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RESEARCH

Prioritization of prognostic biomarkers regulated by calorie restriction in colon cancer through integrated biosignature analysis

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Abstract

Colorectal cancer (CRC) remains a critical global health challenge, ranking second in cancer-related mortality and third in cancer incidence as of 2018, with risk increasing with age. Addressing its rising burden requires early diagnosis, prognostic biomarkers, and effective therapeutic strategies. Emerging evidence suggests that calorie restriction may mitigate aging-related functional decline and influence CRC progression, yet the molecular markers and mechanisms remain poorly understood. In this study, we analyzed the GSE24432 dataset, using multiple computational databases to screen differentially expressed genes (DEGs) associated with calorie restriction in CRC. Functional annotations, including Gene Ontology (GO), KEGG pathway analysis, and gene set enrichment analysis (GSEA), were undertaken to explore potential underlying mechanisms and pathways in CRC pathogenesis. Kaplan Meier and Cox proportional hazards regression analyses were conducted to establish the diagnostic and prognostic significance of the hub genes. The validation test was conducted via multiple databases. Our investigation identified 50 DEGs, using the cutoff criteria, $p.\,\text{adj} < 0.05$, $|\log2FC| > 0.3$. GO and functional analysis results revealed extensive crosstalk of cellular and molecular components and pathways associated with mRNA and ribosome biogenesis, AMPK signaling, and p53 signaling pathway following calorie restriction. To understand how these DEGs drive biological reactions, we sorted the genes according to gene score > 3 and GO term > 3 and obtained 14 DEGs most relevant to the GO terms. Further analysis with GO CHORD showed that most genes are enriched in ribosome biogenesis and protein synthesis. Gene set enrichment analysis (GSEA) revealed the involvement of the hub genes in several hallmarks, such as tissue invasion and metastasis ($p < 0.001$), tumor-promoting inflammation ($p < 0.001$), resisting cell death ($p < 0.01$), and replicative immortality ($p < 0.05$). Survival analysis showed that higher expression of 7 hub genes, *CDKN2A* ($p < 0.05$), RPL9 ($p < 0.02$), *TUBB6* ($p < 0.01$), and *RPS15A* ($p < 0.01$), and lower expression of *CDKN1B* ($p < 0.01$), *NPM1* ($p < 0.01$), and *RALA* ($p < 0.01$), correlated to shorter survival of colon cancer. However, cross-reference of these genes revealed that calorie restriction decreased the expressions of *CDKN2A* and *TUBB6* while *CDKN1B* and *NPM1* were increased ($p < 0.05$). Several validation tests from multiple databases showed that high *CDKN2A* is associated with shorter overall survival rates, indicating *CDKN2A* is a therapeutic target and could serve as a more reliable biomarker for CRC prognosis. These findings could potentially facilitate the development of precision-based energy restriction interventions for CRC management, offering promising prospects for targeted therapeutic strategies for CRC patients.

Keywords Calorie restriction · Colorectal cancer · CDKN2A · Prognosis · Biomarker · Obesity

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Abbreviations

CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
COAD	Colorectal adenocarcinoma
NPM1	Nucleophosmin 1
TUBB6	Tubulin beta 6 class V
RPL5	Ribosomal protein L5
CRC	Colorectal cancer
AMPK	5′ Adenosine monophosphate-activated protein kinase
SREBP	Sterol regulatory element-binding protein
SIRT1	Sirtuin 1
PI3K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
IGF	Insulin growth factor
MCC	Maximal clique centrality
LCD	Low-calorie diet
PD-1	Programmed cell death-1
RFS	Relapse-free survival

Introduction

McCay et al. [1] first demonstrated that calorie restriction initiated post-puberty in rats significantly increased both median and maximum life span while simultaneously attenuating the progression or severity of chronic pathologies. This pioneering study provided foundational evidence for dietary intervention's potential long-term physiological impacts during post-pubertal development. Subsequent studies have systematically demonstrated that calorie restriction, which is defined as a sustained 20–50% reduction in daily energy intake without inducing starvation, slows down aging and extends the maximum lifespan of various species, including unicellular organisms (yeast), invertebrates (nematodes, Drosophila) and vertebrates (fish, rodents) [2–4]. Throughout human history, prolonged periods without food posed a significant threat to survival. In response to food deprivation, the body undergoes a series of metabolic adaptations to optimize energy utilization and sustain vital functions [5].

Although dietary treatments have been the subject of much research, calorie restriction is the basis of obesity management. A recent study on approaches to managing obesity in older adults [6] showed that caloric restriction was safe and beneficial by protecting against the molecular and cellular damage associated with functional decline in obesity and aging. Excess calories raise reactive oxygen species (ROS) formation and impair mitochondria, endoplasmic reticulum, and nucleus functions, such as telomere shortening and epigenesis alteration. This process accelerates cellular senescence by upregulating p16 (*CDKN2A*) and p21 (*CDKN1A*), leading to age-related functional decline [7]. In the Comprehensive Evaluation of Long-Term Effects of Reducing Intake of Energy (CALERIE™) phase 2 trial among healthy young-to-middle-aged persons, calorie restriction decreased several senescence biomarkers at 12 and 24 months [8].

Recent epidemiological evidence indicates obesity as a critical determinant of carcinogenesis, demonstrating associations with multiple malignancies [9]. Specifically, substantial human-based research has established obesity as a significant predisposing factor to neoplastic development in several organ systems, including endometrial, esophageal adenocarcinoma, gastric cardia, hepatic, renal, and colorectal tissues. Moreover, the global health impact is profound, with cancer ranking as the primary or secondary cause of premature mortality before the age of 70 in 112 of 183 countries [10].

Colorectal cancer (CRC) is the second most deadly cancer worldwide, with more than two-thirds of cases occurring at age 65 or older [11]. The five-year survival rate for patients with metastatic colorectal cancer is under 15%, which is a serious concern [12]. The propensity of CRC to metastasize to a variety of organs, including the liver, lungs, peritoneum, bones, and the nervous system, further complicates treatment options and increases mortality risk [13]. Recent studies have explored the feasibility of liver transplantation combined with chemotherapy as a treatment option for CRC metastasis to the liver [14, 15]. While this approach has demonstrated potential benefits, it does not guarantee complete recovery, as recurrence or metastasis to other sites, such as the lungs or lymph nodes, remains possible [14]. This highlights the need for a more systematic approach to identifying reliable prognostic markers for monitoring CRC progression and metastasis risk. This could ultimately enhance treatment strategies and improve patient outcomes.

An emerging therapeutic landscape for gastrointestinal (GI) tumors is evolving with increasing attention on systemic prognostic markers. One emerging biomarker is the neutrophil-to-eosinophil ratio (NER), a non-invasive marker associated with poorer overall survival and progression-free survival in cancer patients [16]. Immune checkpoint inhibitors such as anti-PD-1 and anti-PD-L1 therapies have also shown associations with increased relapse-free survival (RFS) across various cancer types, with no significant impact from gender and age disparities on treatment efficiency [17]. These advances highlight the importance of integrating prognostic and therapeutic biomarkers to refine treatment protocols and improve clinical outcomes for patients with CRC and other GI malignancies. Although calorie restriction has been posited to prevent or delay aging hallmarks, some reports suggest it promotes a proliferative-to-invasive phenotypic change in cancer cells due to starvation in the tumor microenvironment [18]. So far, no specific clinical trial has examined the effect of energy restriction diet on CRC. This indicates

the need for further research on key molecular markers of CRC likely affected by calorie restriction.

Understanding pathogenic mechanisms or evaluating therapy options for various diseases has benefited from identifying gene-specific expression patterns [19]. Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) is a comprehensive public repository for high-throughput gene expression comprised of gene profiles produced primarily utilizing DNA microarray technology. In recent years, microarray technology has been extensively used to probe gene expression in several tissues from experimental animals or humans. Next-generation sequencing technologies have advanced aging research by accelerating the evaluation of the genetic and epigenetic alterations accumulated by individual cells in an aging organism [20].

This study analyzed the transcriptomic signatures associated with obesity induced by calorie restriction during early adulthood through microarray and bioinformatic analysis. The aim is to understand better the molecular mechanisms linking caloric restriction to obesity management and the implications for CRC prognosis. The findings from this study have the potential to contribute to developing an evidence-based strategy for biomarker development, more effective dietary recommendations, interventions, and improved health outcomes for obesity and related diseases.

Materials and methods

Retrieval of datasets

The microarray dataset GSE24432 was acquired from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The microarray studies were searched systematically for calorie restriction intervention in *Homo sapiens*. The dataset was included according to the following eligibility criteria: (1) Containing at least 80 total samples; (2) Comparing before and after low-calorie dietary intake; (3) Raw data or gene expression profiles by array were available in GEO; (4) Significant weight loss following a low-calorie diet. The dataset was based on the GPL4133 platform comprising 40 women (aged between 20 and 50) who followed an 8-week low-calorie diet (LCD) and a 6-month weight maintenance phase. Participants were classified as weight maintainers (0–10% weight regain) and weight regainers (50–100%). Subcutaneous adipose tissue (scAT) biopsies were taken before and after LCD. GeneCards database (https://www.genecards.org/) was retrieved by searching "cellular aging" to explore the relevance between featured genes and low-calorie diet-induced transcriptome.

Screening of DEGs between datasets

To identify the differentially expressed genes (DEGs) in GSE24432, GEO2R was used to analyze the raw microarray data by using the cutoff criteria $p.\ \text{adj} < 0.05$, $|\log_2 FC| > 0.3$. Benjamin and Hochberg's method was utilized to regulate the false discovery rate (FDR). A relevance score > 1.35 was used to screen "cellular aging" genes on GeneCards. Subsequently, overlapping genes were identified using an online tool, Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/).

Gene ontology and KEGG pathway analysis

Database for annotation, visualization, and integrated discovery (DAVID) is the most common online biological information database for functional enrichment analysis and gene list annotation (https://davidbioinformatics.nih.gov/). The overlapping genes were further analyzed on SRplot and visualized on Chiplot using the set criteria of adjusted $p < 0.01$ [21]. The Gene Ontology (GO) CHORD combined the GO from biological process, cellular component, and molecular function using the $p < 0.01$ with additional criteria. All the genes were arranged and sorted according to total gene score > 3 and GOterm > 3.

Construction of protein–protein interaction (PPI) network

The functional interaction between proteins is necessary to understand molecular mechanisms. The database of the Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/) created the PPI network. This online platform allows the collection and integration of protein information from known interactions (curated databases and experimental determination), predicted interactions (gene neighborhood, gene fusions, and gene co-occurrence), and others like text mining, co-expression, and protein homology. A confidence score cutoff of ≥ 0.4 was chosen to perform the PPI network analysis on the overlapping DEGs screened after GO analysis. The Cytoscape software (version 3.10.3) and CytoHubba plugin (version 0.1) were utilized to visualize, identify, and rank the nodes from the PPI network based on the Maximal Clique Centrality (MCC) method (http://js.cytoscape.org). The MCC method was used to identify and retrieve top-ranked nodes. The MCC outperformed the other methods by capturing more essential high-degree and low-degree proteins. In Cytoscape, a node represents a gene or protein, while edges represent interactions.

Validation of hub genes

First, the drug–gene interaction database was utilized to identify likely drug–gene interactions for the hub genes. The

DGIdb (https://dgidb.org/) is a publicly accessible platform that integrates genes or gene products, drugs, and drug–gene interaction records to facilitate hypothesis generation and discovery and possible therapeutic benefits in interactions that might not otherwise be visible. Next, gene enrichment and overrepresentation analysis were performed to compare the distribution of the hub genes across different cancer hallmarks (www.cancerhallmarks.com). A robust analysis was conducted using KM plotter (https://www.kmplot.com/) to establish and validate the survival-associated genes in a new integrated database. The KM plotter platform allows for future analysis and validation of newly found gene expression-based biomarkers and signatures in diverse solid tumors and patient subgroups, including some that have not been studied before [22]. The colon cancer analysis subsystem was integrated into the Kaplan–Meier plotter to mine the entire database with the potential to identify and prioritize promising biomarkers and therapeutic targets in several solid tumors. Lastly, we analyzed the prognostic markers associated with colon adenocarcinoma from the University of Alabama at Birmingham Cancer Data Analysis Portal database (UALCAN) (https://ualcan.path.uab.edu/), the Gene Expression Profiling Interactive Analysis (GEPIA2) database (http://gepia2.cancer-pku.cn/#index) and TCGA PanCancer Atlas Studies (https://www.cbioportal.org/) to verify the identified potential prognostic markers.

Results and discussion

Differential expression analysis

The GSE24432 microarray dataset from 40 obese women was analyzed for this study. After processing with GEO2R, 664 genes were obtained with criteria DEGs adj. $p < 0.05$ and $|\log_2(FC)| > 0.3$, with a FC range of 0.244 to 1.816. "Cellular Ageing" on GeneCards yielded 1892 protein-coding genes with a relevance score > 1.35. Venny reveals 50 intersecting genes (Fig. 1).

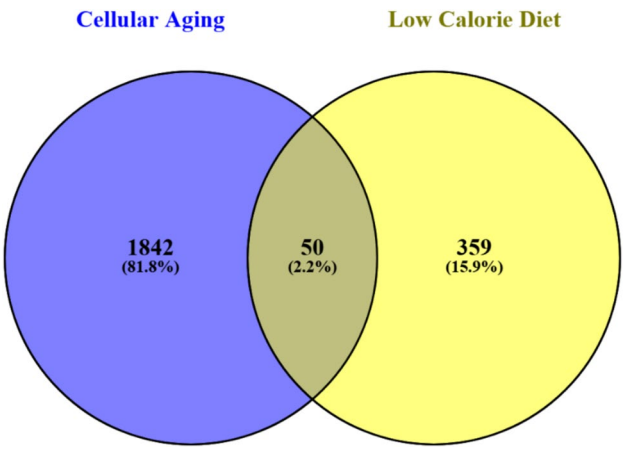

Fig. 1 Identification of differentially expressed genes (DEGs) of GSE24432 and cellular aging datasets

GO and KEGG pathway enrichment analysis of DEGs

To understand the biological functions of the 50 DEGs, we mapped the DEGs across different biological processes, cellular components, and molecular functions. The GO functional analysis was separated into three parts: Biological process (BP), cellular component (CC), and molecular function (MF). This provides a broader functional context of the DEGs from a mechanistic point of view based on the hierarchical structure of the GO terms. Based on the study, the DEGs enriched in BP are associated with processes such as translational initiation, SRP-dependent co-translational protein targeting to membrane, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, protein targeting to endoplasmic reticulum (ER), and establishment of protein localization to the ER (Fig. 2A). Regarding cellular components (CC), DEGs were primarily enriched in the cytosolic ribosome, ribosome, focal adhesion, cell-substrate junction, cytosolic large ribosomal subunit, and large ribosomal subunit (Fig. 2B). DEGs enriched in Molecular Function include structural constituent of the cytoskeleton, cyclin-dependent protein serine/threonine kinase regulator activity, structural constituent of ribosome, protein kinase regulator activity, GTPase activity, kinase regulator activity, 5S rRNA binding, cyclin-dependent protein serine/threonine kinase inhibitor activity, GTP binding, purine ribonucleoside binding, purine nucleoside binding, enzyme inhibitor activity, ubiquitin-protein transferase regulator activity, and ubiquitin-protein ligase binding (Fig. 2C). SRplot shows the combined GO from BP, CC, and MF by enrichment score (Fig. 3A) using the set criteria of $p < 0.01$. As we conducted the KEGG pathway analysis, we found that the DEGs are mainly enriched in Phagosome, Coronavirus disease, *Salmonella* infection, Cell cycle, ribosome, p53 signaling pathway, Chronic myeloid leukemia, Gap junction, Endocrine resistance, Bladder cancer, Motor proteins, Focal adhesion, Pathogenic *Escherichia coli* infection, AMPK signaling pathway, Melanoma, Glioma, and Pancreatic cancer. The 10 pathways with the corresponding genes are shown in Table 1.

The nutrient-sensing network, which includes extracellular ligands like insulins and IGFs, receptor tyrosine kinases with which they interact, and intracellular signaling cascades such as the PI3K-AKT and Ras-MEK-ERK pathways, and transcription factors FOXOs and E26, has been remarkably conserved throughout evolution [23]. Tyrosine kinase (TK) receptors bind extracellular growth factors like insulin or IGF1, activating mitogen signaling pathways via several downstream mediators, including PI3K, AKT, and

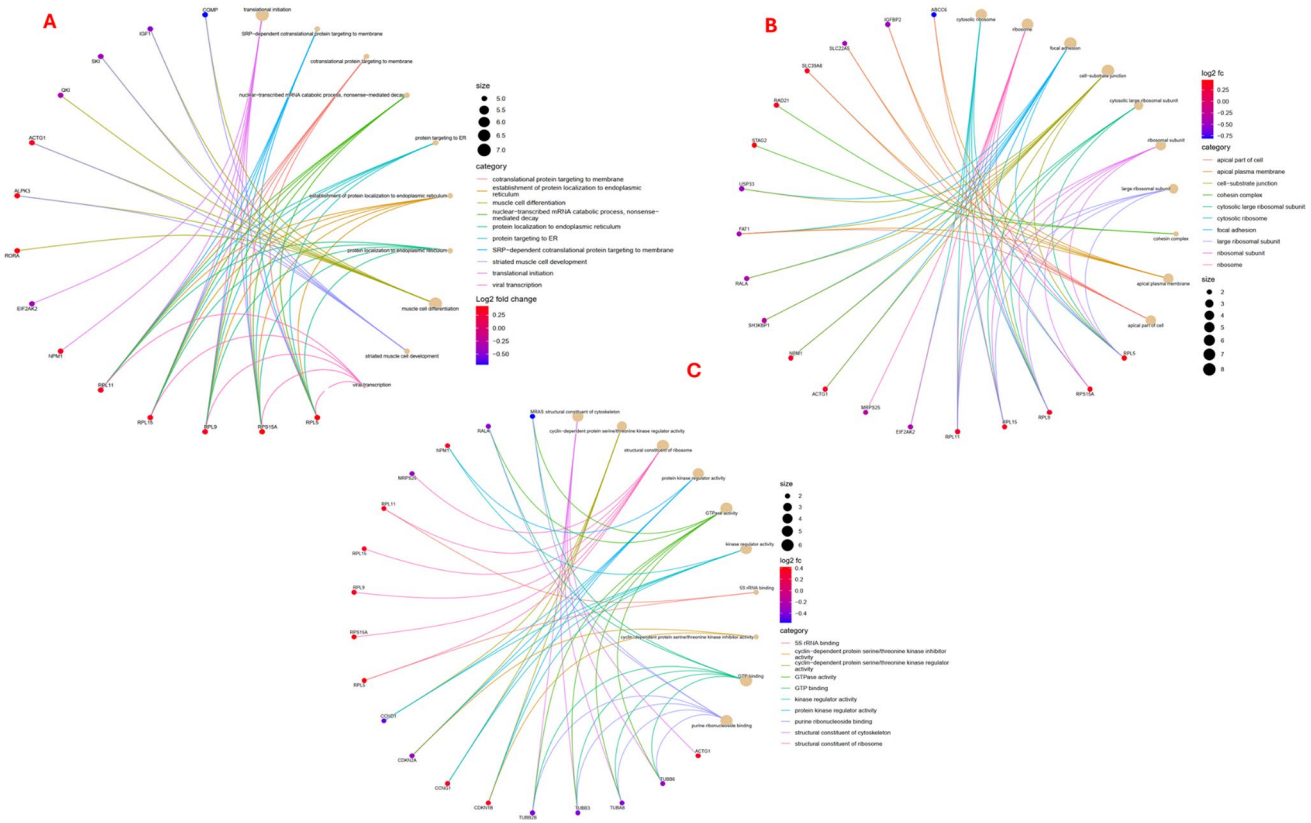

Fig. 2 Gene ontology analysis for biological process **A**, cellular components **B**, and molecular function **C**

the molecular machinery involved in protein translation and ribosome biogenesis (mTORC1, 4EBP1, S6K1, RPS6) [24]. The mechanistic target of rapamycin complex 1 (MTORC1) is responsive to various nutrients, stressors, and low energy conditions, thereby regulating the activity of numerous proteins, including transcription factors such as sterol regulatory element-binding protein (SREBP) and transcription factor EB (TFEB) [25]. This network is a principal regulator of cellular functions, encompassing autophagy, mRNA and ribosome biogenesis, protein synthesis, glucose metabolism, nucleotides, and lipids. Additionally, it plays a crucial role in mitochondrial biogenesis and proteasomal activity [26]. GO and functional analysis results revealed extensive crosstalk of cellular and molecular components and pathways associated with mRNA and ribosome biogenesis, AMPK signaling, and p53 signaling pathway after calorie restriction, as shown in Figs. 2 and 3A. AMPK and SIRT1 are essential nutrient sensors regulated by the ER, impacting cellular metabolism and energy homeostasis linked to cell proliferation, autophagy, and apoptosis [27]. While the PI3K/Akt pathway promotes mTOR-dependent cell growth and proliferation by stimulating nutrient uptake, the AMPK signaling pathway induces the p53-dependent stress response when energy levels are low [28]. Dietary restriction inhibits MTORC1, activates AMPK, SIRT1, and SIRT3, and increases adaptive cellular stress responses while suppressing the somatotrophic axis, extending longevity [29]. Given that cancer is linked to a metabolic state characterized by high energy demand, this implies that calorie restriction offers a possible cancer treatment.

PPI network

Protein–protein interaction (PPI) networks can be used to understand better the molecular and cellular mechanisms associated with disease onset and progression and identify therapeutic targets. To understand how these DEGs drive biological reactions, we sorted the genes according to gene score > 3 and GO term > 3 to show which DEGs are significantly enriched in BP, CC, and MF. We obtained 14 DEGs most relevant to the GO terms (Fig. 3B). The GO CHORD shows how the gene interacts with the different GO terms from BP, CC, and MF and is ordered based on their expression levels (log_2FC) to highlight upregulated or downregulated pathways. Thick ribbons connect to specific GO terms, indicating a high concentration of genes associated with a biological function. The GO CHORD showed that most genes are enriched in ribosome biogenesis and

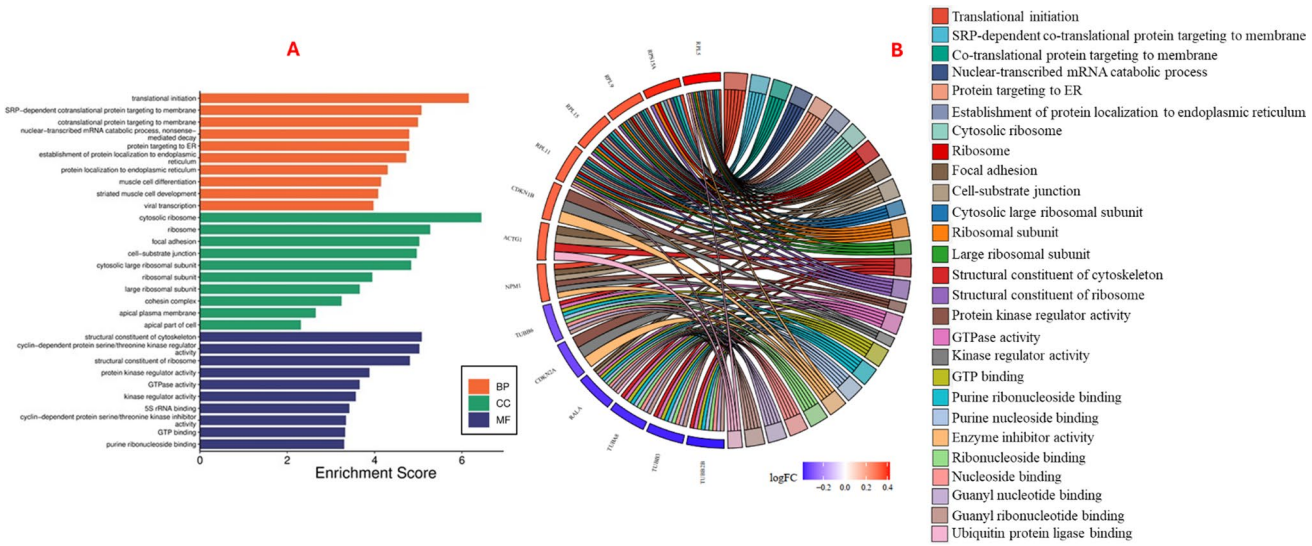

Fig. 3 GO result for three ontologies and functional analysis plot. **A** The horizontal plot of combined gene ontology and functional analysis from biological processes, cellular components, and molecular functions by enrichment score. **B** GO CHORD shows the relationship between GO term and 14 DEGs, which meet the cutoff criteria: $p < 0.01$, gene score ≥ 3, and GOterm ≥ 3. The DEGs are ordered based on their expression levels ($\log_2$FC) to highlight upregulated or downregulated pathways. Thick ribbons connect to specific GO terms, indicating a high concentration of genes associated with a biological function. The DEGs correspond to the combined gene ontology and functional analysis in **B** as follows: Ribosomal Protein, *RPL*; Nucleophosmin, *NPM1*; Tubulin Beta 6 Class V, *TUBB6*; Ribosomal Protein S15a, *RPS15A*; Tubulin Alpha 8, TUBA8; Tubulin Beta 2B Class IIb, *TUBB2B*; Cyclin-dependent Kinase Inhibitor 2A, *CDKN2A*; Actin Gamma 1, *ACTG1*; Tubulin Beta 3 Class III, *TUBB3*; RAS Like Proto-Oncogene A, *RALA*; Cyclin Dependent Kinase Inhibitor 1B, *CDKN1B*

Table 1 KEGG pathway analysis of overlapping DEGs (adj. $p < 0.05$) was considered significantly enriched

ID	Description	*p*. adj	Gene
hsa04145	Phagosome	0.009598295	*ACTG1/TUBB6/TUBA8/TUBB3/TUBB2B/COMP*
hsa05171	Coronavirus disease—COVID-19	0.014418485	*RPL5/RPS15A/RPL9/RPL15/RPL11/EIF2AK2*
hsa05132	*Salmonella* infection	0.014418485	*ACTG1/TUBB6/RALA/TUBA8/TUBB3/TUBB2B*
hsa04110	Cell cycle	0.014418485	*STAG2/CDKN1B/RAD21/CDKN2A/CCND1*
hsa03010	Ribosome	0.014418485	*RPL5/RPS15A/RPL9/RPL15/RPL11*
hsa04115	p53 signaling pathway	0.014418485	*CCNG1/CDKN2A/CCND1/IGF1*
hsa05220	Chronic myeloid leukemia	0.014418485	*RUNX1/CDKN1B/CDKN2A/CCND1*
hsa04540	Gap junction	0.014418485	*TUBB6/TUBA8/TUBB3/TUBB2B*
hsa01522	Endocrine resistance	0.014418485	*CDKN1B/CDKN2A/CCND1/IGF1*
hsa05219	Bladder cancer	0.014418485	*DAPK2/CDKN2A/CCND1*
hsa04814	Motor proteins	0.022212302	*ACTG1/TUBB6/TUBA8/TUBB3/TUBB2B*
hsa04510	Focal adhesion	0.022212302	*ACTG1/CCND1/IGF1/LAMB3/COMP*
hsa05130	Pathogenic *Escherichia coli* infection	0.022212302	*ACTG1/TUBB6/TUBA8/TUBB3/TUBB2B*
hsa04152	AMPK signaling pathway	0.022212302	*CCND1/IGF1/LEP/SREBF1*
hsa05218	Melanoma	0.041372899	*CDKN2A/CCND1/IGF1*
hsa05214	Glioma	0.042403339	*CDKN2A/CCND1/IGF1*
hsa05212	Pancreatic cancer	0.042403339	*CDKN2A/RALA/CCND1*

protein synthesis. Next, the 14 DEGs were submitted to STRING.db to generate functional and specific interactions between the proteins. The number of edges generated was 30, the average node degree was 4.29, and the average local clustering coefficient was 0.698. The PPI enrichment value was $p = 2.61 \times 10^{-7}$, showing that the nodes have more interactions among themselves than what would have been expected for a random set of proteins

of the same size and degree distribution drawn from the genome. This indicates that the proteins are biologically connected (Fig. 4A). *CytoHubba* ranked the proteins based on their importance within the interactive PPI network. Using the MCC method to increase sensitivity and specificity, the proteins were ranked as follows: RPL5 and RPL11 = 1, RPL9 and RPL15 = 3, NPM1 = 5, RPS15A = 6, TUBB6 = 7, TUBA8 and TUBB2B = 8, CDKN2A = 10, ACTG = 11, TUBB3 = 12, RALA = 13 and CDKN1B = 14 (Fig. 4B). The result revealed that ribosomal proteins are highly ranked in the network, which suggests their involvement in the molecular mechanisms associated with calorie restriction. Furthermore, we observed upregulated expressions of *RPS15A*, *RPL15*, *RPL11*, *RPL9*, and *RPL5,* as well as *CDKN1B* and *NPM1,* while *CDKN2A* was downregulated by calorie restriction (Fig. 4C). These ribosomal genes encode proteins that are essential to ribosome synthesis. Ribosomes are crucial in protein synthesis, cell survival, growth, and proliferation. Nutrient-sensing pathways, such as insulin/IGF signaling, regulate ribosome biogenesis by stimulating rRNA transcription in the nucleolus. A modest reduction of these nutrient-sensing pathways extends lifespan across species, thereby linking ribosome biogenesis to aging [30]. Dysregulation of ribosome biogenesis at different stages is linked to cell cycle arrest, senescence, or apoptosis by impairing the expression of ribosomal proteins, leading to age-related degenerative diseases like cancer [31]. The NPM is another multifunctional protein whose depletion impairs ribosome biogenesis at multiple levels [32]. Our result showed that following calorie restriction intervention, expressions of *NPM1* and other ribosomal protein genes, *RPS15A*, *RPL15*, *RPL11*, *RPL9*, and *RPL5*, showed consistent patterns of elevated expression (Fig. 4C). This agrees with the PPI network in Fig. 4A, showing the co-expression of NPM1 with the ribosomal proteins and suggesting potential regulatory function, association, and tissue-specific processes. As a histone chaperone that can stimulate rRNA transcription, NPM has been implicated in numerous cellular processes such as centrosome duplication, cell cycle regulation and genome stability maintenance [33]. The NPM exhibits a bidirectional role in apoptosis, which frequently vary according on the circumstances. Alterations in the *NPM1* have been reported in numerous hematological malignancies [34]. The CDKN2A promotes NPM degradation, inhibiting ribosome biogenesis [35] (Fig. 4B). Moreover, in cases where NPM is upregulated, it is reported to be an attractive target for cancer therapy [31]. Our findings suggest that calorie restriction may regulate several molecular mechanisms involved in protein synthesis to increase life span and mitigate chronic disease by downregulating ribosome biogenesis and protein translation, regulating global proteostasis, and reducing disease severity.

Validation of hub genes and identification of prognostic biomarkers for colon cancer

To determine which of the DEGs genes could serve as a prognostic biomarker for colon cancer, we run the hub

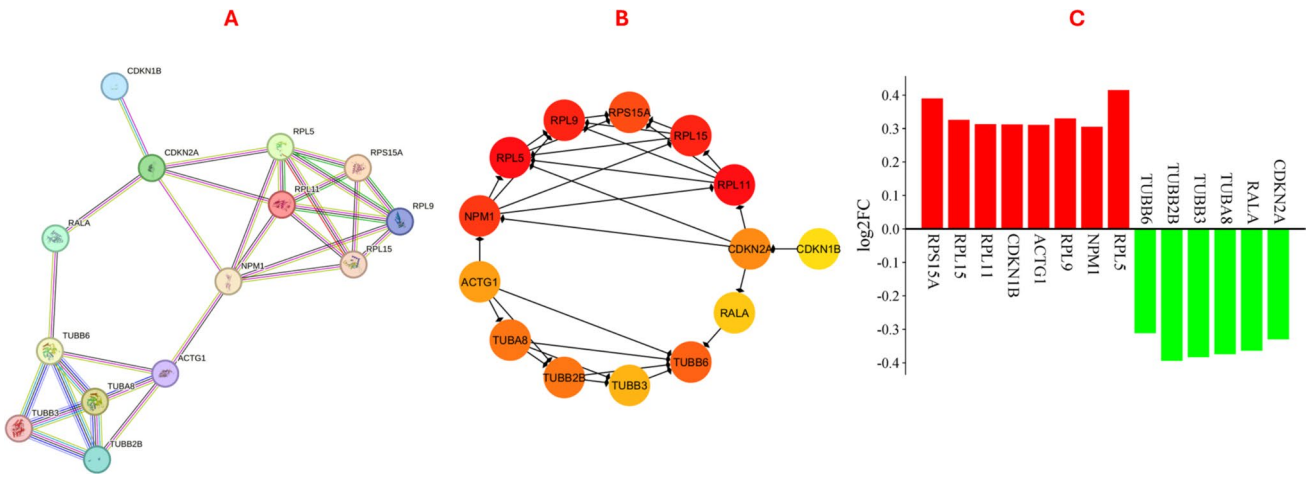

Fig. 4 PPI network from STRING.db **A** and Cytoscape **B** and differential expressions of DEGs represented by log_2FC values **C**. The *CytoHubba* plugin identifies and ranks the nodes from the PPI network based on the Maximal Clique Centrality (MCC) method. Rank as follows: RPL5, 1; RPL11, 1; RPL9, 3; RPL15, 3; NPM1, 5; RPS15A, 6; TUBB6, 7; TUBA8, 8; TUBB2B, 8; CDKN2A, 10; ACTG1, 11; TUBB3, 12; RALA, 13; CDKN1B, 14. Ribosomal Protein, RPL; Nucleophosmin, NPM1; Tubulin Beta 6 Class V, TUBB6; Ribosomal Protein S15a, RPS15A; Tubulin Alpha 8, TUBA8; Tubulin Beta 2B Class IIb, TUBB2B; Cyclin-dependent Kinase Inhibitor 2A, CDKN2A; Actin Gamma 1, ACTG1; Tubulin Beta 3 Class III, TUBB3; RAS Like Proto-Oncogene A, RALA; Cyclin Dependent Kinase Inhibitor 1B, CDKN1B. PPI (**A**) color codes: from curated databases (aqua), experimentally determined (purple), gene neighborhood (green), gene fusions (red), gene co-occurrence (navy), text mining (yellow) co-expression (black), protein homology (maya)

genes through the drug–gene interaction database (DGIdb) to understand the gene druggability information and available drugs and therapeutics for the hub genes. We identified several FDA-approved drugs from DGIdb for the genes. This step revealed that profiled drugs associated with these genes are treatments for different types of cancer, as shown in Table 2, with various side effects such as anemia, ascites, alopecia, constipation, edema, and fertility issues in girls and women. Next, we compared the distribution of the hub genes across different cancer hallmarks. Gene set enrichment analysis (GSEA) revealed the involvement of the hub genes in several hallmarks, such as tissue invasion and metastasis ($p < 0.001$), tumor-promoting inflammation ($p < 0.001$), resisting cell death ($p < 0.01$), and replicative immortality ($p < 0.05$) (Fig. 5 and Table 3). To validate the hub genes and discover potential prognostic biomarkers, we utilized the Kaplan Meier analysis and Cox proportional hazards regression to assess the correlation between the genes and survival of colon cancer. The study included 1167 patients and was not restricted by gender, adjuvant chemotherapy, or molecular subtypes of CRC. Although most patients receive conventional treatments like chemotherapy, radiation, or surgery, adenocarcinoma formation involves multiple genes and pathways [36]. This highlights the need for a comprehensive biomarker discovery to identify molecules involved in disease progression. Our results showed that higher expression of 4 hub genes, *CDKN2A* (FDR < 5%), *RPL9* (FDR < 2%), *TUBB6* (FDR < 1%), and *RPS15A* (FDR < 1%), and lower expression of *CDKN1B* (FDR < 1%), *NPM1* (FDR < 1%), and *RALA* (FDR < 1%), correlated to shorter survival (Fig. 6). We cross-referenced these genes to the scAT expression of participants with obesity. We found that calorie restriction intervention decreased the expressions of *CDKN2A* and *TUBB6* but increased *CDKN1B* and *NPM1*, which implies their mechanistic involvement in linking obesity to colon cancer. Genome-wide association studies (GWAS) revealed the association of *CDKN2A* locus with obesity-related diseases, including epicardial adipose tissue development, gestational diabetes, rapid decline in beta cell function, diabetic nephropathy progression, and coronary heart disease [37]. Since colon adenocarcinoma (COAD) is a common age-related digestive system tumor associated with obesity, this may support *CDKN2A* as a prominent biomarker for different CRC conditions and may be significant for the prognosis of cancer predisposition during early adulthood. Our result agrees with a previous study that showed considerably higher levels of *CDKN2A* expression in CRC tissues than in normal tissues, and elevated expression of *CDKN2A* was associated with poor CRC prognosis [38]. A similar experiment also revealed that *CDKN2*A was highly expressed ($p < 0.001$) in COAD compared to normal tissues, and the survival time was shorter for high CDKN2A

Table 2 FDA-approved drugs associated with hub genes

Genes	FDA-approved drugs	Target	Ref
CDKN2A	Palbociclib	Breast cancer	NCT01740427
CDKN2A	Abemaciclib	Breast cancer	NCT02308020
NPM1	Venetoclax	Acute myeloid leukemia, Chronic lymphocytic leukemia	NCT01328626
CDKN2A	Ribociclib	Breast cancer	NCT01958021
NPM1	Ceritinib	Lung cancer	NCT01283516
NPM1	Midostaurin	Acute myeloid leukemia	NCT00651261
NPM1	Crizotinib	Lung cancer	NCT01154140
CDKN2A	Dabrafenib	Melanoma	NCT01597908
ACTG1	Plerixafor	Myeloma	NCT00103662
CDKN2A	Vemurafenib	Melanoma	NCT01006980
CDKN2A	Cobimetinib	Melanoma	NCT01689519
CDKN2A	Temozolomide	Glioblastoma	NCT00006353
CDKN2A	Everolimus	Renal cell carcinoma	NCT01668784
CDKN2A	Letrozole	Breast cancer	NCT01740427
CDKN2A	Cetuximab	Colorectal cancer	NCT00154102
CDKN2A	Panitumumab	Colorectal cancer	NCT00364013
TUBB3	Paclitaxel	Lung cancer	NCT00021060
NPM1	Daunorubicin liposomal	Acute myeloid leukemia	NCT01696084
TUBB3	Ixabepilone	Breast cancer	NCT000080301
TUBB3	Vinorelbine	Lung cancer	NCT01405079
CDKN2A	Carboplatin	Lung cancer	NCT02367781
CDKN2A	Gemcitabine	Pancreatic cancer	NCT00844649

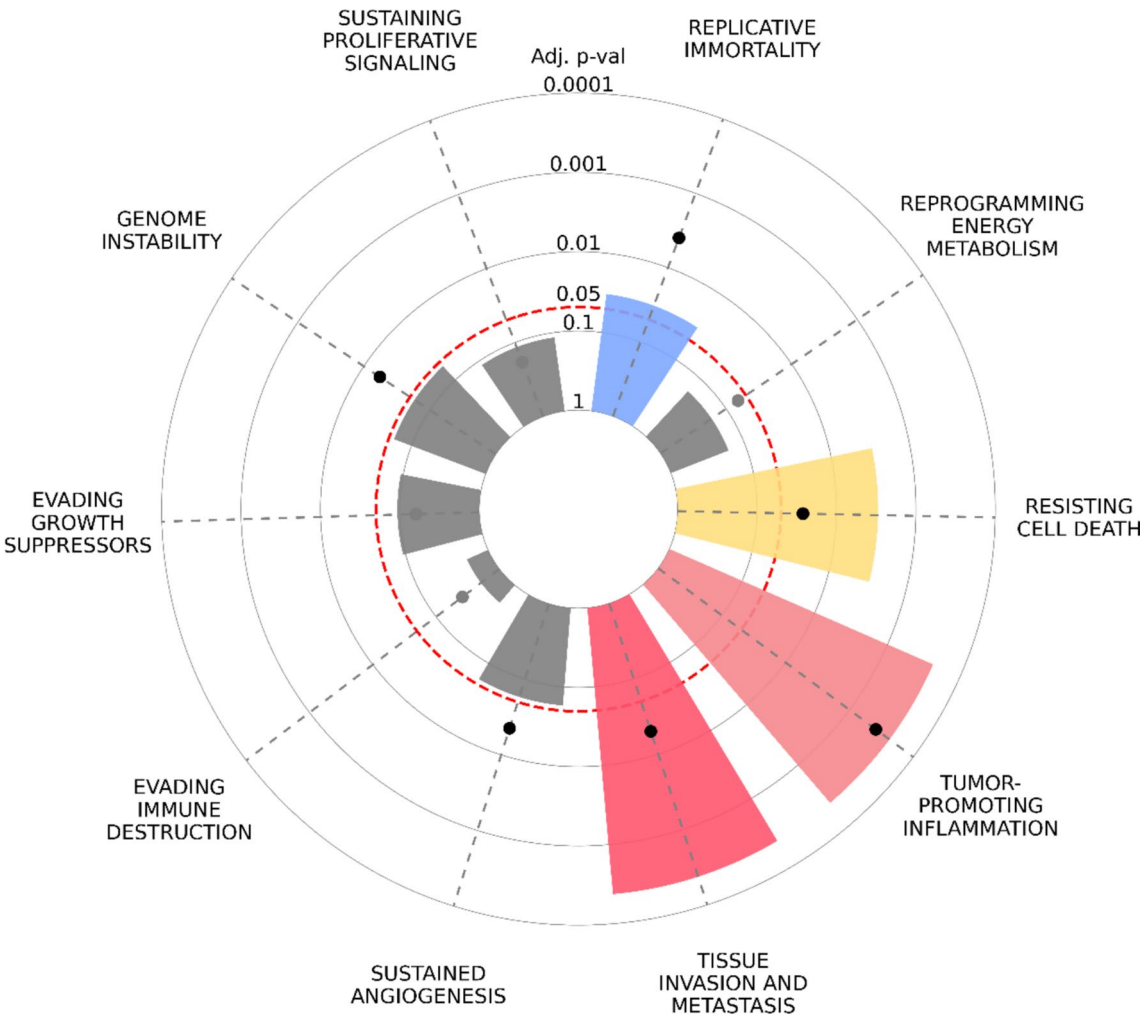

Fig. 5 Cancer hallmark enrichment plot shows the enrichment of the cancer hallmarks when compared to the integrated cancer hallmark gene set and the distribution of genes across the different hallmarks to each other. Significant hallmarks are colored ($p < 0.05$)

Table 3 Genes in Hallmark Genesets

Cancer hallmarks	Genes
Sustained angiogenesis	*CDKN1B, ACTG1, CDKN2A*
Tumor-promoting inflammation	*CDKN1B, TUBB6, TUBB3, TUBB2B, ACTG1, TUBA8*
Genome instability	*CDKN1B, NPM1, CDKN2A*
Sustaining proliferative signaling	*CDKN1B, RPL5, RPS15A, RALA, NPM1, ACTG1*
Evading immune destruction	*ACTG1*
Replicative immortality	*CDKN1B, NPM1, CDKN2A*
Resisting cell death	*CDKN1B, CDKN2A, RPL5, RALA, NPM1, ACTG1, TUBA8*
Evading growth suppressors	*CDKN1B, CDKN2A, RPL5, RALA, NPM1, ACTG1*
Reprogramming energy metabolism	*CDKN1B, NPM1*
Tissue invasion and metastasis	*CDKN1B, TUBB6, CDKN2A, TUBB3, TUBB2B, RALA, NPM1, ACTG1, TUBA8*

expression when compared to low expression [36]. We further validated the genes on the UALCAN Data Analysis Portal, cBioPortal, and GEPIA2 databases. Among the four genes (*CDKN2A*, *TUBB6*, *CDKN1B,* and *NPM1*) whose expressions were regulated by calorie restriction intervention, we found that only *CDKN2A* was consistent throughout

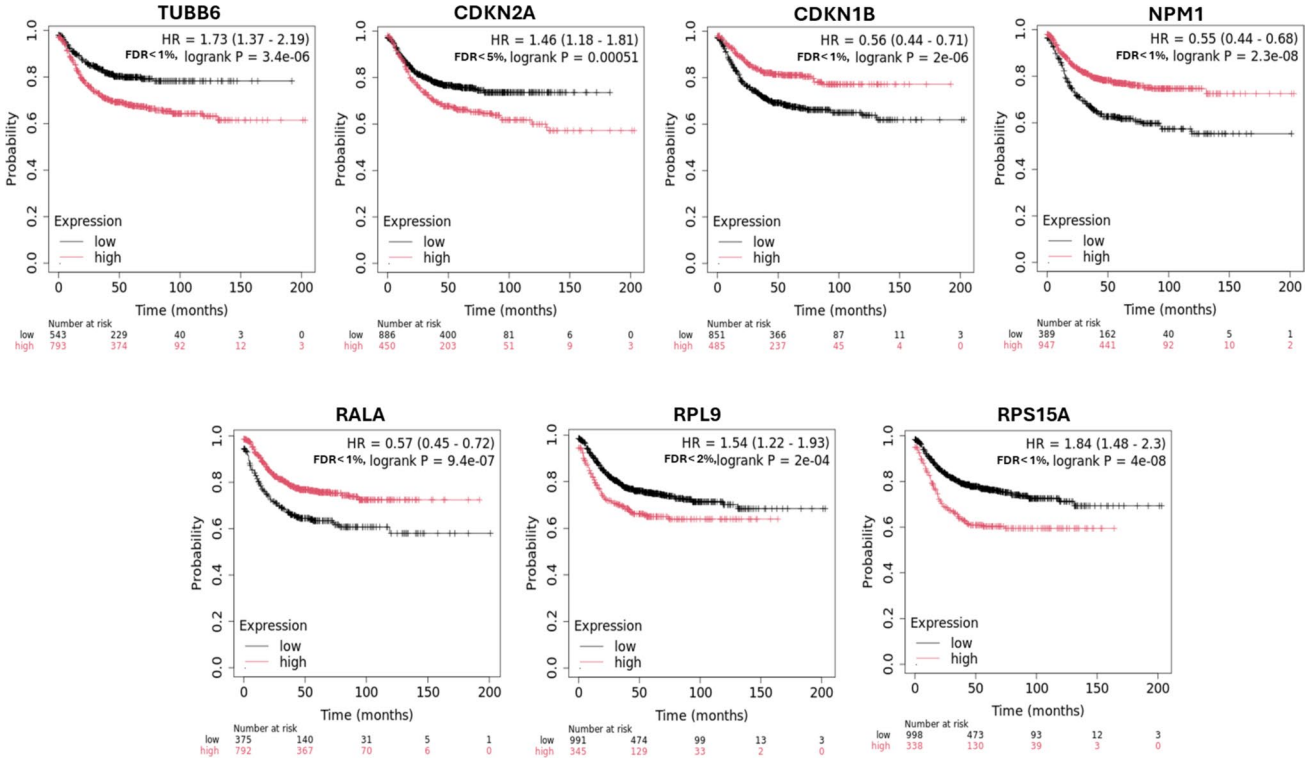

Fig. 6 Survival probability of colon cancer-associated genes *TUBB6*, *CDKN2A, CDKN1B*, *NPM1, RALA, RPL9,* and *RPS15A*. High expressions of 4 hub genes, *CDKN2A* (FDR < 5%), *RPL9* (FDR < 2%), *TUBB6* (FDR < 1%), and *RPS15A* (FDR < 1%), and low expressions of *CDKN1B* (FDR < 1%), *NPM1* (FDR < 1%), and *RALA* (FDR < 1%), correlated to shorter survival. The four genes *TUBB6*, *CDKN2A, CDKN1B,* and *NPM1* showed significant changes in gene expression ($p < 0.05$, $|\mathrm{Log_2FC}| > 0.3$) of participants with obesity after calorie restriction intervention compared to before. HR = hazard ratio, FDR = False Discovery Rate

multiple databases with high expression significantly associated ($p < 0.05$) with shorter survival in COAD patients. This highlights *CDKN2A* as a more reliable prognostic molecular marker for COAD and a target for calorie restriction intervention (Fig. 7). However, based on the functional annotations and PPI analysis (Figs. 3 and 4), CDKN2A may also act as a master regulator of other hub genes by promoting or repressing their activity, thereby regulating several downstream molecular mechanisms and biological processes.

The *CDKN2A* gene encodes multiple tumor suppressor 1 (*MTS1*), which belongs to the INK4 family [39]. p16INK4a inhibits CDK4/6 to prevent the phosphorylation of retinoblastoma protein, a tumor suppressor protein that promotes binding to transcription factor E2F and blocks G1 phase exit [37]. *CDKN2A* gene mutation results in cyclin D-CDK4 inhibition, resulting in abnormal cell proliferation [40], which explains the lower survival rates in CRC with increased *CDKN2A* levels. In addition, the INK4a-ARF locus encodes p16INK4a and p19ARF, which regulate Rb and p53, respectively. As a tumor suppressor protein, p53 mutations have been linked to ribosomal protein deletions (RPL5 and RPL11), increasing human cancer susceptibility [41]. Our study revealed that ribosomal protein genes are regulated by energy restriction intervention (Fig. 4C) and may be essential for p53 signaling by regulating cell stress response, inducing apoptosis, cell cycle arrest, or senescence. This agrees with a previous study which showed that energy restriction differentially regulated functional pathways like focal adhesion, apoptosis and p53 signaling (Mutch et al. 2011).

CDKN2A is a prototypical marker of cellular senescence. It is an independent prognostic factor that promotes the progression of CRC through epithelial–mesenchymal transition (EMT), a significant hallmark in tumor invasion and the process behind metastasis initiation that is involved in the apoptotic regulation of HT-29 cells [42]. The EMT has also been linked to beta-tubulin [43]. Tissue invasion and metastasis are among the cancer hallmarks associated with tubulin isotypes, according to Fig. 5 and Table 3. These isotypes regulate oncogenesis and possess prognostic influence in various solid tumors [44]. In bladder urothelial carcinoma cell lines, *TUBB6* depletion reduced cell migration and invasion [45]. Several other studies showed the correlation of *TUBB6* overexpression to tumor aggressiveness and shorter survival [46–48], as seen in Fig. 6. However, calorie restriction reduced the expression of *TUBB6* and other

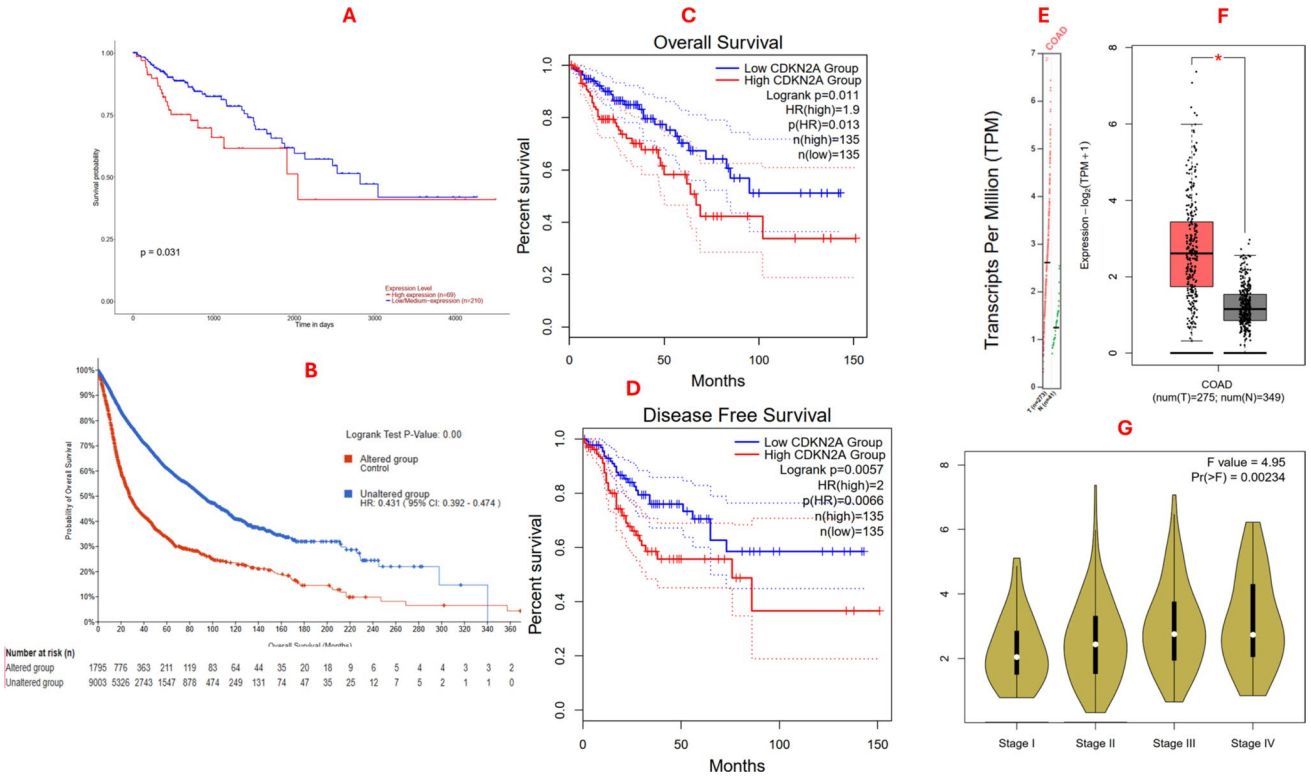

Fig. 7 Effect of *CDKN2A* expression level and prognostic significance analysis of COAD from UALCAN Data Analysis Portal **A**, cBioPortal **B**, and GEPIA2 databases **C** and **D**, gene expression profile **E** and boxplot **F** ($p < 0.01$) for normal (N) and tumor (T), and stage plot **G** ($p = 0.00234$). High expression of CDKN2A is associated with shorter survival in COAD patients. COAD = colon adenocarcinoma, HR = hazard ratio

tubulin isotypes (*TUBB2B*, *TUBB3*, and *TUBA8*) (Fig. 4C), which indicates that these genes could offer therapeutic targets for colon cancer by targeting specific cancer hallmarks like tumor-promoting inflammation, tissue invasion, and metastasis, resisting cell death and replicative immortality (Fig. 5). Because the overall survival rates of patients with high *CDKN2A* and *TUBB6* are significantly lower, our result suggests that in addition to *CDKN2A*, *TUBB6* influences patient survival and can be considered a prognostic molecular marker of colon cancer. Our result also showed that elevated expression of *NPM1* was accompanied by the downregulation of *CDKN2A* expression following calorie restriction intervention, as shown in Fig. 4C. A recent study revealed that overexpression of NPM1 inhibits CDKN2A and increases cell proliferation in esophageal squamous cell carcinoma [49]. Other studies have also demonstrated the elevated expression of NPM1 in cancers associated with obesity, including CRC [50], kidney cancer [51], and liver cancer [52]. However, a major study revealed that nuclear retention of *NPM1* increases *CDKN1A* and *CDKN2A* genes in acute myeloid leukemia [53]. This suggests that *NPM1* exhibits context-dependent regulation of transcription, acting either as a transcriptional coactivator or corepressor, and a substantial tumor cell line depends on the expression [54]. The differential expressions of *NPM1* and *CDKN2A* (Fig. 6) in colon cancer support the antagonistic effect of *NPM1* on *CDKN2A*, which may indicate that the overexpression of *NPM1* suppresses *CDKN2A* and limits its cell cycle functions.

Calorie restriction is well established as a longevity intervention in flies, rodents, and nonhuman primates, with a potential benefit in preventing malignancies and enhancing the efficacy of cancer treatments. However, to our knowledge, no single pharmacological agent has been developed to replicate all its benefits. This limitation stems from the incomplete understanding of the intricate molecular processes by which calorie restriction influences several biochemical pathways and regulates systemic metabolism. Several factors associated with calorie restriction intervention, including macronutrient sources, time of intake, optimal timing and duration, feeding schedule, inter-individual variability influenced by behavioral and environmental factors, and nutrigenetics, must be considered as significant variables that may have an impact on the outcomes. Preclinical models have provided valuable insights into how organisms adapt to fasting and how cancer cells respond to nutrient restriction. These discoveries have underscored calorie restriction as a feasible therapy for the prevention

of cancer and chemotherapy toxicity across multiple malignancies [55]. However, the prolonged latency period for CRC presents challenges in determining the optimal window for dietary intervention [56]. Additionally, the risk of cancer-related weight loss may affect patient compliance and recruitment for prospective clinical studies. Since calorie restriction can inhibit tumor progression by altering cancer cell energy metabolism, delaying disease advancement, and improving survival outcomes, it has the potential as a therapeutic adjunct through metabolic reprogramming, ultimately enhancing treatment efficacy and patient prognosis.

A major limitation of our study is the small sample size, which may reduce its generalizability with the broader population. Further, the statistical power of the analysis is diminished by the small sample size, which increases the likelihood of Type II errors (failure to detect actual effects) and, when significant results are obtained, may lead to an overestimation of effect sizes. Additionally, the small sample size hinders the study's ability to control confounding variables and reduces the generalizability of the findings. Future research should incorporate larger, more diverse cohorts to validate and expand these preliminary findings. Given the molecular heterogeneity of CRC and confounding factors such as age, tumor source, and size, these results should be interpreted with caution until validated through large-scale integrated analyses, in vivo studies, and clinical trials. However, a key strength of this study is its integration of multiple bioinformatics platforms for biomarker identification, enabling a rigorous stepwise comparison of existing and emerging databases. While colonoscopy remains the gold standard for CRC diagnosis, identifying non-invasive and more reliable early-stage diagnostic tools via biomarker discovery is essential for enhancing early detection and developing more personalized, effective treatment strategies.

Conclusion

This study demonstrates that high *CDKN2A* expression is associated with shorter survival in colorectal cancer (CRC) but is downregulated following calorie restriction intervention. These findings highlight the importance of leveraging multiple bioinformatics databases to enhance CRC prognosis prediction. Additionally, while *TUBB6* emerged as a potential therapeutic target, contradictory validation results from other databases underscore the need for more rigorous experimental research. Overall, our study contributes to prioritizing key genes and therapeutic targets for CRC biomarker discovery in the context of dietary interventions. It also emphasizes the need for further investigations to identify the specific mechanisms through which calorie restriction and other energy restriction strategies may confer therapeutic benefits in CRC treatment.

Author contributions All authors contributed to the study conception and design. OEA and JJK contributed to manuscript writing, software, validation, and resources. OEA and IDB contributed to data curation, review & editing. OEA and EEN contributed to validation and data interpretation. All authors reviewed the manuscript.

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Data availability The GSE24432 dataset was retrieved from the GEO database.

Declarations

Conflict of interest The authors declare no competing interests.

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