


Low *BIN3* Expression is an Independent Predictor of Unfavorable Survival in Patients With Primary Colorectal Cancer

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Abstract

This study aimed to explore the mechanisms of *bridging integrator-3* dysregulation, its prognostic value, and related signaling pathways in colorectal cancer. Bioinformatic analysis was performed based on the data from The Cancer Genome Atlas–colorectal cancer and Human Protein Atlas. Colorectal cancer cell lines, LoVo and HT29 cells, were used as *in vitro* cell model to assess the effect of demethylation on *bridging integrator-3* expression. Results showed that *bridging integrator-3* was downregulated in colorectal cancer tissues compared to normal colon and rectum tissues. Heatmap of *bridging integrator-3* messenger RNA expression, exon expression, and DNA methylation indicated a negative correlation between *bridging integrator-3* expression and methylation of some CpG sites within the coding sequence. Demethylation treatment significantly increased *bridging integrator-3* expression in LoVo and HT29 cells. Low *bridging integrator-3* messenger RNA and exon expression were associated with significantly worse overall survival ($P = .015$ and $.013$, respectively). Multivariate analysis confirmed that low *bridging integrator-3* messenger RNA expression was an independent prognostic factor of unfavorable overall survival (Hazard Ratio (HR) = 1.596, 95% confidence interval: 1.024–2.486; $P = .039$). High *bridging integrator-3* DNA methylation was also associated with significantly worse overall survival ($P = .013$). Kyoto Encyclopedia of Genes and Genomes analysis indicated that the genes correlated with *bridging integrator-3* (absolute Pearson $r \geq 0.3$, $n = 121$) were enriched in sphingolipid signaling pathway, natural killer cell-mediated cytotoxicity, p53 signaling pathway, and apoptosis. Based on these findings, we infer that DNA hypermethylation might be an important mechanism of suppressed *bridging integrator-3* expression in colorectal cancer. Its low expression is an independent predictor of unfavorable survival in patients with primary colorectal cancer. *Bridging integrator-3* might act as a tumor suppressor via modulating natural killer cell-mediated cytotoxicity, p53 signaling pathway, and apoptosis.

Keywords

BIN3, hypermethylation, overall survival, colorectal cancer, KEGG pathway

Abbreviations

BAR, Bin, Amphiphysin, Rvs; *BIN3*, bridging integrator-3; CDC42, cell division control protein 42; CRC, colorectal cancer; CSMD1, Cub and Sushi multiple domains 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA; OS, overall survival; qPCR, quantitative real-time polymerase chain reaction; TCGA, The Cancer Genome Atlas; 5-AZA-dC, 5-Aza-2'-deoxycytidine.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world.¹ During the past decades, survival of patients with CRC have improved significantly due to early diagnosis and advances in therapeutic strategies.¹ However, a large proportion (about 40%-50%) of patients with CRC still develop local

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or distant recurrence after surgery.² Currently, the American Joint Committee on Cancer (AJCC) tumor, node, metastases classification is the most valuable tool in staging CRC and for making corresponding therapeutic strategies.³ However, the therapeutic outcomes of patients at similar clinical stages varied significantly.⁴ Therefore, it is necessary to explore new predictive markers for individualized therapies.

BIN3 is a gene-encoding bridging integrator-3 in human, which is a member of the Bin, Amphiphysin, Rvs (BAR) domain protein family.⁵ This protein has a conserved role in activating cell division control protein 42 (CDC42) in human myocytes and muscle cells, thereby promoting correct cell division.^{5,6} This gene has an interesting location at human chromosome 8p21.3, a region that has been implicated widely in cancer suppression.⁷ Recent studies suggest that *BIN3* is a tumor-suppressive gene in some cancers. Mice with *BIN3* knockdown had increased susceptibility to lymphomas and lung cancers.⁸ Primary mouse embryonic fibroblasts from *BIN3*-null mice had stronger potentials in proliferation, invasion, and resistance to anoikis, which are common characteristics of cancerous cells.⁸ Another recent study also observed the tumor suppressive effect of *BIN3* in breast cancer.⁹ Its inactivation can protect the cancer cells from anoikis.⁹ These findings suggest that *BIN3* might play a tumor suppressor role in multiple cancers.

In this study, we explored *BIN3* expression profile and its prognostic value in CRC and studied the possible mechanisms of its dysregulation. In addition, we also investigated the potential signaling pathways it might be involved in.

Materials and Methods

Data Mining Using FireBrowse

Bridging integrator-3 messenger RNA (mRNA) expression in digestive system neoplasms and in the adjacent normal tissues was examined using data from The Cancer Genome Atlas (TCGA). Data analysis was performed by using FireBrowse (<http://firebrowse.org/>) that provides access to analyze data generated by TCGA.

Data Mining in the Human Protein Atlas

Bridging integrator-3 protein expression in cancer tissues and in the corresponding normal tissues was reviewed by data mining in the Human Protein Atlas (<http://www.proteinatlas.org/>).¹⁰ The images of *BIN3* immunohistochemistry staining in CRC tissues and in normal colon and rectum tissues were also obtained from this database.

Data Mining Using UCSC Xena browser

Heatmap of *BIN3* mRNA expression, *BIN3* exon expression, and *BIN3* DNA methylation in patients with primary CRC (TCGA-CRC) was reviewed using University of California, Santa Cruz (UCSC) Xena browser. Kaplan-Meier plots of overall survival (OS) of the patients were also generated by using

UCSC Xena browser. The patients were divided into high and low *BIN3* expression groups according to median *BIN3* mRNA or median *BIN3* exon expression and were divided into high and low DNA methylation groups according to median DNA methylation.

KEGG Analysis

Bridging integrator-3 correlated genes (absolute Pearson $r \geq 0.3$) in TCGA-CRC were identified by using cBioPortal for Cancer Genomics. Then, these genes were loaded into ClueGo in Cytoscape¹¹ for analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Cell Culture

Human CRC cell lines, LoVo and HT29 cells, were obtained from Shanghai Cell Biology, Institute of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum (Invitrogen, Carlsbad, California).

Demethylation Treatment

Demethylation treatment was performed by using 5-Aza-2'-deoxycytidine (5-AZA-dC; Sigma-Aldrich, St Louis, Missouri). LoVo and HT29 cells were subjected to 1 or 2.5 μ M 5-AZA-dC treatment for 24 hours.

Quantitative Real-Time PCR

Total RNA from the cell samples was isolated using the Trizol Reagent (Invitrogen) and then was reversely transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California). Then, quantitative real-time polymerase chain reaction (qPCR) was performed to detect gene expression using gene-specific primers and TaqMan Master Mix (Applied Biosystems, Foster City, California), with an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers for *BIN3* were forward, 5'-CTTACTCTCCAATCCCCTCT-3' and reverse, 5'-TCGATCACAGTCTTCTGGA-3'. The relative mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

Statistical analysis was performed by using SPSS 19.0 (SPSS Inc, Chicago, Illinois). Continuous variables were reported as means (standard deviation [SD]). The association between *BIN3* RNA expression and the clinicopathological features was evaluated using χ^2 tests. Log-rank test was performed to determine the significance of the difference between OS curves. Prognostic values were analyzed by univariate and multivariate Cox regression models. The group difference was compared by

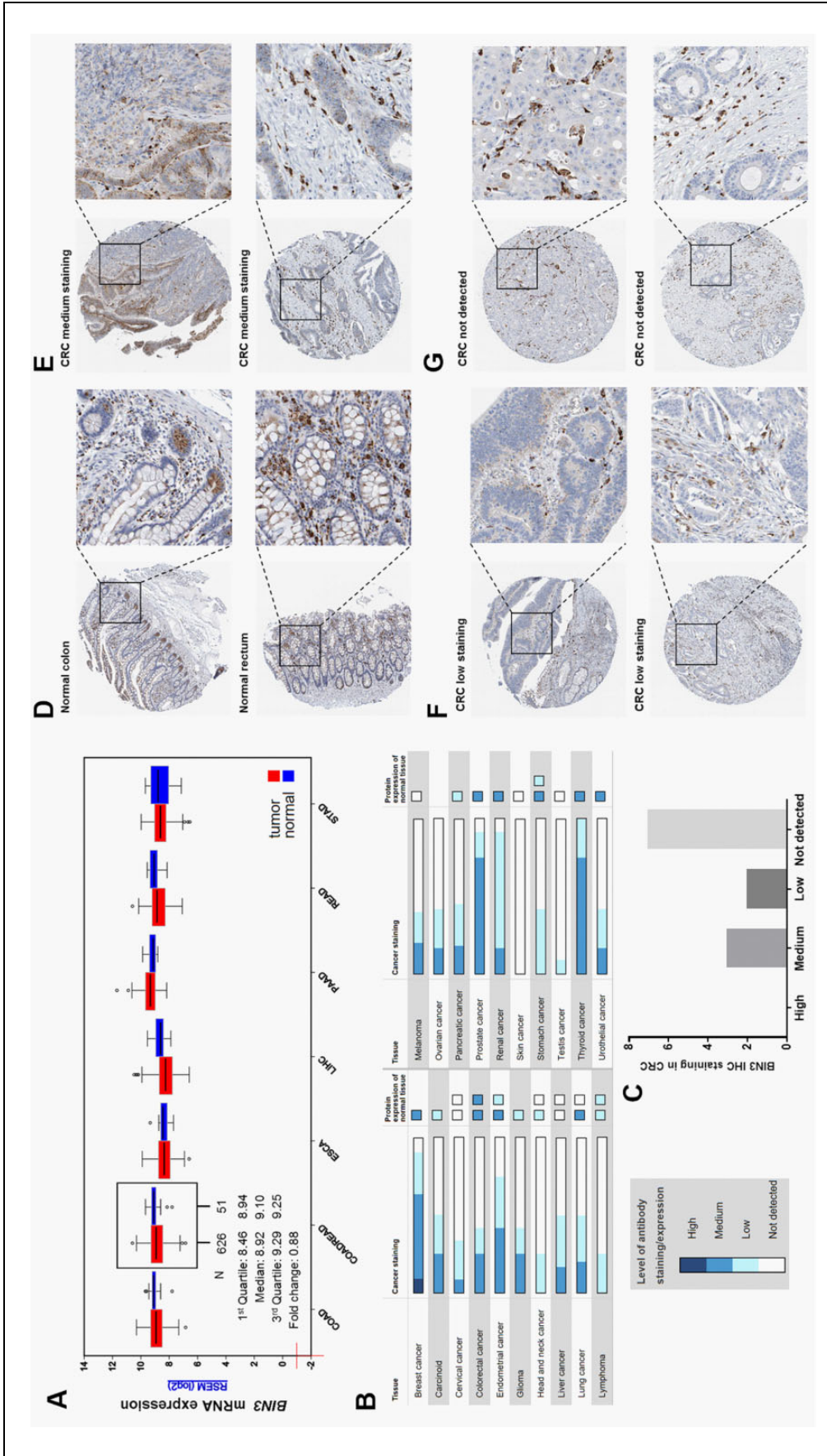


Figure 1. Bridging integrator-3 mRNA and protein expression in CRC. A, *BIN3* mRNA expression in some digestive system neoplasms. Box plots were obtained by data mining in FireBrowse. B, BIN3 IHC staining summary in some cancers and in the corresponding normal tissues. Data were obtained from the Human Protein Atlas. C, BIN3 IHC staining summary in 12 cases of CRC. D-G, Representative images of BIN3 IHC staining in normal colon and rectum (D), Medium (E), low (F), and not detectable (G) BIN3 IHC staining in CRC tissues. Images were obtained from the Human Protein Atlas. BIN3 indicates bridging integrator-3; CRC, colorectal cancer; IHC, immunohistochemistry; mRNA, messenger RNA.

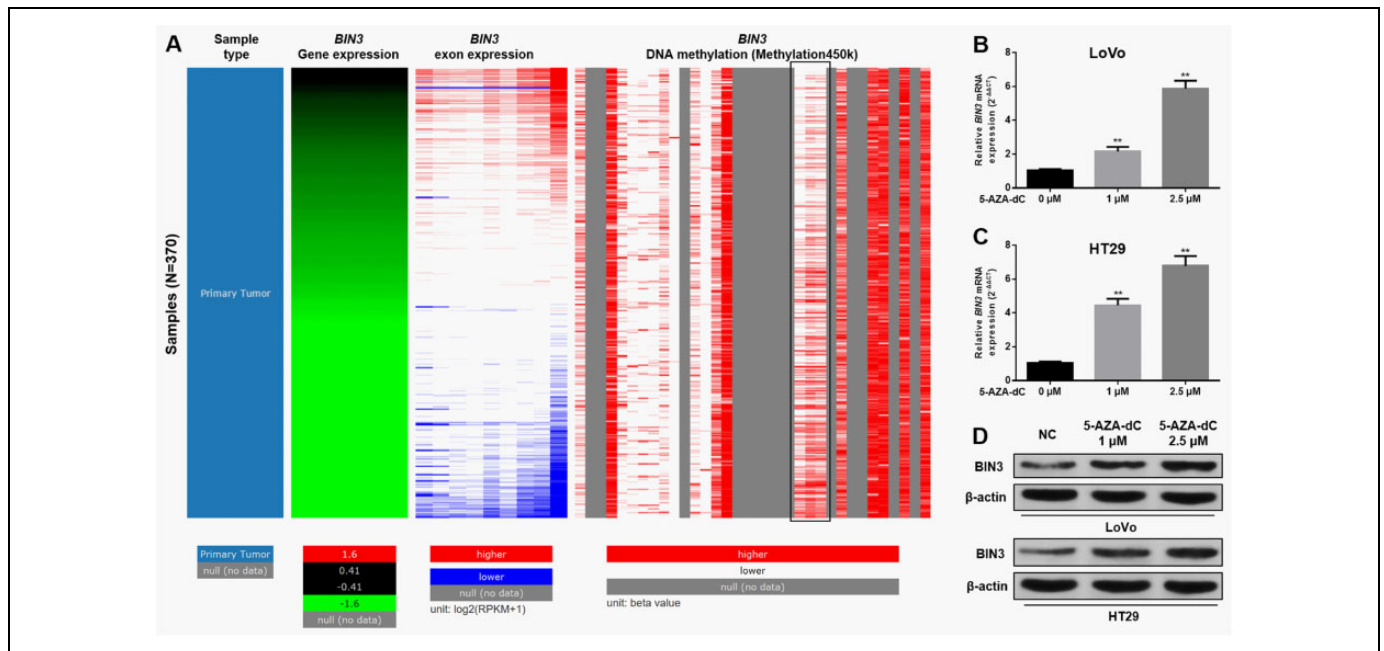


Figure 2. Bridging integrator-3 downregulation is a result of DNA hypermethylation. A, Heatmap of *BIN3* mRNA expression, exon expression, and DNA methylation. Only data from patients with primary CRC were used. Analysis was performed using UCSC Xena Browser. B-D, qPCR (B-C) and Western blotting (D) of *BIN3* expression in LoVo and HT29 cells 24 hours after 5-AZA-dC treatment. ** $P < .01$. *BIN3* indicates bridging integrator-3; CRC, colorectal cancer; mRNA, messenger RNA; qPCR, quantitative real-time polymerase chain reaction; 5-AZA-dC, 5-Aza-2'-deoxycytidine.

2-tailed Student *t* test or analysis of variance with Student Newman-Keuls test as a post hoc test. $P < .05$ was considered statistically significant.

Results

BIN3 is Downregulated in CRC

By retrieving data in TCGA-CRC, we characterized *BIN3* mRNA expression in some digestive system neoplasms (Figure 1A). *BIN3* expression was slightly lower in CRC tissues than that in normal tissues (fold change: 0.88; Figure 1A). To further characterize its expression at the protein level, data mining was performed in the Human Protein Atlas. Results showed that a majority of breast, prostate, thyroid, and endometrial cancers had moderate granular cytoplasmic staining, whereas the other cancer tissues were negatively or weakly stained (Figure 1B). The normal colon and rectum usually had medium *BIN3* staining (Figure 1B and D). In comparison, among 12 cases of CRC tissues, only 3 cases had medium *BIN3* staining (Figure 1C and E), whereas 2 cases had weak staining (Figure 1C and F) and 7 cases had no detectable *BIN3* expression (Figure 1C and G).

BIN3 Downregulation is a Result of DNA Hypermethylation

Then, we further investigated the possible underlying mechanisms of suppressed *BIN3* expression in CRC. By data mining in TCGA-CRC ($N = 370$), we generated a comparable heatmap showing *BIN3* mRNA expression, exon expression, and DNA

methylation (Figure 2A). In the heatmap, *BIN3* mRNA expression was positively correlated with exon expression (Figure 2A). Interestingly, the methylation status of some CpG sites (black frame, Figure 2A) was negatively correlated with the expression of *BIN3* mRNA and *BIN3* exon. Therefore, we hypothesized that the expression of *BIN3* might be suppressed by DNA hypermethylation. To verify this hypothesis, LoVo and HT29 cells were treated with 1 or 2.5 μM 5-AZA-dC. After the treatment, the cells were subjected to qPCR and Western blotting assay of *BIN3* expression. Following studies showed that demethylation significantly restored *BIN3* expression at both mRNA and protein level in the cells (Figure 2B-D).

Low *BIN3* Expression is an Independent Indicator of Unfavorable OS of CRC

The association between demographic/clinicopathological parameters and *BIN3* RNA expression was summarized in Table 1. Low *BIN3* expression group had a significantly lower ratio of early stages (I/II) patients (81/175, 46.3%) than high *BIN3* expression group (107/175, 61.1%; $P = .0053$; Table 1). Besides, the low *BIN3* expression group also had a significantly higher proportion of lymphatic invasion (58/161, 36.0% vs 41/161, 25.5%, $P = .04$) and death (52/184, 28.3% vs 33/183, 18.0%, $P = .02$) compared with the high *BIN3* expression group (Table 1). To further assess the prognostic value of *BIN3*, we examined the association between *BIN3* expression and OS in patients with CRC. In TCGA-CRC, low *BIN3* mRNA expression was associated with significantly worse

Table 1. The Association Between Demographic/Clinicopathological Parameters and *BIN3* Expression in Patients With Primary CRC in TCGA-CRC.

	<i>BIN3</i> Expression High (N = 183)	<i>BIN3</i> Expression Low (N = 184)	χ^2	P Value
Age			0.137	.711
>64	100	97		
≤64	83	87		
Gender			3.353	.0671
Female	91	74		
Male	92	110		
Primary site			1.379	.240
Colon	142	133		
Rectum	41	51		
Clinical stage			7.769	.0053
I/II	107	81		
III/IV	68	94		
Null	8	9		
Venous invasion			1.765	.184
No	126	116		
Yes	32	42		
Null	25	26		
Lymphatic invasion			4.215	.0401
No	120	103		
Yes	41	58		
Null	22	23		
CEA level			0.142	.706
<10	88	94		
≥10	25	30		
Null	70	60		
Living status			5.393	.0202
Living	150	132		
Dead	33	52		

Abbreviations: CEA, Carcino-Embryonic Antigen; CRC, colorectal cancer; TCGA-CRC, The Cancer Genome Atlas–colorectal cancer.

OS ($P = .015$, Figure 3A). Since *BIN3* is a protein-coding gene, we also assessed the association between *BIN3* exon expression and OS in the patients. Results of the log-rank test showed that low *BIN3* exon expression was also associated with unfavorable OS ($P = .013$, Figure 3B). In univariate analysis, older age (>64), late stage (III/IV), venous invasion, lymphatic invasion, and low *BIN3* expression were associated with unfavorable OS (Table 2). Multivariate analysis confirmed that low *BIN3* mRNA expression was an independent prognostic factor of unfavorable OS (HR = 1.596, 95% confidence interval [CI]: 1.024–2.486, $P = .039$; Table 2). Besides, age and clinical stage were also independent prognostic factors of OS (Table 2).

High DNA Methylation Might be an Indicator of Unfavorable OS of CRC

Since we confirmed that *BIN3* expression is regulated by DNA methylation, we then examined whether its DNA methylation is associated with OS among the patients. Log-rank test showed that high *BIN3* DNA methylation was associated with significantly worse OS ($P = .013$, Figure 3C).

KEGG Pathway Analysis of *BIN3* Correlated Genes in TCGA-CRC

To further investigate the possible functional role of *BIN3* in CRC, we identified its correlated genes (absolute Pearson $r \geq 0.3$) in TCGA-CRC ($n = 121$, Supplemental Table 1). Then, the genes were loaded into the online software ClueGo to identify their enrichment in KEGG pathways. Results showed that the genes were enriched in sphingolipid signaling pathway, natural killer cell–mediated cytotoxicity, p53 signaling pathway, and apoptosis (Figure 4 and Supplemental Table 2).

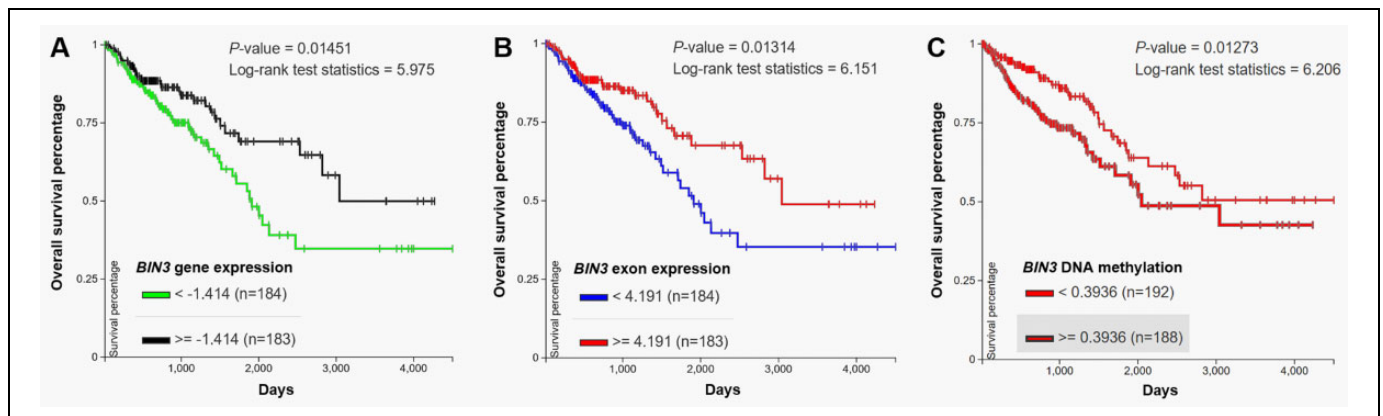


Figure 3. Low *BIN3* expression is associated with unfavorable OS of patients with CRC. A-C, Kaplan-Meier plots of OS of patients in TCGA-CRC. Patients were divided into high and low *BIN3* expression groups according to median *BIN3* mRNA expression (A) or median *BIN3* exon expression (B) or were divided into high and low *BIN3* DNA methylation groups according to median DNA methylation (C). *BIN3* indicates bridging integrator-3; CRC, colorectal cancer; mRNA, messenger RNA; OS, overall survival; TCGA-CRC, The Cancer Genome Atlas–colorectal cancer.

Table 2. Univariate and Multivariate Analyses of OS in Patients With Primary CRC in TCGA-CRC.

Parameters	Univariate Analysis				Multivariate Analysis			
	P	HR	95% CI (Lower/Upper)		P	HR	95% CI (Lower/Upper)	
Age > 64 vs ≤ 64	.002	2.146	1.338	3.443	.003	2.063	1.273	3.343
Female vs male	.367	0.819	0.531	1.263				
Primary site colon vs rectum	.522	1.191	0.697	2.035				
Clinical stage III/IV vs I/II	<.001	2.714	1.704	4.324	.001	2.313	1.382	3.872
Venous invasion yes vs no	<.001	2.357	1.459	3.809	.052	2.284	0.992	5.256
Lymphatic invasion yes vs no	.020	1.766	1.095	2.849	.844	0.918	0.390	2.159
Carcino-Embryonic Antigen (CEA) level ≥ 10 vs <10	.072	1.977	0.942	4.148	.340	1.450	0.676	3.107
<i>BIN3</i> expression low vs high	.016	1.714	1.107	2.654	.039	1.596	1.024	2.486

Abbreviations: BIN3 indicates bridging integrator-3; CI, confidence interval; CRC, colorectal cancer; OS, overall survival; TCGA-CRC, The Cancer Genome Atlas–colorectal cancer.

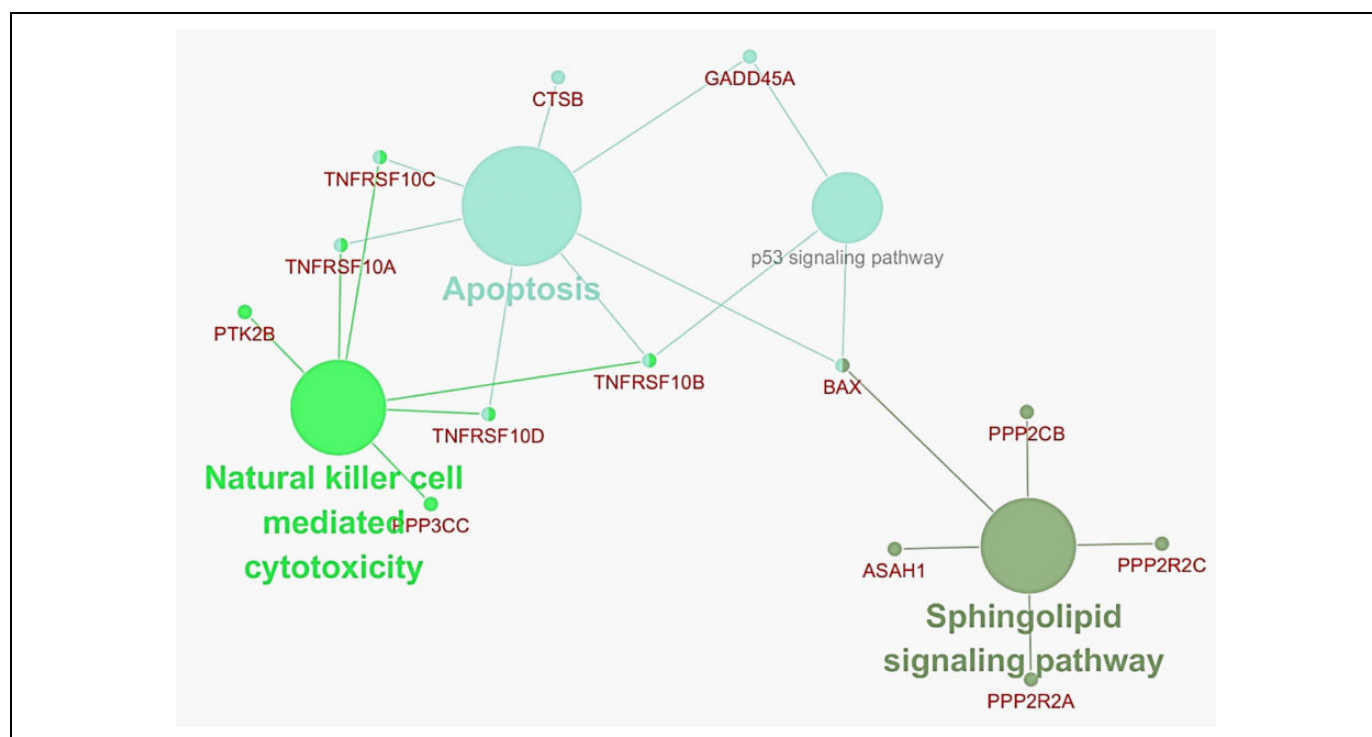


Figure 4. Kyoto Encyclopedia of Genes and Genomes pathway analysis of *BIN3* correlated genes in the TCGA-CRC. BIN3 indicates bridging integrator-3; TCGA-CRC, The Cancer Genome Atlas–colorectal cancer.

Discussion

In this study, we firstly characterized *BIN3* expression in CRC. Bioinformatic results indicated that *BIN3* was slightly downregulated at the mRNA level and was significantly downregulated at the protein level in some CRC cases. *Bridging integrator-3* maps to human chromosome 8p21.3.⁷ In fact, chromosome 8 (8p) is a host region of many cancer suppressor genes.¹² Its deletion is common in some types of cancers and is one of the most frequent events in cancer progression.^{13,14} Besides the chromosomal loss, epigenetic modification, such as DNA hypermethylation, is also an important mechanism of repressed gene expression in this region. For example, secreted

frizzled-related protein 1, which maps to 8p11, is a negative regulator of the Wnt pathway.¹⁵ Secreted frizzled-related protein 1 downregulation caused by promoter methylation is common in bladder, colon, breast, cervical, and ovarian cancers.¹⁵⁻¹⁸ Deleted in liver cancer 1, located at 8p22, encodes a Rho-GTPase activating protein.¹⁹ Deleted in liver cancer 1 can negatively regulate the Rho/ROCK/MLC2 pathway and suppress metastasis and migration of cancer cells.¹⁹ Its promoter methylation was observed in lung, breast, and colon cancers.¹⁹ *Cub and Sushi multiple domains 1 (CSMD1)* is a tumor suppressor gene in CRC, which maps to 8p23.²⁰ One recent study found the majority of CRC tumors were methylated at

one or more CpG loci within the *CSMD1* coding sequence,²⁰ thereby had downregulated *CSMD1* expression. Although we observed suppressed *BIN3* expression in some CRC cases, the underlying mechanisms have not been revealed. In this study, by comparing *BIN3* mRNA expression, exon expression, and DNA methylation, we observed a negative correlation between *BIN3* expression and methylation of some CpG sites within the coding sequence. In addition, by using 5-AZA-dC, a demethylation reagent, we further demonstrated that demethylation increased *BIN3* expression in CRC cells. Based on these findings, we infer that DNA hypermethylation might be an important mechanism of suppressed *BIN3* expression in CRC.

Loss of *BIN3* was correlated with poor prognosis characteristics in patients with breast cancer, such as higher tumor grade, lymph node infiltration, and decreased survival.⁹ In this study, we also assessed the prognostic value of *BIN3* in patients with CRC. Survival data in TCGA-CRC showed that low *BIN3* expression and high *BIN3* DNA methylation were associated with unfavorable OS. Moreover, by performing univariate and multivariate analysis, we confirmed that low *BIN3* mRNA expression was an independent prognostic factor of unfavorable OS (HR = 1.596, 95% CI: 1.024-2.486, $P = .039$).

Bin, Amphiphysin, Rvs adapter proteins have been implicated in many cellular processes, such as actin organization, cell polarity, endocytosis, vesicle fusion, and trafficking, specialized membrane organization, stress signaling, transcription, and tumor suppression.^{21,22} As an archetypal member of the BAR adapter gene family, the signaling pathways in which *BIN3* is involved in cancers have not been fully revealed. In epithelial cancer cells, *BIN3* owns the ability to relocate to the cell membrane after cell detachment and to induce a proapoptotic cascade.⁹ Mechanistically, via its BAR domain, *BIN3* can form a complex with TUBA (the human homolog of *gef1p*) and localize TUBA to the curved membrane.⁹ Once located at the membrane, TUBA activates CDC42 and subsequently leads to the activation of P38 α and cell death.⁹ Colorectal cancer is also a disease originating from the epithelial cells lining the colon or rectum, but the possible regulative effect of *BIN3* in CRC has not yet been reported.

In this study, we identified the genes correlated with *BIN3* in CRC and then performed KEGG pathway analysis. Among the identified pathways, natural killer cell-mediated cytotoxicity, p53 signaling pathway, and apoptosis are closely related to tumorigenesis and malignant progression. These findings provide important insights and clues about the functional role of *BIN3* and its upstream and/or downstream genes in the pathological development of CRC. Therefore, it is meaningful to further investigate its regulative networks in these pathways in the future.

Conclusion

DNA hypermethylation might be an important mechanism of suppressed *BIN3* expression in CRC. Its low expression is an independent predictor of unfavorable survival in patients with primary CRC. *Bridging integrator-3* might act as a tumor

suppressor via modulating natural killer cell-mediated cytotoxicity, p53 signaling pathway, and apoptosis.

Declaration of Conflicting Interests

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Supplemental Material

Supplementary material for this article is available online

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