Association of B-cell lymphoma 2/microRNA-497 gene expression ratio score with metastasis in patients with colorectal cancer: A propensity-matched cohort analysis

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Abstract

Background: Deregulated microRNAs (miRs) significantly impact cancer development and progression. Our in silico analysis revealed that miR-497 and its target gene B-cell lymphoma-2 (BCL2) could be related to poor cancer outcomes.

Purpose: To investigate the BCL2/miRNA-497 expression ratio in colorectal cancer (CRC) and explore its association with the clinicopathological characteristics and CRC prognosis.

Methods: Archived samples from 106 CRC patients were enrolled. MiR-497 and BCL2 gene expressions were detected by Taq-Man Real-Time quantitative polymerase chain reaction in propensity-matched metastatic and nonmetastatic cohorts after elimination of confounder bias.

Results: B-cell lymphoma-2 gene was upregulated in metastatic samples (median = 1.16, 95%CI = 1.09–1.60) compared to nonmetastatic (median = 1.02, 95%CI = 0.89–1.25, p < 0.001). In contrast, lower levels of miR-495 were detected in specimens with distant metastasis (median = 0.05, 95%CI = 0.04–0.20) than nonmetastatic samples (median = 0.54, 95%CI = 0.47–0.58, p < 0.001). Estimated BCL2/miR-497 ratio yielded a significant differential expression between the two cohort groups.
Higher scores were observed in metastasis group (median = 1.39, 95%CI = 0.9–1.51) than nonmetastatic patients (median = 0.29, 95%CI = 0.19–0.39, p < 0.001). Receiver operating characteristic curve analysis showed BCL2/miR-497 ratio score to have the highest predictive accuracy for metastasis at presentation. The area under the curve was 0.90 (95%CI = 0.839–0.964, p < 0.001) at cut-off of >0.525, with high sensitivity 81.1% (95%CI = 68.6%–89.4%) and specificity 92.5% (95%CI = 82.1%–97.0%). Also, the ratio score was negatively correlated with disease-free survival \( r = -0.676, p < 0.001 \) and overall survival times \( r = -0.650, p < 0.001 \). Kaplan–Meier curves showed lower survival rates in cohorts with high-score compared to low-score patients.

**Conclusion:** The BCL2/miR497 expression ratio is associated with poor CRC prognosis in terms of metastasis and short survival.

**Key Words**

BCL2, colorectal cancer, gene expression, metastasis, miR-497, Real-Time qPCR

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**1 | INTRODUCTION**

Colorectal cancer (CRC) substantially influences cancer-related death worldwide.\(^1\) Despite the recent advances in CRC management, the associated morbidity and mortality remain high.\(^2\) The last decade has witnessed a massive growth in our understanding of CRC genetic etiopathology.\(^3\) Identifying and highlighting such genetic contribution may help better understand the molecular basis of cancer patient prognosis with potential future targeted therapy.\(^4\)

Noncoding RNAs have emerged as central genetic/epigenetic players in several cancers, including CRC.\(^5,6\) The noncoding microRNAs (miRNAs) class has been implicated in CRC tumorigenesis/progression and treatment.\(^7,8\) Indeed, their dysregulation may contribute to poor CRC outcomes, including metastasis and short survival.\(^9-11\)

Our in silico analysis has revealed the microRNA-497 (miR-497) as one of the most iterated miRNAs in CRC, as will be detailed later on, and the B-cell lymphoma 2 (BCL2) gene as one of its target genes that proved previously to play a central role in the regulation of apoptosis and was implicated in colorectal carcinogenesis, progression, and treatment resistance.\(^12\) Interestingly, previous studies have reported that miR-497 can suppress proliferation and induce apoptosis via the Bcl2-related molecular axis in several tissues and cancers, including neuronal cells, the “human umbilical vein endothelial cells,” breast cancer, and multiple myeloma.\(^13-15\) Zhu et al. found that miR-497 could decrease the resistance to cisplatin in “human lung cancer cell lines” by targeting BCL2.\(^16\) Also, a recent study by Zheng et al. has proved that miR-497/BCl2 axis could suppress cisplatin resistance in CRC cells.\(^17\) Nevertheless, no previous study demonstrated the impact of BCL2/miR-497 expression ratio score on CRC prognosis and outcome. In this sense, the authors were interested in exploring the association of the BCL2/miR-497 expression profile with the clinic-pathological characteristics and outcomes of CRC patients to help their prognostic stratification and future individualized therapeutic management.

**2 | SUBJECTS AND METHODS**

**2.1 | Bioinformatic selection of microRNA**

Analysis of 2 TCGA datasets (TCGA-COAD for colon adenocarcinoma and TCGA-READ for rectal adenocarcinoma) from Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/) and 16 microarray public datasets (GSE2564, GSE10259, GSE38389, GSE18392, GSE30454, GSE35602, GSE38389, GSE33125, GSE49246, GSE35834, GSE5088, GSE41012, GSE46555, GSE48267, GSE73487, GSE77380) from Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) revealed significant microRNAs in each comparison (Table 1). Log fold change and adjusted \( p \)-values were identified for each experiment using the Database of Differentially Expressed miRNAs in Human Cancers (dbDEMC v3.0) (https://www.biosino.org/dbDEMC/index). The average fold change of microRNAs was estimated, the direction of expression across all studies was identified, and the total number of comparisons with significant expression was calculated. MiR-497-5p was selected because it was the most frequently downregulated microRNA across datasets. Functional enrichment analysis and gene targets of miR-497-5p identification in CRC KEGG pathway were identified using the DIANA-miRPath v3.0 (http://www.microrna.gr/miRPathv3); a “miRNA pathway analysis-based webserver”\(^18\).

**2.2 | Study population and tissue sampling**

This retrospective study enrolled an eligible 53 pairs of “formalin-fixed, paraffin-embedded, FFPE” colorectal tissue samples archived...
in the Suez Canal University hospital pathology lab, Ismailia, Oncology Center of Mansoura Hospital, Mansoura, and El-laban Pathology Laboratory, Port-Said, Egypt, between January 2008 and December 2018. The inclusion criteria included archived paired primary CRC samples with no history of chemotherapy/radiotherapy before the surgery and availability of the related clinicopathological data from the medical records, including the survival follow-up information. The stage system of the tumors was according to the International Union Against Cancer TNM staging system (8th ed.)\textsuperscript{19}

Samples with incomplete clinical and/or follow-up data, history of receiving any therapeutic modality before resection, secondary CRC as well as samples without available paired noncancer tissue, tiny size tissue specimen available for molecular work, and those with low concentration or the extracted total RNA did not have enough quality to proceed in the downstream real-time qPCR steps, were excluded as showed in Figure 1. The ethical/legal guidelines adopted by the Declaration of Helsinki were followed. The local Medical Research Ethics Committee granted ethical approval for this study, and the patient consent was waived as the enrolled samples in this retrospective study were archived.

### 2.3 Clinical assessment and follow-up

Patient information was obtained from the medical records. These included patients’ demographic data, primary cancer site, pathology

| GEO ID             | Sample case                  | Sample control   | Number cases | Number controls | Up   | Down  |
|--------------------|------------------------------|------------------|--------------|-----------------|------|-------|
| Colon cancer       | GSE2564 Colon tumor          | Normal colon     | 10           | 5               | 3    | 4     |
|                    | GSE38399 Colon tumor         | Normal colon     | 85           | 85              | 19   | 8     |
|                    | GSE18392 Colon tumor         | Normal colon     | 116          | 29              | 157  | 153   |
|                    | GSE18392 Colon tumor TNM stage 2 | Normal colon | 44           | 29              | 147  | 153   |
|                    | GSE18392 Colon tumor TNM stage 3 | Normal colon | 38           | 29              | 134  | 137   |
|                    | GSE18392 Colon tumor TNM stage 4 | Normal colon | 15           | 29              | 82   | 103   |
|                    | GSE33125 Colon cancer        | Normal colon     | 9            | 9               | 22   | 25    |
|                    | GSE49246 Colon cancer stage 2 | Normal colon     | 40           | 40              | 407  | 437   |
|                    | GSE35834 Colon cancer        | Normal colon     | 31           | 23              | 37   | 50    |
|                    | GSE48267 Colon cancer        | Normal colon     | 61           | 61              | 44   | 53    |
|                    | GSE73487 Colon cancer        | Normal tissue    | 64           | 23              | 0    | 9     |
|                    | GSE73487 Tubulovillous adenoma | Normal tissue | 35           | 23              | 45   | 40    |
|                    | GSE73487 Serrated adenoma    | Normal tissue    | 3            | 23              | 29   | 1     |
|                    | TCGA-COAD Colon adenocarcinoma | Normal tissue    | 441          | 8               | 158  | 181   |

Colorectal cancer

| GEO ID             | Sample case                  | Sample control   | Number cases | Number controls | Up   | Down  |
|--------------------|------------------------------|------------------|--------------|-----------------|------|-------|
|                    | GSE10259 Colorectal cancer   | Normal colon     | 9            | 20              | 61   | 88    |
|                    | GSE10259 Hereditary nonpolyposis colon cancer | Normal colon | 9            | 20              | 61   | 88    |
|                    | GSE30454 Lynch syndrome tumor | Normal colon     | 13           | 20              | 51   | 77    |
|                    | GSE35602 Colorectal cancer   | Normal colon     | 17           | 4               | 3    | 19    |
|                    | GSE38389 Rectal tumor        | Normal rectal mucosa | 69           | 71              | 137  | 130   |
|                    | GSE40102 Colorectal cancer   | Normal tissue    | 9            | 10              | 2    | 2     |
|                    | GSE41655 Colorectal adenocarcinoma | Normal colon | 33           | 15              | 61   | 88    |
|                    | GSE41655 Colorectal adenoma  | Normal colon     | 59           | 15              | 71   | 109   |
|                    | GSE77380 Rectum adenocarcinoma | Normal rectum    | 3            | 5               | 46   | 619   |
|                    | TCGA-READ Rectum adenocarcinoma | Normal tissue    | 158          | 3               | 147  | 174   |

Note: All experiments are microarray except the two TCGA datasets (microRNA sequencing). Up and down are the number of microRNAs found to be deregulated in the experiment.
reports, and treatment modalities if available. Relapse, recurrence, further metastasis, and death reported during the follow-up were reported. Overall survival was defined as the time from treatment to death (for any reason). Disease-free survival represented the time from treatment to the recurrence (local, regional, distant) or death (for any reason). Survival times were categorized into short and prolonged times; short survival times were defined if ≤24 months after initial treatment.

2.4 | Propensity scores matching analysis

The survival outcomes of metastatic and nonmetastatic colon cancer patients and the impact of transcriptomic signature of selected markers were compared via a propensity score matching analysis. This analysis was performed to adjust confounder variables using the MatchIt R package. The following covariates were adjusted: age, sex, obesity, tumor site, histopathological diagnosis, pathological grade, tumor size, lymph node metastasis, and lymphovascular invasion. Multivariate logistic regression was applied to create a balancing score as a distant measure for each patient. Next, metastatic and nonmetastatic cohorts were allocated using a one-to-one nearest neighbor algorithm without caliper adjustment to find pairs of patients that have the closest match in the two study groups. The quality of the matches in the two datasets (N = 53 patients in each group) were evaluated by estimating mean difference and average absolute standardized difference of covariates.

2.5 | BCL2/miR-497 expression analysis

Total tissue RNA, including miRNAs, was isolated from the CRC samples using miRNeasy FFPE Kit (217504, Qiagen, Hilden, Germany) following the manufacturer’s instructions. To ensure DNA-free extracts, each sample was subjected to DNase I treatment (for 2 h at 37°C). RNA concentration/purity and integrity were tested by "NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc.)" and "agarose gel electrophoresis," respectively.

Reverse transcription (RT) for the total RNA was carried out by a high-capacity complementary DNA RT kit (Applied Biosystems, P/N 4368814) in the case of BCL2 gene expression quantification (assay ID Hs04986394_s1) compared to GAPDH gene (assay ID Hs02786624_g1). The RT reaction contains the RNA extract (5 μl), 100 mM of each dNTP (0.15 μl), "MultiScribe reverse-transcriptase" (50 U/μl; 1 μl), 10 × RT buffer (1.5 μl), ribonuclease inhibitor (20 U/ml; 0.19 μl), gene-specific TaqMan® forward and reverse primers (3 μl of each) and nuclease-free water (4.16 μl) was prepared.
for each RNA sample. For miR-497 quantification, the total RNA was reverse transcribed using TaqMan MicroRNA RT kit (P/N 4366596; Thermo Fisher Scientific, Applied Biosystems) and either the miR-497 specific stem-loop primers (assay ID 001043) or the endogenous control RNU6B primers (assay ID 001093). The RT reactions of BCL2 and miR-497 were done on the "T-Professional Basic, Biometra PCR System" (Biometra, Gottingen, Germany). Non-template and non-RT enzyme negative controls were run with each experiment to exclude amplicon contamination. Then the quantitative Real-Time PCR was carried out in duplicate in "StepOne Real-Time PCR System" (Applied Biosystems) as described in detail previously.10,11 All the steps of the qRT-PCR were run following the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)" guidelines.21 The relative expression levels were calculated using the delta–delta CT (cyclic threshold) method.22

### 2.6 Statistical analysis

Relative expression levels of microRNA and genes were stratified by metastasis and plotted as box plots. Expression data were nonparametric; therefore, log transformation was employed. The Wilcoxon signed-rank test was applied to compare cancer and its paired normal tissues, while the Mann-Whitney U test was carried out to test the difference between metastatic and nonmetastatic groups. To decipher the diagnostic accuracy of BCL2, miR-497, and its ratio score, Receiver Operator Characteristic (ROC) curve analysis was performed, and area under the curve (AUC) was estimated for metastatic and nonmetastatic groups. Optimum cut-off values with high sensitivity and specificity were identified. Univariate analysis was performed to identify variables influencing survival, followed by Cox regression analysis to identify independent risk factors for overall survival. Hazard ratio (HR) and 95% confidence interval (CI) were reported. Two-sided p-values <0.05 were regarded as significant. Spearman's correlation analysis was applied to identify the correlation between BCL2/miR-497 ratio score and survival times. Kaplan-Meier curves were generated to compare patients with high- and low-ratio scores based on the median value. Log-Rank test with Benjamini and Hochberg adjustment for p-value was applied. Under R version 4.0.5, ggplot2 and survminer R packages were used for plotting. Finally, a Cox regression model was employed to construct a prognostic nomogram using regplot and survival R packages. Statistical analysis was performed using SPSS v27.0 (IBM Corp.), GraphPad prism v9.1.1 software (GraphPad, Inc.), and RStudio 1.4.1106 (R Foundation).
3 | RESULTS

3.1 | In silico data analysis

Analysis of 29 comparisons revealed a total of 2050 unique significant microRNAs in at least one analysis of CRC specimens. One of the most iterated microRNAs was miR-497-5p (Figure 2). It was downregulated in 16 different comparisons for cancer versus normal tissues (Figure 3). Similarly, the meta-profiling of miR-497 highlighted its putative tumor suppressor role in other types of cancers (Table S1). The expression level was the least in pancreatic cancer (GSE28955: FC = −4.69), sarcoma (GSE28423: FC = −4.49), and lymphoma (GSE45264: FC = −3.16). Lower miRNA expression was also noted in the circulation of the prostate (GSE31568: FC = −1.44) and renal (GSE38419: FC = −0.88) cancer patients. Furthermore, miR-497 was two-fold downregulated in tissues of CRC patients with poor outcomes (GSE33961: FC = −2.14) (Table S1).

3.2 | Functional enrichment analysis

Pathway enrichment analysis revealed the involvement of miR-497-5p in cancer-related pathways including proteoglycans in cancer (hsa05205|p = 1.45e-11). hippo
signaling pathway (hsa04390) \( p = 1.11 \times 10^{-6} \), mTOR signaling pathway (hsa04150) \( p = 2.69 \times 10^{-4} \), TGF-beta signaling pathway (hsa04350) \( p = 7.64 \times 10^{-4} \), and p53 signaling pathway (hsa00310) \( p = 8.27 \times 10^{-4} \). In particular, miR-497-5p was significantly enriched in the CRC KEGG pathway [05210]. It has 12 gene targets: BRAF, BCL2, PIK3R2, SMAD3, BIRC5, AKT2, AKT3, CCD1, MAPK1, MAPK8, MAP2K1, MYC, and PIK3CA (Figure S1).

### 3.3 Baseline characteristics of the study population

The study population included 69 males and 37 females, 53.8% over 55 years old, and 62.3% were obese. Detailed information about clinical characteristics of propensity-matched metastatic and nonmetastatic cohorts is described in Table 2. There were no
significant differences in demographic and pathological features of both groups. However, a higher frequency of mortality was reported in 49.1% of metastatic cohorts compared to 18.9% in nonmetastatic cancer patients ($p = 0.002$). In addition, patients with metastasis at presentation showed shorter survival ($p < 0.001$), as expected.

### 3.4 | Expression profile of miR-497-5p and BCL2 in colon cancer tissues

B-cell lymphoma-2 gene was upregulated in metastatic samples (median = 1.16, 95%CI = 1.09–1.60) compared to nonmetastatic (median = 1.02, 95%CI = 0.89–1.25, $p < 0.001$). In contrast, lower levels of miR-495-5p were found in specimens with distant metastasis (median = 0.05, 95%CI = 0.04–0.20) than nonmetastatic samples (median = 0.54, 95%CI = 0.47–0.58, $p < 0.001$). Estimated ratio score between BCL2 and miR-497-5p yielded a significant differential expression between the two cohort groups. Higher scores were noted in metastasis group (median = 1.39, 95%CI = 0.9–1.51) than nonmetastatic patients (median = 0.29, 95%CI = 0.19–0.39, $p < 0.001$) (Figure 4A-C). ROC curve analysis showed BCL2/miR-497 ratio score to have the highest predictive accuracy for metastasis at presentation. AUC was 0.90 (95%CI = 0.839–0.964, $p < 0.001$) at cut-off of $>0.525$, with high sensitivity 81.1% (95%CI = 68.6%–89.4%) and specificity 92.5% (95%CI = 82.1%–97.0%) (Figure 4D-F).

### 3.5 | Prognostic value of miR-497-5p and BCL2 in colon cancer

Table 3 demonstrated the association between the expression levels and demographic, clinical, and pathological parameters. Tested genes were significantly associated with metastasis, clinical stage, and mortality. In univariate analysis, expired patients were three to four times more likely to be obese (80.6% versus 52.9%, $p = 0.006$), have metastasis at presentation (72.2% vs. 38.6%, $p = 0.002$), have lymphovascular invasion (55.6% vs. 28.6%, $p = 0.011$), and have higher ratio score (66.7% vs. 41.4%, $p = 0.023$). Cox regression model revealed that high-risk score was nearly three times more likely to die (HR = 2.82, 95%CI = 1.22–6.55) (Table 4). The ratio score
### Table 3: Univariate association analysis of MIR-497 and BCL2 expression with clinic-pathological features

| Characteristics | No. of cases | BCL2 log2FC | p-value | mIR-497 log2FC | p-value | Log10 ratio score | p-value |
|-----------------|--------------|-------------|---------|----------------|---------|-------------------|---------|
| **Age (years)** |              |             |         |                |         |                   |         |
| ≤55             | 49 (46.2)    | 1.12 (0.95–1.28) | 0.28    | 0.45 (0.05–0.56) | 0.75    | 0.4 (0.28–1.33)  | 0.90    |
| >55             | 57 (53.8)    | 1.13 (1.01–1.52) | 0.44    | 0.52 (0.25–1.42) |         |                   |         |
| **Sex**         |              |             |         |                |         |                   |         |
| F               | 37 (34.9)    | 1.11 (0.97–1.45) | 0.97    | 0.52 (0.25–1.44) | 0.56    | 0.46 (0.27–1.38) | 0.86    |
| M               | 69 (65.1)    | 1.13 (0.98–1.36) | 0.45    | 0.46 (0.27–1.38) | 0.86    | 0.46 (0.27–1.38) | 0.86    |
| **Obesity**     |              |             |         |                |         |                   |         |
| Negative        | 40 (37.7)    | 1.07 (0.95–1.34) | 0.44    | 0.52 (0.27–1.38) | 0.86    | 0.46 (0.27–1.38) | 0.86    |
| Positive        | 66 (62.3)    | 1.14 (1.03–1.42) | 0.45    | 0.46 (0.27–1.38) | 0.86    | 0.46 (0.27–1.38) | 0.86    |
| **Location**    |              |             |         |                |         |                   |         |
| Ascending       | 49 (46.2)    | 1.13 (0.96–1.48) | 0.66    | 0.52 (0.27–1.44) | 0.58    | 0.52 (0.27–1.44) | 0.58    |
| Transverse      | 6 (5.7)      | 1.21 (0.95–1.61) | 0.56    | 0.36 (0.2–1.35)  |         |                   |         |
| Descending      | 51 (48.1)    | 1.11 (0.98–1.29) | 0.45    | 0.46 (0.27–1.35) | 0.81    | 0.46 (0.27–1.35) | 0.81    |
| **Type**        |              |             |         |                |         |                   |         |
| Adenocarcinoma  | 69 (65.1)    | 1.11 (0.94–1.33) | 0.29    | 0.62 (0.25–1.44) | 0.86    | 0.62 (0.25–1.44) | 0.86    |
| Mucinous        | 14 (13.2)    | 1.24 (1.01–1.68) | 0.52    | 0.61 (0.28–1.31) |         |                   |         |
| Signet ring     | 14 (13.2)    | 1.13 (1.06–1.28) | 0.44    | 0.47 (0.22–1.21) |         |                   |         |
| Undifferentiated| 9 (8.5)      | 1.13 (1.07–1.82) | 0.5     | 0.49 (0.32–1.29) |         |                   |         |
| **Grade**       |              |             |         |                |         |                   |         |
| G1              | 13 (12.3)    | 1.13 (0.88–1.34) | 0.23    | 0.24 (0.19–1.37) | 0.89    | 0.24 (0.19–1.37) | 0.89    |
| G2/3            | 93 (87.7)    | 1.12 (0.98–1.46) | 0.46    | 0.46 (0.27–1.35) |         |                   |         |
| **Tumor size**  |              |             |         |                |         |                   |         |
| T1/2            | 61 (57.5)    | 1.13 (0.95–1.34) | 0.53    | 0.72 (0.27–1.4)  | 0.81    | 0.72 (0.27–1.4)  | 0.81    |
| T3/4            | 45 (42.5)    | 1.12 (1.01–1.57) | 0.45    | 0.66 (0.23–1.38) |         |                   |         |
| **LN invasion** |              |             |         |                |         |                   |         |
| Negative        | 66 (62.3)    | 1.11 (0.97–1.4) | 0.76    | 0.033 (0.21–1.3) | 0.08    | 0.033 (0.21–1.3) | 0.08    |
| Positive        | 40 (37.7)    | 1.13 (0.98–1.38) | 0.59    | 0.53 (0.29–1.42) |         |                   |         |
| **Metastasis**  |              |             |         |                |         |                   |         |
| Negative        | 53 (50)      | 1.02 (0.9–1.25) | <0.001  | 0.29 (0.2–0.4)  | <0.001  | 0.29 (0.2–0.4)  | <0.001  |
| Positive        | 53 (50)      | 1.16 (1.09–1.6) | 0.05    | 1.39 (0.9–1.51) |         |                   |         |
| **Site of metastasis** |          |             |         |                |         |                   |         |
| Liver           | 44 (83)      | 1.15 (1.06–1.51) | 0.12    | 1.35 (0.78–1.51) | 0.53    | 1.35 (0.78–1.51) | 0.53    |
| Lung            | 9 (17)       | 1.33 (1.15–2.12) | 0.05    | 1.41 (0.96–1.59) |         |                   |         |
| **LVI**         |              |             |         |                |         |                   |         |
| Negative        | 66 (62.3)    | 1.13 (0.97–1.59) | 0.21    | 0.43 (0.26–1.37) | 0.71    | 0.43 (0.26–1.37) | 0.71    |
| Positive        | 40 (37.7)    | 1.12 (0.97–1.32) | 0.39    | 0.5 (0.27–1.4)   |         |                   |         |
| **Dukes**       |              |             |         |                |         |                   |         |
| A/B             | 24 (22.6)    | 1.04 (0.9–1.2)  | 0.026   | 0.28 (0.19–0.38) | <0.001  | 0.28 (0.19–0.38) | <0.001  |
| C/D             | 82 (77.4)    | 1.14 (1.02–1.43) | 0.34    | 0.72 (0.29–1.45) |         |                   |         |
| **Relapse**     |              |             |         |                |         |                   |         |
| Negative        | 66 (62.3)    | 1.09 (0.95–1.39) | 0.17    | 0.42 (0.23–1.31) | 0.18    | 0.42 (0.23–1.31) | 0.18    |
| Positive        | 40 (37.7)    | 1.15 (1.03–1.43) | 0.41    | 0.57 (0.3–1.44)  |         |                   |         |
| **Died**        |              |             |         |                |         |                   |         |
| Negative        | 70 (66)      | 1.07 (0.94–1.26) | 0.008   | 0.33 (0.23–1.29) | 0.004   | 0.33 (0.23–1.29) | 0.004   |
| Positive        | 36 (34)      | 1.28 (1.06–1.59) | 0.2     | 0.99 (0.33–1.49) |         |                   |         |
was negatively correlated with disease-free survival ($r = -0.676$, $p < 0.001$) and overall survival times ($r = -0.650$, $p < 0.001$). Patients with metastasis exhibited lower survival times (Figure 5A-B). When patients were categorized according to the median ratio score into high-score and low-score groups, Kaplan–Meier curves showed lower survival rates in cohorts with high-score compared to low-score patients (Figure 5C-D). A prognostic nomogram to predict metastasis at presentation was generated using the ratio score with demographic characteristics of patients, which showed good agreement with the actual outcome (Figure 6).

4 | DISCUSSION

CRC’s tendency to invasion/metastasis is one of the major factors leading to poor prognosis. Identifying new genetic/epigenetic biomarkers associated with CRC metastasis and survival could help improve cancer management.

In this work, we explored the association of $BCL2$, miR-479, and $BCL2$/miR-479 ratio with poor prognosis in terms of metastasis and short survival in patients with CRC. We found that $BCL2$ was upregulated in metastatic samples compared to nonmetastatic ones. In contrast, miR-495-5p downregulation was found in specimens with distant metastasis than nonmetastatic samples. The estimated ratio score between $BCL2$ and miR-497-5p yielded a significant differential expression between the two cohort groups. Also, ROC curve analysis showed $BCL2$/miR-497 ratio score to have the highest predictive accuracy for metastasis at presentation. Furthermore, the ratio score showed a negative correlation with disease-free survival and overall survival, as well as included in a newly generated prognostic nomogram to predict metastasis, among other parameters. These results are consistent with previous studies that reported the implication of $BCL2$ and miR-497 in cancer, including the CRC. and support that analyzing combined markers is better than an individual molecule in cancer diagnostics and/or prognostication.

The pro-survival $BCL2$ is one of the “anti-apoptotic $BCL2$ family proteins” implicated in promoting cancer cell proliferation, metastatic spread, and resistance to anticancer therapy. Several mechanisms have been proposed to explain the $BCL2$ gene overexpression, including increasing the rate of gene transcription, gene amplification (increased gene copy number), and posttranscriptional–translational modifications that augment the prosurvival activity of the specified proteins. Accumulating evidence proved that deregulated $BCL2$ family expression is not provided to occur only in the tumorigenesis stage of cancer but can be observed in all stages of cancer progression, including metastasis and even in the anticancer therapeutic resistance stage.

A meta-analysis of 40 articles showed a significant association of $BCL2$ expression with pathological grade, clinical stage, overall, and disease-free survival in patients with CRC. $Bcl-2$ has been shown to prolong cell survival by inhibiting apoptosis. Abnormal activation of the Bcl-2 gene appears to be an early event in colorectal tumorigenesis. It is worth noting that cancer development and

| Characteristics | No. of cases | BCL2 log2FC | p-value | miR-497 log2FC | p-value | Log10 ratio score | p-value |
|-----------------|-------------|-------------|---------|----------------|---------|------------------|---------|
| Short DFS       | 84 (79.2)   | 1.07 (0.95–1.29) | <0.001  | 0.49 (0.13–0.57) | <0.001  | 0.33 (0.23–1.04) | <0.001  |
| Short OS        | 90 (84.9)   | 1.07 (0.95–1.28) | <0.001  | 0.49 (0.06–0.57) | <0.001  | 0.33 (0.24–1.62) | <0.001  |

Note: The expression level is shown as median (quartiles). Mann–Whitney U test was used. Bold values indicate significant $p < 0.05$. LN: lymph node; LVI: lymph-vascular invasion; DFS: disease-free survival; OS: overall survival.
progression rely on the overexpression of antiapoptotic gene players and underexpression of the proapoptotic ones. The outcome of the interplay between these signatures varies according to the cancer type and even could be different within the same cancer type.43,44 This could partly explain the heterogeneity/controversy between the observed prognostic signature of \( BCL2 \) in different cancer types, including CRC, in the present study and previous reports.

MiR-497 dysregulation reflects a complex network that is influenced by several factors.27 Interestingly, miR-497 downregulation in this study agrees with many independent online gene expression omnibus (GEO) experiments, including the GSE41655 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41655), GSE35834,45 and GSE68204,46 among others in which miR-497 downregulation was observed in CRC tissues compared to the adjacent noncancerous mucosa (all \( p < 0.001 \)). Additionally, several previous studies have uncovered the molecular role(s) by which miR-497 can impact CRC tumorigenesis and/or progression. For example, Guo et al. reported that miR-497 downregulation could upregulate “insulin-like growth factor 1 receptor” with subsequent increase of “PI3K/Akt” signaling, contributing to the malignant behavior of CRC cells.47 Zhang et al. also explored miR-497 overexpression can reduce the ability of CRC cells to invade tissues, and this inhibition was mediated through “Fos-related-antigen-1” regulation.48 Similarly, Xu et al. reported that miR-497 targeted upregulation in CRC tissues can suppress the proliferation and migration/invasion of CRC cells by “insulin receptor substrate-1” degradation.49 

### TABLE 4

| Characteristics | Levels                      | Survived (\( N = 70 \)) | Died (\( N = 36 \)) | \( p \)-value | HR (95%CI) |
|-----------------|-----------------------------|--------------------------|---------------------|--------------|------------|
| Age (years)     | \( \leq 55 \)               | 33 (47.1)                | 16 (44.4)           | 0.83         | Reference  |
|                 | >55                          | 37 (52.9)                | 20 (55.6)           | 1.11 (0.49–2.50) |            |
| Sex             | Female                      | 22 (31.4)                | 15 (41.7)           | 0.39         | Reference  |
|                 | Male                        | 48 (68.6)                | 21 (58.3)           | 0.64 (0.27–1.47) |            |
| Obesity         | Negative                    | 33 (47.1)                | 7 (19.4)            | 0.006        | Reference  |
|                 | Positive                    | 37 (52.9)                | 29 (80.6)           | 3.69 (1.43–9.54) |            |
| Location        | Ascending                   | 32 (45.7)                | 17 (47.2)           | 0.65         | Reference  |
|                 | Transverse                  | 3 (4.3)                  | 3 (8.3)             | 1.88 (0.34–10.3) |            |
|                 | Descending                  | 35 (50)                  | 16 (44.4)           | 0.86 (0.37–1.98) |            |
| Type            | Adenocarcinoma              | 45 (64.3)                | 24 (66.7)           | 0.69         | Reference  |
|                 | Mucinous carcinoma          | 11 (15.7)                | 3 (8.3)             | 0.51 (0.13–2.01) |            |
|                 | Signet ring carcinoma       | 9 (12.9)                 | 5 (13.9)            | 1.04 (0.31–3.45) |            |
|                 | Undifferentiated carcinoma  | 5 (7.1)                  | 4 (11.1)            | 1.50 (0.36–6.11) |            |
| Grade           | G1                           | 32 (88.9)                | 32 (88.9)           | 0.79         | Reference  |
|                 | G2/3                         | 32 (88.9)                | 32 (88.9)           | 1.18 (0.33–4.13) |            |
| Tumor size stage| T1/2                        | 37 (52.9)                | 24 (66.7)           | 0.21         | Reference  |
|                 | T3/4                         | 33 (47.1)                | 12 (33.3)           | 0.56 (0.24–1.29) |            |
| LN invasion     | Negative                    | 28 (40)                  | 17 (47.2)           | 0.53         | Reference  |
|                 | Positive                    | 42 (60)                  | 19 (52.8)           | 0.74 (0.33–1.67) |            |
| Metastasis      | Negative                    | 43 (61.4)                | 10 (27.8)           | 0.002        | Reference  |
|                 | Positive                    | 27 (38.6)                | 26 (72.2)           | 4.14 (1.72–9.92) |            |
| LVI             | Negative                    | 50 (71.4)                | 16 (44.4)           | 0.011        | Reference  |
|                 | Positive                    | 20 (28.6)                | 20 (55.6)           | 3.12 (1.35–7.21) |            |
| Duke stage      | A/B                          | 20 (28.6)                | 4 (11.1)            | 0.042        | Reference  |
|                 | C/D                          | 50 (71.4)                | 32 (88.9)           | 3.2 (1.0–10.2) |            |
| Ratio score     | Low score                   | 41 (58.6)                | 12 (33.3)           | 0.023        | Reference  |
|                 | High score                  | 29 (41.4)                | 24 (66.7)           | 2.82 (1.22–6.55) |            |

Note: Data are presented as frequency and percentage. A two-sided Chi-square test was performed. \( p \)-value less than 0.05 was set to be significant (bold values). Univariate Cox regression analysis was performed and shown in the last column. Hazard ratio (HR) and 95% confidence intervals (CI) are reported. Log10 Ratio score at >0.45 (median value) was set as a high score, based on ROC curve analysis. N: number; LN: lymph node; LVI: Lymph-vascular invasion.
expression induced by Wang et al. was found to suppress the CRC cell oncogenic hallmarks and augment the sensitivity of these cells to the chemotherapeutic agents via “kinase suppressor of Ras-1” oncogene regulation.50 Also, Zou et al. concluded the same miR-497 downregulated signature in patients with CRC, but in the sera of patients, which was an independent parameter for CRC.26 These findings supported the potential suppressor role of miR-497 that plays in CRC.

Some limitations should be addressed in this study. The sample size of eligible cohorts was considerably small; thus, multivariate analysis including many confounders was challenging. However, propensity matching in nature reduces the bias of confounding variables and mimics randomization leading to analysis of balanced groups. To the best of our knowledge, our study shows for the first time the relationship between the two study molecules in a group of CRC patients with metastasis and nonmetastasis.

5 | CONCLUSION

In summary, our findings in this study suggest the essential role of the BCL2/miR-497 ratio as a prognostic ratio for CRC in terms of association with metastasis and poor survival indices. However, it is worth noting that our study lacks the functional studies that
prove the exact mechanism by which miR-497 and its molecular
target BCL2 could play in CRC samples. Thus, future studies to as-
sess the exact mechanistic roles of the BCL2/miR-497 ratio in vivo
and clinical context are warranted. The present findings could have
important implications for the prognosis of patients with CRC and
could be assigned in future anticancer therapeutic management
protocols.

FIGURE 6 Nomogram for predicting metastasis. (A) The nomogram was constructed based on demographic features of patients and ratio
risk score. The outcome measured was metastasis. The logistic regression model was applied. (B) Example for using the nomogram. Assumed
having a 45-year-old obese male patient whose tissue microRNA risk score was high at 0.45. Each variable will be scored on its points scale.
The scores for all variables are added to obtain the total score “of 123,” and a vertical line is drawn from the total points’ row to estimate the
probability of metastasis “23.7%”

CONFLICT OF INTEREST
The authors report no conflicts of interest.

AUTHOR CONTRIBUTION
All authors contributed to data analysis, drafting, or revising the
article, have agreed on the journal to which the article has been
submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this submitted article and supplementary materials.

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