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Expression of RNA-binding Motif Protein 3 (*RBM3*) and Cold-inducible RNA-binding protein (*CIRP*) Is Associated with Improved Clinical Outcome in Patients with Colon Cancer

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Abstract. *Background: Recent studies have shown a potential role of RNA-binding proteins (RBPs) in a variety of biological pathways, including cancer progression, whilst their expression in various tumor types may be associated with patient prognosis. However, the role of the RBP family members has not been explored in colon cancer and their possible use as prognostic biomarkers is largely unknown. Materials and Methods: To determine the prognostic role of three RBP genes: insulin-like growth factor-binding protein 2 (IGFBP2), RNA-binding motif protein 3 (RBM3), and cold-inducible RNA-binding protein (CIRP) in colon cancer. Results: We examined the RNA expression of IGFBP2, RBM3, and CIRP in 94 human colon cancer samples along with matched normal tissue samples from each patient using quantitative real-time polymerase chain reaction (qRT-PCR). No significant associations were observed between RNA expression of RBPs and the studied clinical features. The estimated 5-year disease-free survival rate was significantly better for patients with higher expression of RBM3 and CIRP, while patient survival was not significantly correlated to IGFBP2 expression. Conclusion: RBM3 and CIRP may be useful prognostic biomarkers of colon cancer.*

Colon cancer accounts for 10-15% of all cancers and is the second leading cause of cancer-related deaths in most Western countries. It is also the third most common cancer affecting the Korean population (1). Currently, pathological tumor staging is considered to be the most reliable prognostic factor in colon cancer. However, while the prognosis for each patient is largely dependent upon the extent of the disease, the actual survival rate, recurrence after surgery, and other predictive information for each individual vary widely, even for patients diagnosed with the same stage of cancer. This is especially true for those with stage II and resectable stage IV cancer (2-4). Thus, in order to improve patient survival rates, other predictive markers of patient outcome and tumor recurrence should be evaluated.

Various biomarkers have been identified as playing a role in cancer development, metastasis, or tumor recurrence. However, the use of these biomarkers as prognostic or predictive indicators is limited (1). Furthermore, the routine clinical use of these prognostic biomarkers during the decision to initiate adjuvant treatment has not been validated with a sufficient level of evidence. This ultimately results in a number of patients receiving unnecessary treatment that will not alter their survival. It is, therefore, essential to continue uncovering additional biomarkers for clinical use.

RBPs are involved in the expression of a diverse set of genes responsible for cell growth and proliferation (2). Altering the expression of these RBPs has been shown to cause defects in cell physiology and lead to cancer development. For example, it was reported that reduced expression of RBM3 is correlated with poor prognosis in several forms of human cancer, including ovarian, breast, prostate, and malignant melanoma (3, 4). Furthermore, cold-inducible RNA-binding protein (CIRP) plays diverse tissue-specific roles, such as the maintenance of normal cellular function and morphogenesis. On the other hand, insulin-like growth factor-binding protein 2 (IGFBP2) is known to function in transcriptional regulation, induction of apoptosis, and DNA damage repair, all of which are intimately involved in tumor development, progression, and resistance to treatment (5). It is likely that this family of proteins plays a

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significant role in cancer progression. However, to the best of our knowledge, no report has been issued regarding the expression of these proteins in colon cancer.

In the present study, our primary objective was to assess and compare the possible prognostic use of three specific RBPs [CIRP, RNA-binding motif protein 3 (RBM3), and IGFBP2] in colon cancer. To do so, we evaluated the RNA concentration of each RBP in 94 patients with colon cancer using quantitative real-time polymerase chain reaction (PCR) in addition to monitoring the post-surgical clinicopathological features of the patients, as well as their long-term/disease-free survival using follow-up information.

Materials and Methods

Patients and samples. Samples of colon cancer tissue were obtained from September 2009 to September 2011. Ninety-four consecutive patients were diagnosed with stage II to III colon cancer and underwent curative resection at the Gachon University, Gil Cancer Center (Incheon, Korea). Informed consent was obtained from each patient according to the guidelines issued by the Ethical Committee of our institution. All of the experimental methods were approved by Gachon Univerisity Gil Hospital Institutional Review Board (GIRB#2016-198). The samples were collected immediately after the surgical procedure and stored at −80˚C.

Surveillance. Surveillance following surgery was conducted as follows: physical examination, serum carcino-embryonic antigen (CEA) determination, carbohydrate antigen 19-9 (CA19-9) measurement, chest radiography, and spiral abdominal computed tomography were performed every 6 months for the first 3 years and annually thereafter. Other examinations, such as colonoscopy and abdominal ultrasound, were selected and performed every 6 to 12 months depending on the status of the patient. Median follow-up duration was 51.1 months (ranging from 3.1-57.1 months). All patients were staged according to the seventh edition of the American Joint Committee on Cancer staging system (6). All the CEA and CA19-9 assays were performed in a single laboratory using the ADVIA Centaur™ CEA/CA19-9 Lite Reagent & Solid Phase (Siemens, Germany) (7).

Total RNA isolation from human tissues and quantitative real-time PCR (qRT-PCR). Total RNA was obtained from both normal and cancerous tissues of the 94 patients examined in this study. Using a spatula, pieces of the same tissue were combined and placed in a tube containing TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The tissues were then homogenized on ice. Homogenates were clarified by centrifugation at 10,000 × *g* for 15 min at 4˚C to remove cellular debris and insoluble material. The resulting supernatants were collected in RNase-free tubes. Glycogen (1 μl, 20 mg/ml) and an equal volume of chloroform were then added to each tube, which eves then shaken vigorously by hand for 30 s, allowed to stand at room temperature for 10 min, and then centrifuged at 10,000 × *g* for 20 min at 4˚C. Aqueous layers (top, clear layers) were then transferred to fresh RNase-free tubes, an equal volume of isopropanol was added, mixed, and tubes mere then incubated for 20 min at 4˚C. After a final centrifugation, supernatants were removed and pellets were washed with cold 70% ethanol. The RNA pellets obtained were then dissolved in RNase-free water and stored until cDNA synthesis.

Table I. *Clinical characteristics of patients included in the study (n=94).*

Characteristic	Value	%
Median age, years	64.0	
Gender: M:F	57:37	
Median CEA (range), ng/ml	5.9 (1.4-103.2)	
Median CA19-9 (range) U/ml	22.9 (3.4-340.5)	
T-Stage		
T1+T2	15	16.0
T3+T2	79	84.0
N-Stage		
Negative	68	72.3
Postive	26	27.7
M-Stage		
M0	85	90.4
M1	9	9.6
Lymphatic invasion		
No	57	60.6
Yes	37	39.4
Perineural invasion		
No	90	95.7
Yes	4	4.3
Tumor location		
Right colon	25	26.6
Left colon	69	73.4
Cell type		
Well-differentiated	5	5.3
Moderately differentiated	80	85.1
Poorly differentiated	9	9.6
Microsatellite instability		
High	19	20.2
Stable or low	75	79.8
KRAS status		
Wild-type	73	77.7
Mutant	21	22.3

M:F: Male:female; CA19-9: carbohydrate antigen 19-9; CEA: carcino-embryonic antigen.

RNA samples were treated with DNase I and first-strand cDNA was synthesized from 1 μl aliquots of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). The expression of *CIRP, RBM3*, and *IGFBP2* was quantified by real-time PCR using SYBR Premix Ex Taq II (Takara, Otsu, Shiga, Japan) and an ABI 7900HT Fast Real-time PCR System, according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). Each sample was tested in triplicate, and gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The following PCR primer sequences were used: human CIRP, forward 5'-AGGGCTGAGTTTTGACACCAA-3' and reverse 5'-ACAAA CCCAAATCCCCGAGAT-3'; human *RBM3*, forward 5'-GAGG GCTCAACTTTAACACCG-3' and reverse 5'-GACCACCTCAGA GATAGGTCC-3'; human *IGFBP2*, forward 5'-AGTGGAATT GCATGGGAAAATCA-3' and reverse 5'-CAACGGCGGTTT CTGTGTC-3'; and human *GAPDH*, forward 5'-AATCCCATCACC ATCTTCCA-3' and reverse 5'-TGGACTCCACGACGTACTCA-3'.

Table II. *Association between RNA-binding motif protein 3 (RBM3), cold-inducible RNA-binding protein (CIRP), and insulin-like growth factor-binding protein 2 (IGFBP2) expression and clinicopathological factors.*

	RBM3			*CIRP*			*IGFBP2*		
	Low	High	*p*-Value	Low	High	*p*-Value	Low	High	*p*-Value
Age, years			0.310			0.461			0.257
≤60	24	13		30	6		27	9	
>60	42	15		50	8		48	10	
Gender			0.330			0.504			0.307
Male	39	18		48	9		31	6	
Female	27	10		32	5		44	13	
CEA, ng/ml			0.038			0.427			0.571
≤5	49	25		63	12		60	15	
>5	17	3		17	2		15	4	
CA19-9 U/ml			0.238			0.341			0.380
≤20	50	23		64	10		58	16	
>20	16	5		16	4		17	3	
T-Stage			0.371			0.071			0.139
T1+T2	11	5		15	0		14	1	
T3+T2	55	24		65	14		61	18	
N-Stage			0.503			0.332			0.341
Negative	48	19		59	9		53	15	
Positive	18	9		21	5		22	4	
M-Stage			0.719			0.861			0.871
M0	58	25		71	13		67	17	
M1	8	2		9	1		8	2	
Lymphatic invasion			0.099			0.496			0.491
No	43	13		49	8		46	11	
Yes	23	14		31	6		29	8	
Cell type			0.275			0.579			0.370
Well-differentiated	2	3		5	0		5	0	
Moderately+poorly	64	25		75	14		70	19	
Tumor size, cm			0.065			0.562			0.563
≤5	39	10		41	7		38	11	
>5	28	17		38	7		36	9	
Tumor location			0.003			0.041			0.127
Right colon	5	20		7	18		7	18	
Left colon	40	29		43	26		36	33	
Microsatellite instability			0.043			0.051			0.115
High	4	15		6	13		12	7	
Stable or low	45	30		47	28		63	12	
KRAS status			0.422			0.534			0.237
Wild-type	29	45		34	39		38	35	
Mutant	8	13		11	10		14	7	

CA19-9: Carbohydrate antigen 19-9; CEA: carcino-embryonic antigen.

Statistical analysis. Comparisons between groups were performed using Pearson's chi-square test. The cutoff points were the mean values of RBP expression. The primary endpoint of the study was disease-free survival (DFS). DFS and overall survival (OS) were estimated using the Kaplan–Meier method. DFS was measured from the date of surgery to the date of the first evidence of recurrence. OS was measured from the date of diagnosis to the date of death or the last follow-up visit. Survival rates were compared using a log-rank analysis. Survival curves were plotted using the Kaplan–Meier product-limit method and significant differences between the curves were determined using the log-rank test. Differences with *p*-values less than 0.05 were considered significant.

Results

The clinical characteristics for the patients enrolled in this study are described in Table I. There were 57 men (58.8%) and 37 women (41.2%) with a median age of 64 years (range=41-83 years). In this study, we relied on qRT-PCR to investigate the expression of three RBPs (*CIRP, RBM3*, and *IGFBP2*) in normal tissue and tumor samples isolated from 94 patients. The expression of each RBP was normalized using the housekeeping gene *GAPDH*, and the normalized

expression levels were compared between the normal and cancerous tissue samples. In the following analysis, higher expression of a gene means that there was higher expression in the tumor compared to the normal tissue, and lower expression of a gene indicates lower expression in the tumor compared to the normal tissue. All cases were classified according to their mean expression into either a low- or high-density group for each of the biomarkers.

Correlation of expression of RNA-binding proteins with clinicopathological features. The correlations found between the expression of each RBP and the clinicopathological features of the patient are described in Table II. No significant associations were observed between any of the RBPs investigated in this study and the listed clinical features. Right-sided colon cancer and microsatellite-high cancer were correlated with higher RBM3 expression with statistical significance (p=0.003 and 0.043, respectively). Right-sided colon cancer was correlated with high expression of *CIRP* (p=0.041) but was not significantly related with microsatellite-high status (p=0.051). None of the RBPs were correlated with *KRAS* status.

Survival. The 5-year OS rate was 78.7% for patients overall. Univariate analysis revealed that greater depth of tumor invasion (T stage) and higher nodal status (N stage) were significant predictors of poor prognosis (p=0.043 and 0.001, respectively; Table III). The estimated 5-year DFS rate for patients with high expression of *RBM3* was 92.6% compared to 74.2% for patients with lower expression (Figure 1A, p=0.049). However, the estimated 5-year OS rates for patients with high and low expression of RBM3 did not significantly differ (p=0.135) (Figure 2A). The estimated 5-year DFS rate for patients expressing a higher level of *CIRP* was 100% compared to 76.3% for patients with lower levels of expression (Figure 1B, p=0.048). Notably, the estimated 5-year OS rates for patients expressing high and low levels of *CIRP* were not significantly different (p=0.087) (Figure 2B).

Table III. *Univariate analyses of factors associated with 5-year overall (OS) and disease-free (DFS) survival in patients with colorectal cancer (n=94).*

	N at start, OS (%)	*p*-Value	N at start, DFS (%)	*p*-Value
Age, years		0.870		0.870
≤60	36 (82.4)		36 (84.4)	
>60	58 (81.8)		58 (83.3)	
Gender		0.973		0.808
Male	57 (78.1)		57 (84.1)	
Female	37 (81.1)		37 (83.7)	
CEA, ng/ml		0.980		0.601
≤5	74 (83.8)		74 (84.2)	
>5	20 (84.2)		20 (78.7)	
CA19-9 U/ml		0.597		0.959
≤20	73 (85.0)		73 (87.4)	
>20	21 (80.0)		21 (81.0)	
T-Stage		0.043		0.043
T1+T2	15 (90.0)		15 (95.0)	
T3+T2	79 (70.7)		79 (75.9)	
N-Stage		0.001		0.001
Negative	64 (85.7)		64 (89.7)	
Positive	27 (61.0)		27 (63.1)	
M-Stage		N/A		N/A
M0	85		85	
M1	10		10	
Lymphatic invasion		0.071		0.071
No	57 (89.2)		57 (89.2)	
Yes	37 (75.7)		37 (75.7)	
Tumor size, cm		0.056		0.007
≤5	49 (89.4)		49 (91.7)	
>5	45 (77.3)		45 (68.9)	
Tumor location		0.289		0.276
Right colon	25 (90.0)		25 (86.7)	
Left colon	69 (81.0)		69 (76.6)	
RBM3		0.135		0.049
Low	67 (80.0)		67 (74.2)	
High	27 (92.6).		27 (92.6)	
CIRP		0.087		0.048
Low	80 (81.1)		80 (76.3)	
High	14 (98.1)		14 (99.7)	
IGFBP2		0.172		0.087
Low	75 (81.1)		75 (76.0)	
High	19 (94.7)		19 (94.7)	

CA19-9: carbohydrate antigen 19-9; CEA: carcino-embryonic antigen; RBM3: RNA-binding motif protein 3; CIRP:cold-inducible RNA-binding protein; IGFBP 2: insulin-like growth factor-binding protein 2; N/A: Not available.

Discussion

A number of recent studies implicate distinct roles for RBPs, including RBM3, CIRP, and IGFBP2, in cancer. Interestingly, even though they have been shown to function in cancer progression and treatment resistance, the prognostic value of this gene family in determining the survival of patients with colon cancer has not been investigated. In the present study, tumor staging still appeared to be the most accurate predictor of patient outcome. However, our data also indicate that the expression levels of *RBM3* and *CIRP* RNA can be used as independent prognostic markers, as higher expression of these genes was associated with improved DFS.

CIRP, also known as heterogeneous ribonucleoprotein particle (hnRNP) A18 (8), and RBM3 belong to the hnRNP subgroup of the RBP family. While CIRP is expressed ubiquitously, tissue distribution of RBM3 appears to be more restricted (9). It has been shown that expression of the stress-induced RBP CIRP is increased in breast cancer cells, where it contributes to increased levels of the RBP HuR and the G_1-S

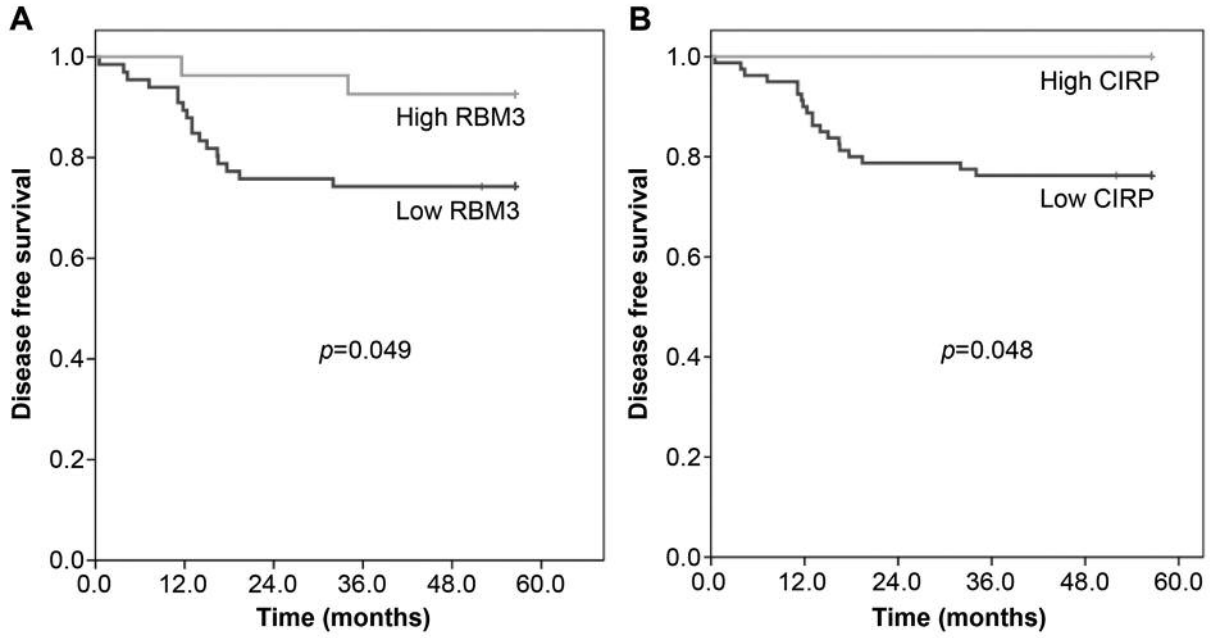

Figure 1. *Disease-free survival curves for patients with colon cancer according to high and low expression of RNA-binding motif protein 3 (RBM3) and cold-inducible RNA-binding protein (CIRP).*

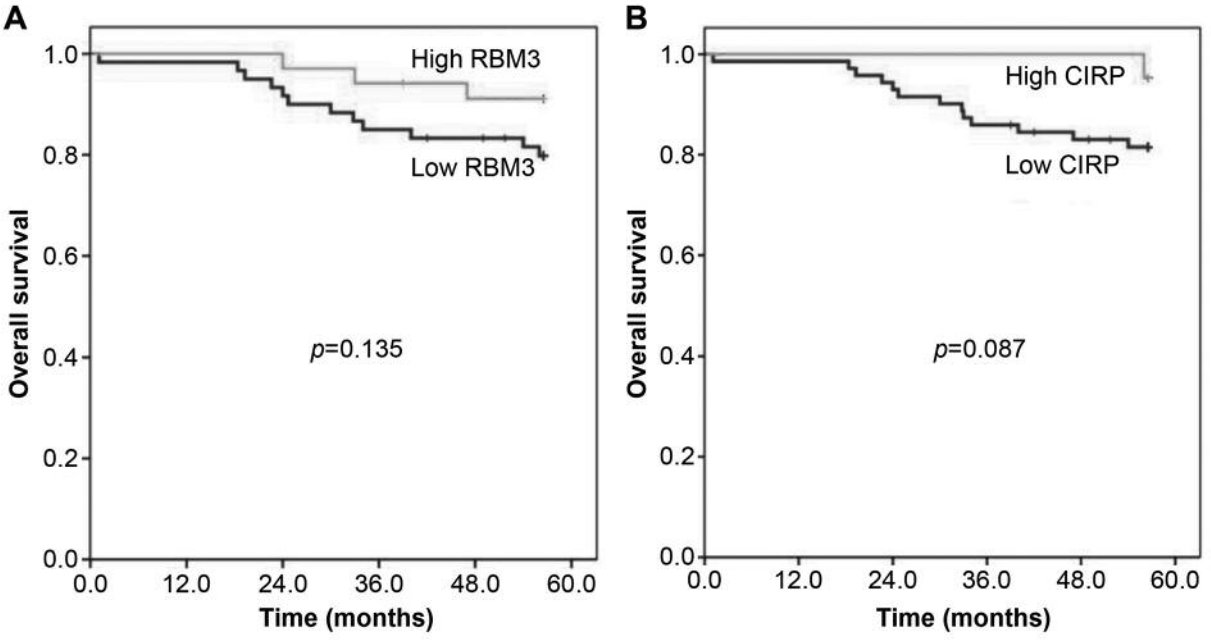

Figure 2. *Overall survival curves for patients with colon cancer according to high and low expression of RNA-binding motif protein 3 (RBM3) and cold-inducible RNA-binding protein (CIRP).*

regulator cyclin E1 (10). Aberrant expression of HuR or cyclin E1 contributes to malignant transformation and tumorigenesis in breast and other cancer types, including colon cancer (11, 12). We suspect that the expression of both *RBM3* and *CIRP* is higher in patients with more favorable outcomes because these proteins are structurally related in terms of their RNA-binding ability (13). Furthermore, both genes have been shown to be up-regulated in hypoxic environments (14).

Notably, RBM3 has also been identified as a cold-shock protein (15). Cold-shock proteins have been suggested to be important mediators of a novel type of mitotic cell death, known as caspase-independent mitotic death (16); however, the exact role of RBM3 in this process remains unknown. Notably, Dresios *et al*. (17) showed that RBM3 may influence tumor progression by controlling global protein expression within the cell *via* altering microRNA levels. Furthermore, RBPs have also been described as a novel family of apoptosis modulators (18), and a correlation between the X chromosome-related *RBM* genes (*RBMX, RBM3*, and *RBM10*) and the pro-apoptotic BCL2-associated X (*BAX*) gene was shown in two independent breast cancer cohorts (19). The association between *RBM3* and more favorable clinicopathological parameters that was observed in that study is supported by our data, although our results were not significant. In addition, we also observed that higher *RBM3* expression was associated with an improved DFS rate. However, due to the small sample size, we did not find any significant correlation between *RBM3* and the OS of the patients. Of note, the results are contradictory to previously published data which suggested increased RBM3 expression is associated with more aggressive colorectal cancer cell lines (20). Sureban *et al*.'s study is based on small sample size (n=15) with no prognostic information (20). Hjelm *et al*. analyzed 270 colon cancer samples and found positive prognostic role of high RBM3 expression using tissue microarray-based immunohistochemical analysis (21). Our study finding is concordant with that of Hjelm *et al*. (21).

High microsatellite instability is commonly associated with good prognosis and right-sided colon cancer (22). This study found a positive relationship between microsatellite instability-high tumor with high expression of *RBM3*. This may be indirect evidence that high expression of *RBM3* is associated with good DFS. Further investigation is needed to clarify this finding.

Similarly to RBM3, CIRP has been poorly characterized, but may participate in transcriptional and post-transcriptional events known to regulate gene expression in cancer cells. Our data indicate that higher *CIRP* expression is associated with an improved DFS rate. Unfortunately, the small number of patients included in this study again limited the significance of findings for this gene and OS rate. Thus, additional investigation using a larger subset of patients is warranted to fully elucidate the possible use of *RBM3* and *CIRP* as prognostic biomarkers of colon cancer survival.

Taken together, our findings suggest that *RBM3* and *CIRP* may be markers of good prognosis in colon cancer, while *IGFBP2* does not appear to be correlated to patient outcome. While future in-depth studies are needed in order to fully elucidate the functional mechanisms underlying the protective roles of these RBPs in colon cancer, we believe the relationship between *RBM3* and *CIRP* expression and patient survival may be related to tumor hypoxia. Notably, as far as we are aware of, this is the first study to address the importance of *RBM3* and *CIRP* expression in a uniform curatively resected colon cancer population, and thus, the first to identify *RBM3* and *CIRP* as independent prognostic factors. Assessment of these RBPs in combination with N staging may be more useful in stratifying curatively resected cancer, allowing patients to acquire the best possible adjuvant treatment.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study. The Authors alone are responsible for the content and writing of this article.

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