Research Article

High expression of MRE11A, the gene involved in the resection step during homologous recombination, is associated with shorter survival and a higher risk of death in CRC patients.

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Background: Homologous Recombination (HR) is the most accurate repair pathway for double strand breaks and replication fork disruption that is capable of faithfully restoring the original nucleotide sequence of the broken DNA. The deficiency in this mechanism is a frequent event in tumorigenesis. Therapies that exploit defects in HR have been explored essentially in breast, ovarian, pancreas, and prostate cancers, but poorly in colorectal cancers (CRC) although CRC ranks second in mortality worldwide. **Methods:** Tumor specimens and matched healthy tissues from 63 patients with CRC were assessed for gene expression of HR key components and MMR status, which were correlated with clinicopathological features, progression-free survival, and overall survival (OS). **Results:** Enhanced expression of MRE11A, the gene encoding a key molecular actor for resection, is significantly overexpressed in CRC, is associated with the occurrence of primary tumors particularly T3-T4 and is found in more than 90% of the right-side of CRC, the location with worst prognostic. Importantly, we also found that high MRE11A transcript abundance is associated with 16.7 months shorter OS and a 3.5 higher risk of death. **Conclusion:** Monitoring MRE11 expression could be used both as a predictor of outcome and as a marker to select CRC patients for treatments thus far adapted for HR deficient cancers.

Keywords: colorectal cancer; homologous recombination; prognosis.

1. Introduction

Among the multiple DNA lesions that occur recurrently in human cells, the DNA double-strand breaks (DSBs), which result from direct environmental chemical attacks or the collapse of stalled replication forks, constitute the greatest threat to genomic stability. Several conserved, mechanistically distinct pathways of DSB repair have evolved to repair these DNA damages. These include homologous recombination (HR), non-homologous end joining (NHEJ), alternative end joining, and single-strand annealing [1,2]. While the NHEJ, alternative end joining, and single strand annealing pathways often cause deletion or insertion of several nucleotides and can trigger chromosome translocations, HR is the most accurate DSB repair mechanism capable of faithfully restoring the original configuration of the broken DNA molecule. HR is the most conservative process that uses a sister chromatid as a template to accurately fix the DSBs. In contrast to NHEJ, the first essential step of HR is

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DNA resection at the break termini, which is controlled by breast cancer-associated gene 1 (BRCA1) in a cell-cycle–dependent manner by restricting access of the NHEJ factor 53BP1 to the ends of DSBs, thereby permitting the generation of recessed DNA with 3'overhangs [3]. Such 5' to 3' end resection, which is performed by the highly conserved MRN complex comprising the MRE11, RAD50, and NBS1 factors in conjunction with CtIP (or RBBP8), is of paramount importance for the subsequent HR steps which consist in the binding of PALB2 to BRCA1 which in turn directs BRCA2 to promote RAD51 filament formation on the recessed DSB, a critical step necessary for homology searching, strand invasion, and repair synthesis [4].

The deficiency of such a conservative repair pathway is a frequent event in tumorigenesis which provides a selective growth advantage to tumor cells as this results in replicative stress, genetic instability, and enhanced mutation rates, driving forces for tumor evolution. Multiple hereditary or somatic cancers are deficient in HR. This has been particularly well described in tumors with germline mutations in BRCA1/2, such as breast and ovarian cancers [5]. HRD has been documented to be associated with higher sensitivity to alkylating or platinum-based agents due to the generation of non-processed and highly toxic DSBs [6,7]. Importantly, the use of poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi) was approved by the US Food and Drug Administration and the European Medicines Agency to implement these classical drugs to treat ovarian and breast HR-deficient tumors through a synthetic lethality concept [8].

Generally, therapies that exploit defects in HR have been explored essentially in breast, ovarian, pancreas, and prostate cancers, but poorly in colorectal cancers (CRC), although it is a commonly diagnosed malignant neoplasm which ranks third among all cancers in terms of incidence and second in mortality worldwide [9]. Discrepancies within TNM (Tumor-node-metastasis) stages of CRC patients have been widely reported, primarily due to contradictions in the survivorship of patients harboring stages IIB/C or IIIA disease [10–12]. Moreover, the therapeutic repertoire for CRC remains limited, with few targeted agents and companion diagnostics endorsed for clinical use [13]. Despite the efforts to identify a molecular signature to improve prognosis prediction and CRC patients' selection [14–16], a biomarker-enriched classification has not yet reached clinical translation. Recent data have demonstrated that between 10-30% of CRC harbor somatic mutations in genes involved in DNA Damage Response (DDR), which could explain some aspects of resistance to therapy and a poor prognosis [17,18]. Among these, germline pathogenic variants of BRCA1, ATM, and PALB2 have been associated with greater CRC risk, and up to 15% of all CRC present germline or somatic alterations in HR repair genes [19]. Moreover, it has been suggested that a subset of CRC patients harbors mutations in HR genes, including ATM, BRCA1/2, MRE11A, FANCC, NBN, and PALB2 [20].

Here, we further explored the link between HR genes and CRC by examining the prognostic significance of the expression of genes encoding proteins involved in key steps of HR in a series of CRC patients.

2. Materials and Methods

Patients and tissue collection

This study was conducted in Santa Rita Hospital (Irmandade Santa Casa de Misericórdia de Porto Alegre, Brazil). Patients with histologically confirmed adenocarcinoma of the colon and rectum and who were admitted to colorectal surgery were eligible. Patients with non-primary CRC, familial adenomatous polyposis, or inflammatory bowel disease were excluded. Variables of interest included age, gender, date of surgical resection, primary location, tumor grade, laterality, vascular invasion, perineural invasion, preoperative carcinoembryonic antigen (CEA) levels, and treatment. Histopathological data (such as tumor subtype, depth of invasion, lymph node and/or metastasis distance, and staging) were also extracted from the pathological reports. The pathological TNM stage was used as the staging scale for prognosis [21]. Outcomes of interest were disease-free survival (DFS) and overall survival (OS). DFS was defined as the time from diagnosis to first recurrence. OS was defined as the time from diagnosis to last follow-up or death.

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A total of 63 primary colorectal neoplasms from sporadic CRC patients were collected between March 2013 and July 2016. These patients were followed from the surgical tumor resection date until December 2022. At least two fresh tissue samples were collected from each patient's surgical specimen: full-thickness colorectal tumor and adjacent normal colon or rectum tissue at least 10 cm away from the largest tumor. Neoplastic and healthy tissues were immediately embedded in RNAlater™ stabilization solution (Invitrogen) for 24 hours and then frozen for subsequent analyses. The CRC series data were collected prospectively; patients were informed and signed a written consensus for collecting data and biological samples. Ethical committees of participating institutions approved the study, and written informed consent was obtained from patients before study enrollment. The research conformed to the Helsinki Declaration and was approved by the Regional Committee for Medical and Health Research Ethics (CAAE: 58299916.3.3001.5345).

Gene expression analysis and HRD assessment

Total RNA was extracted using RNeasy Mini kit (Qiagen), and cDNA synthesis was performed in a 20-µl reaction with 1 µg of total RNA using RT2 FirstStrand Kit (Qiagen) according to the manufacturer's instructions. Quantitative RT-PCR was performed in duplicates by RT2 Profiler™ PCR Array (SABiosciences/Qiagen) using RT2 SYBR Green qPCR Mastermix (Qiagen) and StepOne Plus apparatus (Applied Biosystems™). Custom RT2 Profiler PCR Array (#CLAH-32033-9619-6, Qiagen) included MRE11A, RAD50, NBN, BRCA1, BARD1, RBBP8, and PALB2 genes. Threshold cycle (Ct) values for each duplicate were normalized by the geometric average of housekeeping genes ACTB and PPIA. Averages of the resulting values were calculated to obtain ΔCt values for biological replicates. Relative mRNA (ratio between ΔCt in neoplastic tissue/ΔCt in healthy tissue) was calculated and a log2 transformation was applied. Gene expression was dichotomized into "high" and "low" according to the median fold change of each HRR gene (expression ≤ median = low; expression > median = high). We also classified a gene with a fold change > - 2.0 as "deficient", i.e. the gene expression was significantly reduced in tumor tissue [22].

Investigation of microsatellite instability

Immunohistochemical analysis for the expression of MMR proteins (MLH1, PMS2, MSH2, and MSH6) was evaluated by immunohistochemistry. Briefly, 3-µm thick FFPE tissue sections were deparaffinized in xylene, rehydrated in graded alcohols, washed in double-distilled water, and pretreated with DAKO solution (EnVision FLEX Target Retrieval Solution, High pH) at 97 ◦C. The slides were then incubated with primary monoclonal antibodies against MLH1 (clone ES05, DAKO), PMS2 (clone EP51, DAKO), MSH2 (clone FE11, DAKO), and MSH6 (clone EP49, DAKO) plus EnVision FLEX+ Mouse (LINKER) for 30 min. The analysis was performed on the automated platform Autostainer Link 48 (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. The antigen-antibody reaction was inspected with the EnVision FLEX kit with diaminobenzidine as chromogen; slides were counterstained with hematoxylin and, finally, covered. MMR protein expression was categorized as retained (i.e., proficient MMR; pMMR) when a moderate to strong nuclear protein expression was detected in tumor cells as well as in internal control; and lost (i.e., deficient MMR; dMMR), when a complete loss of nuclear expression in tumor cells was observed but retained in normal cells.

Inflammation-Related Peripheral Blood Measurements

The absolute count of circulating neutrophils,, lymphocytes and platelets are associated with inflammatory responses, which are key factors in recognizing pathways for tumorigenesis and growth [23]. Thus, we calculated neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), and platelet-to-lymphocyte ratio (PLR) from absolute counts of pretreatment peripheral blood tests. subgroups were divided using the median expression value (expression ≤ median = low; expression > median = high).

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Statistical Analyses

Statistical analyses were performed with IBM SPSS Statistics 29.0. OS and DFS rates were estimated using the Kaplan-Meier method. The log-rank test was used to assess significant differences between subgroups by univariate analysis. To investigate independent prognostic factors for OS and DFS, factors with a $p < 0.2$ in univariate analyses were entered into multivariate analysis. The Cox proportional hazards regression model was used to identify factors that were independently associated with OS. Pearson's chi-squared test and Fisher's exact test were used to evaluate distributions of categorical variables. P-values less than 0.05 were considered statistically significant.

Availability of data and materials

Any supplementary supporting data relating to the clinical and pathological analysis details are available upon request from the corresponding author and can be found in the electronic medical record system of Irmandade of Santa Casa of Misericórdia of Porto Alegre.

3. Results

3.1. Characteristics of CRC patients

A total of 63 patients with histologically confirmed sporadic colorectal adenocarcinoma and who underwent surgical tumor resection between March 2013 and July 2016 were included. Clinicopathological features are summarized in Table 1. Forty patients (63.5%) were male, the mean age at CRC diagnosis was 64 (range 35-87) years old, and 52% were 65 years old or less. Forty patients (64.0%) presented primary tumor location in the colon. Among these, twenty-seven patients (67%) harbored left-sided CRC. Eight (13%) patients presented with metastatic disease at diagnosis. Preoperative CEA < 5 ng/dL was observed in 45 patients (71%), and forty (63%) presented moderate or high tumor grade. MMR deficiency was present in 13% (8 patients) of primary tumors. Representative patterns of MMR protein staining are depicted in Supplementary Figure 1. At the data analysis time with a mean follow-up of 61 (range 6-113) months, 18 patients (29%) had reported disease progression, and 34 (54%) were still alive.

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Table 1. Clinicopathological features of CRC patients.

Variable	n (%)	*p value*
Total cases	63 (100)	
Age (years; mean ± SD)	64 ± 11	
Age (years)		
≤ 65	33 (52)	0.705
> 65	30 (47)	
Gender		
Female	23 (36.5)	0.032
Male	40 (63.5)	
Primary Tumor location		
Colon	40 (64)	0.033
Rectum	23 (36)	
Laterality (Colon)		
Right-sided	13 (33)	0.027
Left-sided	27 (67)	
Preoperative CEA, ng/mL		
≤ 5	45 (71)	<0.001
> 5	18 (29)	
pT		
T1-T2	16 (25)	<0.001
T3-T4	47 (75)	
Nodal metastasis		
No	36 (57)	0.257
Yes	27 (43)	
Tumor Grade		
Low	23 (54)	0.432
Moderate/high	40 (46)	
Lymphatic invasion		
No	34 (54)	0.529
Yes	29 (46)	
Perineural invasion		
No	47 (75)	<0.001
Yes	16 (25)	
TNM stage		
I	12 (19)	
II	23 (36)	0.378
III	20 (32)	
IV	8 (13)	
Chemotherapy		
Neoadjuvant	17 (27)	0.131
Adjuvant	23 (36)	
Both	24 (36)	
MMR status		
pMMR/MSS	55 (87)	<0.001
dMMR/MSI	8 (13)	
Disease recurrence		
No	45 (71)	0.060
Yes	18 (29)	
Survival		
Alive	34 (54)	0.259
Dead	29 (46)	

Abbreviations: CEA: Carcinoembryonic Antigen; pMMR/MSS: mismatch repair proficient/microsatellite stable; dMMR/MSI: mismatch repair deficient/microsatellite instable. Proportion test.

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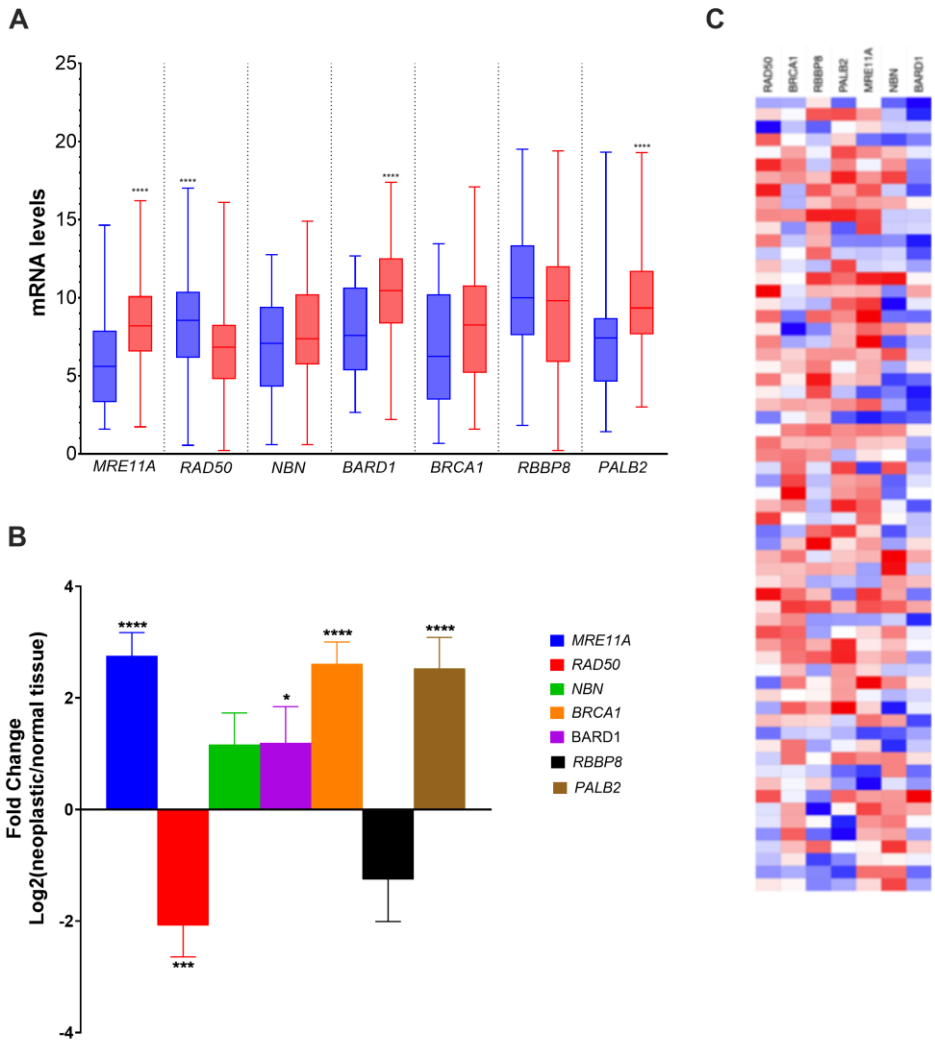

Figure 1. Changes in gene expression of representative HRR components in neoplastic colorectal specimens and matched healthy tissues. (A) mRNA levels (mean ⊙ SEM); (B) Fold change between neoplastic and normal tissues (mean ⊙ SEM). (C) Heatmap showing the individual gene expression fold-change of HRR key components in colorectal tumors. Blue represents the expression ≤ median = low; red represents the expression > median = high; Gene expression means between normal and neoplastic tissues and folds change were compared using Mann-Whitney's test and Wilcoxon test, respectively; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.2. *Alteration of HR gene expression in CRC*

The mRNA levels for key actors involved in important steps of HRR, MRE11A, RAD50, NBN, BARD1, BRCA1, RBBP8, and PALB2 were quantified using qPCR in 63 pairs of primary sporadic colorectal tumors and matched adjacent tissues. MRE11A (p< 0.001), BARD1 (p< 0.001) and PALB2 (p< 0.001) were significantly overexpressed (Fig. 1A) with a significant mean fold induction of 3.28 (p< 0.0001) for MRE11A, 2.83 (p< 0.0001) for BARD1 and 2.09 (p=0.373) for PALB2 (Fig. 1B). In contrast, RAD50 (p< 0.001) mRNA levels were significantly reduced (Fig.1A) in comparison to healthy tissues with a mean -2.15-fold decrease (p< 0.001) (Fig. 1B). For NBN, BRCA1, and RBBP8, no difference in gene expression between tumoral and adjacent normal tissues was observed (Fig. 1B). When we analyzed the proportion of CRC patients according to the number of reduced HRR gene expression in tumor samples (fold change > -2.0), we found that about 50% of the patients harbored 1 or 2

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altered gene expression, 8% of the patients showed deficiency of expression for 5 HRR genes concomitantly, and 16% of the patients have no deficiency in HRR gene expression (Fig.2A). We observed that RAD50 expression was the most affected among the HRR genes (57,1%), while BARD1 was deficient in only 9.5% of the patients. Interestingly, when the modified HR gene expression was explored according to MSI status, only MRE11A was significantly affected in MSI colorectal tumors (-2.43, IC 95% -4.96 – 0.10; p= 0.039) (Fig. 2C). More details in fold-change difference and T-test results for equality of means are given in Supplementary Table 1.

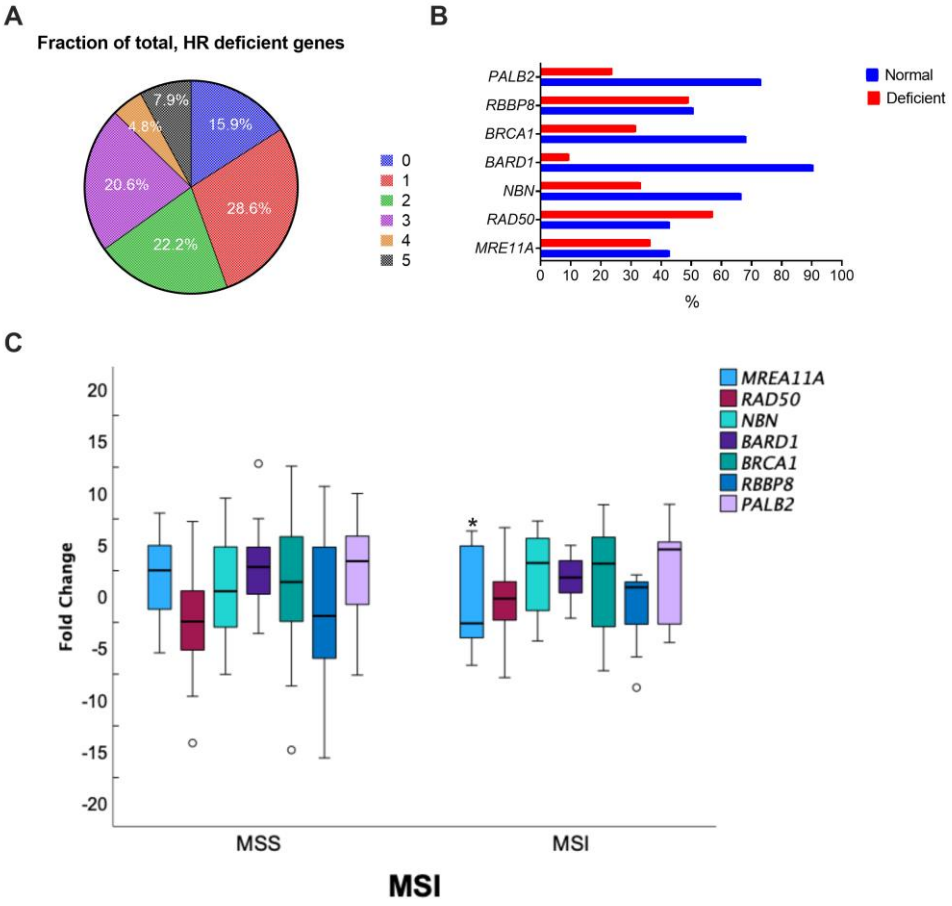

Figure 2. Characterization of HRR profile in CRC patients. (A) proportions of patients according to the number of deficient HRR genes in tumor samples (fold change > -2.0); (B) proportions of each HR gene according to normal or deficient expression; (C) boxplots of HR genes fold change means according to MMR status. T-test for equality means. *p (2-sided) <0.05.

3.3. *Associations between HR gene expression and clinicopathological features in CRC*

Next, we investigated the possible associations between the clinicopathological features of CRC patients and gene expression of MRE11A, RAD50, NBN, BRCA1, RBBP8, and PALB2 (Table 2). Low expression of RAD50 was found in 68% of left-sided CRC (p= 0.024) and 75% (p=0.044) of tumors with no perineural invasion. Low expression of NBN was found in 75% (p=0.044) of tumors in the initial stages, T1-T2. RBBP8 was not associated with any clinicopathological feature. High expression of BARD1 was found in young patients (65 years old or less) (p=0.046) and in patients with low TNM staging (p=0.044), TNM II-III. Normal or high expression of BRCA1 was correlated with patients with no regional lymph nodes affected (p=0.040). For PALB2, we found relatively active high expression of this gene was associated with age at diagnosis (p=0.022), absence of nodal metastasis

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(p=0.002), TNM I-II (p=0.001), and absence of lymphovascular invasion (p=0.015). Finally, we found that high expression of MRE11A was associated with the occurrence of primary tumors in the colon (p=0.008) and 92% of these cases with high MRE11A abundance were found in the right side (p= 0.042) of the colon, which corresponds to the worst prognosis side. Enhanced MRE11A expression was also associated with T3-T4 tumors (p=0.04). Collectively, these data support that only MRE11A high expression shows a critical correlation with the most pejorative features of CRC.

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Table 2. Associations between fold-change of MREA11, RAD50, NBN, BRCA1, BARD1, RBBP8, PALB2 gene expression and clinical features of CRC patients.

		MRE11A	***RAD50***	***NBN***	***BARD1***	***BRCA1***	***RBBP8***	***PALB2***
Gender	χ2	.535	.205	.034	.521	.535	.476	.877
	p	.464	.650	.853	.471	.464	.490	.349
Age at diagnosis	χ2	1.801	.005	.000	**3.391**	.067	.014	**5.219**
	p	.180	.942	1.000	**.046a***	.796	.904	**.022***
Tumor site	χ2	**7.747**	1.284	.548	.029	.154	.128	.877
	p	**0.008***	.257	.459	.865	.695	.721	.349
Sidedness	χ2	**3.790**	2.196	.005	.620	.005	.014	.114
	p	**.042***	.138	.941	.431	.941a	.906	.73
CEA	χ2	1.802	1.429	0.800	0.022	0.022	1.429	0.089
	p	.148	.185	.276	.560	.560	.185	.489
Tumor size	χ2	**3.704**	0.406	1.429	1.429	1.429	0.229	1.429
	p	**.040***	.360	.180	0.180	.180	.421	.180
Histological tumor grade	χ2	2.301	.365	2.191	.521	.912	1.471	.877
	p	.129	.546	.139	.471	.340	.225	.349
Tumor invasive depth	χ2	**3.298**	.251	**5.028**	.220	.328	2.767	.017
	p	**.039***	.616	**.024***	.639	.567	.096	.897
Nodal metastasis	χ2	.001	.022	.829	2.017	**2.872**	.024	**9.314**
	p	.972	.882	.363	.156a	**.040***	.877	**.002***
TNM stage	χ2	.004	.000	2.057	**4.062**	1.322	.013	**10.080**
	p	.952	1.000	.151	**.044***	.250	.910	**.001***
Lymphovascular invasion	χ2	1.435	.085	.032	1.137	.186	.019	**5.907**
	p	.231	.770	.858	.286a	.666	.891	**.015***
Perineural invasion	χ2	0.961	**3.379**	0.168	0.220	0.002	0.423	1.516
	p	0.610	**0.044***	0.682	0.639	0.961	0.358	0.173
MSI	χ2	**3.706**	.770	.305	.003	.305	.580	2.094
	p	**0.049***	.380	.581	.959	.581	.446	.148

χ2 test and Fisher's exact test. *Correlation is significant at the 0.05 level (2-tailed). CEA: Carcinoembryonic Antigen; LMR: Lymphocyte-to-monocyte-ratio; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio

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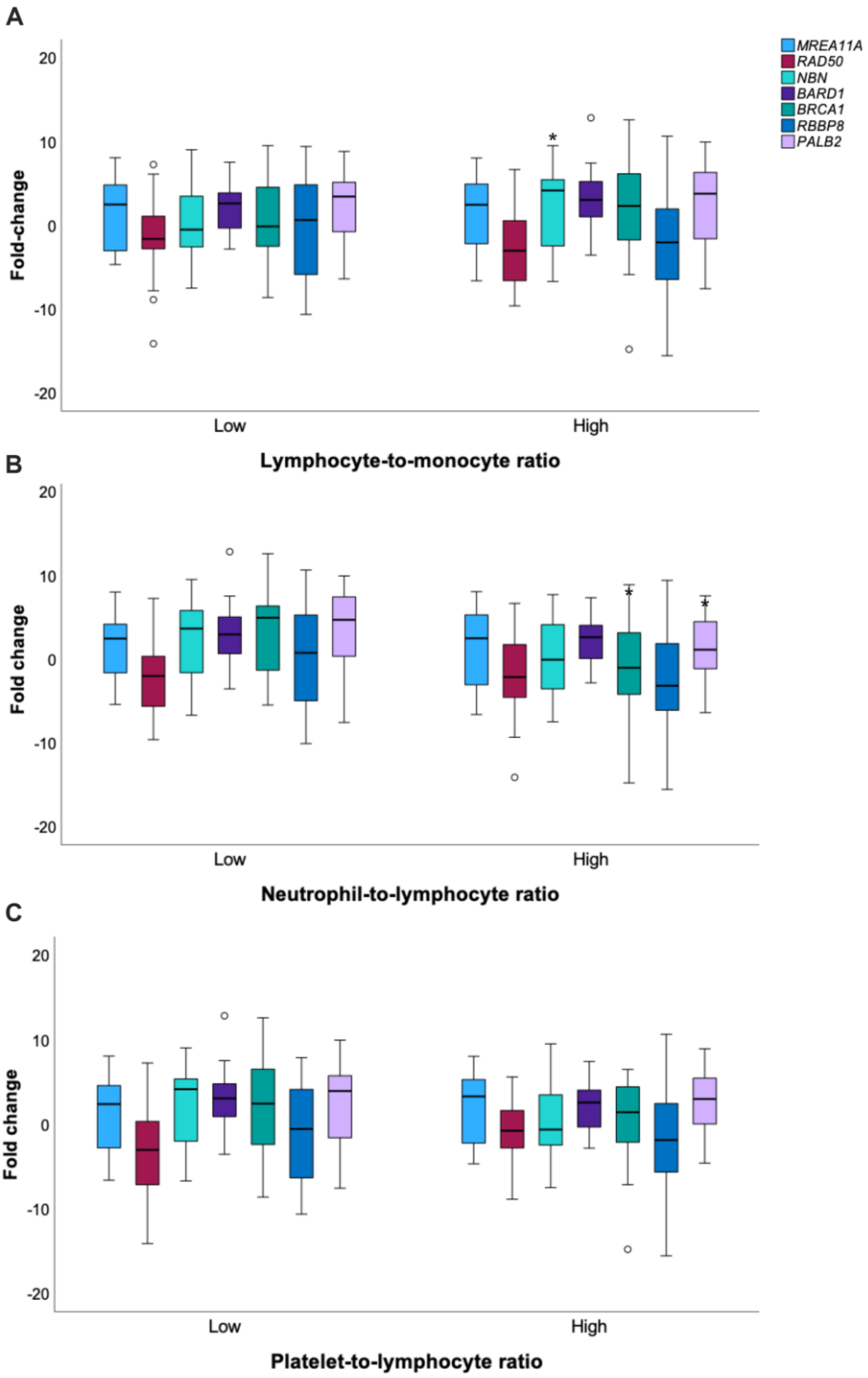

Figure 3. Fold-change of HRR representative genes according to composite inflammatory blood indexes. T-test for equality means. *p <0.05.

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3.4. *Analysis of inflammatory features in CRC patients and crosstalks with HRR*

Considering that systemic inflammatory factors also promote cancer growth and metastasis (34) and crosstalk between DNA damage response and inflammation has been evidenced (35), we investigated the possible associations of perioperative absolute counts of lymphocytes, neutrophils, monocytes, and platelets and their derived inflammation-based indexes with HRR gene expression profile (Fig. 3). Median NLR, LMR, and PLR values (2.1, 2.7, and 131.3, respectively) were used as a cut-off to determine subgroups with low (≤ median) or high (> median) inflammatory blood indexes. Mean NBN fold-change was significantly higher in patients harboring high LMR (2. vs 18 0.11 p= 0.039). In counterpart, both BRCA1 and PALB2 fold-induction were inversely correlated to NLR, i.e., these genes were found overexpressed in patients who presented low neutrophil-to-lymphocyte ratios (Supplementary Table 2).

Associations of inflammatory blood indexes with clinicopathological features of CRC patients were also explored (Supplementary Table 3). High LMR was observed in 63% of the patients younger than 65 years old with a diagnosis (p= 0.029, while low LMR was present in 70% of patients harboring tumors < 3 cm (p= 0.043) and with perineural invasion (p= 0.044). NLR was associated with tumor size (p= 0.021), tumor invasive depth (p= 0.008), and perineural invasion (p=0.040). Almost 80% and 70% of tumors diagnosed with more than 3 cm and perineural invasion, respectively, occurred in patients with NLR > 2.1. Conversely, 81.3% of the patients with low NLR were staged as pT1 or pT2.

Table 3. Univariate and multivariate analyses of disease free survival.

Variable	P	Variable	HR (95% CI)	p
Age	**0.148**	**< 65 years**	1.00	0.061
		> 65 years	3.79 (0.94-5.32)	
CEA	**0.087**	**< or = 5 ng/mL**	1.00	0.299
		> 5 ng/mL	3.18 (0.47-11.65)	
Tumor location	0.710			
Sidedness	**0.098**	**Left-sided CRC**	1.00	0.500
		Right-sided CRC	2.15 (0.23-10.08)	
Tumor size	0.325			
Tumor grade	0.202			
Lymphovascular invasion	**0.029**	**No**	1.00	0.588
		Yes	1.73 (0.24-12.68)	
Perineural invasion	0.206			
TNM stage	**0.035**	**I-II**	1.00	0.102
		III-IV	3.75 (0.94-8.54)	
NLR	0.465			
LMR	0.528			
PLR	0.207			
MMR status	**0.140**	**MSS**	1.00	0.380
		MSI	2.65 (0.31-3.41)	
MRE11A	**0.183**	**Relative low expression**	1.00	0.384
		Relative high expression	2.31 (0.35-5.06)	
RAD50	0.830			
NBN	0.475			
BARD1	0.633			
BRCA1	0.278			
RBBP8	0.636			
PALB2	0.269			

Abbreviations: CEA: Carcinoembryonic Antigen; LMR: Lymphocyte-to-monocyte- ratio; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio.

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Table 4. Univariate and multivariate analyses of overall survival of CRC patients.

Univariate Analysis		Multivariate Analysis		
Variable	**p**	**Variable**	**HR (95% CI)**	**p**
Age	0.306			
CEA	0.803			
Tumor location	0.201			
Sidedness	**<0.001**	**Left-sided**	1.00	**0.006**
		Right-sided	4.57 (1.55-13.49)	
Tumor grade	0.736			
Lymphovascular invasion	0.971			
Perineural invasion	0.970			
TNM stage	**0.076**	**I-II**	1.00	0.187
		III-IV	2.55 (0.63-10.30)	
NLR	0.548			
LMR	0.454			
PLR	0.877			
MMR status	**0.157**	**MSS**	1.00	0.884
		MSI	0.907 (0.24-3.36)	
MRE11A	**0.121**	**Relative low expression**	1.00	**0.046**
		Relative high expression	3.11 (1.64-15.08)	
RAD50	0.498			
NBN	0.615			
BARD1	0.607			
BRCA1	0.608			
RBBP8	0.558			
PALB2	**0.159**	**Relative low expression**	1.00	**0.044**
		Relative high expression	2.06 (1.67-17.63)	

Abbreviations: CEA: Carcinoembryonic Antigen; LMR: Lymphocyte-to-monocyte- ratio; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio.

3.5. *High MRE11A expression is associated with poor survival in CRC*

Using univariate and multivariate analysis, we explored the characteristics in the CRC patient cohort which influence the prognosis value as DFS and OS. Univariate analysis of DFS (Table 3) identified as potential independent prognostic features: age (p= 0.148), CEA levels at diagnosis (p=0.087), sidedness (p= 0.098), lymphovascular invasion (p= 0.029), TNM stage (p= 0.035), MMR status (p= 0.140) and MRE11A (p=0.183). Cox regression tests of coefficients did not confirm any of these features as independent prognostic factors for composite outcome disease progression in sporadic CRC patients. Mean OS was 51.9 months IC 95% (44.8-58.9). Univariate analysis of molecular and clinicopathological variables identified tumor location (p=0.201), sidedness (p<0.001), TNM stage (p=0.076), MMR status (p=0.157), MRE11A status (p=0.121) and PALB2 status (p=0.259) as candidates to independently predict the risk of death in CRC patients (Table 4). Tests of the model reached statistical significance (p= 0.007). Cox regression confirmed independent prognostic value for sidedness (HR=4.57 IC95% [1.55-13.49], p=0.006), MRE11A status (HR=3.11 IC95% [1.64-15.08], p=0.046) and PALB2 status (HR 2.06 IC95% [1.67-17.63], p=0.044). Kaplan-Meier plots for OS adjusted by MRE11A and PALB2 status are shown in Figure 4. MRE11A status (HR= 2.80 IC95% [1.23-6.36], p=0.016) and PALB2 status (HR= 3.33 IC95% [1.44-7.70] p=0.005) adjusted for sidedness presented as independent prognostic factors for overall survival in patients with CR

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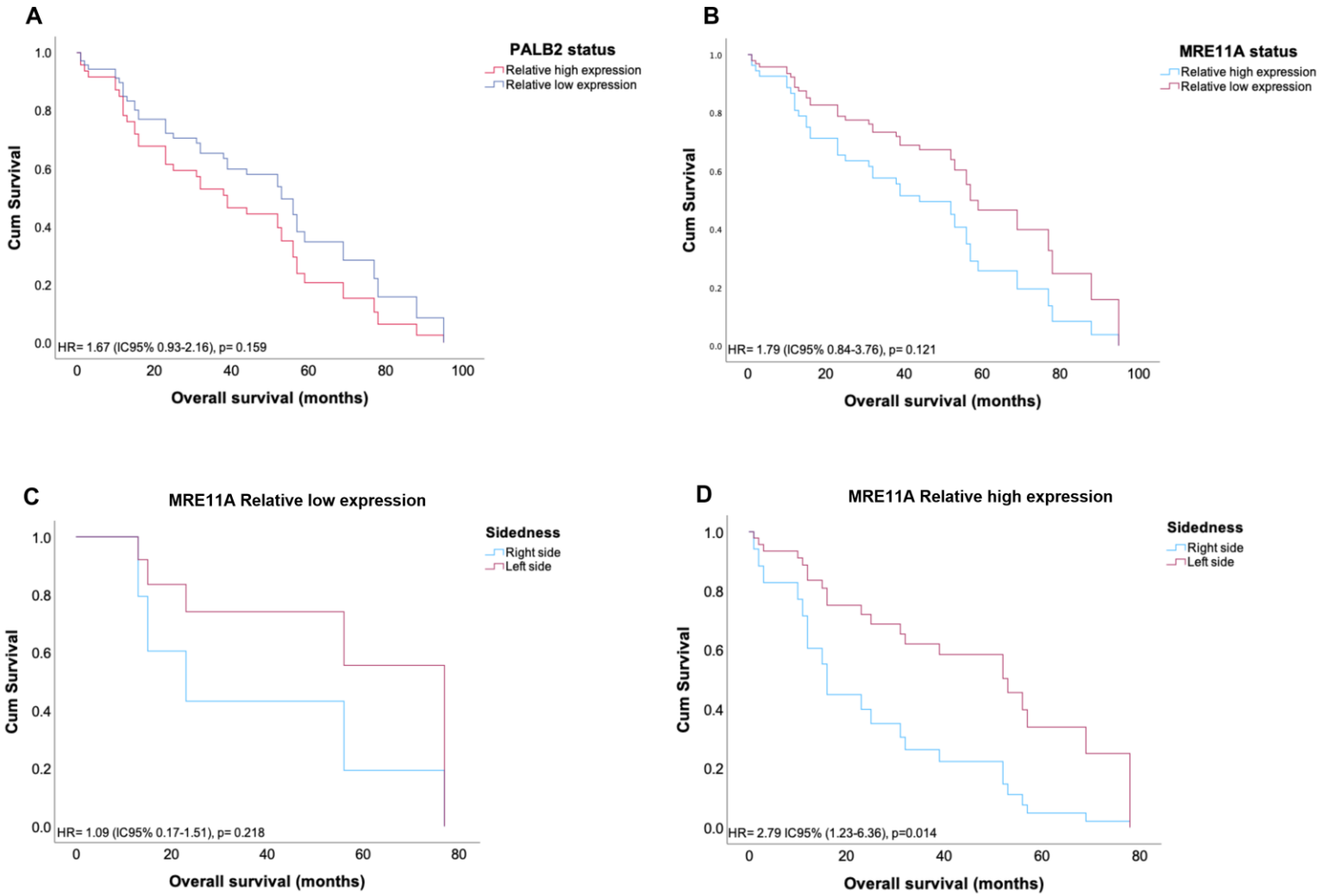

Figure 4. Kaplan-Meier plots of Cox Regression for progression free survival of CRC patients. Survival curves of (A) PALB2, (B) MRE11A, (C) MRE11A relative low expression adjusted by sidedness and (D) MRE11A relative high expression adjusted by sidedness.

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4. Discussion

HRR is a multistep pathway that starts with the complex formation of BRCA1 and BARD1, leading to the removal from the DSB of the 53BP1-RIF1, a complex known to recognize and protect DSB, and access of nucleases that extensively resect the 5′ DNA ends. This resection step generates single-stranded DNA (ssDNA) several kilobases long on each side of the break. This resection step is essential as ssDNA hybridizes with complementary sequences on a sister chromatid and is then extended to allow efficient and accurate repair. The MRN complex is involved in this resection step and includes the critical nuclease factor MRE11 (meiotic recombination 11) together with RAD50 and NBS1 (Nijmegen breakage syndrome 1). C-terminal binding protein (CtBP)–interacting protein (CtIP) contributes to this process by stimulating the exonuclease activity of MRE11. After CtIP-stimulated DNA resection, the stretches of ssDNA are rapidly coated by RPA, protecting them from degradation. BRCA2 associated with PALB2 then promotes the loading of the RAD51 recombinase, replacing RPA and the RAD51 molecules to form nucleoprotein filaments, which catalyze invasion and homology search on the sister chromatid. Variants in key genes of HRR such as BRCA1, BRCA2, PALB2, BARD1, RAD51C, and RAD51D, altering their function and/or their expression, have a significant association with breast cancer risk [24,25]. Variants of some of these genes are also associated with ovarian, pancreas, and prostate cancers. In CRC, HRR alterations have also been found in up to 20% of CRC cases [17,19,20,29–31]. Importantly, none of these gene mutations were found in whose encoding factors participating to the resection step of HRR.

Here, we report alteration of HRR gene expression in CRC, including BRCA1, BARD1, PALB2, but also genes involved in the resection such RAD50 and MRE11. We report notably that for one of them, MRE11, its relative high expression associated with pejorative features of CRC, i.e. right-sided colon tumors, increased tumor invasive depth, higher tumor grade, MMR deficiency, and finally poorer overall survival. Interestingly, high expression of Mre11 was previously shown to be associated to poor prognosis in rectal cancer patients treated with radiotherapy, and in patients with right-side-localized CRC [32]. In colon cancer cohort (TCGA-COAD) data analysis, it is observed a tendency of correlation between high MRE11 and worse survival probability. Mechanistically, why overexpression of MRE11 would lead to such unfavorable outcomes for CRC patients? In this cohort, more than 90% of the patients with right-sided CRC also harbored MRE11A upregulation. Right-sided CRC have a higher incidence of dMMR/MSI and increased tumor mutational burden, which favor the local recruitment of tumor-infiltrating lymphocytes. While MRE11 has been described as disrupted in over than 60% of the dMMR/MSI CRC, it has been suggested that MRE11 expression is strongly correlated with the activation of the immune response as evidenced by higher levels of tumor-infiltrating inflammatory cells [33]. This could be explained by the short pieces of DNA generated in the cytosol by enhanced ssDNA degradation which in turn can trigger strong innate immune responses, including the production of type I interferons (IFNs) and other inflammatory cytokines through the binding to these DNA fragments of cGAS, the major sensor for cytosolic DNA [34]. Thus, a high level of Mre11 could be a potential predictive biomarker for response to immunotherapy in CRC. Furthermore, high abundance of Mre11 would interfere and compete with the normal process of HRR and lead to exacerbated resection on DSB, creating excessive ssDNA and in turn excessive DSB, a source of chromosome instability and consequently enhanced cell variability, explaining why excess Mre11 is associated with poor prognosis. Such high resection levels by overexpressed MRE11A may sensitize CRC cells to agents that interfere with the progression of the replication forks, including the PARPi, whose toxicity has been recently associated with the presence of gap DNA at the forks [35], as these agents would exacerbate ssDNA gap accumulation.

Another aspect that should be considered for CRC prognosis is the primary tumor sidedness. Recent studies have been demonstrating that patients with right-side tumors have a worse prognosis [36,37]. In a systematic review and meta-analysis published by Petrelli et al, it was demonstrated that laterality has prognostic value independently of the stage, race, and adjuvant chemotherapy [38]. We have identified that more than 90% of the cases with high expression of MRE11A are located in the

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right side of the colon. Additionally, in Kaplan–Meier analyses, we identified that MRE11A high expression within right-sided CRC was significantly correlated with worse OS. Taken together, these results suggest that MRE11A overexpression may have a prognostic value for poor outcomes in CRC.

Overall, our study reveals that among the key HRR genes that are differentially expressed in our cohort of patients with sporadic CRC, only MRE11A up-regulation has a strong clinical value in terms of tumor aggressiveness, sidedness, and survival prediction. After validation of these data in larger CRC cohort, whether Mre11 high-expressing tumors could be good candidates for immunotherapy or agents that target DNA replication need to be explored in the future.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., Figure S1: Representative images of MSI panel by immunohistochemistry; Table S1: Mean fold-change of HRR gene according to MMR status.; Table S2: Mean fold-change of HRR representative genes according to composite inflammatory blood indexes. Table S3: Associations between inflammatory blood indexes and clinicopathological features of CRC patients.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional committee for Medical and Health Research Ethics (CAAE: 58299916.3.3001.5345).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and analysed during the current study are not publicly available due to privacy/ethical restrictions, but are available from the corresponding author at reasonable request. Quantitative RT-PCR was performed using Custom RT2 Profiler PCR Array (#CLAH-32033-9619-6, Qiagen), the primers and sequences were generated by an experimentally verified, proprietary computer algorithm and therefore are propriety of QIAGEN.

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