

BNIP3-Related Mitophagy-Associated Signaling in Cancer-Induced Myotube Atrophy: Modulation by 5-Fluorouracil and *In Vitro* Mechanistic Validation

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Background: Chemotherapy, an indispensable treatment for patients with colorectal cancer (CRC), often results in some adverse effects, such as sarcopenia. This study investigates the role of Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3)-related mitophagy signaling in tumor cell-mediated myotube atrophy and its modulation by 5-fluorouracil (5-FU)-induced alterations in tumor secretory phenotype *in vitro*. It aims to understand the causal relationship between chemotherapy and myotube atrophy in CRC.

Methods: Gene expression datasets (GSE142340, GSE153412, GSE167186) and The Cancer Genome Atlas (TCGA) data were evaluated using differential expression analysis and weighted gene co-expression network analysis (WGCNA) to identify key candidate genes linking CRC chemotherapy and sarcopenia. *In vitro*, C2C12 myotubes were exposed to conditioned medium from C26 colon cancer cells to establish a cancer-induced atrophy model. Myotube morphology was assessed by hematoxylin and eosin (H&E) staining. The expression of BNIP3, myotube atrophy-related proteins (MHC, MyoD, MuRF-1, Atrogin-1), and mitophagy regulators (PINK1, PRKN) was examined by western blotting. BNIP3 overexpression was performed to validate its functional role.

Results: BNIP3 was identified as a candidate gene shared by CRC chemotherapy-related transcriptome changes and the age-related sarcopenia dataset. *In vitro*, 5-FU significantly reduced C26 cell viability. In the cancer-induced myotube atrophy model, 5-FU treatment increased myotube diameter ($p < 0.05$), downregulated BNIP3 expression ($p < 0.01$), restored MHC and MyoD levels ($p < 0.001$), suppressed MuRF-1 and Atrogin-1 ($p < 0.001$), and was associated with reduced expression of the mitophagy-related proteins PINK1 and PRKN ($p < 0.01$). Rescue experiments using BNIP3 overexpression reversed these effects ($p < 0.01$), highlighting its functional role.

Conclusion: BNIP3 is a molecular node in tumor cell-mediated myotube atrophy through mitophagy-associated signaling *in vitro*. 5-FU attenuates cancer cell-conditioned medium-induced myotube atrophy via downregulation of BNIP3-related signaling, consistent with a tumor cell-conditioned medium-mediated effect, although residual 5-FU and direct effects cannot be fully excluded.

Keywords: CRC; chemotherapy; sarcopenia; weighted gene co-expression network analysis; BNIP3; mitophagy

Introduction

Colorectal cancer (CRC) comprises malignant epithelial tumors of the colon and rectum. Approximately 95% of CRCs are adenocarcinoma [1]. Research showed that CRC accounts for over 1.8 million new cases and approximately 881,000 deaths worldwide annually [2]. In recent years, the treatment methods for CRC have been continuously advanced, improving the survival rate of CRC, with a 5-year survival rate of 65% and a 10-year survival rate of 58% [3]. Among the available treatment modalities, chemotherapy remains an indispensable treatment for CRC, as it can eliminate residual cancer cells that cannot be de-

tected or completely removed during the surgical process, thereby improving postoperative survival [4]. However, the adverse effects of chemotherapy limit the clinical application of chemotherapy agents [5].

Sarcopenia is one of the adverse effects of chemotherapy, manifested as a loss of muscle mass and function, which can lead to adverse consequences such as falls, weakness, and death in patients [6]. Research revealed that 12%–60% of metastatic CRC patients also suffer from sarcopenia, which is related to malnutrition caused by cancer-induced metabolic disorders and the damage to skeletal muscles caused by chemotherapy drugs [7]. Research reported that CRC patients with sarcopenia have

a poorer 3-year postoperative survival compared to those without sarcopenia [8]. However, the relationship between chemotherapy and sarcopenia is not unequivocal. Clinical observations revealed that patients responding favorably to chemotherapy often exhibit weight gain, improved nutritional status, and preservation or restoration of skeletal muscle function [9,10]. Campelj et al. [11] administered 5-fluorouracil (5-FU) to male Balb/c mice and demonstrated that 5-FU did not affect body composition, skeletal muscle mass, or function. Collectively, these findings indicate that the causal relationship between chemotherapy and sarcopenia remains controversial, and elucidating this relationship is of great significance for improving the prognosis of CRC patients.

Weighted gene co-expression network analysis (WGCNA) is a widely used analytical method in bioinformatics for capturing complex relationships between genes and phenotypes [12]. The principle of WGCNA is to aggregate genes with similar expression patterns into a module, effectively facilitating the preliminary identification of target hub genes. The advantage of WGCNA compared to conventional differential expression analysis is that it can divide genes from multiple samples into modules based on their expression patterns, allowing subsequent analyses to be performed at the module level. This not only reduces computational complexity but also improves accuracy. Currently, WGCNA has been frequently used to investigate potential molecular targets for Sarcopenia and diseases, such as osteoporosis or neuroticism [13]. However, WGCNA has not been used to explore the relationship between Sarcopenia and CRC chemotherapy.

To address this gap, this study first screened chemotherapy-related differentially expressed genes (DEGs) using multiple datasets from public databases, followed by WGCNA analysis of sarcopenia transcriptomic data to identify potential key targets. Subsequently, *in vitro* experiments were performed using a myotube atrophy model induced by conditioned medium (CM) from CRC cells to investigate the effects of the chemotherapeutic agent 5-FU on cancer-induced myotube atrophy and the underlying molecular mechanisms. This study aims to provide new experimental evidence for understanding the pathogenesis of chemotherapy-associated sarcopenia in CRC.

Methods

Data Sources

For CRC analysis, two datasets, namely GSE142340 and GSE153412, were downloaded from Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>). The GSE142340 dataset contains a comparative analysis of chemotherapy for six CRC cell lines, involving the drugs Regorafenib, Selumetinib, Vemurafenib, Vatalanib, AZD-4547, and GDC-09945. GSE153412 contains a compar-

ative analysis of chemotherapy for three CRC cell lines, involving the drugs 5-FU and Uracil (control). In addition, clinical information and tumor tissue gene expression data of 166 rectum adenocarcinoma (READ) patients and 461 colon adenocarcinoma (COAD) patients were obtained from The Cancer Genome Atlas (TCGA) (<https://cancer.genome.nih.gov/>) and analyzed using the TCGAblinks package. The tumor transcriptome samples were grouped according to the chemotherapy status documented in the corresponding TCGA clinical records. For the analysis of sarcopenia, the GSE167186 dataset was used, which contains transcriptome data from lower-limb muscle biopsies from 72 young adults, elderly adults, and patients with sarcopenia. The data were normalized using the edgeR package.

Data Processing and Quality Control

Each dataset was processed and analyzed independently using platform-appropriate pipelines to minimize cross-platform batch effects. For microarray datasets (GSE142340, GSE153412, GSE167186), raw expression data were background-corrected, log₂-transformed, and quantile-normalized using the limma or edgeR packages. For TCGA RNA-seq datasets (TCGA-READ, TCGA-COAD), raw count data were normalized using the trimmed mean of M-values (TMM) method via the edgeR package as implemented in TCGAblinks. Differential expression analysis was performed independently for each dataset, and the resulting gene lists were intersected bioinformatically. Cross-dataset integration at the raw expression level was not performed due to platform heterogeneity (microarray vs. RNA-seq) and distinct experimental designs.

Identification of DEGs

The DEGs were identified using the limma package in R software (version 4.2.1, R Foundation for Statistical Computing, Vienna, Austria) with an adjusted *p*-value < 0.05 and |log₂ fold change (FC)| > 0.3.

Identification of WGCNA Co-Expression Modules and Functional Analysis

A co-expression module was established using the WGCNA package, and the soft threshold power and adjacency relationship were calculated by executing the pickSoftThreshold function. A topological overlap matrix was derived using adjacency matrix data, and a dynamic tree-cutting algorithm was used to identify gene modules. Modules related to sarcopenia were identified through correlation analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis were conducted using the R package clusterProfiler. The KEGG pathway was visualized through the pathview package.

Cell Culture

Murine colon carcinoma C26 cells (CBP60044, Cobioer, China) and murine skeletal muscle C2C12 myoblasts (CBP60252, Cobioer, China) were routinely maintained in their specific medium (CBP60044M/CBP60252M, Cobioer, China) at 37 °C in a humidified atmosphere containing 5% CO₂. Both cell lines were authenticated by short tandem repeat (STR) profiling and were regularly tested for mycoplasma contamination.

Preparation of Cancer Cell-CM

C26 cells were seeded at 70–80% confluence and treated with 13.62 μM 5-FU for 48 h. Following treatment, cells were washed twice with phosphate-buffered saline (PBS) to remove residual 5-FU, and subsequently incubated in fresh, drug-free serum-free medium for an additional 48 h to generate the CM. The culture supernatant was collected, centrifuged at 1000 ×g for 10 min to remove cell debris, and filtered through a 0.22 μm membrane. The resulting CM was stored at –80 °C for subsequent use.

Cell Viability Assay

C26 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and allowed to adhere overnight. Cells were then treated with various concentrations of 5-FU (0–25 μM, HY-90006, MedChemExpress, NJ, USA) for 48 h. Subsequently, 10 μL of Cell Counting Kit-8 (CCK-8, HY-K0301, MedChemExpress, NJ, USA) solution was added to each well, followed by incubation at 37 °C for 2 h. Absorbance at 450 nm was measured using a microplate reader (SpectraMax 190, MD, California, USA). The half-maximal inhibitory concentration (IC₅₀) was calculated by nonlinear regression analysis using GraphPad Prism software.

Plasmid Construction and Cell Transfection

The full-length coding sequence (CDS) of mouse Bnip3 (NM009760.3) was synthesized and subcloned into the pcDNA3.1 (+) mammalian expression vector (VectorBuilder, China) under the control of the CMV promoter. The negative control (NC) plasmid was the empty pcDNA3.1(+) vector without insert (VectorBuilder, China), which was transfected in parallel to control for vector backbone effects.

For transfection, C2C12 myoblasts were seeded in 6-well plates at a density of 2.5×10^5 cells per well and differentiated into myotubes over 5–6 days as described above. Fully differentiated myotubes were then transfected directly in the same plates using Lipo8000 transfection reagent (C0533, Beyotime, China) according to the manufacturer's protocol. Briefly, 2.5 μg of plasmid DNA and 5 μL of Lipo8000 reagent were diluted separately in 125 μL of serum-free Opti-MEM medium, mixed gently, and incubated at room temperature for 15 min to allow com-

plex formation. The DNA-lipid complexes were then added dropwise to each well containing 2 mL of differentiation medium. After 6 h of incubation at 37 °C, the transfection medium was replaced with fresh differentiation medium. Transfection efficiency and Bnip3 overexpression were validated 48 h post-transfection by quantitative real-time PCR (qRT-PCR), as described below.

Myotube Differentiation and Atrophy Induction

C2C12 myoblast differentiation was induced by switching to differentiation medium (DMEM with 2% horse serum) for 5–6 days, with medium renewal every other day as previously described [14]. Fully differentiated myotubes were then treated for 48 h with either normal differentiation medium (Blank group), C26 cell-CM mixed 1:1 with differentiation medium (Control group), or CM collected from C26 cells that had been pre-treated with 13.62 μM 5-FU for 48 h (5-FU group). For Bnip3 overexpression experiments, myotubes were transfected with pcDNA3.1-Bnip3 or NC plasmid as described above, followed by the same treatment protocol.

Hematoxylin and Eosin (H&E) Staining

After treatment, myotubes were fixed with 4% paraformaldehyde (E672002, Sangon, China) for 15 min at room temperature, washed with PBS, and stained with hematoxylin for 5 min followed by eosin for 2 min using an H&E kit (HY-K0315A, MedChemExpress, China). Images were captured using an inverted light microscope (KD-810A, KDYXBIO, China) at 200× magnification. Myotube diameter was measured at three randomly selected fields per well using ImageJ software.

Western Blotting

Total protein was extracted from treated C2C12 myotubes using RIPA Lysis Buffer (AP01L013, Life-iLab, China). Protein concentration was quantified using a BCA assay (YWB002, YFXBIO, China). Equal amounts of protein (30 μg per lane) were separated by SDS-PAGE (190916-40, YaJi Biological, China) and transferred to PVDF membranes (WJ001S, epizyme, China). After blocking with a dedicated buffer (AP36L118, Life-iLab, China) for 1 h at ambient temperature, the membranes were incubated overnight at 4 °C with primary antibodies (listed in Table 1). Following washes, the blots were reacted with HRP-conjugated secondary antibodies (Table 1). Chemiluminescent signals were developed using an ECL kit (YWB003, YFXBIO, China) and documented with a SynGene imaging system (G:BOX F3, UK). Band densities were analyzed using ImageJ software, and target protein levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

Table 1. Antibodies used in this study.

Name	Catalog	Dilution	Manufacturer
Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3)	ab109362	1/1000	abcam, UK
Major histocompatibility complex (MHC)	ab139365	1/1000	abcam, UK
Myoblast determination protein 1 (MyoD)	ab203383	1/1000	abcam, UK
Muscle RING-finger protein 1 (MuRF-1)	DF7187	1/1000	Affinity, USA
F-box protein 32 (Atrogin-1)	ab168372	1/1000	abcam, UK
PTEN-induced kinase 1 (PINK1)	DF7742	1/1000	Affinity, USA
Parkin RBR E3 ubiquitin protein ligase (PRKN)	AF0235	1/1000	Affinity, USA
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	ab8245	1/10,000	abcam, UK
Goat anti-rabbit	ab205718	1/2000	abcam, UK
Goat anti-mouse	ab205719	1/2000	abcam, UK

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from C2C12 myotubes using a standard RNA extraction kit (AG21023, Agbio, China), and cDNA was synthesized using a reverse transcription kit (A232, GenStar, China). Quantitative real-time PCR was carried out using a fluorescent quantitative detection system (HM-P32, Hengmei, China). The relative expression of BNIP3 mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal control. The following primer sequences were used: BNIP3 (forward: 5'-AGGCGTCTGACAACTTCCA-3', reverse: 5'-TTACAGATAACCCGCACGCA-3') and GAPDH (forward: 5'-AGGTCGGTGTGAACGGATTTG-3', reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'). All primers were designed using NCBI Primer-BLAST and synthesized by Sangon Biotech (Shanghai, China). Primer specificity was confirmed by melting curve analysis, and amplification efficiency was validated using standard curve analysis.

Statistical Analysis

All bioinformatics analyses in this study were conducted using R software (version 4.1.2). All experiments were performed at least three independent times. Data are presented as mean $\pm$ standard deviation (SD). Prior to parametric testing, normality of distribution was assessed using the Shapiro-Wilk test, and homogeneity of variance was evaluated using Levene's test. All data met the assumptions for parametric analysis. Multiple group comparisons were performed by one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant.

Results

Analysis of DEGs in CRC Cells Treated With Chemotherapy

To investigate the gene expression changes in CRC cells induced by chemotherapy, we used two datasets, GSE142340 and GSE153412. The former is a comparative analysis of chemotherapy for 6 CRC cell lines, while the latter is a comparative analysis of chemotherapy for 3

CRC cell lines. The DEGs in the two datasets are shown as UpSet diagrams in Fig. 1a,b. As shown in Fig. 1a, the intersection of six DEG sets comprised six common DEGs. In Fig. 1b, compared with 5-FU-sensitive cells, 5-FU-resistant cells showed almost no changes in gene transcription levels. Therefore, among the initial 1958 DEGs, DEGs derived from the 5-FU-resistant group in the GSE153412 dataset were excluded. The remaining identified in the 5-FU-sensitive cells yielded a final set of 1895 DEGs.

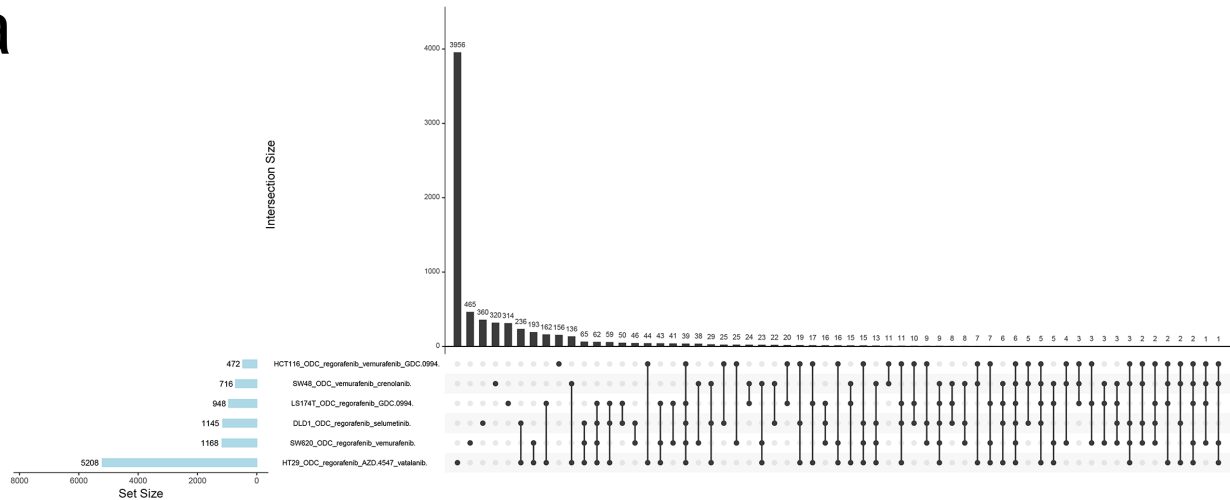
Analysis of Gene Expression in TCGA Clinical Samples With or Without Chemotherapy

Next, the impact of chemotherapy on CRC gene transcription at the individual level was explored. Transcriptome data and response clinical data were obtained from the TCGA database for READ and COAD. The TCGA-READ data included 166 patients, of which 40.4% have not received chemotherapy, 41% have received chemotherapy, and 18.7% have not reported their chemotherapy status (Fig. 2a). After excluding unreported data, the remaining data were divided into untreated and chemotherapy-treated groups, obtaining a total of 456 DEGs, as shown in Fig. 2b. The TCGA-COAD dataset included 461 patients, of whom 51.8% did not receive chemotherapy, 32.3% received chemotherapy, and chemotherapy status was not reported for 15.8% (Fig. 2c). Similarly, after excluding unreported data, the remaining data were divided into untreated and chemotherapy-treated groups, obtaining a total of 964 DEGs, as shown in Fig. 2d.

Gene Co-Expression Analysis of Sarcopenia

WGCNA was used to analyze sarcopenia and the control group to establish a co-expression network. The soft threshold power was determined to be 2, with relatively good mean connectivity (Fig. 3a). The data were clustered into 12 modules (Fig. 3b), and the correlations between each module and sarcopenia were calculated (Fig. 3c), identifying a total of 6460 genes belonging to significantly associated modules. At the same time, a total of 1956 genes were calculated for DEGs in the sarcopenia and control groups. Candidate hub genes were identified by select-

a



b

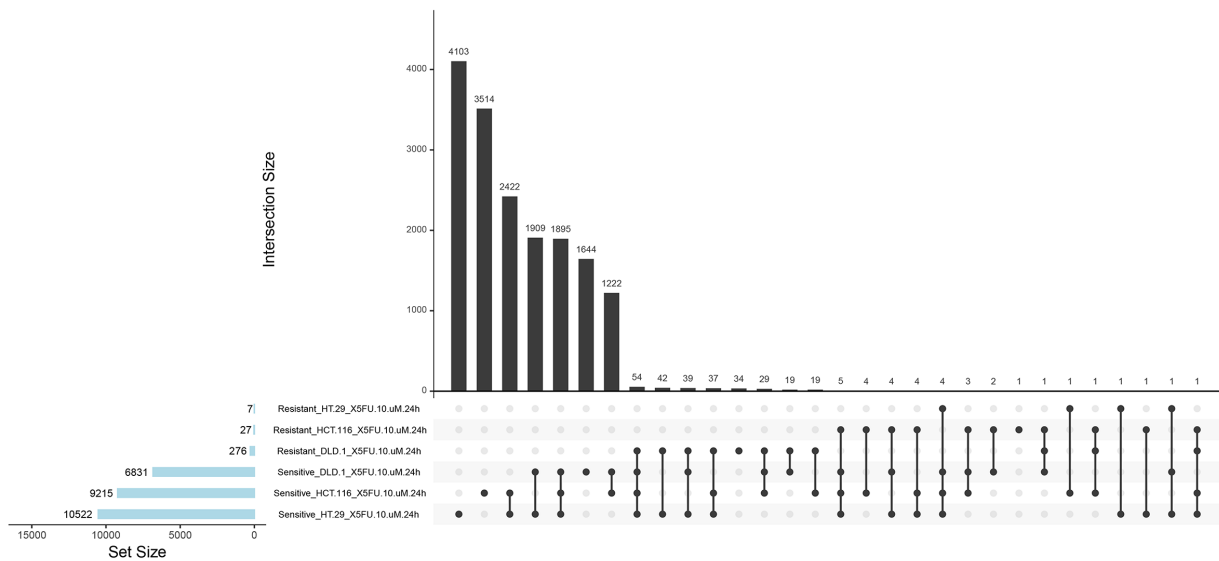


Fig. 1. DEGs obtained from two GEO datasets about colorectal chemotherapy. (a) UpSet diagram of DEGs from the GSE142340 dataset. (b) UpSet diagram of DEGs from the GSE153412 dataset. DEGs, differentially expressed genes.

ing genes with high module membership in the sarcopenia-associated module and high gene significance for sarcopenia status. A total of 1779 genes met both criteria (Fig. 3d) and were subsequently intersected with the sarcopenia DEGs.

Enrichment Analysis of Genes Related to CRC-Sarcopenia

The number of DEGs at the CRC cell and individual levels is shown in Fig. 4a, with a total of 3384 genes identified across datasets. After merging datasets and removing 187 duplicate genes, 3197 unique CRC DEGs were obtained for subsequent intersection analysis with sarcopenia-associated genes. The intersection of these 3197 CRC DEGs and 1779 sarcopenia hub genes resulted in 184 overlapping genes. (Fig. 4b). KEGG enrichment analysis showed that these 184 genes were enriched in autophagy- and mitophagy-related pathways (Fig. 4c), while GO en-

richment analysis also showed that these 184 genes were enriched in mitochondrial-related pathways, including mitochondrial protein processing, mitochondrial inner membrane and autophagosome (Fig. 4d). Fig. 4e shows the key genes of the autophagy pathway, with 8 genes (*REDD1*, *BNIP3*, *HMGB1*, *ATG9L*, *CTSL*, *HOPS*, *RAB7A* and Receptor) overlapping with 184 genes (shown in red).

Identification of *BNIP3* as a Shared Candidate Gene Across CRC Chemotherapy and Age-Related Sarcopenia Transcriptomes

Clinical validation of the six mitophagy-related genes (*ATF4*, *JUN*, *BNIP3*, *ATG9A*, *CALCOCO2/NDP52*, and *RAB7A*) across the three datasets revealed dataset-specific differential expression patterns (Fig. 5a). In the sarcopenia dataset, *BNIP3* ($p = 0.035$) and *JUN* ($p = 0.0051$) were significantly differentially expressed between the sarcopenia and control groups, whereas *ATF4* ($p = 0.6$), *ATG9A*

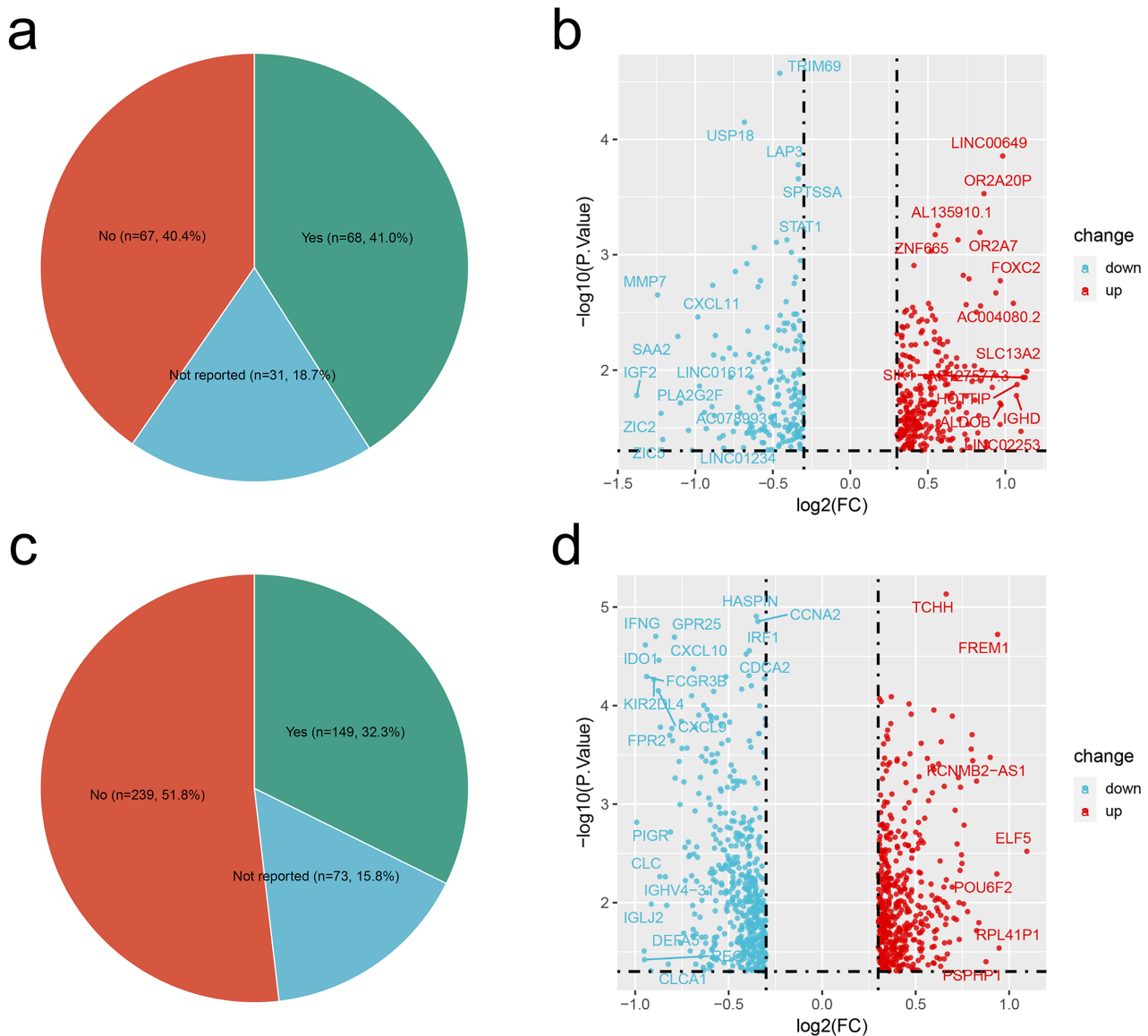


Fig. 2. DEGs obtained from two TCGA project datasets about colorectal chemotherapy. (a) Distribution of chemotherapy in patients with rectal adenocarcinoma ($n = 166$; yes, $n = 68$; no, $n = 67$; not reported, $n = 31$) in the TCGA database (TCGA-READ). Percentages: yes, 41.0%; no, 40.4%; not reported, 18.7%. (b) Volcano plot obtained by differential analysis of TCGA-READ patients' data between the non-chemotherapy group and chemotherapy group. (c) Distribution of chemotherapy in patients with colon adenocarcinoma ($n = 461$; yes, $n = 149$; no, $n = 239$; not reported, $n = 73$) in the TCGA database (TCGA-COAD). Percentages: yes, 32.3%; no, 51.8%; not reported, 15.8%. (d) Volcano plot obtained by differential analysis of TCGA-COAD patients' data between the non-chemotherapy group and chemotherapy group. Note: In panels (a) and (c), "yes" denotes patients who received chemotherapy, "no" denotes patients who did not receive chemotherapy, and "not reported" denotes patients with undocumented chemotherapy status in the TCGA clinical metadata. Patients in the "not reported" category were excluded from subsequent differential expression analysis. TCGA, The Cancer Genome Atlas; READ, rectum adenocarcinoma; COAD, colon adenocarcinoma.

($p = 0.059$), *CALCOCO2* ($p = 0.28$), and *RAB7A* ($p = 0.26$) showed no significant differences (Fig. 5b). In the TCGA-READ dataset, none of the six genes reached statistical significance for differential expression between chemotherapy-treated and untreated patients: *BNIP3* ($p = 0.26$), *JUN* ($p = 0.32$), *ATF4* ($p = 0.22$), *ATG9A* ($p = 0.45$), *CALCOCO2* ($p = 0.46$), and *RAB7A* ($p = 0.5$) (Fig. 5c).

In contrast, in the TCGA-COAD dataset, only *BNIP3* ($p = 0.018$) was significantly differentially expressed, while *JUN* ($p = 0.61$), *ATF4* ($p = 0.98$), *ATG9A* ($p = 0.64$), *CALCOCO2* ($p = 0.45$), and *RAB7A* ($p = 0.31$) did not show significant differential expression (Fig. 5d). Collectively, these results indicate that *BNIP3* is the only gene among the six mitophagy-related candidates that is consistently dif-

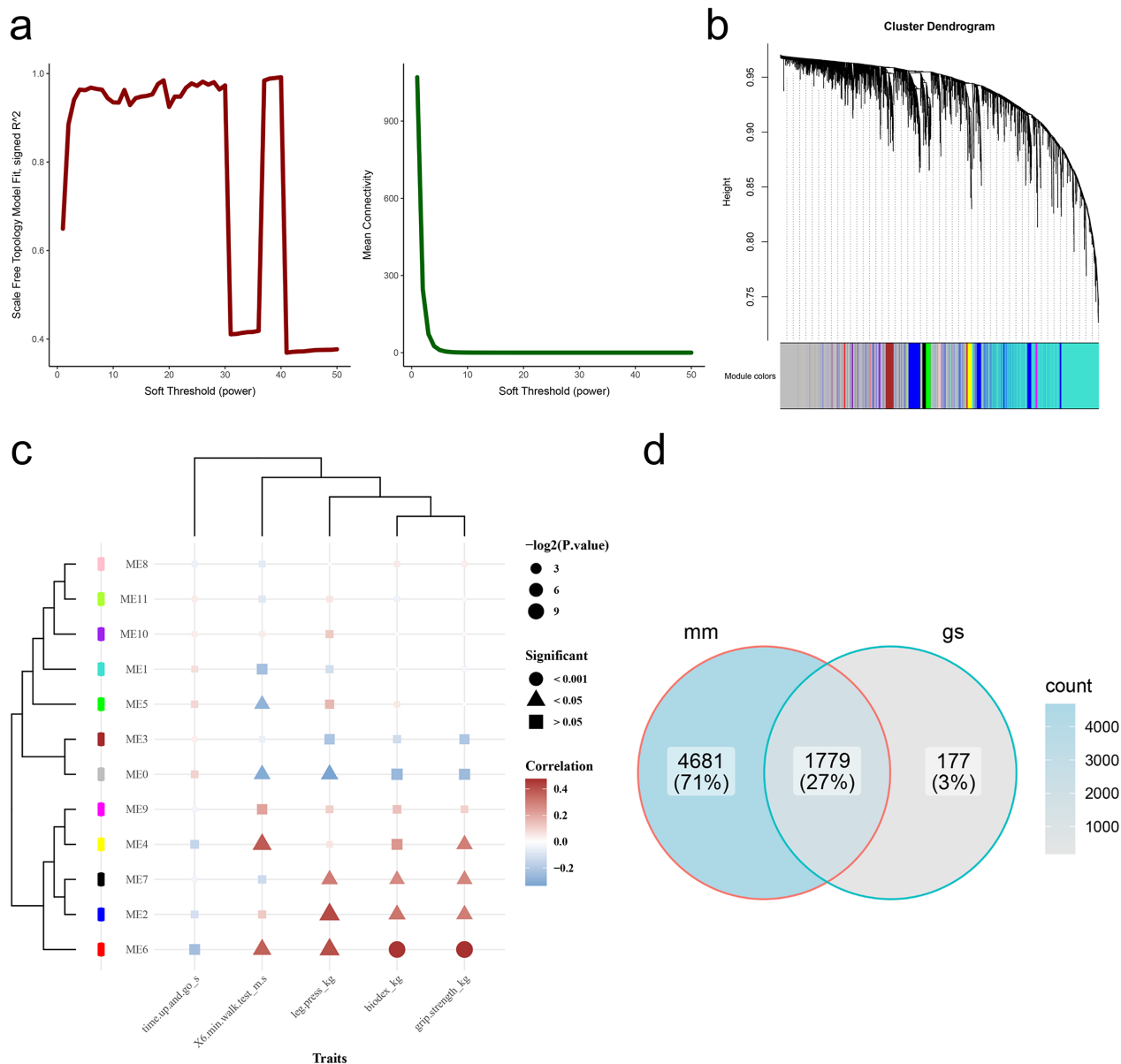


Fig. 3. Significant genes obtained by WGCNA analysis of the GEO Sarcopenia dataset. (a) Analysis of network topology for various soft-thresholding powers. The left panel shows the scale-free fit index (y-axis) as a function of the soft-thresholding power (x-axis). The right panel displays the mean connectivity (degree, y-axis) as a function of the soft-thresholding power (x-axis). (b) Clustering dendrogram of genes, with dissimilarity based on topological overlap, together with assigned merged module colors and the original module colors. (c) Screening significant gene modules based on clinical data. (d) Identification of candidate hub genes by integrating module membership (MM) and gene significance (GS). Genes with high module membership in the sarcopenia-associated module and high gene significance for sarcopenia status were selected as candidate hub genes. WGCNA, weighted gene co-expression network analysis; GEO, Gene Expression Omnibus.

ferentially expressed in both the sarcopenia dataset and at least one CRC chemotherapy dataset (TCGA-COAD), supporting its selection as the key candidate gene linking CRC chemotherapy and sarcopenia through the mitophagy pathway.

5-FU Modulates Cancer Cell-Mediated Myotube Atrophy In Vitro by Suppressing the BNIP3-Mediated PINK1/PRKN Mitophagy-Related Signaling

The CCK-8 assay showed that 5-FU reduced C26 cell viability in a concentration-dependent manner with an IC_{50} of 13.62 μ M (Fig. 6a). In C2C12 myotubes, exposure to C26-CM significantly decreased myotube diameter,

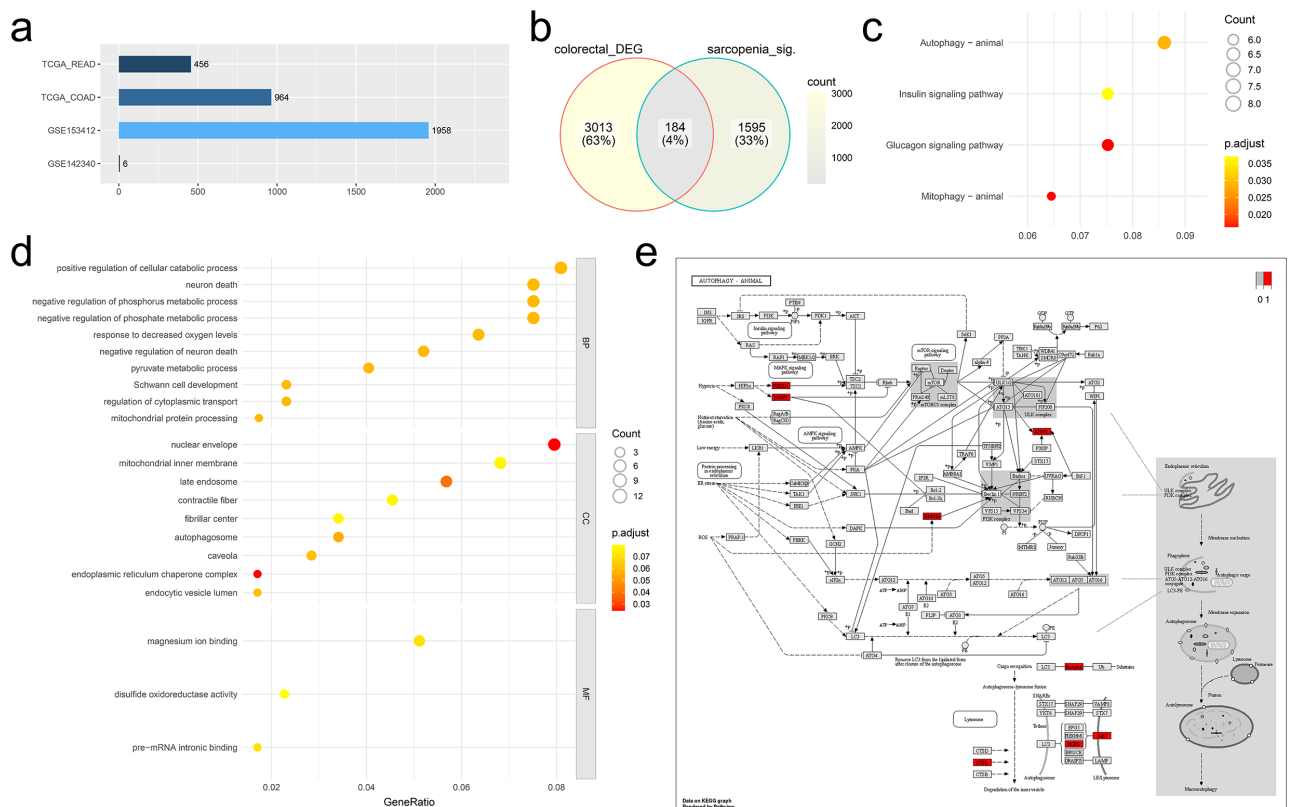


Fig. 4. The intersected genes enriched in autophagy- and mitophagy-related pathways. (a) The number of DEGs in TCGA-READ, TCGA-COAD, GSE153412 and GSE142340 databases. (b) Intersection of colorectal cancer DEGs and sarcopenia-significant genes. (c) KEGG enrichment analysis. (d) GO enrichment analysis. (e) Hits in the autophagy pathway visualized by the pathview R package (version 1.38.0, Bioconductor; <https://bioconductor.org/packages/release/bioc/html/pathview.html>). DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; COAD, colon adenocarcinoma.

whereas 5-FU treatment effectively reversed this atrophy (Fig. 6b,c, $p < 0.05$). Mechanistically, 5-FU downregulated BNIP3 in C26-CM induced C2C12 myotubes (Fig. 6d,e, $p < 0.01$). qRT-PCR confirmed successful BNIP3 overexpression (Fig. 6f, $p < 0.001$). H&E staining showed that the overexpression of BNIP3 reversed the therapeutic effect of 5-FU on C26-CM-induced myotube atrophy (Fig. 7a,b, $p < 0.01$). Furthermore, 5-FU restored the expression of MHC and MyoD as well as suppressed the MuRF-1, Atrogin-1, and mitophagy regulators PINK1 and PRKN (Fig. 7c-j, $p < 0.001$). Overexpression of BNIP3 abolished the protective effects of 5-FU, reduced MHC/MyoD, elevated MuRF-1/Atrogin-1, and increased PINK1/PRKN expression (Fig. 7c-j, $p < 0.01$).

Discussion

Increasing attention has been paid to sarcopenia in CRC patients. On the one hand, patients with sarcopenia are prone to experience severe chemotherapy-related toxicities, often requiring dose reduction or treatment delay. On the other hand, they are also prone to develop resistance to chemotherapy agents [15,16]. Therefore, understand-

ing the causal relationship between sarcopenia and CRC chemotherapy is particularly important for improving the prognosis of CRC.

Based on our analysis, autophagy and mitophagy were selected as common pathways between sarcopenia and CRC chemotherapy. Autophagy is a cellular degradation process in which eukaryotic cells utilize lysosomes to eliminate damaged organelles [17,18]. In CRC, the role of autophagy is complex, as it can exert both tumor-promoting and anti-tumor effects [19]. It is worth noting that autophagy also plays an indispensable role in chemotherapy resistance in CRC. Accumulating evidence suggests that inhibiting autophagy can enhance the sensitivity of CRC cells to chemotherapy agents [20]. On the other hand, autophagy is also related to sarcopenia. Exercise and nutrition are recognized as effective treatment strategies to improve sarcopenia, and also been reported that they can affect autophagic flux [21,22].

Mitophagy is a selective autophagy, which is mainly manifested by the specific degradation of damaged mitochondria in autophagosomes [23]. Mitochondria play a crucial role in the pathophysiology of CRC, as they can provide energy to cancer cells and regulate the occurrence of

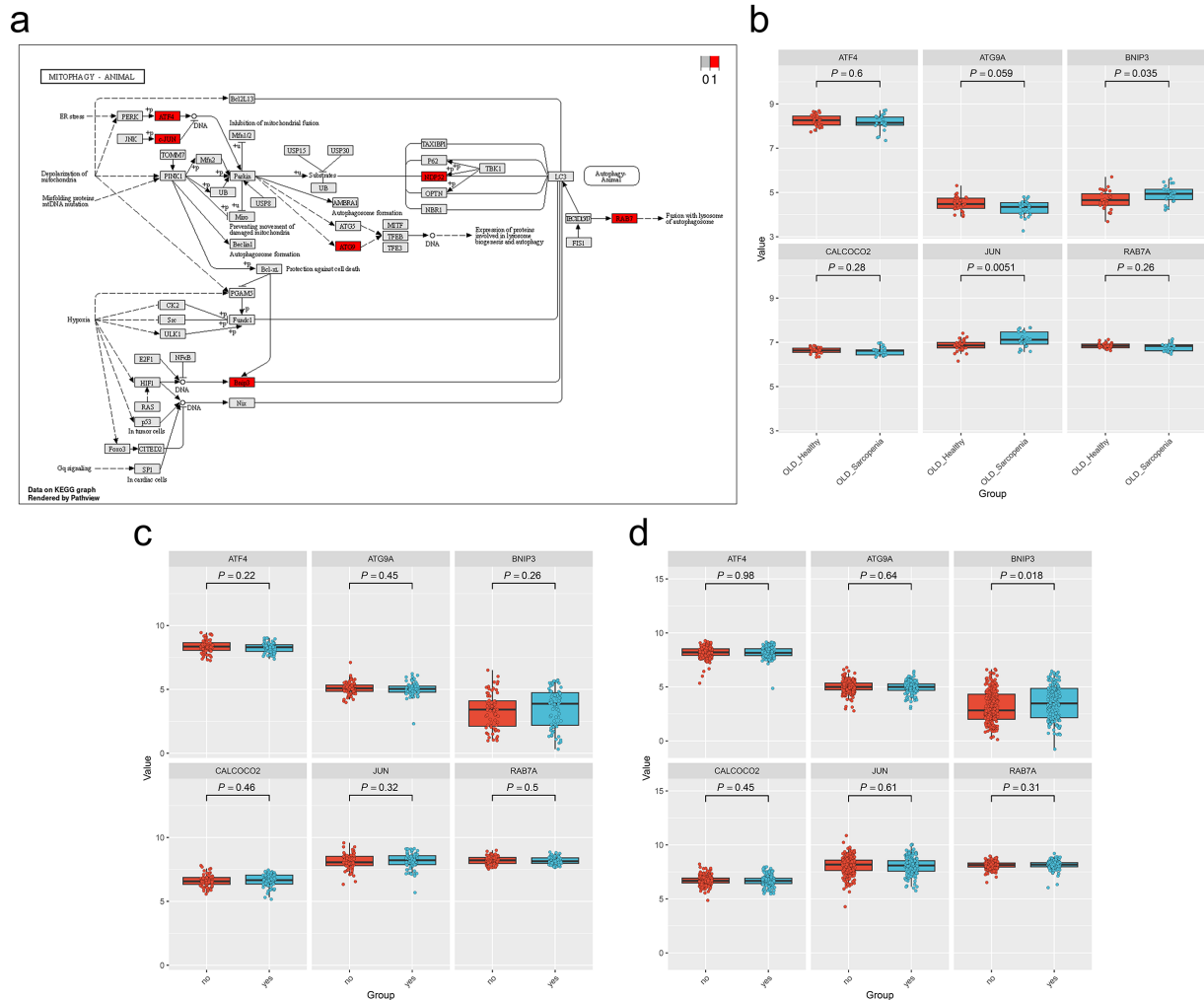


Fig. 5. The expression of mitophagy-related genes in colorectal cancer or sarcopenia. (a) Hits in the mitophagy pathway visualized by the pathview R package (version 1.38.0, Bioconductor; <https://bioconductor.org/packages/release/bioc/html/pathview.html>). (b) Wilcoxon test of mitophagy-related genes in sarcopenia. (c) Wilcoxon test of mitophagy-related genes in TCGA-READ. (d) Wilcoxon test of mitophagy-related genes in TCGA-COAD.

gastrointestinal inflammation [24]. Research showed that mitophagy maintains mitochondrial homeostasis by affecting the accumulation of damaged mitochondria in tumors, thereby regulating the survival of cancer cells [25]. In addition, mitophagy in intestinal epithelial cells triggers adaptive immunity during tumor development [26]. Therefore, previous studies have identified mitophagy-related genes as key genes for CRC prognosis and chemotherapy sensitivity through bioinformatics analysis [27,28]. In addition to its involvement in the pathogenesis of CRC, mitophagy is also closely associated with the development of sarcopenia. It has been reported that excessive or unbalanced mitophagy may lead to muscle degeneration and dysplasia [29].

Next, we focused on BNIP3, which overlaps with the autophagy/mitophagy pathway-related gene set and the key candidate genes of CRC-sarcopenia. BNIP3 is the main regulatory factor of mitophagy, and its expression is often dysregulated in tumor cells [30]. In addition, the re-

sistance of CRC to chemotherapy agents is also related to BNIP3 methylation [31]. Research revealed that approximately 58% of CRC patients exhibit methylation of BNIP3, indicating that these CRC patients are more resistant to chemotherapy and have poorer prognoses [32]. Consistent with this finding, our analysis results indicate that BNIP3 is differentially expressed in COAD patients. In addition, Li et al. [33] reported that inhibition of BNIP3-dependent mitophagy regulates the stress response in CRC cells and then inhibits CRC development. On the other hand, BNIP3 expression in muscle has been reported to increase with age [34]. Consistently, our analysis results show that BNIP3 is upregulated in patients with sarcopenia. Furthermore, inhibiting BNIP3 expression can alleviate myotube atrophy [35].

It is noteworthy that our *in vitro* finding (CM from 5-FU-treated C26 cells increased myotube diameter) does not contradict the clinical observation that chemotherapy

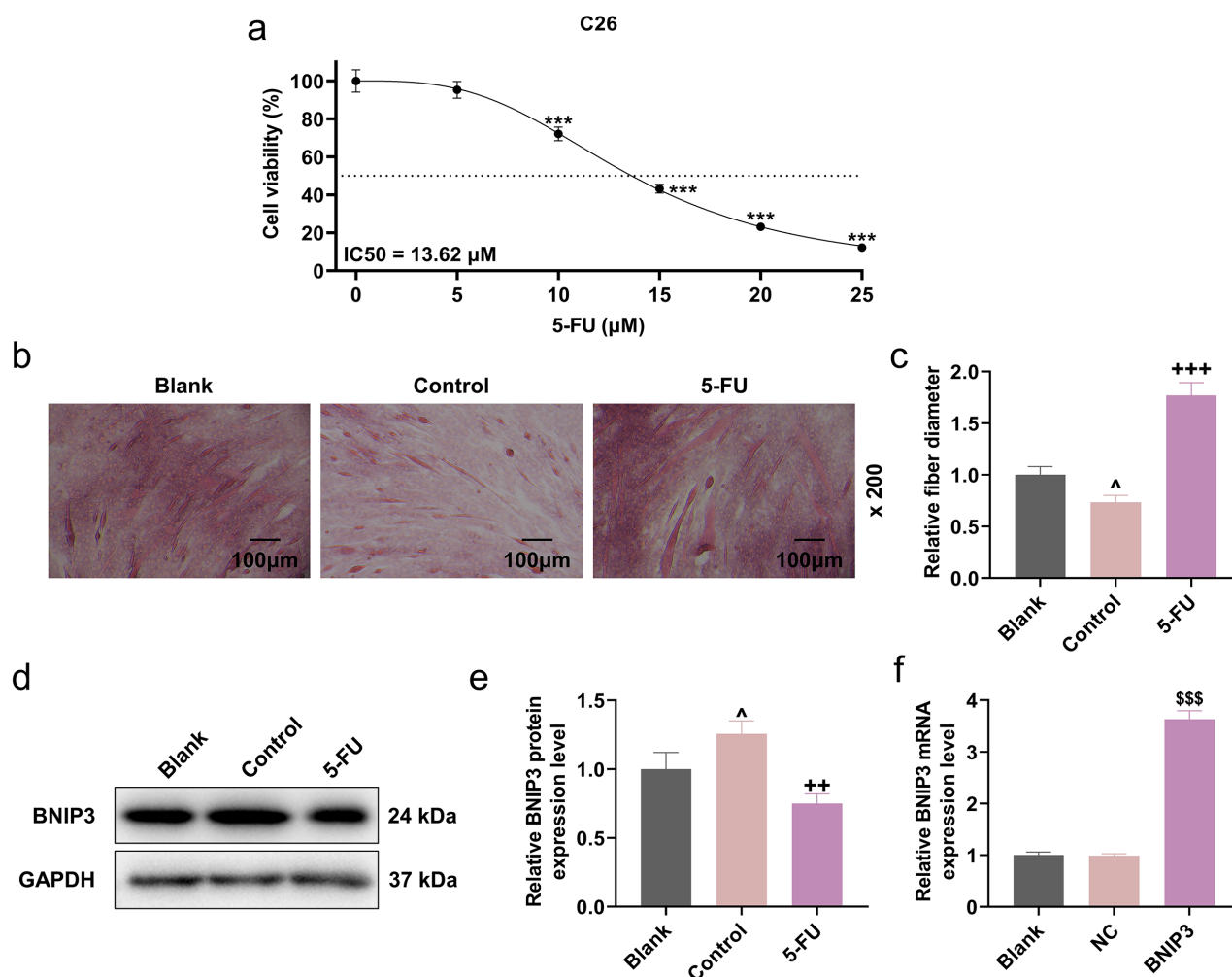


Fig. 6. The effect of 5-FU on cell viability, myotube diameter and BNIP3 expression. (a) CCK-8 assay was performed to evaluate the viability of C26 cells treated with increasing concentrations of 5-FU (0, 5, 10, 15, 20, 25 μM) for 48 h. (b) Representative H&E staining images of C2C12 myotubes (magnification ×200, scale bar = 100 μm). (c) Quantification of relative myotube diameter in C2C12 myotubes after different treatments. (d,e) Western blotting was performed to detect BNIP3 expression. (f) qRT-PCR was used to detect the transfection efficiency of the BNIP3 overexpression plasmid. Data are presented as the mean ± standard deviation (SD). n = 3 independent experiments. ****p* < 0.001 vs. 0 μM. ^*p* < 0.05 vs. Blank. ++*p* < 0.01, +++*p* < 0.001 vs. Control. \$\$\$*p* < 0.001 vs. NC. 5-FU, 5-fluorouracil; BNIP3, Bcl-2/adenovirus E1B 19-kDa-interacting protein 3; CCK-8, Cell Counting Kit-8; qRT-PCR, Quantitative Real-Time Polymerase Chain Reaction.

is often associated with sarcopenia, but rather reflects differences in the levels of action. First, in the *in vivo* setting, chemotherapeutic agents can simultaneously exert direct actions on skeletal muscle cells (e.g., activation of p38 MAPK, induction of oxidative stress) and immune cell-mediated effects on muscle homeostasis [36,37]. VanderVeen et al. [36] demonstrated that 5-FU significantly reduces CD45⁺ immune cells and infiltrating monocytes in skeletal muscle, highlighting the importance of immune mechanisms in chemotherapy-associated sarcopenia. Second, as an anti-metabolite, 5-FU suppresses the metabolic activity of cancer cells, thereby reducing their secretion of atrophy-inducing factors. This likely accounts for the attenuated myotube atrophy observed in our study. Finally, the

differences in dosing regimen and exposure intensity should not be overlooked. Clinical chemotherapy typically employs intermittent high-dose administration, whereas in our experiment, myotubes were exposed to cancer cells' CM, resulting in altered drug concentrations and active components. This scenario may more closely resemble a low-dose, sustained exposure paradigm, leading to distinct biological outcomes [38]. Collectively, the protective effect of 5-FU on myotubes observed in this study reflects tumor cell-mediated indirect pathways, whereas chemotherapy-associated sarcopenia in the clinical setting likely involves direct muscle toxicity and immune-mediated effects. These two perspectives are not contradictory but rather represent the multifaceted actions of chemotherapeutic agents.

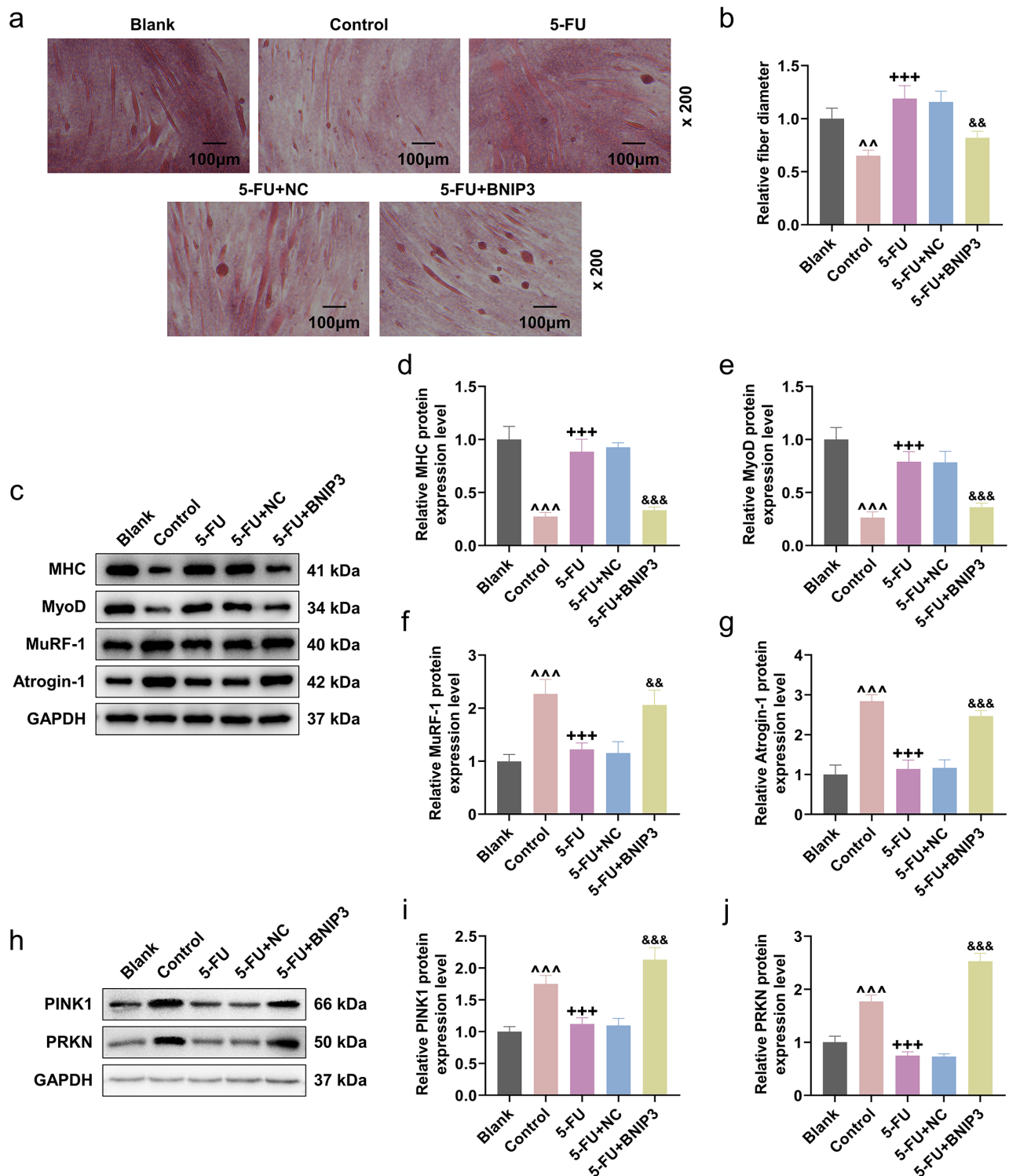


Fig. 7. 5-FU affects cancer-induced myotube atrophy and is associated with downregulation of BNIP3 and reduced PINK1/PRKN expression. (a) Representative H&E staining images of C2C12 myotubes (magnification $\times 200$, scale bar = 100 μm). (b) Quantification of relative myotube diameter in C2C12 myotubes after different treatments. (c–g) Western blotting was performed to detect the expression of myotube atrophy-related proteins (MHC, MyoD, MuRF-1, Atrogin-1) in C2C12 myotubes across different treatment groups. (h–j) Western blotting was performed to detect the expression of mitophagy-related proteins (PINK1, PRKN) in C2C12 myotubes. Data are presented as the mean $\pm$ standard deviation (SD). $n = 3$ independent experiments. ^{^^} $p < 0.01$, ^{^^^} $p < 0.001$ vs. Blank. ⁺⁺⁺ $p < 0.001$ vs. Control. ^{&&} $p < 0.01$, ^{&&&} $p < 0.001$ vs. 5-FU+NC.

We wish to emphasize that the present study was specifically designed to investigate the tumor-muscle crosstalk axis, namely, how chemotherapy-induced alterations in tumor cell secretory behavior indirectly influence muscle cell phenotype. Our CM-based model does not, and was not intended to, recapitulate the direct myotoxic effects of chemotherapeutic agents or the systemic pathophysiology of cancer cachexia. Therefore, our conclusions are strictly confined to the *in vitro* mechanistic evidence demonstrating that BNIP3-related mitophagy-associated signaling serves as a molecular node in tumor-induced myotube atrophy, and that this node can be pharmacologically modulated by 5-FU at the tumor cell level.

Several limitations should be acknowledged in this study. First, our findings were derived exclusively from an *in vitro* conditioned-medium model, which captures only tumor-secretory effects without accounting for direct myotoxicity, immune-mediated damage, or systemic metabolic alterations; thus, extrapolation to *in vivo* pathophysiology or clinical outcomes requires caution. Second, the CRC drug response datasets (GSE142340 and GSE153412) include heterogeneous agents (targeted therapies and cytotoxic chemotherapy), which may introduce bias in identifying chemotherapy-specific DEGs. Third, the CM prepared after 5-FU treatment of C26 cells may reflect decreased total secretory output due to reduced cell viability rather than qualitative changes in specific factors, and residual 5-FU in the CM was not measured. Fourth, BNIP3 overexpression rescue experiments demonstrated that exogenous BNIP3 is sufficient to reverse the protective effects of 5-FU, but they do not formally prove that endogenous BNIP3 is necessary for C26-CM-induced myotube atrophy; knockdown or knockout studies are needed to establish this causal requirement. Fifth, the DEG screening threshold ($|\log_2FC| > 0.3$) was relatively lenient and may include genes with modest expression changes, although this was partially compensated by subsequent WGCNA filtering and multi-dataset validation. Sixth, the sarcopenia dataset (GSE167186) reflects age-related pathology, whereas our *in vitro* model represents cancer-induced atrophy; these conditions involve partially distinct mechanisms, and BNIP3 may function through context-specific pathways. Seventh, mitophagy activity was not directly measured in the present study. Our conclusions regarding the involvement of mitophagy-related signaling are solely based on the expression levels of BNIP3, PINK1 and PRKN proteins, which serve as surrogate markers rather than functional readouts of mitophagic flux. Eighth, the experiments only use the mouse C26/C2C12 system, lacking human models and clinical muscle indicators for verification.

Conclusion

In summary, this study identifies BNIP3 as a molecular node in tumor cell-mediated myotube atrophy through

mitophagy-associated signaling *in vitro*. Our integrated bioinformatics and *in vitro* experiments demonstrate that (1) BNIP3 exhibits dysregulated expression in TCGA-COAD, whereas no significant alteration is found in TCGA-READ; (2) 5-fluorouracil attenuates cancer cell-conditioned medium-induced myotube atrophy; and (3) BNIP3 overexpression partially reversed the effects associated with CM from 5-FU-pretreated C26 cells, as evidenced by rescue experiments. These findings establish BNIP3-related mitophagy signaling as a functional mediator of tumor-muscle crosstalk and suggest that modulation of the BNIP3-PINK1/PRKN axis may represent a potential strategy for preserving skeletal muscle integrity during colorectal cancer therapy.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

HY and BXC designed the research study; HY, BXC, HXM and ZY performed the research; HXM and ZY collected and analyzed the data. HY and BXC have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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