c-Myb promotes growth and metastasis of colorectal cancer through c-fos-induced epithelial-mesenchymal transition

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Abstract
c-Myb is a crucial transcription factor that participates in various biological functions; however, its role in colorectal cancer (CRC) remains poorly investigated. We first analyzed the expression and clinical significance of c-Myb in a retrospective cohort enrolling 132 CRC patients. Then, the CRISPR/Cas9 technique was used to establish c-Myb gene KO CRC cell lines. Cellular functional assays in vitro and in vivo were used to evaluate the impact of c-Myb KO in CRC cells. Finally, RNA sequencing was used to investigate the potential oncogenic mechanisms regulated by c-Myb in CRC progression and related cellular validations were accordingly carried out. As a result, c-Myb is significantly overexpressed in CRC tissues as compared with adjacent normal tissues. High expression of c-Myb is positively correlated with lymph node metastasis and poor prognosis. Univariate analysis and multivariate analysis further identify c-Myb as an independent unfavorable prognostic factor for CRC patients. c-Myb KO inhibits the proliferation, apoptosis resistance, invasion, metastasis, colony formation and in vivo tumorigenesis of CRC cells. Also, the mechanism investigation indicates that c-Myb may promote CRC progression by regulating c-fos. c-fos overexpression can rescue the inhibitory effect of c-Myb KO on the malignant characteristics of CRC cells. Finally, we find that c-Myb KO inhibits the epithelial-mesenchymal transition (EMT) molecular phenotype in CRC cells, whereas c-fos overexpression can rescue this inhibitory effect. This study suggests that c-Myb promotes the malignant progression of CRC through c-fos-induced EMT and has the potential to be a promising prognostic biomarker and therapeutic target.

KEYWORDS biomarker, c-fos, c-Myb, colorectal cancer, epithelial-mesenchymal transition

Abbreviations: CRC, colorectal cancer; DFS, disease-free survival; EMT, epithelial-mesenchymal transition; NC, negative control; OS, overall survival; RNA-seq, RNA sequencing; RT-PCR, real-time polymerase chain reaction.

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1 | INTRODUCTION

Colorectal cancer is one of the most common causes of cancer-related death worldwide. The pathogenesis of CRC is currently thought to be a complicated, multistep process involving multigene regulation. Therefore, a thorough understanding of CRC molecular pathogenesis may benefit the discovery of novel diagnostic markers or therapeutic targets that can improve individualized CRC management strategy.

The protein, c-Myb, a member of the Myb protein family, is a transcription factor that participates in regulating cell cycle, differentiation and proliferation through DNA binding. It is reported to be involved in maintaining colonic crypt homeostasis, which is related to its regulatory role in crypt proliferation, integrity and normal differentiation. Although it was originally described to be hematopoietic lineage-specific, increasing studies have found it is overexpressed in numerous solid tumors and may contribute to the malignant characteristics of cancer cells. For example, c-Myb is able to drive the invasion and metastasis of breast cancer cells through the Wnt/β-Catenin/Axin2 signaling pathway. In liver cancer, it transcriptionally regulates the expression of Yes-associated protein (YAP) to promote the growth of cancer cells in vivo and in vitro. A recent review has attributed its oncogenic role to its interaction with cell cycle proteins, protein kinases and other transcription factors. Previously, we have proven that microRNA-150 suppressed the malignant characteristics of CRC cells by negatively regulating c-Myb. However, the specific clinical significance and biological role of c-Myb in CRC remain poorly studied. Therefore, in the present study, we first detected its expression and analyzed its clinical significance in a retrospective CRC cohort. Then, we established c-Myb gene KO CRC cell lines and accordingly carried out functional validations in vitro and in vivo. Finally, RNA sequencing was used to investigate the potential oncogenic mechanisms regulated by c-Myb in CRC progression and its results were subsequently confirmed by molecular assays.

2 | MATERIALS AND METHODS

2.1 | Patient data

For details, please see Appendix S1. This study was approved by the ethics committees of Shanghai Tongji University Affiliated Tenth People’s Hospital, and informed consents have been acquired.

2.2 | Immunohistochemical staining and scoring

For details, please see Appendix S1.

2.3 | Cell culture

For details, please see Appendix S1.

2.4 | Real-time polymerase chain reaction

For details, please see Appendix S1. All the primer sequences are shown in Table S1.

2.5 | Western blot

For details, please see Appendix S1.

2.6 | Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling analysis

For details, please see Appendix S1.

2.7 | Cell counting kit-8 assay

For details, please see Appendix S1.

2.8 | Apoptosis assay

For details, please see Appendix S1.

2.9 | Transwell migration and invasion assay

For details, please see Appendix S1.

2.10 | Colony formation assay

For details, please see Appendix S1.

2.11 | CRISPR/Cas9 technique

Gene KO was carried out using CRISPR/Cas9. Single-guide RNA (sgRNA) was designed based on the following sequence: 5′-AGTCTGGAAAGCGTCACTTG-3′ and then its vector was constructed. Lentiviral packaging was done using two plasmids, which contain Cas9 and sgRNA, respectively. KO cells and NC cells were selected by puromycin. Lentivirus also carries GFP for monitoring transfection efficiency.

2.12 | Plasmid construction

For details, please see Appendix S1.

2.13 | Xenograft model

For details, please see Appendix S1.

2.14 | RNA sequencing

For details, please see Appendix S1.
2.15 | Chromatin immunoprecipitation

Crosslinking of DNA and proteins was carried out by adding formaldehyde to CRC cells and terminated using glycine. Samples were lysed using lysis buffer and transferred to ultrasound disruption using a Bioruptor Plus sonicator (Diagenode). After DNA purification, the input DNA control samples were prepared. The acquired chromatin fragments were transferred to immunoprecipitation using Dynabeads Protein A magnetic beads (Multi Sciences) and the primary antibody against c-Myb. After protein digestion, the purified DNA was transferred to PCR using the following PCR primers that were designed for the promoter region of c-fos: forward: GCTGCAGCCCGCGAGCAG; reverse: AATCTCGTGAGCATTTCG.

2.16 | Dual-luciferase reporter assay

The binding site of c-Myb on the c-fos promoter was analyzed using an online transcription factor prediction program (http://consite.gene-reg.net/). c-fos promoter (c-fos-p) construct contained the c-Myb-binding sites 1 (c-fos-p site 1: -149--143). Sequences for the c-fos promoter containing the binding site were designed as follows: wild type (WT) c-fos promoter: 5’- CCTCTGAGACAGGAACCTGCG-3’; mutant type (MT) c-fos promoter: 5’- CCTCTGAGACAGGGATTATG-3’. Dual-luciferase reporter assay was carried out using the Dual-luciferase Reporter assay kit (Promega).

2.17 | Statistical analysis

For details, please see Appendix S1.

3 | RESULTS

3.1 | Expression and clinical significance of c-Myb in CRC patients

First, we used RT-PCR (Figure 1A) and western blot (Figure 1B, C) to compare the expression of c-Myb between CRC tissues and paired adjacent normal tissues (n = 20). Results showed that the expression

**FIGURE 1** Expression of c-Myb in colorectal carcinoma (CRC) and matched adjacent normal tissues. A, RT-PCR shows that the mRNA level of the c-Myb gene was significantly higher in CRC tissues than that in matched adjacent normal tissues (n = 20, \( P = .005 \)). B, Western blot shows that the protein level of c-Myb was higher in CRC tissues than that in matched adjacent normal tissues (n = 20, \( P = .008 \)). C, Representative protein bands of c-Myb and GAPDH. D, Representative immunohistochemical staining images of c-Myb in CRC and matched adjacent normal tissues. E, Cut-off value of immunohistochemical staining scores is determined by receiver operating characteristic curve analysis. **\( P < .01 \)**
of c-Myb is significantly higher in CRC tissues than in adjacent normal tissues both at mRNA and protein level. In addition, we used immunohistochemistry to detect its expression in the CRC and paired adjacent normal tissues from a retrospective cohort enrolling 132 patients. Representative staining results are shown in Figure 1D. Using the semiquantitative scoring and ROC analysis, we determined that the cut-off value of staining scores was 3.5 (Figure 1E). Therefore, based on this threshold, the entire cohort was divided into a high-expression group (n = 71) and a low-expression group (n = 61).

As shown in Table 1, we found that c-Myb expression was significantly associated with lymph node metastasis (P < .001), whereas there was no correlation between c-Myb expression and other clinical factors such as gender (P = .542), age (P = .376), tumor location (P = .582), tumor size (P = .674), tumor differentiation (P = .655), and tumor invasion (P = .790), and serum CEA level (P = .655). Then, the Kaplan Meier survival model was used to analyze the impact of c-Myb expression on CRC prognosis (Figure 2). As a whole, patients with high c-Myb expression had a worse OS and DFS than those with low c-Myb expression (both P < .001, Figure 2A). Subgroup analysis based on TNM staging also showed that c-Myb expression could stratify the OS and DFS of CRC patients both in stage II and in stage III (all P < .01, Figure 2B,C). Univariate analysis indicated that both lymph node metastasis and c-Myb expression were significant factors for OS and DFS (all P < .01, Table 2). Multivariate analysis indicated that they were also independent prognostic factors for OS and DFS (all P < .01, Table 3).

### 3.2 Knockout of c-Myb inhibits the malignant characteristics of CRC cells in vitro

First, we used RT-PCR to detect c-Myb expression in five commonly used CRC cell lines (HCT116, SW620, HT29, LOVO, and SW480) and one normal intestinal epithelial cell line (HIEC-6). As a result, we found that the mRNA level of c-Myb was significantly higher in CRC cell lines compared with the normal intestinal epithelial cell line, and was relatively abundant in HCT116 and SW620 cell lines (Figure 3A). Then, we carried out the CRISPR/Cas9 technique to KO c-Myb expression in both the cell lines and western blot confirmed its KO efficiency (Figure 3B).

Next, we carried out functional assays in vitro to assess the impact of c-Myb KO on the malignant characteristics of CRC cells. First, in the CCK8 assay, we found c-Myb KO significantly inhibited the proliferation of HCT116 and SW620 cells (Figure 3C). Apoptosis assay showed that c-Myb KO dramatically increased the apoptosis rate of HCT116 and SW620 cells (Figure 3D). Moreover, in the colony formation assay, c-Myb KO was found to reduce the number of colonies in HCT116 and SW620 cells (Figure 3E). Finally, Transwell assay showed that c-Myb KO also inhibited the migratory and invasive ability of CRC cells (Figure 3F,G). Taken together, these results suggested that c-Myb KO effectively inhibited the malignant characteristics of CRC cells in vitro.

### 3.3 Knockout of c-Myb inhibits the growth of CRC cells in vivo

To assess the impact of c-Myb KO on the growth of CRC cells in vivo, we established a xenograft model by injecting CRC cells s.c. into the right hind of nude mice. Harvested tumors are shown in Figure 4A, and we found that size of tumors in the NC group was significantly larger than that in the KO group. Growth curve showed that c-Myb KO significantly inhibited the growth of xenografts (Figure 4B). In addition, we also found that weights of tumors in the NC group were significantly greater than those in the KO group (Figure 4C). We also carried out immunohistochemical staining to detect proliferating cell nuclear antigen (PCNA) expression in the harvested tumors and the results showed that c-Myb KO significantly reduced its positive rate in tumor cells (Figure 4D). Finally, using TUNEL assay, we found c-Myb KO remarkably increased the apoptotic rate of tumor cells (Figure 4E). Taken together, these data suggested that c-Myb KO also effectively inhibited the growth of CRC cells in vivo.

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**TABLE 1** Correlations between c-Myb expression and clinicopathological characteristics in CRC patients

| Characteristics          | Total | Low   | High  | P value |
|--------------------------|-------|-------|-------|---------|
| Gender                   |       |       |       |         |
| Male                     | 73    | 32    | 41    | .542    |
| Female                   | 59    | 29    | 30    |         |
| Age (y)                  |       |       |       |         |
| ≤60                      | 26    | 10    | 16    | .376    |
| >60                      | 106   | 51    | 55    |         |
| Tumor location           |       |       |       |         |
| Colon                    | 68    | 33    | 35    | .582    |
| Rectum                   | 64    | 28    | 36    |         |
| Tumor size (cm)          |       |       |       |         |
| ≤5                       | 74    | 33    | 41    | .674    |
| >5                       | 58    | 28    | 30    |         |
| Tumor differentiation    |       |       |       |         |
| Well/moderate            | 115   | 54    | 61    | .655    |
| Poor                     | 17    | 7     | 10    |         |
| Tumor invasion           |       |       |       |         |
| T1–T2                    | 14    | 6     | 8     | .790    |
| T3–T4                    | 118   | 55    | 63    |         |
| Lymph node metastasis    |       |       |       |         |
| N0                       | 57    | 36    | 21    | <.001   |
| N1, N2                   | 75    | 25    | 50    |         |
| Serum CEA level (ng/mL)  |       |       |       |         |
| ≤5                       | 60    | 29    | 31    | .655    |
| >5                       | 72    | 32    | 40    |         |

CEA, carcinoembryonic antigen; CRC, colorectal cancer.
The bold values indicate statistically significant.
To further explore the molecular mechanisms regulated by c-Myb in CRC development, RNA-seq was first used to discover significantly expressed genes between the KO group and its negative control in HCT116 cells. Significantly expressed genes were defined as follows: 1, corrected P-value < .05; 2, absolute value of fold change > 2.

The heatmap demonstrated all the significantly expressed genes in c-Myb KO HCT116 cells and negative control (Figure 5A). Gene ontology (GO) functional analysis showed these genes participated in several cancer-related functions such as cell proliferation, cell migration, and cell death (Figure 5B). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed these genes were also involved in several cancer-related signaling pathways such as PI3K protein kinase B (PI3K-Akt) and MAPK signaling pathway (Figure 5C). Among the downregulated genes, the top 10 genes were selected according to their corrected P-values and fold change values (Figure 5D). Then, Cytoscape software was used to predict the interaction between c-Myb and those genes. Results suggested that only the c-fos gene appeared to have an interaction with c-Myb (Figure 6A). We carried out RT-PCR (Figure 6B) and western blot (Figure 6C) to detect c-fos expression in c-Myb KO CRC cells. Results demonstrated that c-Myb KO CRC cells had a significantly lower expression of c-Myb than negative control both at the mRNA and protein levels. Furthermore, immunohistochemical analysis was used to detect the expression of c-fos in the same cohort, and representative staining results are shown in Figure 6D. Spearman correlation analysis showed that the expression of c-Myb and c-fos was positively correlated in CRC tissues ($r = .256$, $P = .003$). For further validation of the interaction between c-Myb and c-fos, we carried out ChiP and the results indicated that c-Myb KO effectively inhibited the binding of c-Myb with the promoter of the c-fos gene (Figure 6E). Finally, using the luciferase reporter gene assay, we found c-Myb overexpression increased the activity of the wild-type promoter of the c-fos gene (Figure 6F). Taken together, these findings suggested that c-Myb may promote CRC progression by regulating c-fos.
### TABLE 2  Univariate analysis for prognostic factors affecting CRC overall survival and disease-free survival

| Variables                      | OS                      | DFS                      |
|-------------------------------|-------------------------|--------------------------|
|                               | HR   | 95% CI      | P value   | HR   | 95% CI | P value |
| Gender                        |      |             |           |      |        |         |
| Male vs female                | 1.442 | 0.819-2.538 | .205     | 0.831 | 0.496-1.392 | .482 |
| Age (y)                       |      |             |           |      |        |         |
| ≤60 vs >60                    | 1.218 | 0.610-2.430 | .576     | 1.296 | 0.656-2.563 | .456 |
| Tumor location                |      |             |           |      |        |         |
| Colon vs rectal               | 0.978 | 0.576-1.688 | .936     | 0.908 | 0.539-1.528 | .715 |
| Tumor size (cm)               |      |             |           |      |        |         |
| ≤5 vs >5                      | 1.117 | 0.647-1.927 | .692     | 1.142 | 0.682-1.914 | .613 |
| Tumor differentiation         |      |             |           |      |        |         |
| Well/moderate vs poor         | 1.251 | 0.533-2.938 | .607     | 1.169 | 0.529-2.585 | .699 |
| Tumor invasion                |      |             |           |      |        |         |
| T1-T2 vs T3-T4                | 1.684 | 0.524-5.410 | .381     | 1.538 | 0.557-4.250 | .407 |
| Lymph node metastasis         |      |             |           |      |        |         |
| N0 vs N1-N2                   | 2.796 | 1.502-5.203 | .001     | 2.112 | 1.210-3.686 | .008 |
| Serum CEA level (ng/mL)       |      |             |           |      |        |         |
| ≤5 vs >5                      | 0.816 | 0.473-1.407 | .465     | 0.947 | 0.563-1.591 | .836 |
| c-Myb expression              |      |             |           |      |        |         |
| Low vs high                   | 4.355 | 2.235-8.487 | <.001    | 3.286 | 1.850-5.837 | <.001 |

CEA, carcinoembryonic antigen; CI, confidence interval; CRC, colorectal cancer; DFS, disease-free survival; HR, hazard ratio; OS, overall survival. The bold values indicate statistically significant.

### TABLE 3  Multivariate analysis for prognostic factors affecting CRC overall survival and disease-free survival

| Variables                      | OS                      | DFS                      |
|-------------------------------|-------------------------|--------------------------|
|                               | HR   | 95% CI      | P value   | HR   | 95% CI | P value |
| Gender                        |      |             |           |      |        |         |
| Male vs female                | 1.396 | 0.774-2.520 | .268     | 0.768 | 0.448-1.317 | .338 |
| Age (y)                       |      |             |           |      |        |         |
| ≤60 vs >60                    | 1.436 | 0.706-2.921 | .318     | 1.380 | 0.678-2.809 | .374 |
| Tumor location                |      |             |           |      |        |         |
| Colon vs rectal               | 0.856 | 0.484-1.515 | .593     | 0.953 | 0.553-1.643 | .863 |
| Tumor size (cm)               |      |             |           |      |        |         |
| ≤5 vs >5                      | 1.225 | 0.686-2.190 | .493     | 1.260 | 0.725-2.192 | .413 |
| Tumor differentiation         |      |             |           |      |        |         |
| Well/moderate vs poor         | 1.431 | 0.580-3.528 | .436     | 1.175 | 0.501-2.754 | .711 |
| Tumor invasion                |      |             |           |      |        |         |
| T1-T2 vs T3-T4                | 1.990 | 0.598-6.623 | .262     | 1.735 | 0.603-4.994 | .307 |
| Lymph node metastasis         |      |             |           |      |        |         |
| N0 vs N1-N2                   | 2.180 | 1.108-4.289 | .024     | 1.914 | 1.048-3.494 | .035 |
| Serum CEA level (ng/mL)       |      |             |           |      |        |         |
| ≤5 vs >5                      | 0.739 | 0.421-1.300 | .294     | 0.936 | 0.548-1.600 | .809 |
| c-Myb expression              |      |             |           |      |        |         |
| Low vs high                   | 3.729 | 1.867-7.450 | <.001    | 2.993 | 1.645-5.447 | <.001 |

CEA, carcinoembryonic antigen; CI, confidence interval; CRC, colorectal cancer; DFS, disease-free survival; HR, hazard ratio; OS, overall survival. The bold values indicate statistically significant.
Overexpression of c-fos rescues the inhibitory effect of c-Myb KO on the malignant characteristics of CRC cells in vitro

To further confirm whether c-fos is responsible for the oncogenic role of c-Myb, we constructed plasmids to upregulate c-fos expression in c-Myb KO CRC cells. Overexpression efficiency of constructed plasmids was validated by RT-PCR (Figure 7A) and western blot (Figure 7B). In the CCK-8 assay, c-fos overexpression effectively accelerated the proliferation of c-Myb KO HCT116 and SW620 cells (Figure 7C). In the apoptosis assay, c-fos overexpression significantly decreased the apoptosis rate of c-Myb KO HCT116 and SW620 cells (Figure 7D). Transwell assay showed c-fos overexpression significantly promoted the migratory and invasive ability of c-Myb KO HCT116 and SW620 cells (Figure 7E,F). Finally, colony formation assay indicated c-fos overexpression also increased the colony numbers of c-Myb KO HCT116 and SW620 cells (Figure 7G). Taken together, these results collectively suggested that c-fos overexpression effectively rescued the inhibitory effect of c-Myb KO on the malignant characteristics of CRC cells in vitro.
3.6 | Overexpression of c-fos rescues the inhibitory effect of c-Myb KO in the EMT phenotype

As EMT is a crucial molecular mechanism regulating CRC growth and metastasis, we used western blot to detect its representative markers (snail, E-cadherin, N-cadherin, Vimentin) in CRC cells (Figure 8). Compared with negative control, c-Myb KO CRC cells had a significantly decreased expression of N-cadherin, Vimentin and snail, but increased expression of E-cadherin. Compared with c-Myb KO CRC cells, c-fos overexpression significantly increased the expression of N-cadherin, Vimentin and snail, but decreased the expression of E-cadherin. Taken together, these findings suggested that c-Myb KO inhibited the EMT phenotype of CRC cells whereas c-fos overexpression could rescue this inhibitory effect.

4 | DISCUSSION

It is well established that members of the MYB family exist in all the eukaryotic lineages and participate in various biological processes by regulating the transcription of target genes. Recently, increasing evidences have suggested they play a crucial role in tumor initiation and progression. For example, B-Myb overexpression was found to promote tumor growth by altering the cell cycle which correlates with unfavorable prognosis. Using in-situ hybridization, researchers found that MYB or MYBL1 gene splits may be associated with local invasion of salivary gland adenoid cystic carcinoma. c-Myb, as a member of the MYB family, has been closely linked to malignant progression of several human malignancies; however, its role in CRC remains poorly investigated.

In this study, we first detected the expression of c-Myb in CRC and its matched adjacent normal tissues. We found its expression was significantly higher in CRC tissues, which is in accordance with previous observations in other tumors including laryngeal, nasopharyngeal, and gallbladder. Correlation analysis showed that c-Myb expression was associated with lymph node metastasis, implying its potential participation in CRC metastasis. To identify its prognostic significance, we constructed Kaplan-Meier survival curves based on follow-up data. We found patients with high c-Myb expression had a significantly worse OS and DFS than those with low c-Myb expression. In addition, using subgroup analysis, we found c-Myb expression could effectively stratify the OS and DFS of patients within stage II or stage III, suggesting that detecting c-Myb expression may help predict patient prognosis within the same TNM
stage. Multivariate analysis showed that c-Myb expression is an independent unfavorable prognostic factor affecting OS and DFS, further confirming its predictive role. Similar findings have also been obtained in other human malignancies. Ma et al.\(^1\) found that high expression of c-Myb was associated with advanced clinical stage and worse OS/progression-free survival in patients with diffuse large B-cell lymphoma. They also proved its same prognostic value in patients with Burkitt lymphoma.\(^1\) Li et al.\(^6\) found that a high mRNA level of c-Myb indicated worse relapse-free survival in patients with breast cancer, and that it can combine with interleukin (IL)-1β to predict OS in patients who are estrogen receptor(−). In CRC, based on the current evidence, we speculated that c-Myb may contribute to poor outcome in two ways. One is that c-Myb may promote postoperative metastasis of CRC cells. The other is that c-Myb may induce chemotherapy resistance in CRC cells, as inspired by our previous work showing that c-Myb is a target of microRNA (miR)-150 and that miR-150 level is correlated with patient response to adjuvant chemotherapy.\(^9\) Pekarčíková et al.\(^20\) also found that c-Myb could

**FIGURE 6** c-Myb promotes colorectal carcinoma (CRC) progression by regulating c-fos. A, Cytoscape software predicts that c-Myb has interaction with c-fos. B, RT-PCR indicates that c-Myb KO decreases the mRNA level of the c-fos gene in HCT116 and SW620 cells. C, Western blot indicates that c-Myb KO decreases the protein level of c-fos in HCT116 and SW620 cells. D, Representative immunohistochemical staining images of c-fos in CRC tissues. E, ChIP assay shows that c-Myb KO effectively inhibited the binding of c-Myb with the promoter of the c-fos gene in HCT-116 and SW620 cells. F, Luciferase reporter gene assay indicates that c-Myb overexpression (OE) increases the activity of the wild type (WT) promoter of the c-fos gene, whereas it has no impact on the activity of the mutant type (MT) promoter of the c-fos gene, as compared with negative control (NC). **P < .01, ***P < .001. Error bar represents standard deviation, n = 3

**FIGURE 7** Overexpression of c-fos rescues the inhibitory effect of c-Myb KO on the malignant characteristics of colorectal carcinoma (CRC) cells in vitro. A, RT-PCR validates that the mRNA level of c-fos in c-Myb KO CRC cells is increased by c-fos overexpression. B, Western blot validates that the protein level of c-fos in c-Myb KO CRC cells is increased by c-fos overexpression. C, c-fos overexpression accelerates the increase of the optical density value in c-Myb KO CRC cells. D, c-fos overexpression decreases the apoptosis rate of c-Myb KO CRC cells. E, c-fos overexpression increases migratory cells in c-Myb KO CRC cells. F, c-fos overexpression increases invasive cells in c-Myb KO CRC cells. G, c-fos overexpression increases colony numbers of c-Myb KO CRC cells. NC, negative control; OE, overexpression. **P < .05, ***P < .01, ****P < .001. Error bar represents standard deviation, n = 3
prevent the cisplatin/oxaliplatin/doxorubicin-induced apoptosis of CRC cells through NOX1/p38 signaling pathways. Taken together, we concluded that c-Myb may serve as a promising indicator for patient prognosis, but this needs multicenter validations based on more clinical samples.

As c-Myb is correlated with clinical outcome, we next carried out assays in vitro and in vivo to investigate its biological role in CRC cells. In vitro, we found that c-Myb KO significantly inhibited proliferation, apoptosis resistance, migration, invasion and colony formation in both HCT-116 and SW620 cells. In vivo, we found that c-Myb KO significantly inhibited xenograft growth, decreased PCNA expression and induced tumor apoptosis. These findings collectively suggest that c-Myb plays a crucial role in promoting the malignant characteristics of CRC cells.

To further investigate the oncogenic mechanisms regulated by c-Myb in CRC, we used RNA sequencing to compare significantly expressed genes between c-Myb KO CRC cells and their negative control. In the biological function analysis, we found these genes were significantly enriched in cell proliferation, cell migration, and cell death. Meanwhile, the KEGG pathway showed that these genes were also significantly enriched in several oncogenic signaling pathways such as PI3K and MAPK. Both results may partly explain why c-Myb KO resulted in impaired malignant characteristics of CRC cells. Correlations of c-Myb with these oncogenic pathways have already been indicated in several previous studies. For example, Huang et al.\textsuperscript{21} found that c-Myb is a downstream transcription factor of PI3K/Akt signaling, contributing to the growth and invasion of gastric cancer. Pekarčiková et al.\textsuperscript{20} found that c-Myb could activate the p38 MAPK signaling pathway to induce chemotherapy resistance of CRC cells. Next, using bioinformatics analysis, we tried to identify a direct downstream gene of c-Myb and found that c-fos might be a candidate. c-fos, also known as Fos proto-oncogene, not only participates in tissue development and cellular stress, but is also involved in malignant transformation and progression in numerous tumors.\textsuperscript{22,23} Overexpression of c-fos promotes the growth and metastasis of osteosarcoma cells by regulating Wnt2 and Fzd9.\textsuperscript{24} Liver-specific c-fos overexpression contributed to premalignant hepatocyte transformation and diethylnitrosamine-induced hepatocellular carcinoma through inflammation and metabolic pathways.\textsuperscript{25} c-fos could also up-regulate P-glycoprotein to induce fluorouracil resistance in laryngeal cancer cells with vincristine resistance.\textsuperscript{26} Subsequent RT-PCR and western blot both indicated that c-Myb KO significantly decreased the expression of c-fos both at the mRNA and protein levels. In addition, we found that expression of c-Myb and c-fos are positively

**FIGURE 8** Overexpression of c-fos rescues the inhibitory effect of c-Myb KO in the epithelial-mesenchymal transition (EMT) phenotype. c-Myb KO increases E-cadherin expression, but decreases N-cadherin, Vimentin and snail expression in HCT-116 (upper panel) and SW620 (lower panel) cells, whereas c-fos overexpression could rescue this effect. NC, negative control; OE, overexpression. *P < .05, **P < .01, ***P < .001, ****P < .0001. Error bar represents standard deviation, n = 3.
correlated in CRC tissues. More importantly, ChIP assay showed that c-Myb KO effectively inhibited the binding of c-Myb with the promoter of the c-fos gene, and luciferase reporter gene assay showed that c-Myb overexpression increased the activity of the wild-type promoter of the c-fos gene. Finally, we found that c-fos overexpression could rescue the inhibitory effect of c-Myb KO in proliferation, apoptosis resistance, invasion, migration and colony formation. Taken together, these findings strongly suggest that c-Myb promotes the malignant progression of CRC through regulating c-fos.

It is well established that EMT is a crucial molecular mechanism involved in tumor growth and metastasis. Molecularly, the EMT phenotype is characterized by downregulation of epithelial markers (E-cadherin) and upregulation of mesenchymal markers (N-cadherin, Vimentin and snail). In hepatocellular carcinoma, c-Myb functions as a target of long non-coding RNA downregulated in hepatocellular carcinoma (DRHC) to promote the EMT phenotype through the MEK/ERK signaling pathway. c-Myb could also cooperate with transforming growth factor beta (TGF-β) to enhance the EMT phenotype of estrogen(+) breast cancer cells. Meanwhile, c-fos has been proven to induce EMT as well as the stem cell phenotype of head and neck squamous cell carcinoma. Therefore, we speculated that c-Myb may promote EMT by regulating c-fos in CRC. To validate this, we detected the expression of fourteen widely used EMT markers in c-Myb KO cells, c-Myb KO + c-fos overexpression cells and negative control. As a result, we found that c-Myb KO increased the expression of E-cadherin, but decreased the expression of N-cadherin, Vimentin and snail. However, this effect of c-Myb KO could be effectively rescued by c-fos overexpression, strongly suggesting that c-Myb promotes CRC progression through EMT by upregulating c-fos.

In summary, the present study found that c-Myb was abnormally overexpressed in CRC tissues and was correlated with lymph node metastasis. High expression of c-Myb is an independent unfavorable factor affecting patient prognosis. Mechanism investigations showed that c-Myb promoted the growth and metastasis of CRC cells through EMT by upregulating c-fos. These findings collectively indicate that c-Myb is a promising prognostic indicator or therapeutic target for CRC patients. Future work is needed to further clarify its clinical value as well as other potential oncogenic mechanisms in CRC.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION
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