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Prognostic value of *BRAF*/MIR-17 signature and B-Raf protein expression in patients with colorectal cancer: A pilot study

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Abstract

Background: Despite the recent improvement in colorectal cancer (CRC) treatment, it still has a poor prognosis with a low survival rate. Genetic and epigenetic mechanisms have proved to play a substantial role in CRC tumorigenesis and progression. According to Gene Ontology and TargetScan analyses, the B-Raf proto-oncogene (*BRAF*) gene is one of the microRNA-17 (miR-17) targets. We aimed to explore the prognostic value of B-Raf protein and *BRAF*/microRNA-17 (MIR-17) gene expression signature in CRC archived samples.

Methods: B-Raf protein expression was identified by immunohistochemistry, while gene expression studies were quantified by real-time qPCR in 53 paired archived CRC specimens.

Results: The *BRAF* showed higher expressions in CRC specimens relative to noncancer tissues ($p = 0.006$). MIR17 expression was inversely and significantly correlated with both B-Raf protein ($r = -0.79$, $p < 0.001$) and gene expression ($r = -0.35$, $p = 0.010$) and showed a significant direct correlation with a high rate of relapse ($p = 0.020$). *BRAF*/miR-17 expression in CRC was associated inversely with tumor size, high grade of colonic carcinoma, lymph node metastasis, and carcinoma subtype. Spearman correlation and Kaplan-Meier survival curve analyses revealed that disease-free survival and overall survival were inversely and significantly correlated with positive B-Raf protein expression ($r = -0.31$ and -0.35, $p = 0.023$ and 0.011, respectively) and directly correlated with log *BRAF*/MIR17 ratio ($r = 0.50$ and 0.41, $p < 0.001$ and $= 0.003$, respectively). Cox hazard regression analysis revealed the *BRAF*/MIR17 ratio could predict both types of patients' survival, among other variables.

Conclusion: *BRAF*/MIR17 ratio could have prognostic utility in patients with CRC. Further larger-scale studies are warranted to confirm this utility.

KEYWORDS
BRAF, colorectal cancer, gene expression, IHC, miR-17, prognosis, qPCR

1 | INTRODUCTION

Colorectal cancer (CRC) represents the third most common malignancy and the second leading cause of cancer-related mortality worldwide.[1] It is characterized by a heterogeneous etiology derived from the constellation of potentially modifiable risk factors and multiple genetic/epigenetic alterations.[2,3] Although surgical resection in the earlier stage of the disease is associated with a relatively high 5-year survival rate, the prognosis of late-stage CRC patients remains poor.[4]

Accumulating evidence revealed the implication of coding and non-coding genes in every CRC tumorigenesis.[5] In this regard, a substantial number of genetic biomarkers, including the serine/threonine kinase B-Raf proto-oncogene (BRAF), DNA mismatch repair (dMMR) defects, the tumor protein p53 (P53), and Kirsten rat sarcoma viral oncogene homolog (KRAS), displayed prognostic impact and survival-predictive potentials for CRC.[6-10] However, "the clinical use of these markers requires further research," proposed by Xia et al.[11]

Given the central role of the protein kinase B-Raf in the "RAS/RAF/MEK/ERK," the "mitogen-activated protein kinase (MAPK)" signaling pathway,[12] its deregulation has been implicated in several aspects of malignant transformation and progression, including cell proliferation and survival, neoangiogenesis, invasion, and metastasis.[13] Several *BRAF* mutations confer important predictive value in the treatment of patients with colorectal carcinomas.[14] However, although *BRAF* gene mutation has been extensively studied (Figure S1A), there were scant (if any) reports exploring *BRAF* gene expression profile and B-Raf protein as diagnostic and/or prognostic biomarker in CRC.

In addition to the genetic markers, the last few decades have witnessed remarkable developments in the field of epigenetic regulators based on the recent advances in high-throughput techniques.[5] As an emerging family of non-coding RNAs, microRNAs (miRNAs) have proved to be implied in several biological and pathological processes.[15] Their dysregulation can contribute to carcinogenesis and metastasis in several cancers, including CRC.[16] Our in silico analysis by searching the Diana pathway database (http://diana.imis.athena-innovation.gr/) has revealed that microRNA-17 (miR-17-5p) transcript ranked the second most important microRNA enriched in colorectal cancer pathway [KEGG ID: 05210] and it has 25 gene targets, including BRAF (Figure S2). Interestingly, Kishore et al also have reported complementarity between the *BRAF* gene and miR-17 in the kidney cell line (HEK293).[17] Also, the Cancer Hallmarks Analytics tool has revealed the important role of B-Raf in invasion and metastasis, replicative immortality, and genomic instability and mutation. At the same time, the miR-17 gene (MIR-17) is highly involved in inducing angiogenesis, resisting cell death, and sustaining proliferative signaling (Figure 1).[18]

Considering that both BRAF and miR-17-5p are closely correlated with cancer, and the multigene prognostic predictors have the theoretical advantages of optimal use of information from continuous variables, according to Győrffy et al,[19] we speculated that the transcriptional pattern of both markers might participate in disease progression and open up a new era for combined targeted therapy. In this sense, the current study aimed to explore the association of B-Raf protein and *BRAF*/miR-17 expression signatures with pathological features and outcomes of CRC patients to assist in the prognostic stratification of CRC patients and, subsequently, the individualized therapeutic management of such lethal disease.
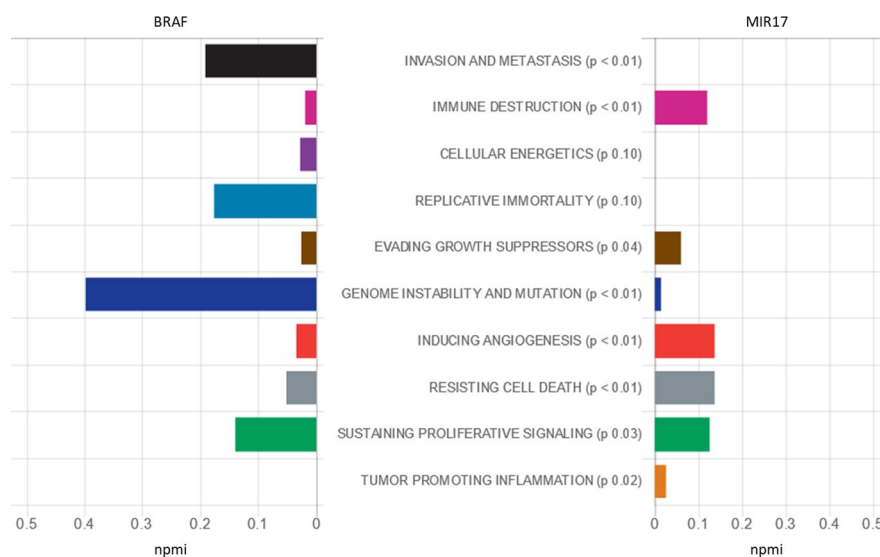

FIGURE 1 Role of *BRAF* and MIR-17 genes in cancer hallmarks. Hallmark annotations were derived from The Cancer Hallmarks Analytics Tool (CHAT) web browser, which analyzes the strength of association between genes of interest and the hallmarks of cancer in the literature based on a text-mining analysis of 26 million PubMed abstracts. npmi: normalized pointwise mutual information of h and q: npmi(h,q) = pmi(h,q)/-log2 p(h,q). The "npmi" metric normalizes "pmi" by dividing by the negative log probability of co-occurrence -log2 p(h,q), which constrains its values to the range [−1, 1]. Zero is a frequency of co-occurrence matching random (no association, as for "pmi") and 1 for complete co-occurrence (perfect association). [Data source: http://chat.lionproject.net]

2 | SUBJECTS AND METHODS

2.1 | Study participants and tissue specimens

The present retrospective study included the available 53 eligible archived primary colorectal carcinoma specimens staged according to the International Union Against Cancer TNM staging system (eighth edition)[20] and their paired non-cancerous tissue, which have complete clinical and survival data. Paraffin sections of resected specimens of colorectal carcinoma and the clinical data were retrieved from archives of the Pathology lab, Mansoura University Hospital, in the last five years. None of the patients have received any neoadjuvant chemotherapy or radiotherapy before sample collection. The clinicopathological data, including patients' age, sex, tumor site, and size, were obtained from patients' medical records. Sections of cancer-free tissues adjacent to the tumor were cut, examined, and collected to serve as controls during the genetic profiling and immunohistochemistry studies. Samples that were not homogeneous or histologically well-characterized primary CRC determined by an experienced pathologist and those with incomplete clinical and/or survival data were excluded. The study was conducted following the guidelines in the Declaration of Helsinki and was approved by the authors' institutions. Informed consent of the patients was not applicable in the present study as achieved samples were retrieved for the study analysis.

2.2 | Immunohistochemistry (IHC) methodology

IHC was performed on formalin-fixed, paraffin-embedded (FFPE) whole tissue sections of colorectal carcinomas for B-Raf using rabbit monoclonal antibody anti-BRAF V (Catalog No. IHC-00607, GeneID 673, Isotype IgG, dilution 1:50, BETHYL laboratory, USA). First, for all cases, the original hematoxylin-and-eosin-stained sections of the lesions were re-examined for histopathological confirmation of the diagnosis and recording of histopathological parameters as histological type, grading, the staging of cancer, depth of tumor invasion in the wall, circumferential margin, lymph node metastasis, lymphovascular, and perineural invasion. Sections of 4 µm thickness were cut and spread on poly-L-lysine–coated slides. The paraffin sections were immersed in three xylene changes and hydrated using a graded series of alcohol solutions. Antigen retrieval was routinely performed by immersing the sections in 0.01 M Tris-EDTA buffer (pH 9.0) (20 minutes). The sections were counterstained for 3 minutes with Meyer's hematoxylin, and then, they were mounted. Sections from breast carcinoma were used as a positive control. Negative controls were obtained by omitting the primary antibodies.

2.3 | Interpretation of the immunohistochemical stain

All immune-stained slides were evaluated two times by the pathologist, blinded to all clinical, histopathological, and genetic data.

Scoring of B-Raf cytoplasmic staining was according to the following scoring system: 0: negative; 1: weak staining if weak staining <20% of tumor cells, 2: moderate to strong staining in <20% of tumor cells, 3: weak staining in 20% to 70% of tumor cells, 4: moderate to strong staining in 20% to 70% of tumor cells; 5: weak staining in >70% of tumor cells, and 6: moderate to strong staining in >70% of tumor cells.[21] Examples of immunohistochemical staining for B-Raf protein in CRC specimens and its intensity evaluation in the overall analysis are illustrated in Figure 2.

2.4 | BRAF/MIR-17 gene expression analysis

Total tissue ribonucleic acid, including small RNA, was extracted from the FFPE tissue sections using Qiagen miRNeasy FFPE Kit (217 504, Qiagen, Hilden, Germany) following the protocol supplied by the manufacturer. Treatment of samples with RNase-free DNase I for two hours at 37°C was applied. RNA concentration and purity were determined using the "NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc Wilmington, DE, USA)" by adjustment of the wavelength-dependent extinction coefficient of the software setting on "33" to measure small RNA(s) in the solution. The integrity of the extracted RNA was checked by running 5 µL of the isolated RNA on agarose gel electrophoresis. The complementary DNA (cDNA) was prepared from total RNA using TaqMan™ MicroRNA Reverse Transcription (RT) Kit (P/N 4366596; Applied Biosystems, Foster City, CA, USA), and either the miRNA-17–specific stem-loop primers (assay ID 002308) or the endogenous control RNU6B primers (assay ID 001093) were used for quantification of miR-17. In BRAF expression quantification, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, P/N 4368814) was used to convert the total RNA into cDNA. For each sample, a 15 µL reaction mixture contains 5µL of RNA extract, 0.15 µL of 100 mM of each deoxynucleotide triphosphate, 1 µL of MultiScribe® reverse transcriptase (50 U/µL), 1.5 µL of 10 × RT buffer, 0.19 µL of RNase inhibitor (20 U/ml), 3 µL of gene-specific TaqMan® primer, and 4.16 µL of nuclease-free water. RT was carried out in a T-Professional Basic, Biometra PCR System (Biometra, Göttingen, Germany) at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes, and then hold at 4°C. Negative controls (ie, non-template and non-reverse transcriptase) were included in each experiment to ensure that PCR products were not due to contamination by genomic DNA.

The real-time PCR amplification reactions were carried out following the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [22] in final volumes of 20 µL, including 1.33 µL RT products, 2 × TaqMan® Universal PCR Master Mix, 1 µL TaqMan® small RNA assay/RNU6B assay. For BRAF quantification, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was applied for data normalization. No-template samples were included as negative controls in each rum. The PCR was performed on StepOne™ Real-Time PCR System (Applied Biosystems) and incubated as follows: 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 60°C for
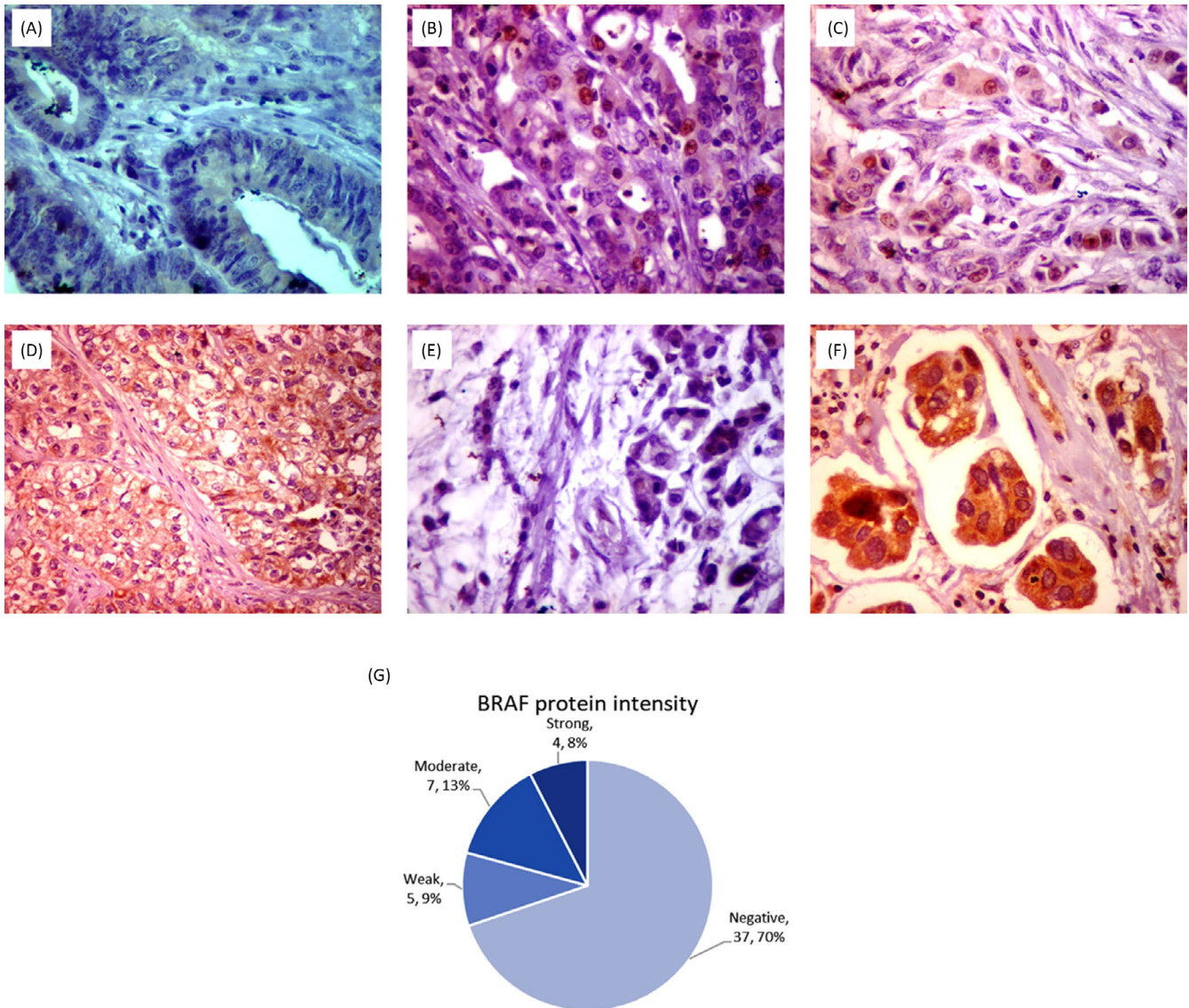
FIGURE 2 Immunohistochemistry of BRAF protein in colorectal cancer specimens. Immunohistochemical staining for B-Raf protein (x400) showed (A) negative staining in colonic adenoma, (B) another colonic adenoma showed weak cytoplasmic staining with non-specific nuclear staining, (C) grade 2 colonic adenocarcinoma showed moderate cytoplasmic staining, (D) grade 3 colonic adenocarcinoma showed strong cytoplasmic staining, (E) mucinous adenocarcinoma showed negative staining, (F) moderately differentiated adenocarcinoma with lymphovascular emboli showed strong cytoplasmic staining, and (G) protein intensity in the overall analysis of CRC samples

1 minute.[23] Duplicate PCR runs were performed, and average values were estimated.

2.5 | Gene expression data analysis

Consistent expression values were obtained with a nearly close cycle of quantification (Cq) values of the same specimen. Fold changes in miR-17 and *BRAF* expression in each patient cancer tissue relative to the corresponding non-cancer adjacent tissues (NAT) were estimated via the Livak method based on the quantification (threshold) cycle ($C_{q \text{ or }} C_T$) value as follows: relative expression $= 2^{-\Delta\Delta Cq}$, where $\Delta\Delta C_q = (C_q \text{ miR-17/BRAF} - C_q \text{ RNU6B/GAPDH})_{cancer} - (C_q \text{ miR-17/} BRAF - C_q \text{ RNU6B/GAPDH})_{NAT}.$[24]

2.6 | Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 26.0 (Armonk, NY: IBM Corp.). The sample size and study power were calculated by G*Power version 3.0.10. The estimated power for the study design (gene expression) was 80% at total sample size = 106, calculated effect size = 0.55, and alpha error probability = 0.05. Means ± standard deviations were generated for continuous variables, and frequencies and percentages were estimated for categorical variables. Chi-square and Fisher's exact, Student t, and Mann-Whitney tests, when appropriate, were used to compare between cases and controls. The correlation coefficient was determined using the Spearman correlation test. Two-sided *p*-values

of <0.05 were considered significant. Multiple Cox proportionate hazard regression analysis was performed to calculate the hazard ratio (HR) and 95% confidence interval (CI) for each putative independent variable. Kaplan-Meier curves were plotted for overall (OS) and disease-free survival (DFS).

3 | RESULTS

3.1 | Baseline characteristics of the study samples

The study included 53 patients with colorectal carcinoma; the patients' characteristics are summarized in Table 1. About 54.7% of tumors were on the right side (cecum and ascending colon), while 39.6% were on the left side (descending and sigmoid colon). The histological type of tumors was 38 (71.7%) adenocarcinoma, signet ring carcinoma 8 (15%), mucinous carcinoma 7 (13.2%). Most of the adenocarcinoma cases were grade 2 (81.6%). According to pTNM 8 staging, 4 cases (7.5%) were pT1 invaded only the mucosa and submucosa, 25 (47.2%) cases were pT2 invaded muscularis propria, 16 (30%) were pT3 invaded through muscularis propria to subserosa, and 8(15.1%) were pT4 invaded the serosa and peritoneum. Tumor deposited was detected in 33 cases of colonic carcinoma, with 22(41.5%) showed tumor deposits in up to 3 of dissected LNs (pN1) and 11 20.8%) showed tumor deposits in 4 or more dissected LNs (pN2). Distant metastasis was detected clinically and radiologically in 10 (18.9%). Detection of the tumor within well-defined vascular and lymphatic spaces (lymphovascular invasion) was detected in 18 (34.0%). On follow-up of patients for three years after surgical removal and post adjuvant therapy, 16 (30.2%) cases showed relapse of the tumor, and 14 (26.4%) died.

3.2 | Expression of the *BRAF* and MIR17 in CRC samples

As depicted in Figure 3, although miR-17 expression in cancer tissues was comparable to that in non-cancer ones (median (interquartile); 3.0 (0.06-4.37), $p = 0.13$), the *BRAF* gene showed higher expressions in CRC specimens relative to NAT; 20.3 (1.6-113.8), $p = 0.006$.

3.3 | Correlation analysis

Although there was a non-significant correlation between *BRAF* gene and protein expressions, MIR17 expression was inversely and significantly correlated with both BRAF protein ($r = -0.79$, $p < 0.001$) and gene expression ($r = -0.35$, $p = 0.010$) (Figure 4).

Correlation of *BRAF* gene/protein expressions and log *BRAF*/MIR17 ratio with the CRC specimens' clinicopathological features is shown in Table 2. High grade of colonic carcinoma showed significant association ($p < 0.001$) and correlation with B-Raf protein positivity ($p < 0.001$, $r = 0.72$), but showed an inverse correlation of MIR17

TABLE 1 Clinicopathologic criteria of 53 cases of colonic carcinoma

Variables	Subtypes	N	%
Age	≥60	32	60.4
	<60	21	39.6
Sex	Male	35	66.0
	Female	18	34.0
Grade	G1	5	13.2
	G2	31	81.6
	G3	2	5.2
	Signet ring	8	
	Mucinous	7	
Site	Cecum and Ascending	29	54.7
	Transverse	3	5.7
	Descending (descending and sigmoid colon)	21	39.6
pT	T1	4	7.5
	T2	25	47.2
	T3	16	30.2
	T4	8	15.1
pN	N0	18	34.0
	N1	22	41.5
	N2	11	20.8
M	M0	43	81.1
	M1	10	18.9
Stage	1	10	18.9
	2	8	15.1
	3	24	45.3
	4	10	18.9
Lymphovascular invasion	No	35	66.0
	Yes	18	34.0
Histopathological type	Adenocarcinoma	38	71.7.
	Mucinous	7	13.2
	Signet cell	8	15.1
Relapse	No	37	69.8
	Yes	16	30.2
Mortality	Survived	39	73.6
	Died	14	26.4

Note: Data are presented as number (N) and frequency (%).

Abbreviations: M, metastasis; N, lymph node; P, pathologic; T, tumor thickness.

expression and the log *BRAF*/MIR17 ratio ($r = -0.49$, $p < 0.001$, and $r = -0.52$, $p < 0.001$, respectively). Simultaneously, there was no significant correlation to *BRAF* gene expression ($p = 0.09$).

Large tumor size was significantly associated with positive B-Raf protein expression ($p = 0.003$, $r = 0.41$). It showed a significant inverse correlation with the log *BRAF*/MIR17 ratio ($p < 0.001$,
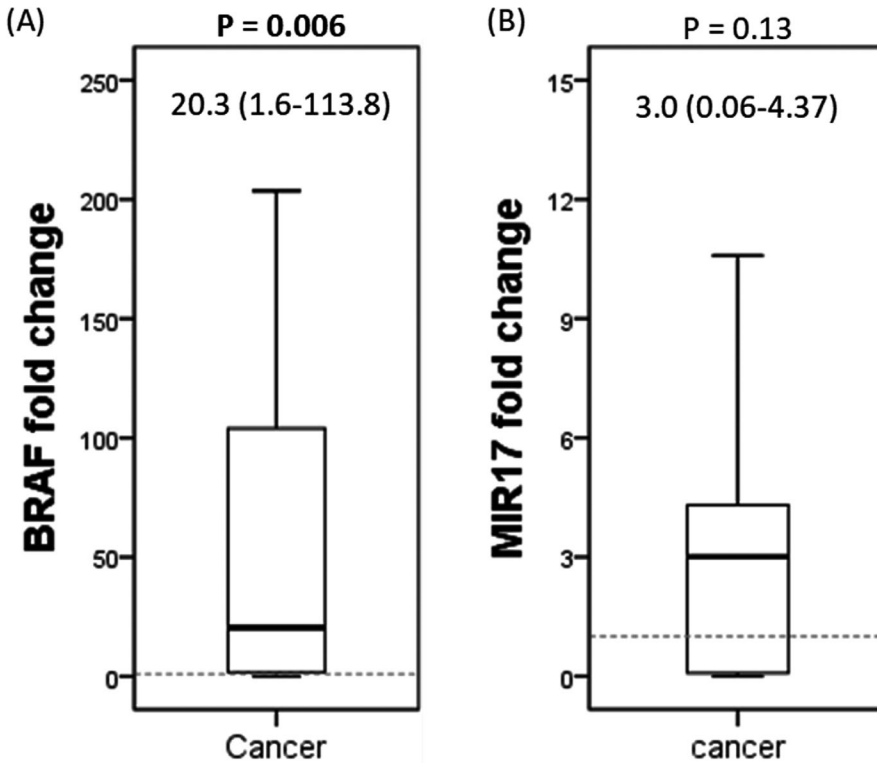
FIGURE 3 The relative expression level of *BRAF* and MIR17 genes in colorectal cancer specimens compared to control tissues. Values are presented as median (Q1-Q3). The Mann-Whitney U test was applied for *p*-value calculation
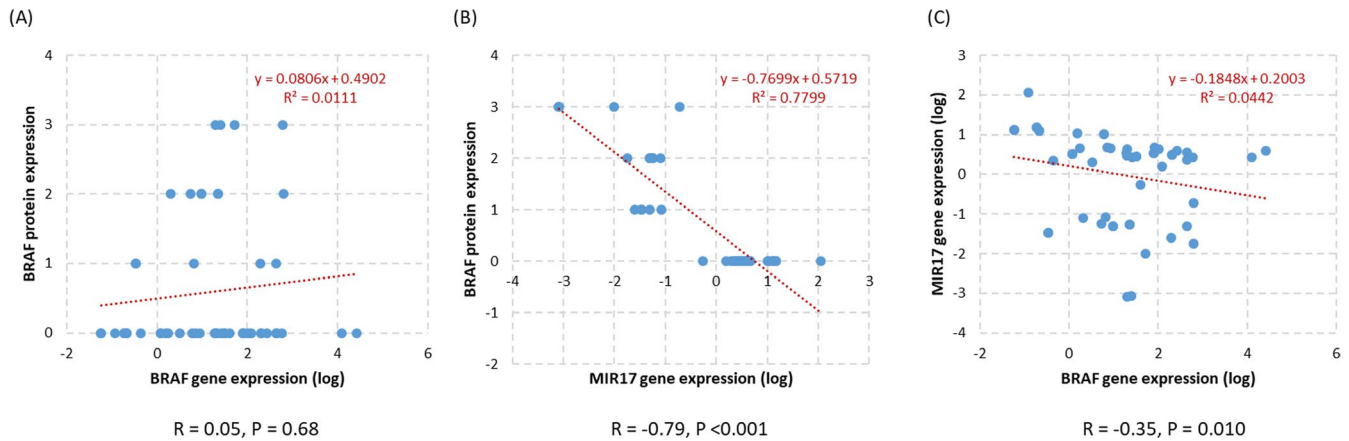

FIGURE 4 Correlation analysis for gene and protein expression in colorectal cancer. Spearman's correlation coefficient is shown

$r = -0.39$) with no significant correlation to *BRAF* gene expression or MIR17 expression.

The lymph node metastasis was inversely correlated with MIR17 expression ($p = 0.045$, $r = -0.28$) and the log *BRAF*/MIR17 ratio ($p = 0.004$, $r = -0.39$), with no significant correlation to B-Raf protein or gene expressions. The lymphovascular invasion (LVI) showed only significant association and correlation to high *BRAF* gene expression ($p = 0.041$, $r = 0.28$).

Although, carcinoma subtype showed significant association and correlation with B-Raf protein positivity ($p < 0.001$, $r = 0.61$), an inverse correlation with MIR17 expression ($p = 0.001$, $r = -0.44$), and the log *BRAF*/MIR17 ratio ($p < 0.001$, $r = -0.53$), it showed no significant correlation with *BRAF* gene expression ($p = 0.25$).

3.4 | Clinical and survival parameters

A high rate of relapse was significantly correlated with MIR17 expression ($p = 0.020$). DFS and OS were inversely and significantly correlated with positive B-Raf protein expression ($r = -0.31$ and -0.35, $p = 0.023$ and 0.011, respectively), as well as directly and significantly correlated with log *BRAF*/MIR17 ratio ($r = 0.50$ and 0.41, $p < 0.001$ and $= 0.003$, respectively). Otherwise, no significant correlations and/or associations were observed between age, sex, site, metastasis, and the number of deceased patients and *BRAF* gene and protein expressions, MIR17 expression, or log *BRAF*/MIR17 ratio in the present specimens (Table 2, and Figure 5). Otherwise, log *BRAF*/MIR17 ratio showed significant association with the

TABLE 2 Correlation of BRAF protein and gene expressions with the clinicopathological features in the colorectal cancer specimens

Variables	Braf protein intensity		BRAF gene expression		MIR17 expression		Log BRAF/MIR17 ratio	
	P_{Ass} value	r (P_{Corr})	P_{Ass} value	r (P_{Corr})	P_{Ass} value	r (P_{Corr})	P_{Ass} value	r (P_{Corr})
Age	0.84	0.03 (0.82)	0.13	-0.09 (0.48)	0.36	-0.02 (0.85)	0.51	0.14 (0.31)
Sex	0.14	-0.20 (0.14)	0.91	-0.016 (0.91)	0.28	0.18 (0.18)	0.91	0.016 (0.91)
Grade	**<0.001**	**0.72 (<0.001)**	0.22	-0.23 (0.09)	**0.030**	**-0.49 (<0.001)**	**0.001**	**-0.52 (<0.001)**
Site	0.74	0.10 (0.45)	0.39	0.09 (0.49)	0.49	-0.19 (0.15)	0.95	0.03 (0.78)
T	**0.003**	**0.41 (0.002)**	0.09	-0.23 (0.17)	0.31	-0.15 (0.28)	**<0.001**	**-0.55 (<0.010)**
N	0.22	0.24 (0.08)	0.21	0.03 (0.81)	0.29	**-0.28 (0.045)**	**0.011**	**-0.39 (0.004)**
M	0.44	0.10 (0.44)	0.85	-0.02 (0.85)	0.52	-0.08 (0.54)	0.07	-0.24 (0.07)
Stage	0.43	0.15 (0.27)	0.28	0.05 (0.71)	0.69	-0.20 (0.14)	0.16	-0.32 (0.019)
LVI	0.58	0.07 (0.59)	**0.042**	**0.28 (0.041)**	0.051	-0.08 (0.52)	0.19	0.18 (0.19)
Subtype	**<0.001**	**0.61 (<0.001)**	0.57	-0.15 (0.25)	0.76	**-0.44 (0.001)**	**0.001**	**-0.53 (<0.001)**
Relapse	0.38	-0.11 (0.39)	0.66	-0.25 (0.06)	**0.020**	**0.33 (0.015)**	0.07	-0.24 (0.07)
Died	0.27	0.15 (0.28)	0.77	-0.03 (0.78)	0.54	-0.05 (0.69)	0.06	-0.25 (0.06)
DFS	**0.027**	**-0.31 (0.023)**	0.75	-0.03 (0.81)	0.17	0.06 (0.63)	**<0.001**	**0.50 (<0.001)**
OS	**0.001**	**-0.35 (0.011)**	1.0	-0.11 (0.42)	**0.016**	0.24 (0.08)	**<0.001**	**0.41 (0.003)**

Note: P_{Ass} (p-values of the association analysis) were calculated by Mann-Whitney U or Kruskal-Wallis tests. Spearman's correlation analysis is expressed as a correlation coefficient (r) and its p-values (P_{Corr}). Bold values are statistically significant at $p < 0.05$.

Abbreviations: DFS, disease-free survival; LVI, lymphovascular invasion; OS, overall survival.
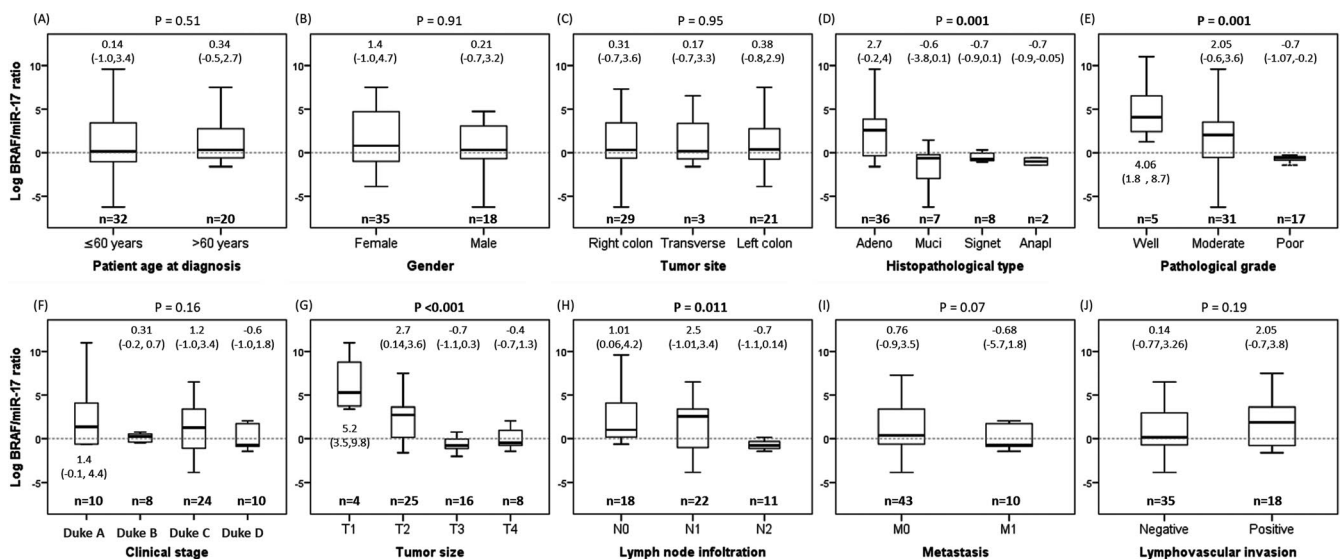

FIGURE 5 Association between BRAF/miR-17 ratio and clinicopathological features. Kruskal-Wallis and Mann-Whitney U tests were applied. The significance level was set at $p < 0.05$

histopathological type and the pathological grade of the samples (both $p = 0.001$), the tumor size ($p < 0.001$), and the lymph node infiltration ($p = 0.011$) (Figure 5).

Analysis of the prognostic value of BRAF/miR-17 ratio for detecting survival (OS and DFS) by Spearman correlation analysis test (Table 2) and Kaplan-Meier survival curve (Figure 6) revealed OS was significantly and inversely correlated with positive B-Raf protein

($p < 0.001$, $r = -0.35$), and directly and significantly correlated with log BRAF/MIR17 ratio ($p < 0.001$, $r = 0.41$), but no significant correlation to BRAF gene expression or MIR17 expression.

Cox proportional hazard regression analysis of OS and DFS among CRC patients revealed that tumor grade, tumor size, and the presence of distant metastasis, as well as increased BRAF/MIR17 ratio, could predict both types of patients survival (Table 3).
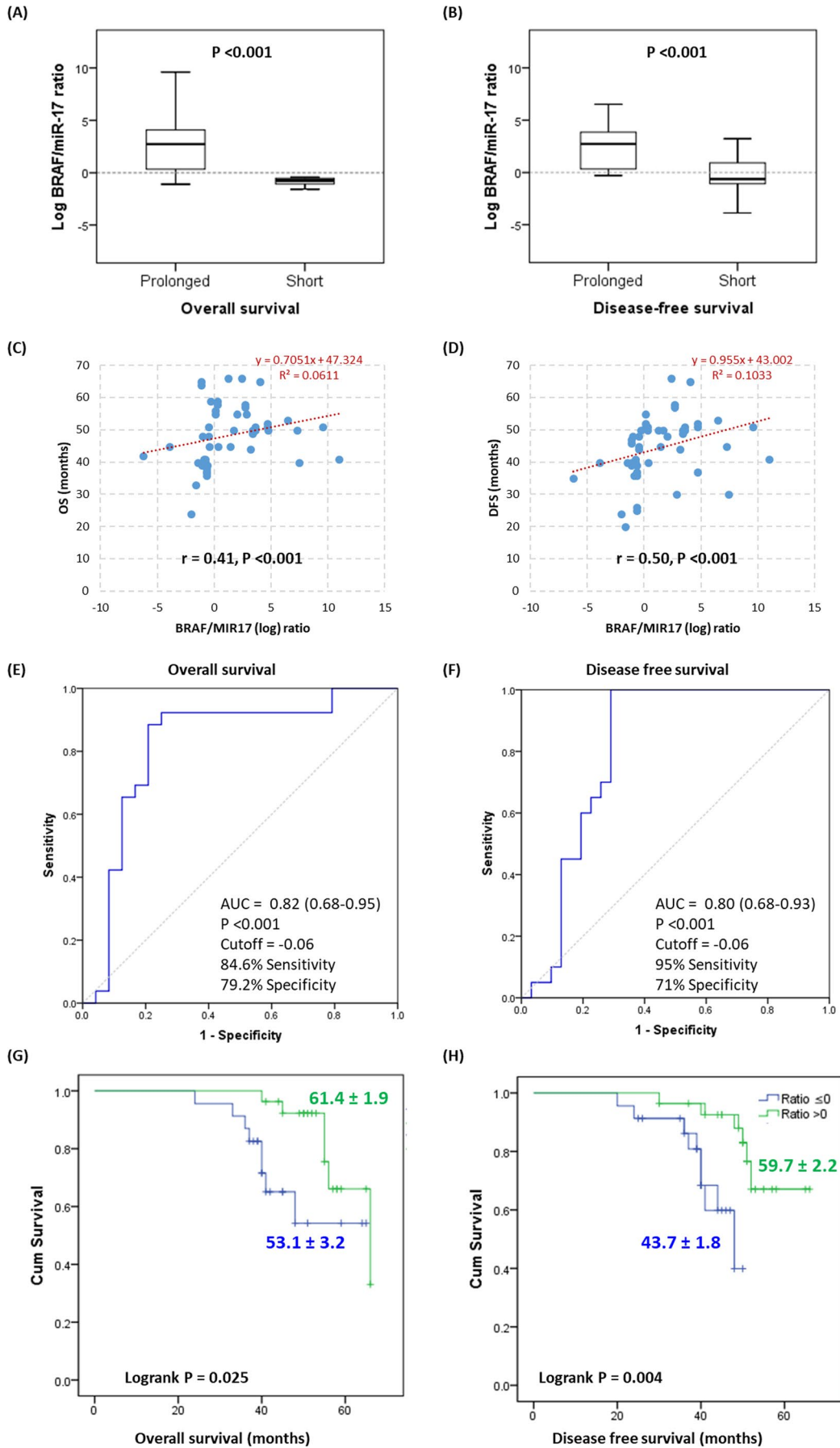
(A) P <0.001

Log BRAF/miR-17 ratio

Prolonged   Short

Overall survival

(B) P <0.001

Log BRAF/miR-17 ratio

Prolonged   Short

Disease-free survival

(C) y = 0.7051x + 47.324
R^2 = 0.0611

OS (months)

BRAF/MIR17 (log) ratio

r = 0.41, P <0.001

(D) y = 0.955x + 43.002
R^2 = 0.1033

DFS (months)

BRAF/MIR17 (log) ratio

r = 0.50, P <0.001

(E) Overall survival

Sensitivity

1 - Specificity

AUC = 0.82 (0.68-0.95)
P <0.001
Cutoff = -0.06
84.6% Sensitivity
79.2% Specificity

(F) Disease free survival

Sensitivity

1 - Specificity

AUC = 0.80 (0.68-0.93)
P <0.001
Cutoff = -0.06
95% Sensitivity
71% Specificity

(G)

Cum Survival

61.4 ± 1.9

53.1 ± 3.2

Logrank P = 0.025

Overall survival (months)

(H)

Cum Survival

Ratio ≤0
Ratio >0

59.7 ± 2.2

43.7 ± 1.8

Logrank P = 0.004

Disease free survival (months)

FIGURE 6 Prognostic value of the *BRAF*/miR-17 ratio for detecting survival. (A-B) Association of BRAF/MIR17 expression ratio with survival. (C-D) Spearman correlation analysis between gene expression and survival. (E-F) Receiver characteristic operator curve to discriminate between prolonged and short survivors. (G-H) Kaplan-Meier survival curve for overall and disease-free survival. LogRank (Mantel-Cox) test was used

4 | DISCUSSION

Colorectal cancer is a heterogeneous disease that manifests through a variable clinical course and with significant inconsistency in response to treatment, even within tumors with similar histopathological features.[25] Although the introduction of cytotoxic drugs has improved the treatment of advanced CRC, the individual response to chemoradiotherapy varies tremendously from one patient to another. However, the observed progress in CRC molecular therapies may provide new insight into treating this disease.[26]

Here in the present study, we explored the role of B-Raf protein and gene expression, in addition to the *BRAF*/MIR17 signature in CRC specimens. The findings indicated that B-Raf protein and gene expression showed higher expression in CRC specimens relative to non-cancerous tissue and significantly associated with

different prognostic indices in terms of a higher grade of colonic carcinoma, large tumor size, and carcinoma subtype (in case of B-Raf protein), and presence of lymphovascular invasion (in case of *BRAF* gene). Also, B-Raf protein expression showed a significant association and inverse correlation with DFS and OS. These findings support the previous studies, which report the significant implication of B-Raf in cancer.[27,28] As a member of the protein kinase family, "serine/threonine-protein kinase," B-Raf is involved in the mitogenic signal transduction from the cell membrane to the nucleus. It phosphorylates the MAP2K1 and thereby activates the highly oncogenic "MAP kinase signal transduction" pathway (Figure S1).[27,28] B-Raf protein is mainly one of the cytoplasmic proteins involved in cell survival, proliferation, and motility while inhibiting differentiation,[29,30] which supports its association with the unfavorable prognostic indices mentioned above. In colorectal

TABLE 3 Cox proportional hazard regression analysis of overall and disease-free survival among patients with colorectal cancer

Variables	OS			DFS		
	HR	(95%CI)	*p*-value	HR	(95%CI)	*p*-value
Age (> 60 vs. ≤ 60)	1.03	0.97-1.09	0.40	1.00	0.94-1.07	0.88
Sex (female vs. male)	2.00	0.42-9.46	0.38	1.27	0.26-6.21	0.77
Location (left/transverse vs. right)	1.14	0.27-4.83	0.85	1.67	0.29-9.54	0.56
Type (Adeno vs. non-adeno)	0.36	0.06-2.27	0.27	0.28	0.04-2.04	0.20
Grade (G3 vs G1/2)	11.47	1.74-75.61	**0.011**	9.13	1.20-69.71	**0.039**
T status (T3/4 vs. T1/2)	0.02	0.00-0.37	**0.008**	0.03	0.00-0.42	**0.009**
LN status (positive vs. negative)	0.43	0.05-3.56	0.43	0.34	0.04-2.68	0.30
Metastasis (positive vs. negative)	28.95	1.42-590.09	**0.029**	46.83	1.82-1,205.20	**0.020**
LVI (positive vs. negative)	2.04	0.43-9.57	0.36	3.04	0.55-16.81	0.20
Staging (duke C/D vs. A/B)	1.38	0.18-10.43	0.75	0.93	0.10-8.85	0.94
BRAF/MIR17 ratio (over- vs. under-expressed)	0.58	0.37-0.90	**0.015**	0.52	0.31-0.89	**0.017**

Note: Bold values indicate statistically significant at $p < 0.05$.

Abbreviations: CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; LN, lymph node; Lt, left; LVI, lymphovascular invasion; OS, overall survival; Rt, right.

cancer, B-Raf plays multifaceted roles in tumor progression, diagnosis, and prognosis and may act as a predictor of response to combined targeted therapies.[31] It was also found to be over-expressed in other types of cancers such as melanoma, thyroid papillary carcinoma, hairy cell leukemia, Langerhans neoplasms, pediatric astrocytoma, and glioma (http://www.cancerindex.org/geneweb/BRAF.htm).

In the present study, the *BRAF* gene and protein expressions showed a negative correlation with the selected miR-17 expression, which supports B-Raf as one of the potential targets of miR-17, as evidenced by our in silico analysis. To mitigate the non-specific tissue expression of *BRAF* expression, the authors were interested in exploring its expression as part of the *BRAF*/MIR17A ratio, which showed a significant inverse correlation with tumor size, high-grade colonic carcinoma, lymph node metastasis, and carcinoma subtype.

Generally, microRNAs are involved in various biological and cellular processes, such as cell proliferation, differentiation, angiogenesis, and apoptosis.[32,33] This cluster of non-coding RNAs has been implicated in several chronic diseases and cancers, including CRC.[34,35] Various genomic data on microRNAs are currently available, including their interactions with related proteins or genes. The key challenge is how to apply these data from myriad sources to investigate its diagnostic/prognostic utility in the clinic.

MiR-17, as a member of the miR-17-92a family, was involved in the deregulation of several players in tumorigenesis.[36,37] It was implicated in regulating epithelial-mesenchymal transition and proliferation in CRC via targeting cytochrome 7 B family, member 1, and salt-inducible kinase-1 genes, respectively.[37,38] The present overall analysis found no significant difference between cancerous and non-cancerous specimens in terms of MIR-17 expression, which contrasts with other studies that showed significant upregulation of this microRNA in CRC tissues.[37,39,40] This difference could be due to the relatively small number of samples that were available in this work, the different clinicopathological characteristics of the patients, or due to different genetic background of the included samples from others as this is the first time to explore the MIR17 relative expression in CRC samples collected from the Middle Eastern population. Further work on larger samples in the same population will be required to confirm this finding.

Otherwise, a high rate of relapse was significantly correlated with MIR17 expression. As a part of the *BRAF*/MIR17 ratio, MIR17 expression showed significant associations and correlations with unfavorable prognostic indices, including DFS and OS. These findings, in part, are consistent with the association of miR-17 with poor cancer prognosis documented in a previous meta-analysis.[41] Furthermore, the circulating miR-17-92a cluster extracted from CRC patient serum was correlated with a high recurrence rate and poor prognosis.[42] Diosdado and colleagues reported a continuous miR-17 upregulation during the progression of adenoma to adenocarcinoma.[43] Other studies also identified miR-17 as a predictor of cancer occurrence and poor prognosis[44,45] and correlated its deregulation with a metastatic hepatic spread in CRC.[39,46] Recently, Fiala et al reported miR-17 deregulation as one of the promising predictive

biomarkers associated with an unfavorable response to anti-epidermal growth factor receptor (EGFR) monoclonal antibody therapy in metastatic CRC patients.[40] Additionally, Ast et al specified matrix metallopeptidase 2 (*MMP2*), fascin actin-bundling protein 1 (*FSCN1*), laminin subunit gamma 1 (*LAMC1*), transforming growth factor-beta 1 (*TGFB1*), dystonin (*DST*), ETS proto-oncogene 1 (*ETS1*), fibrillin 1 (*FBN1*), fibroblast growth factor 2 (*FGF2*), fibronectin 1 (*FN1*), integrin subunit alpha V (*ITGAV*), *ITGB1*, peroxidasin (*PXDN*), and TIMP metallopeptidase inhibitor 2 (*TIMP2*) as potential targets in extracellular matrix remodeling that could mediate some of the essential roles miR-17 play in cancer, including CRC.[47] Interestingly, a recent functional study by Han et al identified the primary miR-17 processing could be regulated via the circular non-coding RNA "circLONP2," another family of non-coding RNAs, which correlated with CRC progression and invasion through modifying the "maturation and the intercellular exosomal dissemination" of miR-17.[48]

It is worth noting that this study is a preliminary one limited by the relatively small sample size, the retrospective nature of the study design, and the lack of in vitro functional studies that clarify the exact molecular mechanisms by which *BRAF*/MIR17 mediates its signature in CRC. Also, it will be interesting to explore the association of *BRAF*/MIR17 signature with the most common *BRAF* V600E mutation by the newly emerged PCR techniques to add to its clinical utility.[49,50] Based on these limitations, future large-scale studies supported by functional in vitro and gene mutation exploring studies are warranted to confirm and validate the results.

5 | CONCLUSION

Collectively, our findings support the prognostic utility of miR-17 and/or *BRAF*/MIR17 ratio in CRC in terms of association with poor prognostic indices and survival. Future prospective large-scale studies are recommended to confirm the study findings.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT
All datasets presented in this study are included in the article/supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.