mRNA Expression and Methylation of the *RAD51*, *ATM*, *ATR*, *BRCA1*, and *BRCA2* Genes in Gastric Adenocarcinoma

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ABSTRACT

BACKGROUND: Immunohistochemical prognostic significance of the homologous recombination-related proteins RAD51, ATM, BRCA1, and BRCA2 is known in gastric adenocarcinoma, one of the deadliest cancers.

OBJECTIVE AND DESIGN: This retrospective cohort study aimed to evaluate mRNA expression and promoter methylation of some homologous recombination-related genes in this neoplasm.

METHODS: We evaluated mRNA expression and methylation of *RAD51*, *ATM*, *ATR*, *BRCA1*, and *BRCA2* in tumor and non-tumor frozen samples from gastrectomy specimens by RT-qPCR and MS-HRM, correlating our results with previous immunohistochemistry data and prognostic features.

RESULTS: *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM* mRNA expression was detected in 93.75% (45/48), 93.75% (45/48), 91.67% (44/48), 83.33% (40/48), and 89.58% (43/48) of the tumors; partial or complete methylation, in 94.87% (37/39), 0 (0/42), 97.56% (40/41), 100% (41/41), and 0 (0/40), respectively. Most gene pairs showed significant weak to moderate positive correlations of tumoral mRNA expression with each other: *RAD51* with *ATR* ($P = .027$), *BRCA1* ($P < .001$), and *BRCA2* ($P < .001$); *ATR* with *BRCA1* ($P = .007$), and *ATM* ($P = .001$); *BRCA1* with *BRCA2* ($P = 0.001$). *BRCA1* mRNA was reduced in tumors compared with non-neoplastic mucosa (0.345 vs 1.272, $P = .015$) and, excluding neoadjuvant therapy cases, in T3 to T4 tumors compared with T2 (0.414 vs 0.954, $P = .035$). Greater tumoral *RAD51* mRNA levels correlated with perineural invasion (1.822 vs 0.725, $P = .010$) and death (1.664 vs 0.929, $P = .036$), but not with survival time. There was an inverse association between nuclear immunohistochemical positivity for ATR and its mRNA levels (0.487 vs 0.907, $P = .032$), and no significant correlation for the other markers.

CONCLUSIONS: Our results suggest *RAD51*, *BRCA1*, and *BRCA2* methylation as a frequent epigenetic mechanism in gastric cancer, support the hypothesis that reduced *BRCA1* expression participates in disease progression, and show an association between *RAD51* mRNA and perineural invasion and mortality that may be considered unexpected, considering the former immunohistochemical studies. The lack of correlation between immunohistochemistry and mRNA, and even the inverse association, for *ATR*, can be seen as indicative of action of post-transcriptional or post-translational regulatory mechanisms, to be better investigated.

KEYWORDS: Stomach neoplasms, homologous recombination, DNA repair, mRNA, methylation

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Introduction

Malignant tumors of the stomach are the fourth major cause of death due to cancer worldwide.[1] More than 90% of them are adenocarcinomas.[2] The pathogenesis of gastric adenocarcinoma is complex, with infection of the gastric mucosa by *Helicobacter pylori* being the main risk factor. The mechanisms by which it induces carcinogenesis are several and include an increased production of reactive species caused by inflammation, which leads to DNA damage,[3] including double-strand breaks.[4]

The most accurate repair mechanism for DNA double-strand breaks is homologous recombination. ATM, BRCA1, BRCA2, ATR, and RAD51 proteins participate in this repair pathway. Initially, DNA breakage activates ATM kinase. Then,

this kinase phosphorylates BRCA1, which acts on the resection of the 5′ ends of the break, a process that activates and is stimulated by ATR via positive feedback.[5] RAD51 covers the newly formed single-stranded ends, in a BRCA2-dependent process, and participates in the search for the homologous sequence in the sister chromatid that serves as a template for DNA repair.[6]

Mutations in genes encoding these proteins are uncommon in gastric adenocarcinoma.[7] However, changes in their expression are frequent and correlate with clinicopathological characteristics, as shown by several previous studies, mostly based on immunohistochemistry.[8-18]

In gastric cancer, the relationship between the methylation of several genes and pathogenesis, prognosis, diagnosis, and resistance to chemotherapy has been reported.[19] However, the methylation of homologous recombination-related genes is still poorly described.

The objective of this work was to evaluate the mRNA expression and promoter methylation of these genes in gastric adenocarcinoma, as well as their possible correlations with clinicopathological characteristics and with the immunohistochemical expression of their respective proteins previously published by our group.[11]

Materials and Methods

Study population

This is a retrospective cohort study that evaluates patients already analyzed in a previous immunohistochemistry-based study of our group,[11] but using different methods (RT-qPCR and MS-HRM). This preceding work evaluated a larger number of patients (n = 121). The procedures and analyses of the former work were conducted between 2018 and 2020, whereas the laboratory procedures of the present study were started in 2019, interrupted in 2020 due to the COVID-19 pandemic, and completed in 2022.

Patients who underwent total or subtotal gastrectomy for gastric adenocarcinoma treatment at the Clinical Hospital of the Ribeirão Preto Medical School at the University of São Paulo (HCFMRP-USP) between April 2008 and June 2017 and who had frozen samples stored were analyzed. Only tumors infiltrating at least the muscularis propria layer were included. Patients who did not survive for at least 30 days after the surgery were excluded.

In total, 73 patients were analyzed, and their characteristics are summarized in Table 1. However, because of the availability of tumoral or non-tumoral frozen material previously stored in the biorepository, loss of samples and the presence of cases with inadequate quality of extracted material, tumor and non-tumor mucosa mRNA expression was evaluated only in 48 and 32 patients, respectively. This number of 48 cases was sufficient for evaluating the correlations between the expressions obtaining coefficients below .40, with a significance level of 5% and a power of 80%. The methylation of gene promoters was evaluated in tumor samples from 44 patients, whereas non-tumor mucosa methylation was evaluated in 49 patients. Of the 48 cases in which tumor mRNA was evaluated, 8 were treated with surgery alone; 13 received neoadjuvant treatment consisting of ECX (epirubicin, cisplatin, and capecitabine) or slight variations of this protocol; and 27 received adjuvant treatment, mainly with the MacDonald protocol or platinum-based schemes. Of these 48 patients, 31 died, with a survival time ranging from 62 to 1646 days (mean 461 ± 371). The other 17 had follow-up times ranging from 60 to 3755 days (mean 1674 ± 1242).

Information on clinicopathological features—histological type, histological grade, depth of invasion, presence of regional lymph node metastasis, distant metastasis, blood or lymphatic vascular invasion, perineural invasion, stage, overall survival, and disease-free survival—was obtained from medical records and histological slides.

Table 1. Clinicopathological characteristics of the patients.

CHARACTERISTICS OF THE STUDY POPULATION (N = 73)	N (%)
Age (years)	62 ± 12.8
Sex	
Male	50 (68.5)
Female	23 (31.5)
Histological type	
Diffuse	42 (57.5)
Intestinal	21 (28.8)
Mixed	10 (13.7)
Histological grade	
1	4 (5.5)
2	13 (17.8)
3	56 (76.7)
Stage	
I/II	31 (42.5)
III/IV	42 (57.5)
pT	
2	14 (19.2)
3-4	59 (80.8)
Lymph node metastasis	
Yes	54 (73.9)
No	19 (26.1)
Distant metastasis	
Yes	13 (17.8)
No	60 (82.2)

This study was approved by the HCFMRP-USP Research Ethics Committee (approval No. 12349/17).

This manuscript was prepared in accordance with the STROBE Guidelines.

DNA and RNA extraction

Gastric adenocarcinoma and gastric mucosa samples were collected from gastrectomy surgical specimens and stored at –80°C. Representative portions of the samples were dissected manually, using HE-stained 4 µm frozen histological sections as reference. Automated DNA extraction was performed with QiaCube (Qiagen, Germantown, MD), according to the manufacturer's instructions. RNA extraction was performed using TRIzol (Invitrogen, Waltham, MA), following the manufacturer's protocol.

Methylation-sensitive high-resolution melting (MS-HRM)

For methylation evaluation of the *ATM*, *ATR*, *RAD51*, *BRCA1*, and *BRCA2* genes, DNA conversion using sodium bisulfite and sample cleanup of 1 µg of genomic DNA were performed using the EpiTect Bisulfite Kit (Qiagen), following the manufacturer's instructions. Afterwards the DNA conversion, each sample was run in duplicate by the MS-HRM technique to determine the methylation pattern of the promoter regions of each gene using primers designed with the aid of MethPrimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi): 5′-TTTTAATA TTGAATTAAGGAAATTG-3′ and 5′-AAATACTCCT CTCTTCAAAAACTAC-3′ for *ATM*; 5′-TTTGTGTTT TTTAGGTTTGAGAATAGTAG-3′ and 5′-TATAAAAA CCAAAACTCCTCCC-3′ for *ATR*; 5′-GAATTTTTGA TTTTAGGTTATTTATT-3′ and 5′-TCACTATCTTAA CCAAACTATTCTC-3′ for *RAD51*; 5′-TGGTTTTTAT TATTTGTTTTTTAAAA-3′ and 5′-TCAACCCCAATA TTTATTATTTTTC-3′ for *BRCA1*; and 5′-GGTGTG GTGGTTTATGTTTGTAAT-3′ and 5′-TCAAATAATT CTCCTACCTCAACCT-3′ for *BRCA2*.

MS-HRM was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using MeltDoctor HRM Master Mix (Applied Biosystems) according to manufacturer's instructions. The PCR reaction conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 58°C for 1 minutes, and 95°C for 1 minutes, followed by the standard melting curve. For the MS-HRM analysis, each sample duplicate was compared to controls, and we employed in each run two controls already treated with sodium bisulfite (Epitect PCR Control DNA Set, Qiagen), one of which was 100% methylated, and another was 100% unmethylated.

RT-qPCR

For cDNA synthesis, reverse transcription (RT) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The cDNA used in the quantitative polymerase chain reaction (qPCR) was previously diluted 1:10 in water treated with diethyl pyrocarbonate (DEPC).

The following assays (ThermoFisher Scientific, Waltham, MA) were used: Hs01556193_m1 (*BRCA1*), Hs00609073_m1 (*BRCA2*), Hs00175892_m1 (*ATM*), Hs00992123_m1 (*ATR*), Hs00153418_m1 (*RAD51*), Hs99999903_m1 (*ACTB*), and Hs99999907_m1 (*B2M*). qPCR amplification reactions were performed in duplicates using Taqman Master Mix (Applied Biosystems, Foster City, CA). Each reaction had a final volume of 10 µl, using 5 µl of the specific Taqman Master Mix reagent, 0.5 µl of each specific probe, and 4.5 µl of diluted cDNA. The amplification conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes (simultaneous annealing and extension). The real-time PCR detection device 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used together with 7500 Sequence Detection System software (Applied Biosystems, Foster City, CA) to obtain Ct values.

The expression levels (fold changes) were given by the formula $2^{(-\Delta\Delta Ct)}$, where ΔCt was the difference between the Ct of the studied gene and the mean Ct of the endogenous *ACTB* and *B2M* for the same sample, and ΔΔCt was the difference between the ΔCt of the sample and the mean ΔCt of non-neoplastic samples. The absence of mRNA amplification of the gene under study, combined with preserved amplification of the endogenous controls, was considered the absence of expression. A fold change of 0 was assigned to cases without gene expression, only for statistical correlation with other variables.

Immunohistochemistry

Immunohistochemistry data that are part of an earlier study published by our group were used for correlations with RT-qPCR results.[11] The following antibodies were used: ATM (St John's Laboratory, London, UK, STJ97797, 1:400), ATR (Abcam, Cambridge, UK, ab178407, 1:100), BRCA1 (St John's Laboratory, STJ113833, 1:300), RAD51 (St John's Laboratory, STJ95330, 1:100), and BRCA2 (St John's Laboratory, STJ91885, 1:100). As previously described, immunohistochemistry results were expressed as histochemical scores (*H*-scores) and were additionally classified as positive or negative. The *H*-score was calculated by multiplying the percentage of stained cells by 1, 2, or 3 for weak, moderate, or strong staining, respectively. Cases with 10% or less stained neoplastic cells were considered negative; those with more than 10% were considered positive.

Statistical analyses

The Shapiro-Wilk test was used to study the normality of the data. The non-parametric Mann-Whitney test was used for comparing two groups, and the non-parametric Kruskal-Wallis test was used for comparing three groups, because the assumption of normality of the data was rejected. For comparing tumor

and non-tumor samples, Wilcoxon's non-parametric test was used, because the assumption of normality of the data was rejected. Spearman's correlation coefficient was used to study correlations. The ROC curve was used in an attempt to define the expression cut-off point to predict the occurrence of death. The chi-square test verified homogeneity between the proportions. Survival was studied using Kaplan-Meier survival curves with log-rank tests and the univariate Cox model. Kaplan-Meier curves were tested with all possible cut-off points to define low and high expression. The significance level was 5%. The software used for the calculations was SPSS 17.0 for Windows. To avoid bias, additional clinicopathological correlation analyzes were carried out excluding cases with neoadjuvant treatment and, in the case of death and survival assessment, with other groupings according to the type of treatment.

Results

Methylation in tumors and non-tumoral mucosa

Considering the tumor samples, total methylation of *RAD51* was detected in 7.7% of the cases (3/39), partial methylation was detected in 87.2% (34/39), and absence of methylation, in 5.1% (2/39); total *BRCA1* methylation was detected in 2.4% of the cases (1/41), partial methylation was verified in 95.1% (39/41), and absence of methylation, in 2.4% (1/41); total *BRCA2* methylation was seen in all cases (41/41); whereas the absence of *ATR* (42/42) and *ATM* (40/40) methylation was seen in all cases.

In non-tumor samples, total methylation of *RAD51* was detected in 6.1% of the cases (3/49), and partial methylation was detected in 93.9% (46/49); total *BRCA1* methylation was detected in 2.1% of the cases (1/48), partial methylation was verified in 95.8% of the cases (46/48), and absence of methylation was observed in 2.1% (1/48); total *BRCA2* methylation was verified in all cases (49/49); and absence of *ATR* (49/49) and *ATM* (49/49) methylation was seen in all cases.

There was agreement in the methylation status of paired tumor and non-tumor samples in 78.4% (29/37) of the cases for *RAD51*, and in 94.6% (35/37) of the cases for *BRCA1* (Table 2). However, it was not possible to apply statistical tests to assess the significance of this correlation because of the great predominance of partial methylation.

Similarly, because of the qualitative nature of the methylation assessment and the reduced number of divergent results, it was not possible to statistically evaluate the correlation between methylation and other variables.

mRNA expression in tumors and non-tumoral mucosa

In tumor samples, the absence of *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM* mRNA expression was detected in 6.25%, 6.25%, 8.33%, 16.67%, and 10.42% of the cases, respectively. More detailed results are shown in Table 3.

Table 2. Cross-analysis of the methylation of *RAD51* and *BRCA1* genes in tumor and non-tumor mucosa, considering the 37 cases with paired tumoral and non-tumoral samples.

	TUMORAL	NON-TUMORAL (%)		
		ABSENT	PARTIAL	TOTAL
RAD51	absent	–	2 (5.4)	0 (0.0)
	partial	–	29 (78.4)	3 (8.1)
	total	–	3 (8.1)	0 (0.0)
BRCA1	absent	0 (0.0)	1 (2.7)	0 (0.0)
	partial	1 (2.7)	34 (91.9)	0 (0.0)
	total	0 (0.0)	0 (0.0)	1 (2.7)

Considering the paired samples, non-tumor mucosa showed significantly higher median level of *BRCA1* mRNA than tumors (1.272 vs 0.345, $P = .015$). No significant difference was observed for the other genes (Figure 1).

The tumor and non-tumor mRNA expression of the studied genes was not statistically different in cases with previous neoadjuvant treatment compared with the other cases (Table 4).

There were positive and significant weak to moderate correlations between tumor mRNA expression levels of *RAD51* and *ATR* ($r = .319$, $P = .027$), *RAD51* and *BRCA1* ($r = .587$, $P < .001$), *RAD51* and *BRCA2* ($r = .502$, $P < .001$), *ATR* and *BRCA1* ($r = .383$, $P = .007$), *ATR* and *ATM* ($r = .449$, $P = .001$), and *BRCA1* and *BRCA2* ($r = .474$, $P = .001$).

Immunohistochemistry and mRNA correlation

Tumors that were positive for nuclear ATR in the immunohistochemical evaluation (more than 10% of neoplastic cells with nuclear staining) had a lower median mRNA fold change than those that were negative (0.487 vs 0.907, $P = .032$). The other immunohistochemical markers showed no significant correlation (Table 5).

mRNA and clinicopathological parameters

Cases with perineural invasion had higher median tumor amounts of *RAD51* mRNA (1.822 vs 0.725, $P = .010$). Size, histological type, histological grade, vascular invasion, depth of invasion, lymph node metastasis, distant metastasis, and stage did not correlate with gene expression in the total population (Tables 6 and 7). Given the possibility of an influence of previous neoadjuvant chemotherapy on mRNA expression, all analyses were also performed excluding patients who received neoadjuvant treatment. This showed that deeper infiltrating tumors (pT3–4, n = 30) had lower median levels of *BRCA1* mRNA than superficial tumors (pT2, n = 5)—0.414 versus 0.954, $P = .035$. The association between high median *RAD51* mRNA and perineural invasion was also maintained in this

Table 3. *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM* mRNA expression in gastric adenocarcinoma and non-tumor gastric mucosa samples.

	MRNA EXPRESSION		MRNA FOLD CHANGE IN CASES WITH EXPRESSION
RAD51			
Tumor	Absent	6.25% (3/48)	
	Present	93.75% (45/48)	Mean 2.0760
			min: 0.1429
			max: 8.6607
			SD: 2.1142
Non-tumor mucosa	Absent	31.25% (10/32)	
	Present	68.75% (22/32)	Mean: 1.6572
			min: 0.0283
			max: 7.6339
			SD: 1.7572
ATR			
Tumor	Absent	6.25% (3/48)	
	Present	93.75% (45/48)	Mean: 1.4332
			min: 0.0871
			max: 17.0522
			SD: 2.6250
Non-tumor mucosa	Absent	32.26% (10/31)	
	Present	67.74% (21/31)	Mean: 5.2802
			min: 0.1427
			max: 83.8923
			SD: 18.0676
BRCA1			
Tumor	Absent	8.33% (4/48)	
	Present	91.67% (44/48)	Mean: 0.6419
			min: 0.0266
			max: 2.8812
			SD: 0.6753
Non-tumor mucosa	Absent	18.75% (6/32)	
	Present	81.25% (26/32)	Mean: 2.1834
			min: 0.0328
			max: 9.2877
			SD: 2.3584
BRCA2			
Tumor	Absent	16.67% (8/48)	
	Present	83,33% (40/48)	Mean: 0.4695
			min: 0.0108
			max: 4.3376
			SD: 0.8572
Non-tumor mucosa	Absent	43.75% (14/32)	
	Present	56.25% (18/32)	Mean: 23.9100
			min: 0.0016
			max: 270.0019
			SD: 65.9849
ATM			
Tumor	Absent	10.42% (5/48)	
	Present	89.58% (43/48)	Mean: 10.3642
			min: 0.4420
			max: 76.1618
			SD: 14.2744
Non-tumor mucosa	Absent	34.37% (11/32)	
	Present	65.62% (21/32)	Mean: 7.0627
			min: 0.3872
			max: 33.9450
			SD: 7.7460

Proportion of cases with no expression and with present expression, mean, minimum (min.), and maximum (max.) fold change values and standard deviations (SD) are shown, calculated considering the cases with present expression.

group (2.028 in the presence of perineural invasion vs 0.456 in the absence of perineural invasion, $P = .024$).

The occurrence of death correlated with higher tumoral *RAD51* mRNA (1.664 vs 0.929, $P = .036$), even if cases with neoadjuvant treatment were excluded (Table 8). However, we were not able to define an expression cut-off point that could be used as a statistically significant predictor of death. Moreover, using the Cox model, we observed no association between overall survival and tumor mRNA expression levels of *RAD51* ($P = .510$), *ATR* ($P = .893$), *BRCA1* ($P = .310$), *BRCA2* ($P = .488$),

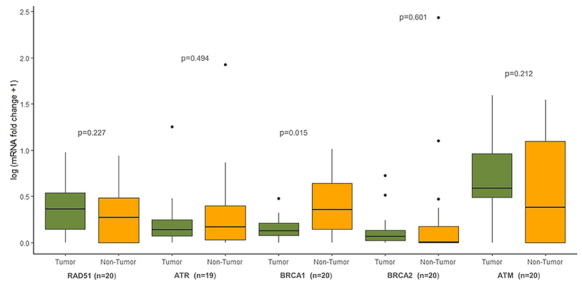

Figure 1. Comparison of *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM* mRNA expression in tumor and non-tumor mucosa, considering the paired samples of the same individuals. The results are presented in box plots, using a logarithmic scale (+1 was added as a constant to allow the logarithmic representation of null values).

and *ATM* ($P = .153$). There was also no association between disease-free survival and *RAD51* ($P = .271$), *ATR* ($P = .474$), *BRCA1* ($P = .566$), *BRCA2* ($P = .288$), and *ATM* ($P = .230$) expression. Separate analyses according to the type of treatment (surgery alone, adjuvant, neoadjuvant, adjuvant + neoadjuvant, and adjuvant + surgery alone) similarly did not show statistically significant correlations. Figure 2 illustrates the absence of a significant correlation between tumor mRNA expression and overall survival.

Discussion

Although the incidence of gastric cancer has decreased in recent decades, it is still the fifth cancer in incidence and the fourth in mortality worldwide.[1] Previous studies have found an association between altered immunohistochemical expression of homologous recombination proteins and clinicopathological features of gastric adenocarcinoma.[8-18]

Specifically concerning the BRCA1 protein, such studies had discrepant results regarding its cellular location—nuclear,[8,9] cytoplasmic,[10] or both.[11-13] The correlation with clinicopathological parameters is also controversial. It was absent in some studies,[11,13] and, in others, low expression was associated with advanced tumors and worse prognosis.[8-10] For Wang et al,[12] not only specifically low cytoplasmic expression but also high nuclear expression is associated with unfavorable characteristics.

Here, we showed that tumors with deeper infiltration of the gastric wall (subserosa or beyond—T3-T4) have lower levels of *BRCA1* mRNA than those limited to the muscularis propria (T2) in the group of patients who had not received previous neoadjuvant therapy. We also demonstrated reduced *BRCA1* mRNA in adenocarcinoma compared with non-tumor mucosa. Regarding survival, although there seems to be a tendency toward greater survival in cases with greater expression, this was not statistically significant. These results, together with some of the previously published data, support the hypothesis that a decrease in *BRCA1* expression favors the development or progression of gastric cancer.

In contrast, Kim et al[13] did not observe any clinicopathological association of *BRCA1* mRNA expression in gastric cancer.

Table 4. Comparison between *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM* mRNA expression in patients with previous neoadjuvant chemotherapy and in those without neoadjuvant chemotherapy, considering tumor samples and non-tumor mucosa samples.

	NEOADJUVANT THERAPY	N	MEDIAN MRNA FOLD CHANGE	*P*
RAD51				
Tumor	Yes	13	1.250	.307
	No	35	1.664	
Non-tumor	Yes	14	0.585	.749
	No	18	0.865	
ATR				
Tumor	Yes	13	0.330	.194
	No	35	0.693	
Non-tumor	Yes	14	0.349	.749
	No	18	0.482	
BRCA1				
Tumor	Yes	13	0.217	.298
	No	35	0.520	
Non-tumor	Yes	14	0.598	.497
	No	18	1.231	
BRCA2				
Tumor	Yes	13	0.121	.928
	No	35	0.081	
Non-tumor	Yes	14	0.001	.849
	No	18	0.069	
ATM				
Tumor	Yes	13	2.998	.057
	No	35	4.811	
Non-tumor	Yes	14	0.927	.255
	No	18	2.011	

According to Zhang et al,[9] the immunohistochemical expression of ATR has no clinicopathological associations. The previous study from our group, in contrast, found a correlation between negative nuclear immunohistochemical expression of ATR and higher histological grade and tumor size in gastric adenocarcinoma.[11] Here, however, there was no correlation between *ATR* mRNA and clinicopathological characteristics. Furthermore, we observed an inverse relationship between immunohistochemical results and mRNA levels, as immunohistochemically positive cases for nuclear ATR had a significantly lower median mRNA fold change than negative cases.

Table 5. Association between immunohistochemistry and mRNA levels in tumor samples.

IMMUNOHISTOCHEMISTRY	N	MEDIAN MRNA FOLD-CHANGE	*P*	*R* (CONSIDERING *H*-SCORE)	*P*
Nuclear RAD51					
Positive	38	1.324	.317	−0.136	.361
Negative	9	1.286			
Cytoplasmic RAD51					
Positive	35	1.359	.726	0.168	.260
Negative	12	1.226			
Nuclear ATR					
Positive	**18**	**0.487**	**.032**	−0.279	.055
Negative	**30**	**0.907**			
Cytoplasmic ATR					
Positive	11	0.651	.826	−0.114	.442
Negative	37	0.602			
Nuclear BRCA1					
Positive	42	0.361	.267	−0.069	.643
Negative	6	0.583			
Cytoplasmic BRCA1					
Positive	43	0.390	.897	−0.027	.855
Negative	5	0.413			
Nuclear BRCA2					
Positive	21	0.124	.624	0.124	.408
Negative	27	0.056			
Cytoplasmic BRCA2					
Positive	13	0.173	.384	0.116	.434
Negative	35	0.081			
Nuclear ATM					
Positive	43	4.638	NA	−0.014	.927
Negative	4	6.541			

Two different analyses are shown: one considers the immunohistochemistry results classified into two categories—positive or negative—, and the other considers the Spearman correlation coefficients between *H*-scores and mRNA fold changes. Bold indicates statistical significance.

This suggests that there may be an effect of post-transcriptional or post-translational regulation on ATR protein expression. In renal carcinoma cell cultures, for example, miR-185 inhibits ATR protein synthesis.[20] In pancreatic cancer cells, ATR undergoes degradation by the E3 ubiquitin ligase FBXO32.[21]

Studies on the prognostic importance of RAD51 in various types of cancer have had conflicting results. Although this protein acts on DNA repair, having therefore certain tumor suppressor character, its overexpression may lead to genomic instability and carcinogenesis.[22] It is also known that RAD51 increases the expression of the EBPβ transcription factor, which favors the epithelial-mesenchymal transition and the synthesis of metalloproteinases.[23] An association between poor prognosis and low immunohistochemical RAD51 expression was noted in glioblastoma,[24] breast carcinoma,[25,26] and non-small cell lung cancer.[27] Conversely, correlation between RAD51 and unfavorable characteristics such as high histological grade, lymph node metastases, or lower survival was described in colon cancer,[28,29] breast carcinoma,[23,30] esophageal squamous cell carcinoma,[31] head and neck tumors,[32] non-small cell lung cancer,[33] and prostatic adenocarcinoma.[34]

Table 6. Correlation between *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM* mRNA expression and histological type, histological grade, perineural invasion, and vascular invasion in tumor samples.

HISTOLOGICAL TYPE	N	MRNA FOLD-CHANGE MEDIAN	*P*	HISTOLOGICAL GRADE	N	MRNA FOLD-CHANGE MEDIAN	*P*	PERINEURAL INVASION	N	MRNA FOLD-CHANGE MEDIAN	*P*	VASCULAR INVASION	N	MRNA FOLD-CHANGE MEDIAN	*P*
RAD51															
Diffuse	31	0.977	.205	1-2	7	2.453	.077	**No**	**24**	**0.725**	**.010**	No	11	1.908	.254
Intestinal	9	1.908		3	41	1.165		**Yes**	**24**	**1.822**		Yes	37	1.165	
Mixed	8	1.570													
ATR															
Diffuse	31	0.581	0.926	1-2	7	1.114	.140	No	24	0.663	.773	No	11	1.062	.173
Intestinal	9	1.062		3	41	0.581		Yes	24	0.627		Yes	37	0.581	
Mixed	8	0.781													
BRCA1															
Diffuse	31	0.259	0.590	1-2	7	0.517	.261	No	24	0.344	.606	No	11	0.519	.291
Intestinal	9	0.517		3	41	0.337		Yes	24	0.524		Yes	37	0.278	
Mixed	8	0.542													
BRCA2															
Diffuse	31	0.063	0.196	1-2	7	0.526	.121	No	24	0.074	.542	No	11	0.327	.113
Intestinal	9	0.327		3	41	0.077		Yes	24	0.131		Yes	37	0.066	
Mixed	8	0.167													
ATM															
Diffuse	31	4.811	0.389	1-2	7	4.228	.942	No	24	4.825	.433	No	11	4.483	.797
Intestinal	9	2.711		3	41	4.811		Yes	24	4.450		Yes	37	4.638	
Mixed	8	8.677													

Bold indicates statistical significance.

Table 7. Correlation between *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM* mRNA expression and depth of invasion (pT), regional lymph node metastasis, distant metastasis, and stage in tumor samples.

	PT	N	MRNA FOLD-CHANGE MEDIAN	*P*	LYMPH NODE METASTASIS	N	MRNA FOLD-CHANGE MEDIAN	*P*	DISTANT METASTASIS	N	MRNA FOLD-CHANGE MEDIAN	*P*	STAGE	N	MRNA FOLD-CHANGE MEDIAN	*P*
RAD51	2	39	1.019	.607	No	13	1.822	.379	No	39	1.288	.802	III-IV	29	1.286	.712
	3-4	9	1.359		Yes	34	1.225		Yes	9	1.286		I-II	19	1.811	
ATR	2	39	0.994	.663	No	13	1.062	.207	No	39	0.651	.782	III-IV	29	0.651	.891
	3-4	9	0.602		Yes	34	0.592		Yes	9	0.536		I-II	19	0.581	
BRCA1	2	39	0.572	.122	No	13	0.517	.468	No	39	0.491	.663	III-IV	29	0.259	.158
	3-4	9	0.298		Yes	34	0.307		Yes	9	0.203		I-II	19	0.519	
BRCA2	2	39	0.081	.361	No	13	0.259	.105	No	39	0.124	.397	III-IV	29	0.063	.311
	3-4	9	0.124		Yes	34	0.065		Yes	9	0.046		I-II	19	0.144	
ATM	2	39	2.997	.362	No	13	4.483	.757	No	39	4.638	.335	III-IV	29	4.811	.744
	3-4	9	4.811		Yes	34	4.724		Yes	9	4.417		I-II	19	4.483	

Table 8. Comparison between the mRNA levels in the tumors of patients who died and those who did not, considering the following groups: total population, excluding neoadjuvant therapy, with only surgical treatment, and with adjuvant or neoadjuvant treatment.

TOTAL POPULATION	DEATH	N	MRNA FOLD-CHANGE MEDIAN	*P*	ONLY SURGERY	DEATH	N	MRNA FOLD-CHANGE MEDIAN	*P*
RAD51	**No**	**17**	**0.929**	**.036**	RAD51	No	3	0.457	n.a.
	Yes	**31**	**1.664**			Yes	5	3.406	
ATR	No	17	0.651	.575	ATR	No	3	0.994	n.a.
	Yes	31	0.602			Yes	5	0.305	
BRCA1	No	17	0.413	.872	BRCA1	No	3	0.521	n.a.
	Yes	31	0.337			Yes	5	0.645	
BRCA2	No	17	0.081	.522	BRCA2	No	3	0.017	n.a.
	Yes	31	0.124			Yes	5	0.144	
ATM	No	17	4.811	.548	ATM	No	3	9.397	n.a.
	Yes	31	4.483			Yes	5	4.638	
Without neoadjuvant					Adjuvant + neoadjuvant				
RAD51	**No**	**10**	**0.405**	**.018**	RAD51	No	14	0.974	.096
	Yes	**25**	**1.855**	Yes		26	1.394		
ATR	No	10	0.823	.596	ATR	No	14	0.539	.992
	Yes	25	0.693	Yes		26	0.647		
BRCA1	No	10	0.520	.674	BRCA1	No	14	0.401	.535
	Yes	25	0.517			Yes	26	0.238	
BRCA2	No	10	0.048	.083	BRCA2	No	14	0.130	.968
	Yes	25	0.142			Yes	26	0.100	
ATM	No	10	4.179	.289	ATM	No	14	3.272	.218
	Yes	25	6.782			Yes	26	4.450	

Bold indicates statistical significance.

Specifically in gastric cancer, our previous published data have shown that immunohistochemically negative cases for nuclear RAD51 had a worse prognosis, a tendency of lymphatic dissemination, and a larger mean tumor size.[11] These cases, however, responded better to chemoradiotherapy.[11,15]

However, in contrast with those previous immunohistochemical findings, in this study, *RAD51* mRNA levels were unexpectedly higher in cases with perineural invasion than in other cases, as well as in the tumors of patients who had died. This may indicate a possible complex role of RAD51 in the pathogenesis of gastric cancer.

Despite this correlation between *RAD51* and the occurrence of death, and although the survival of cases with high expression appears to be lower in the Kaplan-Meier curve, we were unable to demonstrate a statistically significant association with overall or disease-free survival. This may be due to the small number of patients evaluated in this study. It is necessary to consider that a hypothetical association between *RAD51* mRNA and lower survival could be due to resistance to chemoradiotherapy. Our number of untreated patients was too small to allow for reliable statistical analysis.

We also need to consider the possibility of an actual absence of correlation between the nuclear presence of the protein and the amount of mRNA. In fact, we noticed that *RAD51* mRNA even had a slight negative correlation with the nuclear H-score and a positive correlation with the cytoplasmic H-score, both of which were not statistically significant. Nuclear localization of RAD51 depends on a complex cytoplasmic-nuclear transport mechanism mediated by BRCA2 and RAD51C.[35] Similar to ATR expression, RAD51 protein synthesis may be suppressed by post-transcriptional regulation, as demonstrated by Huang et al[36] in neoplastic cell cultures in which miR-103 and miR-107 inhibited RAD51 expression and the occurrence of homologous recombination. In addition, the microRNAs miR-182, miR-221, miR-34a, and miR-766 inhibit the synthesis of the RAD51 protein.[37] Additionally, RAD51 is ubiquitinated and undergoes degradation by FBH1, FBXO5, and RFWD3.[38]

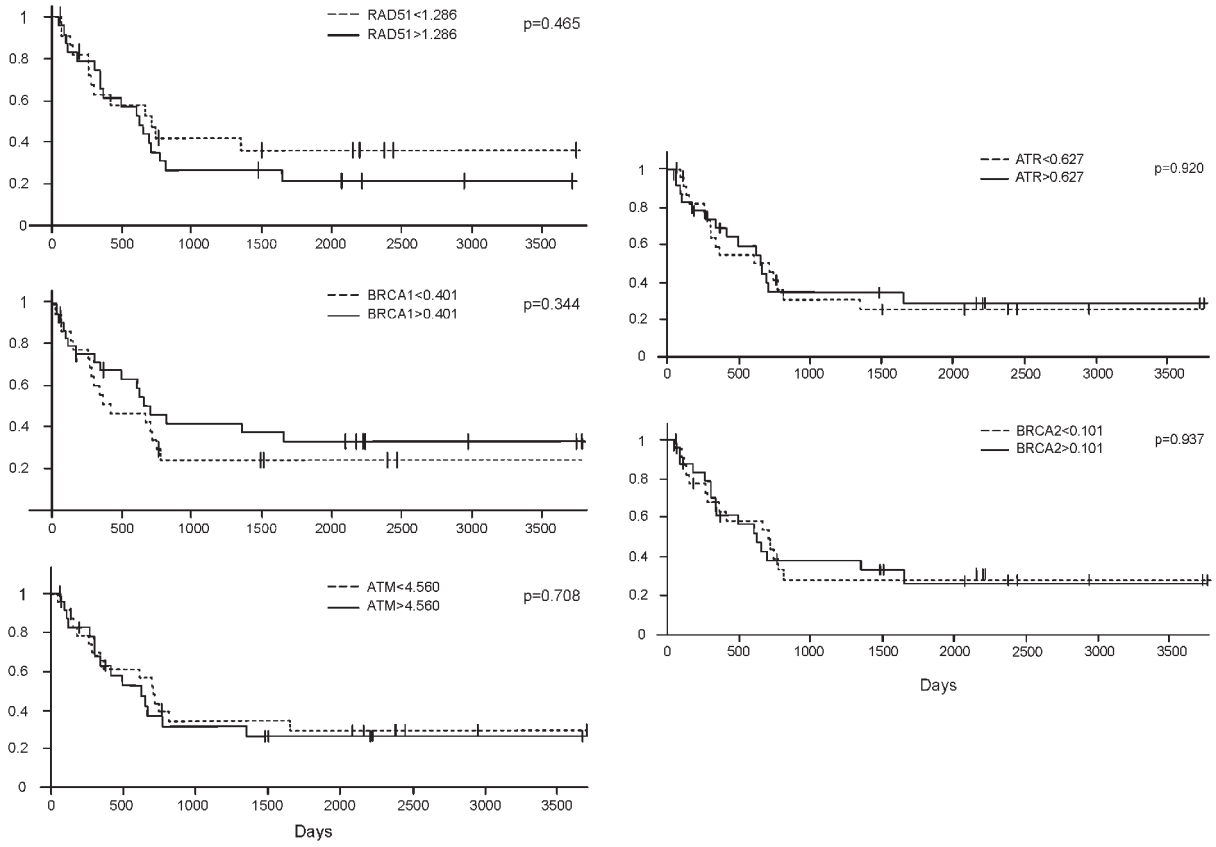

Figure 2. Kaplan-Meier curves comparing the overall survival of the 24 patients with tumor mRNA expression above the median with that of the 24 below the median, for *RAD51*, *ATR*, *BRCA1*, *BRCA2*, and *ATM*.

In contrast to our finding, Redati et al[37] noted that high levels of RAD51 mRNA are associated with greater survival in gastric adenocarcinoma and esophageal squamous cell carcinoma, but with a worse prognosis in hepatocellular carcinoma and esophageal, breast, lung, and gastroesophageal junction adenocarcinomas.

The lack of a significant association between immunohistochemistry and mRNA expression, as observed for *RAD51*, *ATM*, *BRCA1*, and *BRCA2*, may also be due to the semiquantitative and subjective nature of the immunohistochemistry analysis, especially when staining intensity is used to calculate the *H*-score, and to its qualitative and dualistic character when only negative or positive categories are used. Kim et al[13] similarly did not observe a correlation between immunohistochemical results and *BRCA1* or *BRCA2* mRNA expression in gastric cancer.

Previous immunohistochemical studies suggest that high nuclear expression[11,14] or low cytoplasmic expression[12,13] of BRCA2 and low expression of ATM[11,16-18] are characteristics of more advanced gastric tumors and are associated with shorter survival. In the present study, however, we did not observe a correlation between *ATM* or *BRCA2* mRNA levels and clinicopathological parameters or with immunohistochemistry. Likewise, Kim et al[13] did not notice any association between *BRCA2* mRNA levels and clinicopathological characteristics.

BRCA1 promoter methylation was a frequent finding in the work by Bernal et al,[39] who evaluated diffuse-type adenocarcinomas. We detected a high proportion of cases with complete or partial methylation of *BRCA1*, *BRCA2*, and *RAD51*, which suggests that this mechanism participates in regulating the expression of these genes in gastric cancer, but not of *ATM* and *ATR*, where promoter methylation was not detected. In contrast, *ATM* promoter hypermethylation has been observed in breast cancer, gliomas, gastric lymphoma, and colorectal tumors.[40]

Additional findings of this work were the positive weak to moderate correlations between tumor mRNA expression levels of *RAD51* with *ATR*, *BRCA1*, and *BRCA2*; of *ATR* with *BRCA1* and *ATM*; and of *BRCA1* with *BRCA2*. This suggests that there may be a common control of the mRNA synthesis of such genes, possibly occurring in response to DNA damage.

In summary, our findings suggest that alterations in *BRCA1* and *RAD51* mRNA expression play a role in the progression of gastric adenocarcinoma, although, in the case of *RAD51*, there seems to be a discrepancy between the clinical significance of mRNA expression and that of the previously described nuclear immunohistochemical positivity of the protein. Additional studies are needed to define whether there would be prognostic significance in a larger population and to elucidate the mechanisms that influence the relationship between protein and mRNA expression.

The main limitation of this study is the small number of patients analyzed, which did not allow for a definitive assessment of the possible prognostic implications of *RAD51* and *BRCA1*, especially if we consider that they were subjected to different therapeutic modalities. Furthermore, the number of cases in which it was possible to analyze paired tumor and non-tumor expression was limited. Other limitations were the qualitative nature of the method used to evaluate gene methylation, and the possible influence of tumor heterogeneity in the attempt to correlate immunohistochemistry and mRNA levels, as the samples were from different areas of the tumor.

Conclusions

RAD51 mRNA high expression in gastric adenocarcinoma was associated with perineural invasion and death. *BRCA1* mRNA was reduced in tumors compared with non-neoplastic mucosa and, excluding neoadjuvant therapy cases, in deeper infiltrating tumors. Methylation of the *RAD51*, *BRCA1*, and *BRCA2* promoters was highly frequent, whereas that of *ATM* and *ATR* was not detected. ATR nuclear immunohistochemical positivity and mRNA levels were inversely correlated, whereas there was no significant mRNA and immunohistochemistry correlation for the other markers.

Notation

List of Abbreviations

ACTB beta-actin gene
ATM ataxia-telangiectasia mutated
ATR ataxia-telangiectasia and Rad3-related
BRCA1 breast cancer 1
BRCA2 breast cancer 2
B2M beta-2 microglobulin gene
cDNA complementary DNA
Ct cycle threshold
DEPC diethyl pyrocarbonate
EBPβ CCAAT-enhancer-binding protein beta
ECX epirubicin, cisplatin, and capecitabine
FBH1 F-box DNA helicase 1
FBXO5 F-box only protein 5
FBXO32 F-box only protein 32
HCFMRP-USP Clinical Hospital of the Ribeirão Preto Medical School at the University of São Paulo
HE hematoxylin and eosin
H-score histochemical score
miR microRNA
mRNA messenger RNA
MS-HRM methylation-sensitive high-resolution melting
RAD51 radiation-sensitive protein 51
RFWD3 ring finger and WD repeat domain 3
ROC receiver operating characteristic
RT-qPCR reverse transcription-quantitative polymerase chain reaction

Declarations

Ethics approval and consent to participate

The study was approved by the HCFMRP-USP Research Ethics Committee (approval No. 12349/17). Written informed consent was obtained from each participant.

Consent for publication

Not applicable, as the dataset lacks patient images, sensitive information, or any data allowing the identification of patients. It exclusively encompasses the statistical descriptions of medical records and experimental results of groups of patients.

Author contributions

Joel Del Bel Pádua: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing—original draft; Writing—review & editing. Carolline Fontes Alves Mariano: Investigation; Writing—review & editing. Alexandre Todorovic Fabro: Conceptualization; Methodology; Writing—review & editing. Fermino Sanches Lizarte Neto: Formal analysis; Investigation; Methodology; Resources; Software; Validation; Visualization; Writing—review & editing. Rogério Lenotti Zuliani: Investigation; Methodology; Writing—review & editing. Cláudia Tarcila Gomes Sares: Investigation; Methodology; Resources; Software; Validation; Writing—review & editing. José Sebastião dos Santos: Conceptualization; Funding acquisition; Methodology; Writing—review & editing. Ajith Kumar Sankarankutty: Conceptualization; Data curation; Methodology; Writing—review & editing. Daniela Pretti da Cunha Tirapelli: Conceptualization; Methodology; Resources; Writing—review & editing. Vanessa da Silva Silveira: Conceptualization; Methodology; Writing—review & editing. Greice Andreotti de Molfetta: Investigation; Methodology; Resources; Software; Validation; Visualization; Writing—review & editing. Wilson Araújo da Silva Júnior: Conceptualization; Data curation; Methodology; Resources; Writing—review & editing. Mariângela Ottoboni Brunaldi: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing—review & editing.

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Availability of data and materials

The datasets generated in this study are not publicly available, but they may be obtained from the corresponding author upon reasonable request.

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