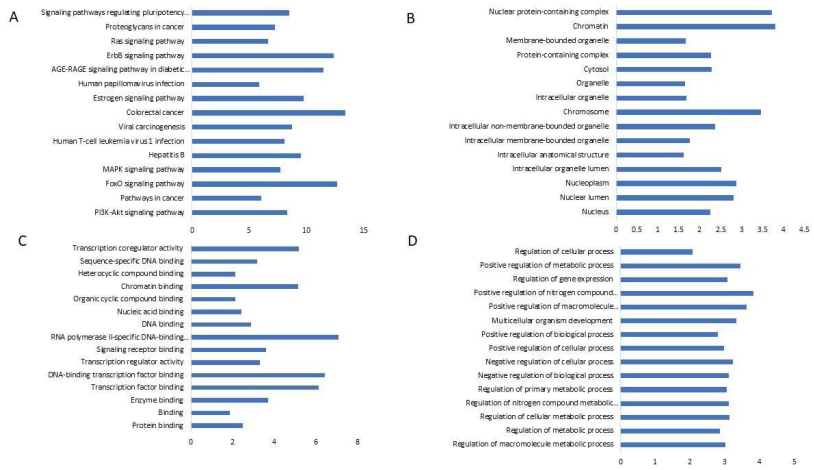

Figure 3: (A) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis; (B) Cellular compartment enrichment analysis; (C) Molecular function enrichment analysis; (D) Biological process enrichment analysis.

Table 5: Piological process of hub proteins.

Term	P-value	Adjusted P-value	Genes
Positive regulation of nucleic acid-templated transcription (GO:1903508)	1.13E-06	4.94E-04	*SMAD4*, *HDAC2*, *CREB1*, *IGF1*, *ESR1*, *YAF2*
Positive regulation of cell population proliferation (GO:0008284)	1.35E-05	0.002498	*HDAC2*, *KRAS*, *IGF1*, *SHANK2*, *EZH2*
Steroid hormone mediated signaling pathway (GO:0043401)	2.04E-05	0.002498	*MAPK1*, *ESR1*
Positive regulation of cell differentiation (GO:0045597)	3.51E-05	0.002498	*SMAD4*, *CREB1*, *IGF1*, *EZH2*
Positive regulation of cellular process (GO:0048522)	3.64E-05	0.002498	*HDAC2*, *KRAS*, *IGF1*, *SHANK2*, *EZH2*
Negative regulation of DNA-templated transcription (GO:0045892)	3.76E-05	0.002498	*SMAD4*, *HDAC2*, *CREB1*, *ESR1*, *YAF2*, *EZH2*
MAPK cascade (GO:0000165)	3.99E-05	0.002498	*MAPK1*, *KRAS*, *IGF1*
Positive regulation of protein phosphorylation (GO:0001934)	1.07E-04	0.004544	*SMAD4*, *MAPK1*, *KRAS*, *IGF1*
Positive regulation of DNA-templated transcription (GO:0045893)	1.11E-04	0.004544	*SMAD4*, *HDAC2*, *CREB1*, *IGF1*, *ESR1*, *YAF2*
Regulation of cardiac muscle hypertrophy (GO:0010611)	1.14E-04	0.004544	*SMAD4*, *IGF1*
Regulation of cell population proliferation (GO:0042127)	1.22E-04	0.004544	*HDAC2*, *KRAS*, *IGF1*, *SHANK2*, *EZH2*
ERK1 and ERK2 cascade (GO:0070371)	1.24E-04	0.004544	*MAPK1*, *IGF1*
Regulation of DNA-templated transcription (GO:0006355)	1.40E-04	0.004609	*SMAD4*, *HDAC2*, *CREB1*, *IGF1*, *ESR1*, *YAF2*, *EZH2*
Cellular response to organic cyclic compound (GO:0071407)	1.47E-04	0.004609	*CREB1*, *CASP3*, *ESR1*

Note: GO: Gene Ontology; *SMAD4*: Suppressor of Mothers Against Decapentaplegic 4; *HDAC2*: Histone Deacetylase 2; *CREB1*: cAMP Responsive Element Binding 1; *IGF1*: Insulin-like Growth Factor 1; *ESR1*: Estrogen Receptor 1; *YAF2*: YY1-Associated Factor 2; *KRAS*: Kirsten Rat Sarcoma; *EZH2*: Zeste Homolog 2; *MAPK1*: Mitogen-Activated Protein Kinase 1; *CASP3*: Cysteine-Aspartic Acid Protease 3; DNA: Deoxyribose Nucleic Acid.

Gene ontology and KEGG enrichment analysis

The search to resolve the complex web of regulatory mechanisms orchestrating the RAS/MAPK signaling pathway during Colorectal Cancer (CRC) tumorigenesis, subnetwork analysis emerges as a powerful tool. By predicting critical pathways and pivotal processes in the interaction network of hub proteins, we aim to explains on the nuanced interplay of microRNAs (miRNAs) within this dynamic cellular framework. To discern the significance of miRNA-targeted genes, we subjected hub protein interactions to comprehensive subnetwork analysis. The exploration of these complex interactions unveils a panoramic view of the molecular choreography governing CRC progression.

Gene Ontology (GO) analysis unveiled a spectrum of 870 Biological Processes (BPs), encompassing diverse cellular regulatory mechanisms. These included metabolic processes, gene expression regulation and both positive and negative modulation of cellular processes. Notably, the regulatory influence extended beyond the nucleus, as evidenced by enrichment in various cell organelles such as intracellular organelles, cytosol, membrane-bounded organelles and protein-containing complexes.

Within the Cellular Components (CCs), 53 entities were identified, with a predominant presence in the nucleus. However, the enrichment spanned across multiple cell organelles, highlighting the multifaceted localization of the involved proteins, including intracellular organelles, cytosol, membrane-bounded organelles and protein-containing complexes.

Delving into the Molecular Function (MF) category of gene ontology, a repertoire of 87 functions was uncovered. This encompassed a diverse set, ranging from chromatin binding and nucleic acid binding to DNA binding, enzyme binding, transcription factor binding and RNA polymerase II specific DNA binding. These findings provide a comprehensive perspective on the functional diversity of the molecular players involved in the complex regulatory landscape revealed by GO analysis.

DISCUSSION

Navigating complexity: Cluster analysis of protein networks resolve functional modules and network biomarkers in colorectal cancer

Cluster analysis of protein networks serves as a powerful tool to enhance our understanding of protein relationships and navigate the wealth of newly discovered protein sequences within databases, particularly in the post-genome era. The sheer volume of protein sequences added to databases has posed challenges in effectively characterizing their structures and roles. In the dynamic landscape of cellular processes, proteins often form complex network clusters, enabling precise control [15]. Identifying proteins within these functional clusters is of great significance (Table 6).

Biological network structures come to light through cluster analysis, a pivotal method for uncovering functional modules, predicting protein complexes and identifying network biomarkers. In this study, Cyto Cluster's algorithms were employed, offering versatility tailored to user needs. Six clustering techniques were applied, including IPCA, a density-based approach spotlighting dense subgraphs in protein interaction networks. IPCA determines edge weight by summing the common neighbors of two nodes linked by an edge. The weight of each node, calculated by adding weights of affected edges, designates the seed. IPCA generates clusters by iteratively adding nodes from neighbors based on node priority, commencing with the seed concept. Two criteria govern node inclusion in a cluster such as the likelihood of a node interacting with others and the shortest route between nodes within the cluster.

In this study, cluster analysis of the subnetwork revealed a total of 817 clusters, with our focus on selecting clusters ranked 1 through 5. The KEGG pathway enrichment of nodes unveils a predominant presence of various cancer types, particularly colorectal cancer, across most of these clusters. This observation robustly supports our research hypothesis, emphasizing the pivotal role of the map kinase pathway in the complex landscape of colorectal cancer development. Among the enriched pathways, the significance of glucose metabolism is prominently highlighted, aligning with its well-established importance for cancer cells. Additionally, a recurrent term in the subnetwork cluster analysis is the Advanced Glycation Endproducts/Receptor for Advanced Glycation Endproducts (AGE/RAGE) signaling pathway in diabetic complications.

AGE/RAGE signaling has been implicated in elevating oxidative stress, thereby promoting diabetes-mediated vascular calcification through NADPH Oxidase 1 (NOX 1) activation and reduced Superoxide Dismutase 1 (SOD 1) expression. This signaling pathway's involvement in diabetes-mediated vascular calcification is further associated with increased oxidative stress, triggering the phenotypic transition of vascular smooth muscle cells to osteoblast-like cells in AGEs-induced calcification. Notably, studies indicate that certain pharmacological agents and antioxidants have demonstrated efficacy in reducing calcium deposition in AGEs-induced diabetes-mediated vascular calcification [16].

Table 6: Top 5 CytoCluster results for Rat Sarcoma/Mitogen-Activated Protein Kinase (RAS/MAPK) signaling pathway targeted by dysregulated microRNA (miRNA) genes.

Cluster	Rank	Nodes	Edges	KEGG pathway enrichment of nodes
1	1	23	180	AGE/RAGE signaling pathway in diabetic complications, MAPK signaling pathway, PI3K/AKT signaling pathway, Pathways in cancer, Colorectal cancer
2	2	23	175	PI3K/AKT signaling pathway, Pathways in cancer, AGE/RAGE signaling pathway in diabetic complications, Breast cancer, Colorectal cancer
3	3	23	175	AGE/RAGE signaling pathway in diabetic complications, Pathways in cancer, PI3K/AKT signaling pathway, MAPK signaling pathway, Ras signaling pathway

4	4	23	177	PI3K/AKT signaling pathway, Pathways in cancer, AGE/RAGE signaling pathway in diabetic complications, Breast cancer, Colorectal cancer
5	5	23	168	AGE/RAGE signaling pathway in diabetic complications, MAPK signaling pathway, PI3K/AKT signaling pathway, Pathways in cancer, Colorectal cancer, Hepatitis B

Note: KEGG: Kyoto Encyclopedia of Genes and Genomes; AGE/RAGE: Advanced Glycation Endproducts/Receptor for Advanced Glycation Endproducts; MAPK: Mitogen Activated Protein Kinase; PI3K/AKT: Phosphatidylinositol 3-Kinase/Protein Kinase B.

Decoding the regulatory symphony: Insights from hub gene promoter motif analysis in colorectal cancer

In the exploration of hub gene promoter motifs, we conducted an analysis of the Upstream Flanking Regions (UFRs) spanning 1 kbp. The Ensemble database provided these UFRs for retrieval. Employing MEME, we identified seven and nine significant motifs targeted by downregulated and upregulated miRNAs, respectively, with lengths ranging from 15 to 22 base pairs.

A comprehensive study using GOMo delved into the Transcription Factor (TF) motifs, encompassing Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) aspects. The results revealed a spectrum of biological roles. GO analysis indicated that Cis Regulatory Elements (CREs) targeted by downregulated miRNAs play important roles in signal transduction, negative regulation of neuron apoptosis, negative regulation of signal transduction and ion transduction.

Promoter analysis exhibit that while most of these regulatory elements are situated in the nucleus, some also extend to the nucleolus, cytosol and dendrites. Biological process terms enriched in the promoter elements included negative regulation of signal transduction, anterior/posterior pattern formation, negative regulation of neuron apoptosis, potassium ion transport and neuron fate commitment. Molecular function terms enriched in the promoter elements encompassed protein heterodimerization activity, protein homodimerization activity, Adenosine Triphosphate (ATP) binding, potassium ion binding, chromatin binding and chromatin binding (Table 4).

Notably, the enrichment of terms such as anterior/posterior pattern formation is intriguing, referring to a regionalization process determining specific areas of cell differentiation along the anterior-posterior axis, influencing cell polarity. Disruptions in cell polarity have been implicated in various cancers. Additionally, terms related to negative regulation of transcription and signal transduction suggest potential implications in increased protein production and cell proliferation [17].

CONCLUSION

In solving the complex blend of colorectal cancer through an integrative systems biology approach, our study has explained on the regulatory complex orchestrated by miRNAs targeting the RAS/MAPK signalling pathway. Through bioinformatics tools, cluster analysis and promoter motif exploration, we uncovered key players, pathways and regulatory elements influencing colorectal cancer development. The identification of hub genes, enriched pathways and regulatory motifs not only strengthens our understanding of the molecular landscape but also highlights potential diagnostic and therapeutic targets. As we navigate the complex network of molecular interactions, this study contributes to the ongoing endeavour to decipher the underlying mechanisms of colorectal cancer, offering valuable insights for future research and clinical applications.

In addition to creating a general picture of how the network of cellular genes is involved by miRNAs targeting the RAS/MAPK signaling pathway, this study introduces important targets in this network that, if inhibited simultaneously, can effective in inhibiting the growth of cancer cells.

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