

miR-200 Modulates EMT in Colorectal Cancer via ZEB1, BBC3, SMAD, and NRAS: Implications for Metastasis through E-Cadherin Suppression and MMP-9 Activation

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Abstract

Background

Colorectal cancer (CRC) remains the second leading cause of cancer-related mortality worldwide, with metastasis driving 90% of deaths. The epithelial-mesenchymal transition (EMT) is a critical process enabling tumor dissemination, yet the molecular networks orchestrating EMT in CRC are incompletely understood. The miR-200 family antagonizes the EMT-inducing transcription factor ZEB1, but the interplay between miR-200 and non-canonical regulators—including BBC3 (PUMA), SMAD2, and NRAS—remains poorly characterized in clinical CRC tissues.

Methods

Fifty formalin-fixed, paraffin-embedded (FFPE) CRC tissues and matched adjacent normal tissues were obtained from the Tumor Bank of Cancer Institute, Imam Khomeini Hospital Complex. Expression levels of miR-200, ZEB1, BBC3, SMAD2, NRAS, and vimentin were quantified by qRT-PCR. Protein expression of E-cadherin and MMP-9 was assessed by Western blot. Expression profiles were correlated with tumor grade (grades 1–3) and stage (stages 1–3).

Results

miR-200 expression was significantly reduced in CRC tissues compared to adjacent normal tissues ($P < 0.05$), with progressive loss observed in advanced grade and stage tumors. Conversely, ZEB1, BBC3, NRAS, and vimentin were significantly upregulated in tumor tissues ($P < 0.05$, $P < 0.0032$, $P < 0.012$, and $P < 0.002$, respectively), and their expression increased progressively from grade 1 to grades 2–3 and from stage 1 to stages 2–3. SMAD2 expression trended higher in tumors but did not reach statistical significance ($P = 0.0847$); however, stratified analysis revealed significantly elevated SMAD2 in grade 2 and stages 2–3 tumors ($P < 0.05$). Western blot confirmed decreased E-cadherin and increased MMP-9 protein expression in tumor tissues, consistent with an EMT phenotype.

Conclusion

Loss of miR-200 in CRC is associated with coordinated upregulation of ZEB1, NRAS, BBC3, and vimentin, alongside E-cadherin suppression and MMP-9 activation—a molecular signature indicative of active EMT. These changes correlate with higher tumor grade and stage, implicating the miR-200/ZEB1 axis as a key driver of CRC progression. SMAD2 exhibits a stage-dependent, non-linear expression pattern, suggesting a nuanced role. Restoration of miR-200 function or therapeutic targeting of ZEB1 may represent promising strategies to suppress EMT and improve outcomes in metastatic CRC.

Introduction

Colorectal cancer (CRC) ranks as the third most commonly diagnosed malignancy and the second leading cause of cancer-related deaths worldwide, with over 1.8 million new cases and 880,000 deaths annually(1, 2). While developments in screening and treatment have improved survival rates in high-income countries, CRC remains a difficult challenge in low-resource settings, where late-stage diagnoses prevail. The disease arises from cumulative genetic and epigenetic alterations in oncogenes (like KRAS, NRAS), tumor suppressors (such as APC, TP53), and DNA repair pathways, often worsened by modifiable risk factors such as obesity, sedentary lifestyles, and diets rich in processed meats(3). Despite therapeutic strides, metastatic CRC—responsible for 90% of CRC mortality—continues to evade strong remission, underscoring the urgency to clarify mechanisms driving tumor dissemination (4).

Metastasis in CRC is promoted by epithelial-mesenchymal transition (EMT), a dynamic process enabling polarized epithelial cells to adopt invasive mesenchymal phenotypes. During EMT, cells downregulate adhesion molecules like E-cadherin, upregulate mesenchymal markers (e.g., vimentin, N-cadherin), and secrete matrix metalloproteinases (MMPs), facilitating extracellular matrix degradation and intravasation into the bloodstream (5). EMT is orchestrated by transcription factors such as ZEB1, Snail, and Twist, which repress epithelial genes while activating pro-migratory pathways. Notably, ZEB1 directly suppresses CDH1 (E-cadherin) and enhances MMP-9 expression, promoting metastatic niche formation (6). However, EMT's pleiotropic regulation—spanning developmental, inflammatory, and oncogenic contexts—complicates its therapeutic targeting, necessitating deeper insights into its molecular mediators (7).

One of the important epigenetic signals in EMT regulation belongs to the miR-200 family, a group of microRNAs that antagonize ZEB1/2, thereby preserving epithelial integrity. The miR-200/ZEB1 axis forms a reciprocal feedback loop: miR-200 represses ZEB1 mRNA, while ZEB1 binds to miR-200 promoters, silencing their transcription (8). In CRC, miR-200 downregulation correlates with advanced tumor stages, chemoresistance, and poor prognosis, implicating its loss as a promoter of EMT-driven metastasis (9). Beyond ZEB1, EMT intersects with oncogenic signaling pathways, including TGF- β /SMAD, RAS/MAPK, and apoptosis regulators like BBC3 (PUMA) (10). For instance, SMAD2 mediates TGF- β 's dual role in CRC—suppressing early tumor growth while promoting late-stage invasion (11)—while BBC3, though pro-apoptotic, may paradoxically enhance survival under genotoxic stress (12). These complexities highlight the need to characterize EMT-related gene expression within CRC's heterotypic tumor microenvironment.

Previous studies have delineated EMT's contribution to CRC progression, yet critical gaps persist. First, the interplay between miR-200 and non-canonical EMT regulators—such as BBC3, SMAD2, and NRAS—remains unknown, particularly in clinical cohorts (13). Second, while miR-200's tumor-suppressive role in tissues is established, its utility as a circulating biomarker or therapeutic agent has not been clarified (14). Third, conflicting reports on SMAD2's role in EMT—as either a tumor suppressor or promoter—demand resolution through stage-specific analyses (15). SLC14A1 and TGF- β signaling: a feedback loop

driving EMT and colorectal cancer metachronous liver metastasis Furthermore, NRAS mutations, though less frequent in CRC than KRAS, drive aggressive phenotypes via MAPK pathway activation, yet their synergy with EMT markers is poorly characterized (16). Addressing these questions could refine prognostic models and unveil novel therapeutic nodes.

This study investigates the expression of miR-200 and its downstream targets—ZEB1, BBC3, SMAD2, NRAS, and vimentin—in CRC tissues, correlating these profiles with EMT markers (E-cadherin, MMP-9) and clinical parameters (tumor grade, stage) .

We hypothesize that miR-200 loss exacerbates EMT by de-repressing ZEB1, thereby suppressing E-cadherin, elevating MMP-9, and activating oncogenic pathways (e.g., RAS/SMAD). By integrating qRT-PCR, Western blot, and clinicopathological data, we aim to (1) delineate miR-200's regulatory network in CRC metastasis, (2) assess BBC3 and SMAD2's contextual roles in EMT, and (3) evaluate the prognostic utility of EMT-related biomarkers. Our findings may inform strategies to restore miR-200 function or disrupt ZEB1-driven EMT, ultimately improving outcomes for metastatic CRC patients.

Materials and Methods

Study Samples and Tissue Preparation

A total of 50 formalin-fixed, paraffin-embedded (FFPE) colorectal cancer (CRC) tissue and non-tumor adjacent tissue samples, along with corresponding clinical-pathological data, were obtained from the tumor bank of the Cancer Institute, Imam Khomeini Hospital Complex. All samples were reviewed and histologically confirmed by a board-certified pathologist. From each paraffin block, 6- μ m thick sections were cut (11 slices per block) for subsequent analyses. This study was conducted in accordance with the ethical standards of the Declaration of Helsinki and was approved by the Ethics Committee (Institutional Review Board) of Imam Khomeini Hospital Complex, Tehran University of Medical Sciences (Approval No. IR.TUMS.IKHC.REC.1401.019). Written informed consent for the use of archived tissue samples and associated clinicopathological data in research was obtained from all patients at the time of sample collection.

RNA Extraction and cDNA Synthesis

RNA Extraction and cDNA Synthesis

Total RNA was extracted from FFPE samples using the RNA X-Plus method. RNA quality and concentration were assessed prior to cDNA synthesis. For gene expression analysis, complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using a cDNA Synthesis Kit (Gene Zist Fannavar Ana, Iran) with a combination of random hexamer and oligo (dT) primers, following the manufacturer's protocol.

For microRNA expression analysis, cDNA was synthesized using a stem-loop-based microRNA synthesis kit (Anacell, Iran).

Quantitative Real-Time PCR (qRT-PCR)

Gene-specific primers for *ZEB1*, *BBC3*, *SMAD2*, *NRAS*, and *Vimentin* were designed using Primer3 software based on nucleotide sequences retrieved from the NCBI database. Primer specificity was verified using NCBI Primer-BLAST. All primers were synthesized by GenSCRIPT (Germany). The primer sequences are listed in Supplementary Table S1.

Quantitative real-time PCR was performed according to the protocols described in Supplementary Tables S2 and S3, following methods from previous studies. PCR reactions were carried out using a Rotor-Gene real-time PCR system (Qiagen, USA).

Protein Extraction and Western Blotting

Protein was extracted from FFPE samples following deparaffinization using lysis buffer. Protein concentration was determined using the Bradford assay with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to established protocols.

Separated proteins were transferred onto nitrocellulose membranes. Membranes were incubated overnight at 4°C with primary antibodies against MMP-9 (rabbit polyclonal, 1:500; Biorbyt, UK) and E-cadherin (rabbit polyclonal, 1:1000; Biorbyt, UK). For normal adjacent tissue samples, the same antibodies were applied. β -actin (mouse monoclonal antibody, 1:1000; Santa Cruz Biotechnology, USA; molecular weight 43 kDa) was used as a loading control.

After primary antibody incubation, membranes were washed several times with Tris-buffered saline containing Tween-20 (TBST) and then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) diluted in TBST for 1 hour at room temperature. Membranes were washed three more times with TBST and then incubated with a Chemiluminescence Detection Kit (Pars Tous Biotechnology, Iran). Protein bands were visualized by exposing membranes to Kodak radiographic film for 1 minute, and band intensities were assessed.

Statistical Analysis

Real-time PCR data were analyzed using LinReg PCR and REST (Relative Expression Software Tool) to calculate gene expression levels. PCR efficiency for each sample was determined from the average amplification curve. Statistical analyses were performed using SPSS software. Differences between groups (e.g., tumor vs. non tumor adjacent tissue) were evaluated using independent or paired Student's t-test, as appropriate. Transcript expression was quantified using the comparative threshold cycle

($2^{-\Delta\Delta C_t}$) method, where C_t values of target genes in experimental samples were compared to those in control samples (e.g., untreated or normal adjacent tissues). A p -value < 0.05 was considered statistically significant.

Bioinformatics Analysis

To further investigate the functional relationships among miR-200 and its downstream targets, a curated gene set relevant to cancer-associated signaling (Supplementary Table) was subjected to pathway enrichment analysis using Enrichr (31) against the KEGG 2021 Human gene-set library; pathways with a nominal $P < 0.05$ were considered significantly enriched. Protein–protein interaction (PPI) networks among the candidate genes were constructed using the STRING database (32) (interaction confidence score ≥ 0.4) to visualize direct and indirect functional associations. In addition, the prognostic relevance of NRAS, SMAD2, BCL2, and ZEB1 mRNA expression was evaluated using the GEPIA2 platform (33), which draws on The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma RNA-sequencing data. Patients were stratified into high- and low-expression groups using the median expression value as the cutoff ($n = 135$ per group), and overall survival was compared using Kaplan–Meier curves and the log-rank test. Putative binding targets of miR-200a-5p and miR-200b-5p were additionally retrieved from TargetScanHuman (release 8.0) (34).

Result

Comparison of genes expression between tumor and normal tissues

Statistical comparison of ZEB1 gene expression in tumor and normal tissue shows the expression of ZEB1 gene in tumor tissue compared to normal tissue. Comparison of adjacent tissue and tumor showed that the amount of ZEB1 in tumor tissue is usually higher than in adjacent tissue ($P < 0.05$). Figure 1A

The evaluation of BBC3 gene expression in tumor and normal tissue statistically illustrates an increase in BBC3 gene expression in tumor tissue compared to normal tissue. Comparison with adjacent tissue showed that BBC3 expression in tumor tissue increased significantly compared to normal tissue ($P < 0.0032$). Figure 1B

SMAD2 gene expression level was evaluated in tumor and normal tissue, which reveals the increased expression level of tumor tissue to normal tissue, although the statistical comparison of the expression level of tumor tissue and normal tissue did not show a significant relationship ($P < 0.0847$). Figure 1C

Comparing NRAS gene expression in tumor and normal tissue demonstrates the level of NRAS gene expression in tumor tissue as opposed to normal tissue. Statistical analysis further indicates a significant correlation between increased expression in tumor tissue to normal ($P < 0.012$). Figure 1D

The results of the analysis show an increase in Vimentin gene expression in tumor tissue compared to normal tissue, and a significant correlation between Vimentin gene expression in tumor tissue and normal tissue can be found ($P < 0.002$). Figure 1E

Statistical analysis has proven a significant reduction in the expression level of miR-200 in colorectal tumor tissue compared to the adjacent normal tissue ($P < 0.05$). Figure 1F

Comparison of genes expression in different grades

The comparison of ZEB1 gene expression in tumor tissue in different grades showed that ZEB1 gene expression in grades 2 and 3 increased significantly compared to grade I ($P < 0.05$). Figure 2A

The evaluation of BBC3 gene expression in tumor tissue with different grades illustrated that the level of BBC3 gene expression in grades 2 and 3 increased significantly compared to grade 1 ($P < 0.05$). Figure 2B

Grade 2 tumor tissue showed higher levels of SMAD gene expression compared with grades 1 and 3 ($P < 0.05$). Figure 2C

Although the analysis of miR-200 expression across tumor tissues of varying grades revealed a significant reduction in miR-200 levels in grade 2 and 3 tumors relative to grade 1 ($P < 0.05$) Fig. 2F, comparing NRAS gene expression in tumor tissues with different grades revealed that it increased significantly in grades 2 and 3 when compared to grade I ($P < 0.05$). Figure 2D

Statistical analysis of vimentin gene expression in tumor tissue in different grades has proven the increase of vimentin expression in grades 2 and 3 relative to grade 1 ($P < 0.05$). Figure 2E.

Western blot results indicated increased MMP-9 protein expression and decreased E-cadherin protein expression in tumor tissue compared with normal tissue (Fig. 3).

The results of the analysis showed that ZEB1 gene expression increases with increasing stage. In this study, ZEB1 expression increased in stage 2 compared to stage 3 ($P < 0.05$). Figure 4A

Also, the results of the analysis showed that in this study, the expression of BBC3 gene increases with increasing stage, so that the level of BBC3 expression in stage 2 is increased compared to stage 3 ($P < 0.05$). Figure 4B

Comparison of SMAD gene expression level in tumor tissue in different stages showed that the expression of this gene increased significantly in stages 2 and 3 compared to stage I ($P < 0.05$). Figure 4C

While Stage 2 and 3 showed significantly higher NRAS expression than stage I when comparing expression of this gene in tumor tissue ($P < 0.05$) Fig. 4D, the analysis of miR-200 expression across

tumor tissues of varying stages revealed a significant reduction in miR-200 levels in stage 2 and 3 tumors compared to stage 1 ($P < 0.05$) Fig. 4F

In addition, different stages of vimentin gene expression were determined as well as investigated by the pathologist to determine the relationship of these genes with cancer progression. The results of comparing the level of vimentin expression in different stages revealed that the expression of this gene increased significantly in stages 2 and 3 compared to stage 1 ($P < 0.05$). Figure 4E

Bioinformatics Analysis

KEGG pathway enrichment analysis showed that the candidate gene set was significantly over-represented in cancer-related pathways, including “Pathways in cancer,” “Ras signaling pathway,” “Colorectal cancer,” and “p53 signaling pathway” (Table 1), all of which are mechanistically linked to EMT, apoptosis, and tumor progression. Notably, SMAD2, BBC3, NRAS, and BCL2 co-occurred across several of the top-ranked pathways, supporting a convergent role for these genes in colorectal cancer signaling.

STRING-based PPI network analysis (Fig. 5) revealed that NRAS and PIK3CA occupy central hub positions within the network, with direct connections to SMAD2, BCL2, BBC3/PMAIP1, and members of the TGF- β receptor family (TGFB1, TGFB2), indicating strong functional interconnectivity among the Ras/PI3K–Akt, TGF- β , and p53-associated apoptotic pathways relevant to colorectal tumorigenesis.

Consistent with these findings, TargetScanHuman (release 8.0) listed BBC3, SMAD2, and NRAS among the predicted targets of miR-200a-5p/miR-200b-5p. BBC3 carried a single 8mer site for miR-200b-5p (total context ++ score = -0.28); SMAD2 carried six seed sites for miR-200a-5p (one 7mer-A1 and five 6mer; total context ++ score = -0.01); and NRAS carried three seed sites for miR-200a-5p (one 7mer-m8 and two 6mer; total context ++ score = -0.14). The near-zero to modestly negative context ++ scores for all three genes indicate weak predicted regulatory potential relative to canonical miR-200-3p/ZEB1 targeting and warrant direct experimental validation. The complete list of TargetScanHuman-predicted targets for miR-200a-5p and miR-200b-5p is provided in Supplementary Table S4.

Kaplan–Meier survival analysis based on TCGA colorectal adenocarcinoma data (GEP1A2) did not reveal a statistically significant association between mRNA expression levels of BCL2 (HR = 0.85, log-rank $P = 0.50$; Fig. 6B), SMAD2 (HR = 1.00, $P = 0.97$; Fig. 6C), or NRAS (HR = 0.78, $P = 0.32$; Fig. 6D) and overall patient survival. ZEB1 expression showed a trend toward reduced survival in the high-expression group (HR = 1.40, $P = 0.18$; Fig. 6A), consistent with its recognized role as a driver of EMT and metastasis, although this trend did not reach statistical significance, possibly reflecting the heterogeneity of colorectal cancer or the limited cohort size.

Table 1
KEGG pathway enrichment analysis of the candidate gene set (Enrichr, KEGG 2021 Human)

KEGG pathway	Gene overlap	P-value	Representative genes
Pathways in cancer	108/531	0.0054	SMAD2, BBC3, NRAS, BCL2, TGFBR1/2, PIK3CA
Ras signaling pathway	53/232	0.0046	NRAS, PIK3CA, SOS1, PLCG1, MAPK8
Colorectal cancer	23/86	0.0082	SMAD2, NRAS, BCL2, BBC3, TGFBR1/2, MSH2, APC
p53 signaling pathway	19/73	0.0204	BBC3, BCL2, PMAIP1, CYCS, MDM2
TGF-beta signaling pathway	20/94	0.1134	SMAD2, TGFBR1/2, TGFB2, BMP2
PI3K-Akt signaling pathway	66/354	0.1112	NRAS, PIK3CA, BCL2, EGF, PDGFA

Discussion

The present study investigated the expression profiles of miR-200 and its potential downstream targets—ZEB1, BBC3, SMAD2, NRAS, and vimentin—in colorectal cancer (CRC) tissues compared to adjacent normal tissues, with further stratification by tumor grade and stage. Additionally, protein expression of E-cadherin and MMP-9 was assessed via Western blot. Our findings reveal a consistent pattern: loss of miR-200 accompanied by upregulation of EMT-associated transcription factors and oncogenic signaling molecules, which correlates with aggressive clinicopathological features.

Downregulation of miR-200 and Upregulation of ZEB1 in CRC

Our results demonstrate a significant reduction in miR-200 expression in CRC tissues compared to adjacent normal tissues ($P < 0.05$), with even greater loss observed in higher-grade (grades 2 and 3) and higher-stage (stages 2 and 3) tumors. Concurrently, ZEB1 expression was significantly elevated in tumor tissues ($P < 0.05$), and its expression progressively increased from grade 1 to grades 2 and 3, as well as with advancing tumor stage. These findings strongly support the existence of a reciprocal feedback loop between miR-200 and ZEB1, as previously described by Bracken et al (8). In the normal colonic epithelium, miR-200 maintains epithelial integrity by repressing ZEB1 translation (17). However, in CRC, downregulation of miR-200 relieves this repression, allowing ZEB1 to accumulate. Elevated ZEB1, in turn, binds to the promoters of miR-200 family members, further silencing their transcription—perpetuating a vicious cycle that drives EMT (18). Our stage- and grade-dependent data suggest that disruption of this axis is an early event that accelerates with tumor progression, consistent with prior reports linking miR-200 loss to advanced tumor stage, chemoresistance, and poor prognosis in CRC (19).

EMT Activation: Vimentin Upregulation with E-Cadherin Loss and MMP-9 Gain

We observed a significant increase in vimentin gene expression in tumor tissues compared to normal tissues ($P < 0.002$), with progressive upregulation across grades 2 and 3 and stages 2 and 3. Western blot analysis further revealed decreased E-cadherin protein expression and increased MMP-9 protein expression in tumor tissues relative to normal controls. This triad—vimentin rise, E-cadherin loss, and MMP-9 elevation—is a hallmark of complete EMT (20). Vimentin, a mesenchymal intermediate filament, confers cellular plasticity and migratory capacity, while loss of E-cadherin disrupts adherens junctions, destabilizing epithelial architecture (21). Simultaneously, MMP-9 degrades type IV collagen in the basement membrane, facilitating local invasion and intravasation. Given that ZEB1 directly suppresses the *CDH1* (E-cadherin) gene and activates *MMP-9* transcription (6), our findings position the miR-200/ZEB1 axis as a master regulator of this mesenchymal switch. The correlation of these molecular changes with higher grade and stage strongly implicates miR-200 loss and subsequent ZEB1-driven EMT in the metastatic progression of CRC.

NRAS Upregulation and Its Synergy with EMT

Our data show that NRAS expression is significantly elevated in CRC tissues ($P < 0.012$), with higher expression in grades 2–3 and stages 2–3 compared to grade 1 and stage 1, respectively. Although NRAS mutations are less frequent in CRC than KRAS mutations (22), our results suggest that NRAS overexpression—independent of mutation status—may contribute to aggressive phenotypes. NRAS activates the MAPK signaling pathway, which can cooperate with ZEB1 to enhance cell migration, survival, and proliferation (23). Furthermore, RAS signaling has been shown to downregulate miR-200 family members indirectly through transcription factors such as ZEB1 (24). Thus, a potential feed-forward loop may exist: miR-200 loss upregulates ZEB1, which promotes NRAS expression or activity, and NRAS/MAPK signaling further suppresses miR-200 transcription. While our gene expression data cannot distinguish between wild-type and mutant NRAS, the significant upregulation across malignant tissues and its correlation with higher grade and stage suggest that NRAS overexpression is a clinically relevant event that may synergize with ZEB1 to drive EMT and metastasis.

BBC3 (PUMA) Upregulation: A Paradoxical Finding

We observed a significant increase in BBC3 expression in tumor tissues ($P < 0.0032$), with progressive elevation from grade 1 to grades 2–3 and from stage 1 to stages 2–3. BBC3 encodes PUMA, a well-characterized pro-apoptotic BH3-only protein(25). At first glance, upregulation of a pro-apoptotic factor in malignancy seems counterintuitive. However, emerging evidence suggests that BBC3 can paradoxically promote tumor survival under conditions of genotoxic or oncogenic stress(26). Chronic BBC3 upregulation may induce apoptosis resistance by triggering the selection of apoptosis-deficient clones or by activating survival pathways such as NF- κ B(27). Alternatively, BBC3 may play context-dependent, non-apoptotic roles—including modulation of autophagy or mitochondrial dynamics—that favor tumor cell adaptation(28). In the setting of ZEB1-driven EMT and NRAS activation, elevated BBC3

might represent a failed compensatory apoptotic response that ultimately contributes to chemoresistance(29). Our grade- and stage-dependent increases suggest that BBC3 upregulation is not a bystander event but may actively co-evolve with aggressive tumor features. Further functional studies are needed to resolve whether BBC3 acts as a tumor suppressor, a pro-survival factor, or a context-dependent modulator in CRC.

SMAD2 Expression: A Trend Without Significance

SMAD2 expression trended higher in tumor tissues compared to normal tissues, but this difference did not reach statistical significance ($P = 0.0847$). However, when stratified by grade, grade 2 tumors showed significantly higher SMAD2 expression than both grade 1 and grade 3 tumors ($P < 0.05$). Similarly, stages 2 and 3 exhibited significantly higher SMAD2 expression compared to stage 1 ($P < 0.05$). This non-linear pattern—peak expression in intermediate-grade tumors—suggests that SMAD2 plays a stage-dependent, dual role in CRC progression. In early stages (grade 1/stage 1), SMAD2 may mediate TGF- β 's tumor-suppressive functions, including growth arrest and apoptosis. As tumors progress to grade 2/stage 2, SMAD2 expression rises, potentially contributing to TGF- β 's pro-invasive effects by activating EMT-related transcription factors. Interestingly, the decline in grade 3 tumors hints at possible loss of SMAD2 function through mutation or epigenetic silencing, allowing tumors to escape TGF- β -mediated growth suppression while maintaining an invasive phenotype through alternative pathways (e.g., ZEB1/NRAS). This pattern aligns with David et al. (2018), who described SMAD2's context-dependent switch from suppressor to promoter (30). Our findings underscore the importance of stage-specific analyses and caution against interpreting SMAD2 as a simple oncogene or tumor suppressor.

Integrated Model and Clinical Implications

Taken together, our results support a cohesive model: in normal colonic epithelium, miR-200 maintains low ZEB1, preserving E-cadherin expression and epithelial integrity. In CRC, miR-200 is progressively lost, releasing ZEB1 from repression. Elevated ZEB1 suppresses E-cadherin, activates MMP-9, and upregulates vimentin, driving EMT. Concurrent upregulation of NRAS (likely via MAPK activation) and BBC3 (potentially as a stress response) further potentiates the invasive phenotype. While SMAD2's role is more nuanced and stage-dependent, its elevation in intermediate stages suggests transient contributory roles in EMT activation.

From a clinical perspective, our findings identify several potential biomarkers and therapeutic nodes. First, reduced miR-200 expression in tissues may serve as a prognostic marker for aggressive CRC, and circulating miR-200 levels could be developed as a liquid biopsy tool. Second, ZEB1 overexpression—particularly in grades 2–3 and stages 2–3—could predict poor outcomes and resistance to conventional therapies. Third, strategies to restore miR-200 function (e.g., using miRNA mimics) or to disrupt ZEB1 (e.g., antisense oligonucleotides or small-molecule inhibitors) represent rational therapeutic approaches. Fourth, targeting the MAPK pathway in NRAS-overexpressing tumors, potentially in combination with EMT inhibitors, may improve outcomes. However, the paradoxical BBC3 upregulation suggests that

simply inducing apoptosis may be insufficient; instead, combination strategies that simultaneously reverse EMT and restore chemosensitivity are warranted.

Limitations of the Study

Several limitations should be acknowledged. First, this study is correlational and does not establish causality; functional experiments (e.g., miR-200 overexpression or knockdown in CRC cell lines) are needed to confirm the regulatory relationships inferred here. Second, sample size ($n = 50$) is modest, and validation in larger, independent cohorts is necessary. Third, our use of FFPE tissues, while clinically relevant, may introduce RNA degradation; however, we observed consistent patterns, suggesting sufficient RNA quality. Fourth, protein validation for all genes (e.g., ZEB1, NRAS, SMAD2, and BBC3) by Western blot or immunohistochemistry would strengthen our conclusions; currently, only E-cadherin and MMP-9 were assessed at the protein level. Fifth, we did not assess KRAS mutation status, which may confound NRAS findings, nor did we measure TGF- β or other upstream regulators of SMAD2. Finally, survival analyses were not performed; future studies should correlate these expression profiles with patient outcomes, including disease-free and overall survival.

Conclusions

In conclusion, this study demonstrates that loss of miR-200 in CRC is associated with upregulation of ZEB1, vimentin, NRAS, and BBC3, along with downregulation of E-cadherin and upregulation of MMP-9—a molecular signature consistent with active EMT. These changes correlate with higher tumor grade and stage, implicating the miR-200/ZEB1 axis as a key driver of CRC progression and metastasis. The non-significant but pattern-driven SMAD2 expression suggests a stage-dependent, dual role that warrants further investigation. Our findings highlight the potential of restoring miR-200 function or targeting ZEB1 as therapeutic strategies to suppress EMT and improve outcomes in patients with metastatic CRC.

Declarations

Ethics Approval and Consent to Participate:

This study was conducted in accordance with the ethical standards of the Declaration of Helsinki and was approved by the Ethics Committee (Institutional Review Board) of Imam Khomeini Hospital Complex, Tehran University of Medical Sciences (Approval No. IR.TUMS.IKHC.REC.1401.019). Written informed consent for the use of archived tissue samples and associated clinicopathological data in research was obtained from all patients at the time of sample collection.

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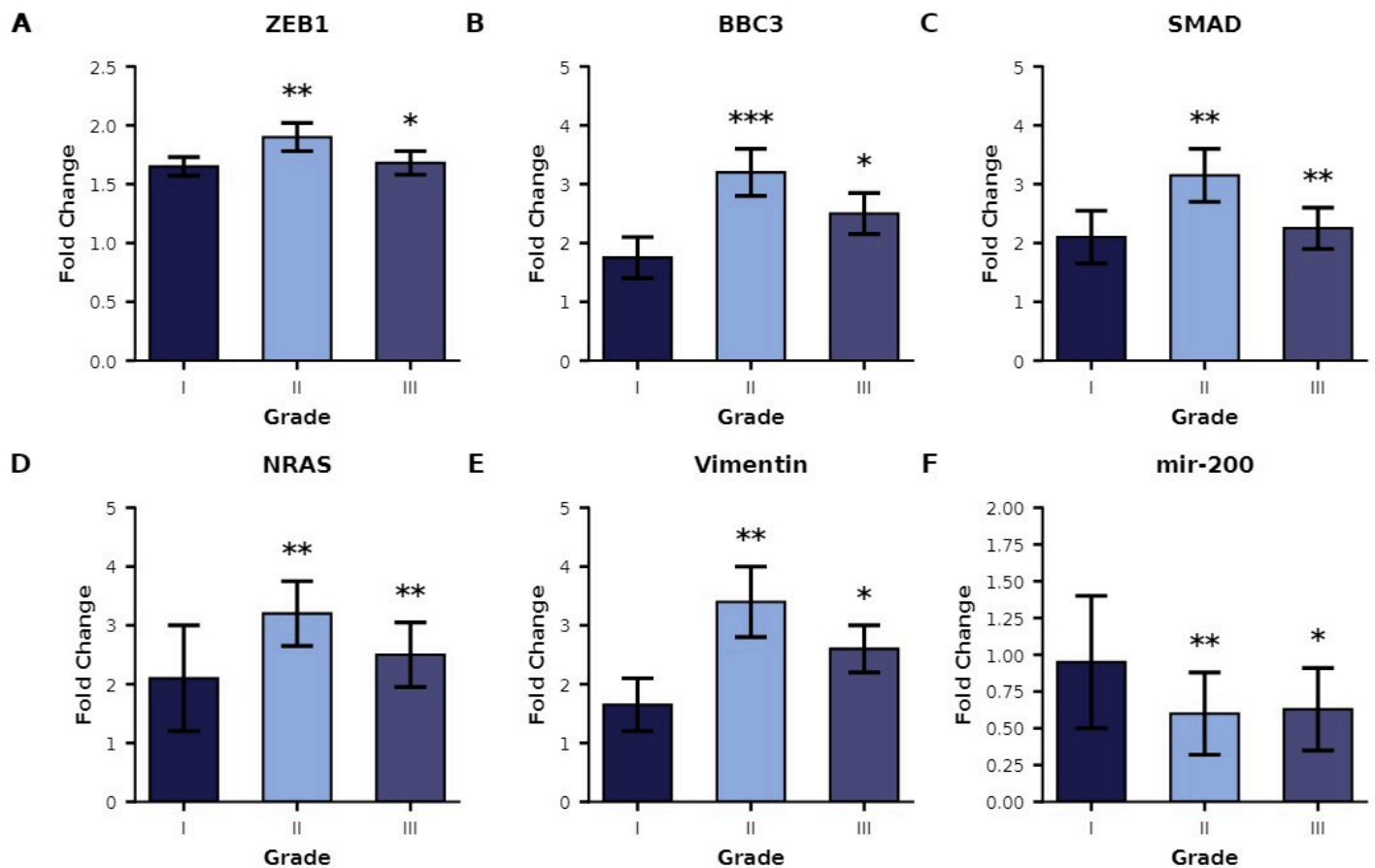
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- # Figures



Shows results of comparing the level of ZEB1, BBC3, SMAD, NRAS, vimentin, and mir200 genes expression in normal and tumor.(*P<0.05 compared to the control group, **P<0.01 compared to the control group, ***P<0.001 compared to the control group. The resulting diagram, in which the results are presented as the mean $\pm$ standard deviation, is derived from the fold change relationship).

Figure 2



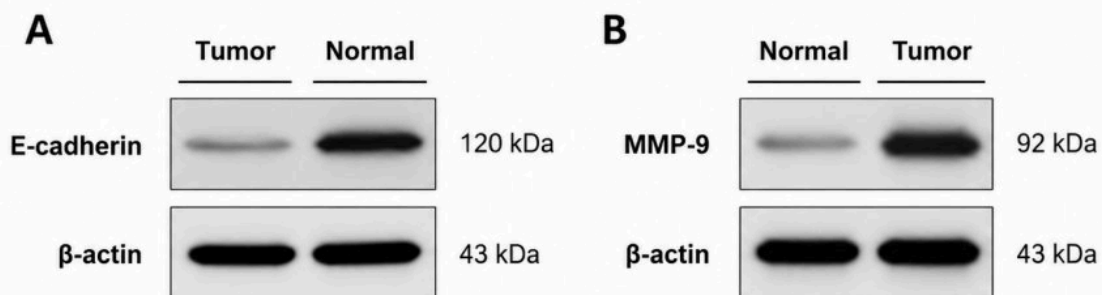
Expression levels of ZEB1, BBC3, SMAD, NRAS, Vimentin, and mir-200 across tumor grades (I, II, III). Data represent fold change relative to grade I control $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2

Shows results of comparing the level of ZEB1, BBC3, SMAD, NRAS, vimentin, and mir200 genes (* $P < 0.05$ compared to the control group, ** $P < 0.01$ compared to the control group, *** $P < 0.001$ compared to the control group. The resulting diagram, in which the results are presented as the mean $\pm$ standard deviation, is derived from the fold change relationship).

Figure 3

Figure 3

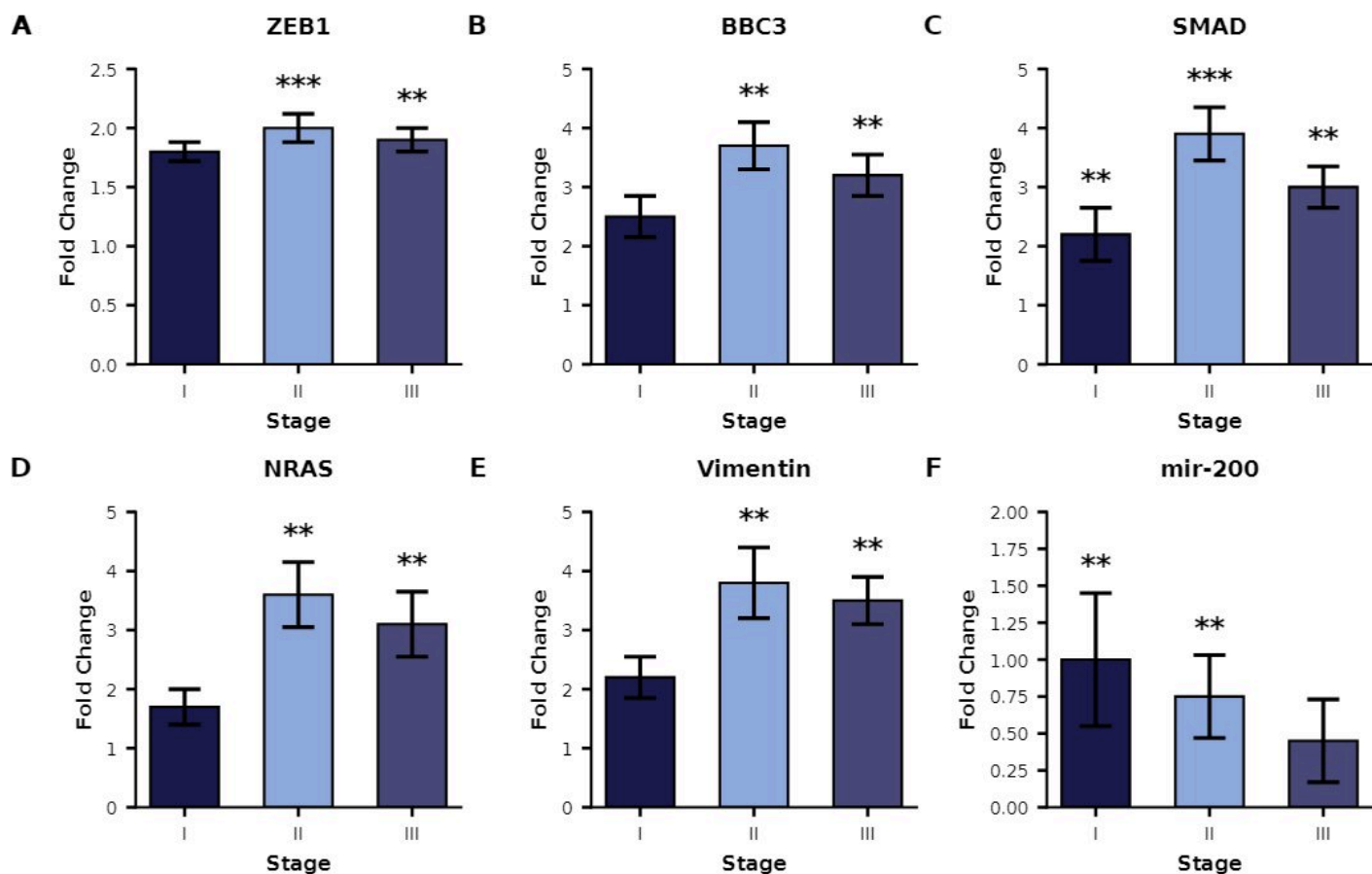


Protein expression levels of E-cadherin and MMP-9 in tumor and non-tumor tissue. Western blot analysis revealed significantly decreased E-cadherin protein expression in tumor tissue compared to non-tumor controls, and significantly increased MMP-9 protein expression in tumor tissue compared to non-tumor controls. β -actin was used as loading control.

Figure 3

Shows expression level of E-Cad and MMP-9 in both tumor and normal.

Figure 4



Expression levels of ZEB1, BBC3, SMAD, NRAS, Vimentin, and mir-200 across tumor stages (I, II, III). Data represent fold change relative to stage I control $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$.

Figure 4

Shows results of comparing the level of ZEB1, BBC3, SMAD, NRAS, vimentin, and mir200 genes expression in different tumor stages 1, 2, and 3. (* $P < 0.05$ compared to the control group, ** $P < 0.01$ compared to the control group, *** $P < 0.001$ compared to the control group. The resulting diagram, in which the results are presented as the mean $\pm$ standard deviation, is derived from the fold change relationship).

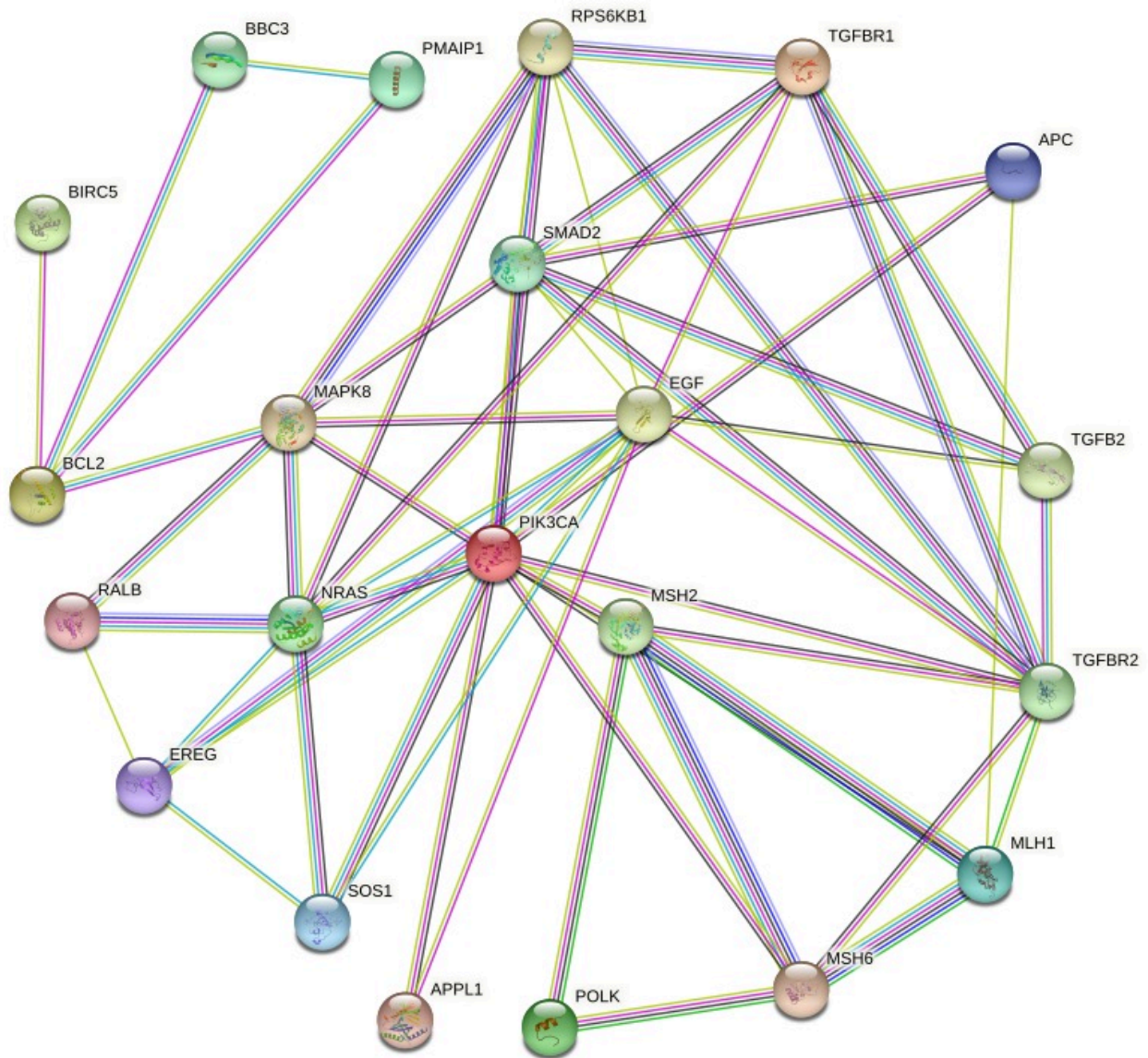


Figure 5

Shows the STRING protein–protein interaction (PPI) network constructed for the candidate gene set (interaction confidence score ≥ 0.4), highlighting NRAS and PIK3CA as central hub nodes with direct connections to SMAD2, BCL2, BBC3/PMAIP1, and members of the TGF- β receptor family (TGFBR1, TGFBR2), reflecting the functional interconnectivity of the Ras/PI3K–Akt, TGF- β , and p53-associated apoptotic pathways in colorectal cancer.

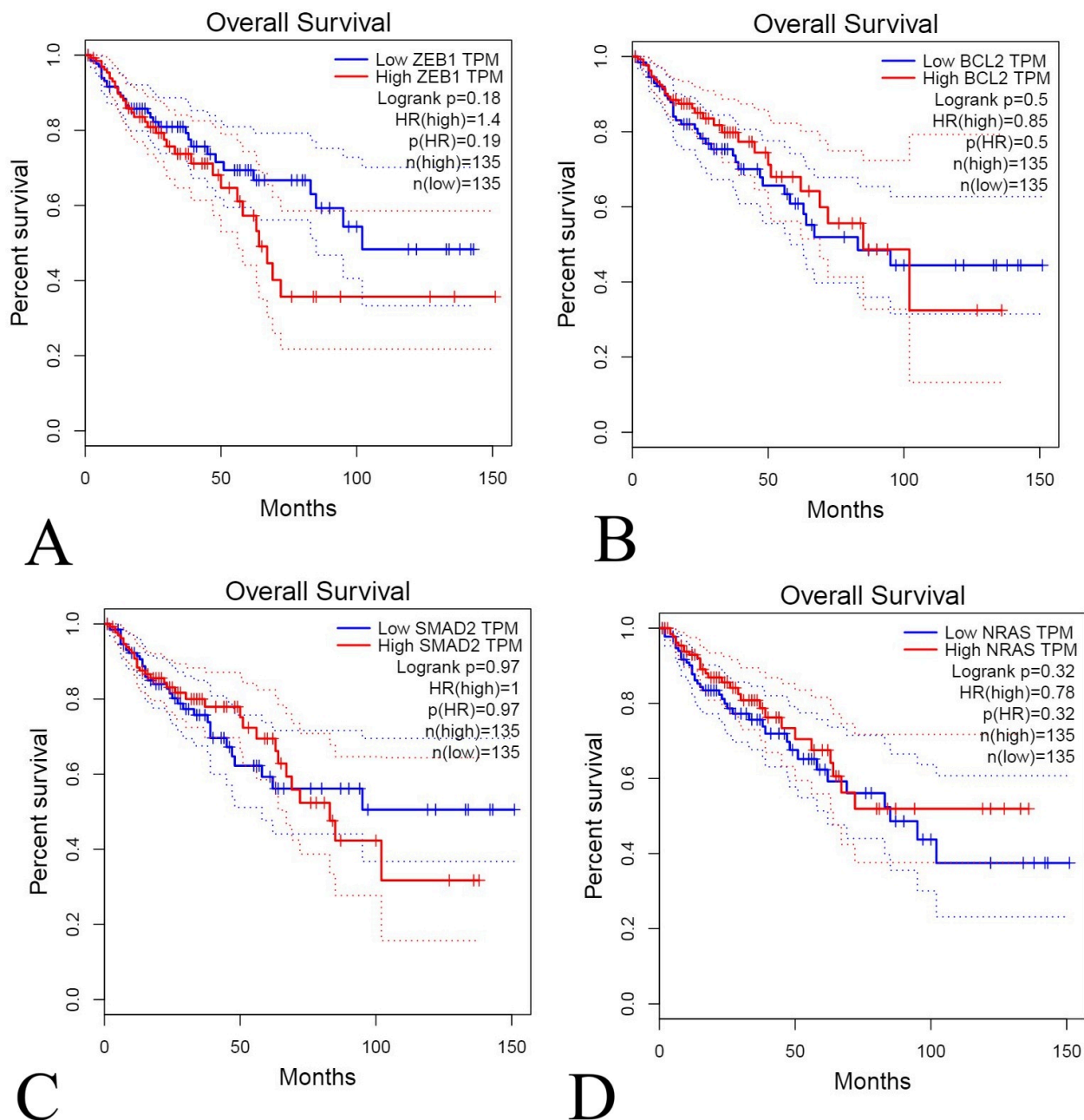


Figure 6

Kaplan–Meier overall survival curves based on TCGA colorectal adenocarcinoma data (GEPIA2) for (A) ZEB1 ($HR = 1.40$, log-rank $P = 0.18$), (B) BCL2 ($HR = 0.85$, $P = 0.50$), (C) SMAD2 ($HR = 1.00$, $P = 0.97$), and (D) NRAS ($HR = 0.78$, $P = 0.32$). None of the four genes showed a statistically significant association with overall patient survival ($n = 135$ per group, median-expression cutoff).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTablesS1S31.docx](#)

- [SupplementaryTableS4TargetScanpredictedtargets.xlsx](#)