



Prognostic impact of mitofusin 2 expression in colon cancer

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Background: Mitofusin 2 (MFN2) is involved in several biological processes, including cancer. MFN2 is downregulated in some types of cancer and inhibits cancer cell proliferation, migration, and invasion. However, the relationship between MFN2 and colon cancer remains unknown.

Methods: In this study, MFN2 expression was investigated using The Cancer Genome Atlas (TCGA) and the Human Protein Atlas (HPA), and the associations between prognostic factors and survival outcomes were assessed via univariate and multivariate analyses. Functional enrichment analyses based on Kyoto Encyclopedia of Genes and Genomes (KEGG) resource and Gene Set Enrichment Analysis (GSEA) were carried out.

Results: MFN2 was downregulated in colon cancer tissues compared with paracancerous colon tissues ($P < 0.001$), and low MFN2 expression was associated with an advanced tumor stage (stage IV *vs.* stage I, $P = 0.03$; stage I–III *vs.* stage IV, $P = 0.003$). MFN2 immunohistochemistry (IHC) staining was medium to high in colon normal tissues, but MFN2 IHC staining was faint or not identified in colorectal cancer (CRC) tumor tissues. MFN2 expression was either low or non-existent in colon cancer distinct cell clusters, according to differential gene analysis. Univariate analysis revealed that MFN2 expression in colon cancer patients was significantly associated with the stage [odds ratio (OR) = 0.29 for stage IV *vs.* stage I, $P = 0.001$], T-stage (OR = 0.20 for T4 *vs.* T1, $P = 0.033$), and distant metastasis (OR = 0.31 for M1 *vs.* M0, $P = 0.000$). Furthermore, Kaplan–Meier survival analysis revealed that patients with colon cancer and high MFN2 expression have a better prognosis than those with low MFN2 expression ($P = 0.002$). MFN2 (hazard ratio = 0.95, 95% confidence interval: 0.92–0.99, $P = 0.007$) was an independent predictor of colon cancer according to univariate and multivariate Cox models. Finally, GSEA results showed that the KEGG GALACTOSE METABOLISM, APOPTOSIS, and VEGF SIGNALING pathways were activated in the high MFN2 mRNA expression group, whereas the KEGG RIBOSOME pathway was inhibited in the low MFN2 expression group.

Conclusions: Our research revealed MFN2 to be a promising predictive biomarker and therapeutic target for colon cancer.

Keywords: Mitofusin 2 (MFN2); colon cancer; prognosis; The Cancer Genome Atlas (TCGA)

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Introduction

Colorectal cancer (CRC), one of the leading causes of cancer-related deaths worldwide, has mortality rates in Europe, the USA, and worldwide of 45%, 35%, and 47.8%, respectively (1,2). Recently, following progress in chemotherapy, targeted therapy, and immunotherapy, the 5-year survival rate of patients with advanced CRC has improved. However, the increase in survival time afforded by comprehensive therapies is modest, and the 5-year survival rate for advanced CRC remains low (3). CRC prognosis is predominantly based on the tumor, node, and metastasis (*TNM*) stage, tumor location, and histologic subtypes. Few molecular biomarkers can predict patient prognosis and serve as therapeutic targets (4). Thus, it is essential to identify novel target biomarkers for CRC, especially advanced CRC.

Mitochondrial dysfunction and the activation of glycolysis are widely accepted as the hallmarks of cancer (5). Mitofusin 2 (MFN2) encodes a GTPase protein located on the mitochondrial outer membrane and is also known as the hyperplasia suppressor gene. It was initially identified in the vascular smooth muscle cells of spontaneously hypertensive rats (6). Previous studies showed that MFN2 disorders are involved in multiple conditions such as hypertension, Charcot-Marie-Tooth disease, obesity, diabetes, atherosclerosis, and cancer (7,8). MFN2 acts as a tumor suppressor in diverse cancers, including cervical cancer, hepatocellular carcinoma, pancreatic cancer, breast cancer, gastric cancer, and bladder cancer (9-11). However, the potential relationship between MFN2 and colon cancer has not been fully investigated to the best of our knowledge.

Previously, we showed that MFN2 is a hyperplasia suppressor gene in CRC cells, and MFN2 overexpression, mediated by an adenoviral vector, exerts an anti-proliferative effect by inducing G2/M-phase arrest in CRC cells (12). This study investigated the relationship between MFN2 expression and prognosis in human colon cancer using data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). We used gene set enrichment analysis (GSEA) to investigate the biological pathways in colon cancer linked to the MFN2 regulatory network. We present the following article in accordance with the REMARK reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-589/rc>).

Methods

Data collection

Transcriptomic expression data of colon cancer and corresponding patient clinical information were downloaded from TCGA (<https://portal.gdc.cancer.gov>). We examined 470 instances of colon cancer and 41 normal samples to investigate the variation in MFN2 expression. We used samples with comprehensive clinical features in logistic regression and Cox analyses, and boxplots were drawn to visualize the correlation between MFN2 expression and the clinical stage of colon cancer. We performed Kaplan-Meier survival analysis using tumor data from TCGA colon cancer samples. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Immunohistochemistry (IHC) staining and single cell RNA-sequencing (RNA-seq) data processing

Immunohistochemical pictures of MFN2 protein expression studies were done in normal and CRC tissues from the HPA (<http://www.proteinatlas.org/>) to determine differences in MFN2 expression at the protein level. For IHC, the antibody HPA030554 was employed. GEO datasets (<https://www.ncbi.nlm.nih.gov/gds>) were used to download single cell RNA-seq data associated with colon cancer (GSE139555 and GSE146771). To identify key cell types, researchers used the Seurat tool in R v.4.1.2 software. Principal component analysis (PCA) was performed using highly variable genes, and primary components were computed using t-distributed stochastic neighbor embedding (t-SNE) analysis.

GSEA

The GSEA program is a robust analytical tool used to evaluate gene expression data that is available as a free software package including an initial database of >1,000 biologically defined gene sets (13). We used GSEA software (version 4.1.0) to identify the biological roles and pathways that differed between sample groups with high and low MFN2 expression. Gene set permutations were conducted 1,000 times for each family-based analysis, with MFN2 expression as a phenotypic marker. Finally, we ranked all enriched pathways by the normalized enrichment score (NES), nominal P value, and false discovery rate (q-value).

Table 1 TCGA colon cancer patient characteristics

Clinical characteristics	Total (n=379), n (%)
Age (years)	
<60	105 (27.7)
≥60	274 (72.3)
Gender	
Female	174 (45.9)
Male	205 (54.1)
Stage	
I	66 (17.4)
II	152 (40.1)
III	101 (26.6)
IV	60 (15.8)
Stage-T	
T1	9 (2.4)
T2	66 (17.4)
T3	262 (69.1)
T4	42 (11.1)
Stage-N	
N0	227 (59.9)
N1	88 (23.2)
N2	64 (16.9)
Stage-M	
M0	319 (84.2)
M1	60 (15.8)

TCGA, the Cancer Genome Atlas; Stage-T, the depth of tumor invasion; Stage-N, lymph node metastasis; Stage-M, distant metastasis.

Statistical analysis

We used R v.4.1.2 for all statistical analyses. A Wilcoxon signed-rank test was used to compare the MFN2 expression between cancerous and paracancerous tissues. Logistic regression analysis was employed to correlate the MFN2 status with clinicopathological parameters. We performed Kaplan-Meier analysis to ascertain survival and calculate the best gene cut-off values using the survminer package in R. Univariate Cox regression analysis was used to examine the relationships between overall survival (OS) and clinical variables. Finally, we performed multivariate Cox regression

analyses to screen out the independent prognostic factors for colon cancer patients. Differences with a P value <0.05 were deemed statistically significant.

Results

Patient characteristics

We searched TCGA on 20 November 2021 for RNA-seq expression data and patient clinical information related to 511 colon cancer samples, including 470 cancerous and 41 paracancerous samples. Invalid data were removed from the final dataset. The study included data from 205 men and 174 women with colon cancer who ranged in age from 31 to 90 years (average age 66.5 years; median age 68 years). According to the American Joint Committee on Cancer (AJCC) TNM staging system, 66 (17.4%) cases were classified as stage I, 152 (40.1%) as stage II, 101 (26.6%) as stage III, and 60 (15.8%) as stage IV. The 9 (2.4%) cases were classified as stage T₁, 66 (17.4%) as T₂, 262 (69.1%) as T₃, and 42 (11.1%) as T₄. Among these colon cancer patients, lymph node metastasis (N₁-N₂) was found in 152 cases, and distant metastasis (M₁) was found in 60 cases. See *Table 1* for a detailed summary of the correlation data.

Differential MFN2 expression

We downloaded information from 470 tumor samples and 41 normal (paracancerous) samples from TCGA. The Wilcoxon signed-rank test was performed to identify significant changes in MFN2 expression. MFN2 expression was considerably lower in colon cancer tissues than in paracancerous colon tissues in the original RNA expression study (P<0.001, *Figure 1A*). Further analysis revealed that the expression levels of MFN2 in tumor tissues were lower compared with matched paracancerous tissues (P<0.001, *Figure 1B*) in 41 paired samples. MFN2 expression was substantially linked with the clinical stage (stage IV vs. stage I, P=0.03, *Figure 1C*; stages I–III vs. stage IV, P=0.003, *Figure 1D*). MFN2 expression, as a categorical dependent variable, was predictive of clinicopathologic outcomes based on univariate analysis (see *Table 2*). In colon cancer patients, MFN2 expression was significantly associated with the AJCC stage [odds ratio (OR) =0.29 for stage IV vs. stage I, P=0.001], T-stage (OR =0.20 for T₄ vs. T₁, P=0.033), and distant metastasis (OR =0.31 for M₁ vs. M₀, P<0.001).

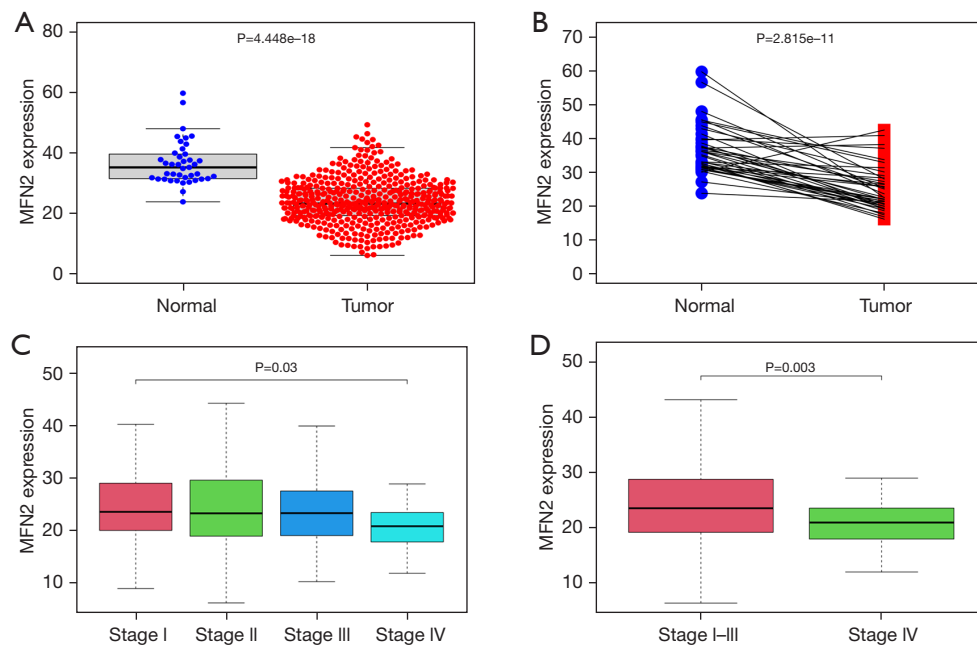


Figure 1 Differential expression of MFN2 based on tumor status (cancer and paracancerous) (A), tumor status of matched cancer and paracancerous samples (B), clinical stage (C), and clinical stages I–III vs. stage IV (D). MFN2, mitofusin 2.

Table 2 Association with MFN2 expression and clinical pathologic variables (logistic regression)

Clinical characteristics	Total	Odds ratio in MFN2 expression (95% CI)	P value
Stage (IV vs. I)	126	0.29 (0.13–0.59)	0.001
Distant metastasis (M ₁ vs. M ₀)	379	0.31 (0.16–0.55)	<0.001
Age (≥60 vs. <60 years)	379	1.07 (0.70–1.63)	0.749
Gender (male vs. female)	379	0.78 (0.53–1.13)	0.184
T stage (T ₄ vs. T ₁)	51	0.20 (0.04–0.81)	0.033
Lymph nodes (N ₂ vs. N ₀)	291	0.62 (0.37–1.02)	0.063

MFN2, mitofusin 2; CI, confidence interval.

In addition, IHC findings were acquired and processed from the HPA to measure MFN2 expression at the protein level. MFN2 IHC staining was medium to high in colon normal tissues (Figure 2A), but MFN2 IHC staining was faint or not identified in CRC tumor tissues (Figure 2B). We employed single cell RNA seq data from the GSE139555 (14) and GSE146771 (15) datasets to explore MFN2 expression in a variety of cells, including colon cancer cells, immunological cells, stromal cells, and vascular endothelial cells. MFN2 expression was either low or non-existent in distinct clusters, according to differential gene analysis (Figure 2C,2D). These findings also revealed

that MFN2 expression was low in colon cancer.

Kaplan-Meier survival analysis

The colon cancer samples were divided into two groups based on the median MFN2 expression level: high- and low-MFN2 expression groups. We performed survival analysis to define the role of MFN2 in the prognosis of colon cancer. Based on Kaplan-Meier survival analysis, colon cancer patients with high MFN2 expression have a better prognosis compared to those with low MFN2 expression (P=0.002, Figure 3A).

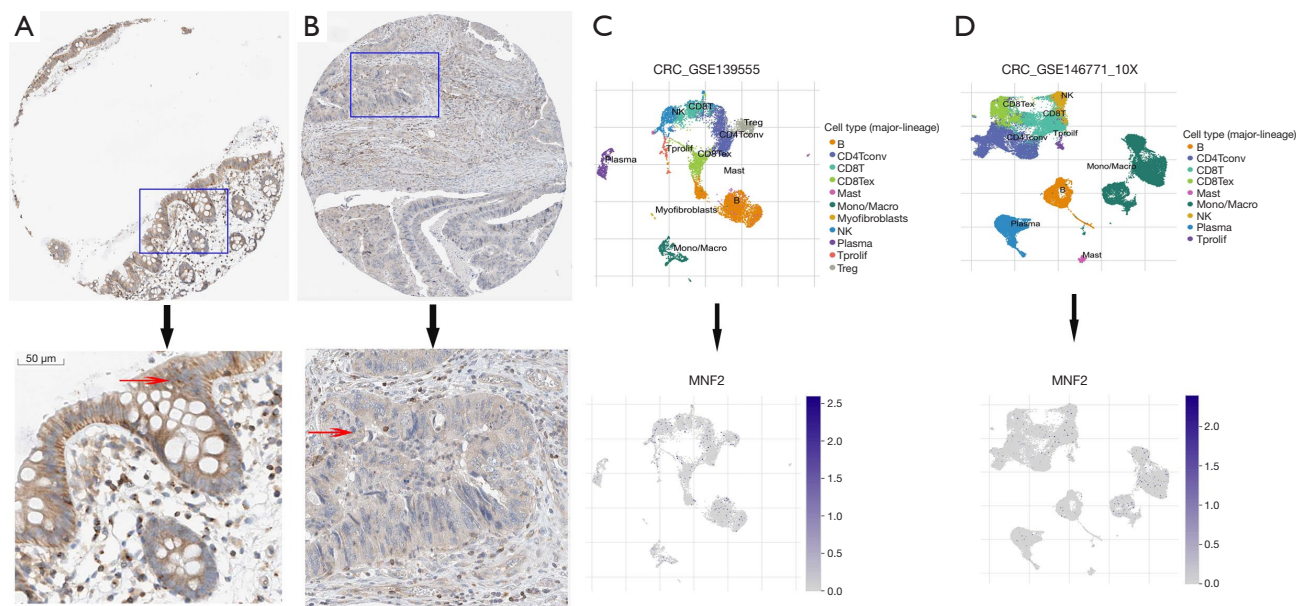


Figure 2 The MFN2 expression detected by immunohistochemistry and single cell RNA-seq analysis of the colon cancer. (A,B) Representative immunohistochemical staining in colon normal tissues (A) and colon cancer tissues (B). The red arrow indicates the stained area of the gland. (C) From dataset GSE139555, an t-SNE plot showing 11 cell clusters (up) and MFN2 expression levels in various clusters (down). (D) The t-SNE plot showing 9 cell clusters (up) and expression levels of MFN2 in different clusters from dataset GSE146771 (down). MFN2, mitofusin 2; CRC, colorectal cancer; RNA-seq, RNA sequencing; t-SNE, t-distributed stochastic neighbor embedding; CD4Tconv, conventional CD4⁺ T cells; CD8T, CD8 T lymphocytes; CD8T_{ex}, exhausted CD8 T cells; Mono/Macro, monocyte/macrophage; NK, natural killer; Tprolif, proliferative T cells; Treg, regulatory CD4⁺ T cell.

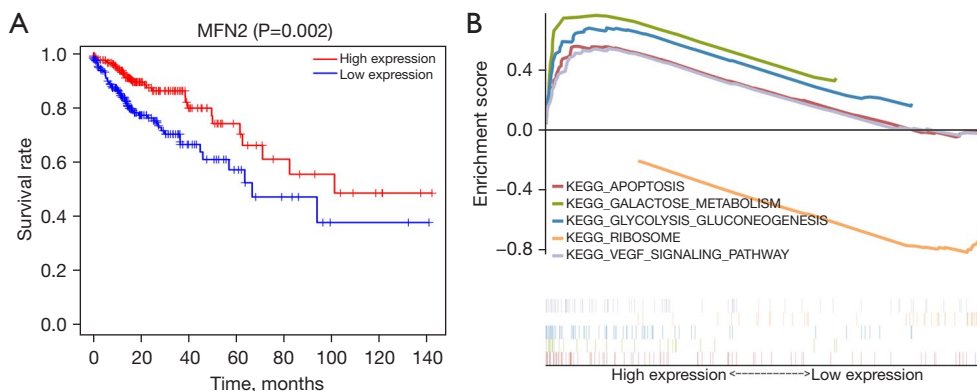


Figure 3 MFN2 affects the prognosis of colon cancer patients. (A) The effect of MFN2 expression on OS in colon cancer cases from TCGA. (B) Results of GSEA of MFN2 mRNA expression. MFN2, mitofusin 2; GSEA, gene set enrichment analysis; TCGA, The Cancer Genome Atlas; OS, overall survival; KEGG, Kyoto Encyclopedia of Genes and Genomes; VEGF, vascular endothelial growth factor.

Univariate and multivariate Cox analyses

We used univariate and multivariate Cox regression analyses to ascertain which characteristics, including age, sex, AJCC stage, TNM stage, and MFN2 expression level, were

independent prognostic predictors for patients with colon cancer. Univariate Cox regression analysis showed that age [hazard ratio (HR) =1.03, 95% confidence interval (CI): 1.01–1.05, P=0.013], the AJCC stage (HR =2.34, 95% CI: 1.78–3.06, P<0.001), the depth of tumor invasion (HR =2.89,

Table 3 Univariate and multivariate Cox proportional hazards analysis of MFN2 expression and OS for patients with colon cancer in the validation cohort

Clinicopathologic variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age	1.03	1.01–1.05	0.013	1.04	1.01–1.06	0.001
Gender	1.11	0.70–1.78	0.653	–	–	–
Stage (AJCC)	2.34	1.78–3.06	7.72E–10	1.68	0.76–3.71	0.20
T	2.89	1.81–4.59	7.92E–06	1.53	0.88–2.67	0.13
M	4.83	2.96–7.87	2.65E–10	1.36	0.47–3.97	0.58
N	2.02	1.54–2.66	4.40E–07	1.23	0.75–2.01	0.41
MFN2	0.94	0.91–0.98	0.001	0.95	0.92–0.99	0.007

AJCC, American Joint Committee on Cancer; Stage-T, the depth of tumor invasion; Stage-N, lymph node metastasis; Stage-M, distant metastasis; MFN2, mitofusin 2; HR, hazard ratio; OS, overall survival; CI, confidence interval.

Table 4 Gene set enrichment analysis of The Cancer Genome Atlas colon cancer with the MFN2 high expression and low expression by using KEGG pathway gene sets

Name	ES	NES	NOM P value	FDR q-value
MFN2 high-expression				
KEGG_GALACTOSE_METABOLISM	0.76	2.32	<0.001	<0.001
KEGG_GLYCOLYSIS_GLUONEOGENESIS	0.68	2.21	<0.001	0.002
KEGG_VEGF_SIGNALING_PATHWAY	0.54	2.11	<0.001	0.006
KEGG_APOPTOSIS	0.53	1.98	0.002	0.010
MFN2 low-expression				
KEGG_RIBOSOME	–0.83	–1.81	0.016	0.137

MFN2, mitofusin 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; ES, enrichment score; NES, normalized enrichment score; NOM, normal; FDR, false discovery rate.

95% CI: 1.81–4.59, $P < 0.001$), distant metastasis (HR =4.83, 95% CI: 2.96–7.87, $P < 0.001$), lymph node metastasis (HR =2.02, 95% CI: 1.54–2.66, $P < 0.001$), and MFN2 expression (HR =0.94, 95% CI: 0.91–0.98, $P = 0.001$) were associated with OS (Table 3). The multivariate Cox model indicated that age (HR =1.04, 95% CI: 1.01–1.06, $P = 0.001$) and MFN2 expression (HR =0.95, 95% CI: 0.92–0.99, $P = 0.007$) can be regarded as independent prognostic variables for colon cancer (Table 3).

GSEA

GSEA was performed using the high- and low-MFN2 expression datasets (false discovery rate $P < 0.05$, nominal

$P < 0.05$) to identify putative signaling pathways involved in colon cancer, and we selected the most significantly enriched signaling pathways based on the NES. Using GSEA, we found that the high expression of MFN2 in colon cancer may be related to three metabolic signaling pathways, respectively KEGG GALACTOSE METABOLISM and KEGG GLYCOLYSIS GLUCONEOGENESIS. In addition, the high expression of MFN2 in colon cancer may be related to apoptosis and VEGF signaling pathways. However, GSEA revealed only one signaling pathway KEGG RIBOSOME, which may be related to the low expression of MFN2 in colon cancer (Figure 3B, Table 4). These results indicate that changes in MFN2 expression may affect signaling pathways involved in the development and progression of colon carcinogenesis.

Discussion

As early clinical symptoms are not obvious in CRC patients, approximately 35% of these patients present with metastatic disease at diagnosis (16). Moreover, over 50% of non-metastatic CRC patients ultimately develop metastatic disease. Aggressive treatment strategies, such as combining systemic chemotherapy with surgery, can sometimes cure patients with metastatic CRC. Molecular targeting with therapeutic drugs has been a landmark development in the treatment of metastatic CRC (17). Thousands of patients with metastatic CRC have received targeted therapies in clinical trials, and many patients experienced prolonged survival times or even a complete cure. However, the relative lack of biomarkers for metastatic CRC has slowed our progress in this area. As CRC-targeting therapies are currently limited, a better understanding of the pathogenesis and mechanisms of CRC is essential to develop targeted therapies.

Based on TCGA, we showed that tumor tissues had lower MFN2 expression levels than non-cancerous tissues ($P < 0.001$), and there was lower expression in tumors at later tumor stages (stages I–III *vs.* stage IV, $P = 0.003$). Besides, patients with low MFN2 expression had a worse prognosis than those with high MFN2 expression. Univariate and multivariate Cox analyses revealed that MFN2 expression may be an independent prognostic factor in colon cancer. A recent study reported low levels of MFN2 immunostaining in hepatocellular carcinoma (HCC) tissues compared to adjacent liver tissues (18). The lower expression of MFN2 was significantly connected to a poorer prognosis for patients with HCC. Overexpression of MFN2 induced apoptosis in HepG2 cells, stimulated Ca^{2+} release from the endoplasmic reticulum into the mitochondria, and increased intracellular reactive oxygen species (18,19). Furthermore, in HepG2 cells, the p53 protein is bound directly to the MFN2 promoter, indicating that MFN2 is a novel p53-inducible target gene (20). More recently, it was hypothesized that MFN2 may play a role in the onset and progression of HCC via a variety of mechanisms, including miRNAs; MFN2 is a direct target of miR-76 in HCC cells, and a miR-761 inhibitor upregulated MFN2, altering mitochondrial function and effectively inhibiting HCC cell growth and metastasis (21). These results suggest that MFN2 may be a potential therapeutic target for treating HCC. Downregulation of MFN2 is also linked to poor prognosis in breast cancer patients. Xu *et al.* used the CRISPR/Cas9 system to knock out MFN2 from MCF7 and A549 cells, stimulating tumor cell invasion *in vitro* and

in vivo. Enhancing the mammalian target of rapamycin complex 2 (mTORC2)/Akt signaling pathway increases tumor cell proliferation and metastasis in MFN2-mutant cancer cells via an AktS437 phosphorylation-induced signal transduction pathway (22). In a separate investigation, MFN2 expression was considerably higher in 30 lung cancer samples compared to paired adjacent normal tissues (23). These contradictory findings suggest that the role of MFN2 in tumor growth is more complex than previously thought.

There are also contradictory reports about the involvement of MFN2 in the development of stomach or gastric cancer (GC). A recent study reported that patients with high MFN2 expression had significantly poorer OS and disease-free survival (DFS) than those with low or negative MFN2 expression. MFN2 overexpression was linked to the depth of invasion and TNM stage of GC, and MFN2 knockdown prevented the migration and invasion of GC cells (9). Zhang *et al.* found lower MFN2 expression in tumor tissue than in normal tissue and that the expression level was inversely connected with tumor size, the clinical stage, lymph node metastasis, and distant metastasis. MFN2 expression had no significant impact on OS or DFS, but MFN2 overexpression reduced GC cell proliferation, clonogenicity *in vitro*, and tumor growth *in vivo*, which were associated with the downregulation of MMP-2 and -9 (24). These inconsistent and conflicting results suggest that the role of MFN2 in GC is also more complicated than initially thought.

As a fusion protein of mitochondria, the specific mechanism of MFN2 involved in the occurrence of CRC is still unclear. Mitochondria have long been regarded as the cell's "powerhouse" as they produce more than 90% of ATP in aerobic settings via oxidative phosphorylation. To maintain optimal mitochondrial activities, each cell maintains a precise balance between fusion and fission. Mitofusin-1 (MFN1), mitofusin-2 (MFN2), and optic atrophy 1 (OPA1) are the three primary dynamin-related proteins that mediate mitochondrial fusion in mammals (25). Abnormal MFN2 function can cause mitochondrial fusion dysfunction, leading to a variety of diseases, such as diabetes, obesity, cardiovascular and cerebrovascular diseases, neurodegenerative diseases, malignant tumors and so on (8,26,27). The increased glucose intake and increased glycolytic rates suggest that metabolic changes help tumor cells grow faster (28). MFN2 reduces cancer cell growth by interacting with pyruvate kinase 2 (PKM2) via the N-terminus and restricting metabolic flow to aerobic glycolysis; phosphorylation of Mfn-2 strengthens this connection (29). The loss of MFN2 activity induces

metabolic changes in mitochondria, including decreased mitochondrial membrane potential, cellular oxygen consumption, and substrate oxidation. Our study also found that high expression of MFN2 in colon cancer may be involved in KEGG GALACTOSE METABOLISM and KEGG GLYCOLYSIS GLUCONEOGENESIS pathway, suggesting that the development of colon cancer may be related to the abnormal mitochondrial energy metabolism caused by MFN2.

In the early stages of intrinsic apoptosis, mitochondria are the principal target (30). Dynamic changes in the mitochondrial location of numerous proteins, including MFN2 and Drp1, as well as the apoptosis regulators Bcl-2 associated X (BAX) and Bcl-2 antagonist/killer (BAK), cause mitochondrial fragmentation during apoptosis (31). Overexpression of either MFN1 or MFN2 can also delay apoptosis by delaying the activation of downstream caspases and apoptosis (32). MFN2 location on the outer mitochondrial membrane is known to be mediated by BAX, while BAK binds to both MFN1 and MFN2 (33). Several investigations have shown that MFN2-induced apoptosis is most likely mediated through the PI3K-Akt pathway (34,35). Our GSEA showed that high expression of MFN2 may promote apoptosis in colon cancer cells, but the specific inhibition needs to be further elucidated. Vascular endothelial growth factor (VEGF) upregulates MFN2 expression in human umbilical vein endothelial cells, and knocking down MFN2 reduces VEGF-induced human umbilical vein endothelial cell migration and differentiation, indicating that MFN2/VEGF connection in endothelial cells plays a role in angiogenesis (36). Another study showed that overexpressed MFN2 has an inhibitory effect on pancreatic cancer, which may be related to the VEGF signaling pathway (37). Our analysis also found that high expression of MFN2 may be involved in the VEGF signaling pathway in colon cancer. VEGF plays a key regulatory role in the growth and invasion of CRC. The VEGF inhibitor bevacizumab provides a significant survival benefit for advanced CRC treatment. Clarification of the relationship between MFN2 and VEGF in colon cancer cells is likely to bring new ideas for targeted therapy of CRC.

Analysis of data from TCGA for rectal cancer (data not shown) did not produce similar results, suggesting that MFN2 may have different functions depending on the site. Due to data heterogeneity or software issues, our results may be biased or erroneous. More data are needed, and further experiments and clinical validation should be conducted to confirm the link between MFN2 and colon cancer.

Conclusions

To the best of our knowledge, this is the first study of the role of MFN2 in colon cancer. Low MFN2 expression implies a poor prognosis and is an independent risk factor for colon cancer prognosis. We also showed that MFN2 may be a potential prognostic biomarker and therapeutic target for colon cancer. A comprehensive evaluation of the mode of action of MFN2 may provide insight into the role of MFN2 in the genesis of colon cancer and lead to targeted molecular therapies.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-589/rc>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-589/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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