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The intricate interplay between MSI and polymorphisms of DNA repair enzymes in gastric cancer *H.pylori* associated

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Abstract

Gastric cancer is the fourth most common type of cancer worldwide. *Helicobacter pylori* is a well-established risk factor and may cause injuries to genomic integrity through an inefficient DNA repair. This study aimed to examine the influence of polymorphisms in DNA repair enzymes using markers for microsatellite instability (MSI). Polymorphisms of DNA repair enzymes were detected by PCR-RFLP and MSI, by high resolution melt (HRM) analysis. *Helicobacter pylori* detection and genotyping were accomplished by PCR. MSI was observed in 47.5% of the cases and it was associated with the ERCC1 polymorphic allele, whereas MSI-H was associated with the XRCC3 heterozygous genotype. MSI was more frequent in intestinal gastric cancer (IGC), where it was associated with ERCC1 or RAD51 polymorphic alleles. Also, MSI-H was associated with the XRCC3 heterozygous. In diffuse gastric cancer (DGC), almost all of MGMT polymorphic genotype carriers showed MSI. *Helicobacter pylori* was positive in 94% of the cases and the most virulent strains were associated with MSI, mainly MSI-H. When the subtypes were considered, these associations were found only in the IGC and associated with more virulent strains. Among the cases with microsatellite instability, IGC showed a correlation between the XPD wild-type and the ERCC1 polymorphic allele, and all of them were infected by the most virulent strains. On the other hand, in DGC, the XPD polymorphic allele was correlated with the XRCC3 wild-type with no prevalence of *H.pylori* virulence. Our data demonstrated that polymorphisms in repair enzymes can interfere with the efficiency of the repair process, but it differs depending on the histological subtype and *H.pylori* involvement. Besides nucleotide excision repair, base excision repair and mismatch repair pathway, the homologous recombination are also involved.

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer deaths worldwide. Adenocarcinomas are the most frequent type of GC, and they are histologically classified as intestinal and diffuse subtypes, according to Lauren (1965)'s classification (1). *Helicobacter pylori* (*H.pylori*) infection is a major risk for GC development, especially concerning the intestinal subtype. It is interesting to point out that this bacterium has co-evolved with humans for at least 50 000 years, indicating a bacteria–host balance (2). Therefore, for cancer development, this balance has to be changed. The presence of bacterial virulence genes seems to be an important factor, since *vac*A (vacuolating cytotoxin A) and *cag*A (cytotoxin-associated gene A), located on the pathogenicity island (*cag*-PAI) are associated with more severe gastric lesions. The *cag* pathogenicity island (*cag*-PAI), a genetic element inserted into the genome of *H.pylori*, is one of the major virulence determinants of this bacteria. The cag-PAI integrity was determinated identifying the genes: *cag*E (cytotoxin-associated gene E), considered, like *cag*A, a marker for the right side of the island and *cag*-PAI integrity, and *vir*B11, located on the left side of the island. Both *cag*E and *virb*11 are present at a high frequency in *H.pylori* presented in GC (3–6). On the host side, gene polymorphisms related to DNA repair could be relevant, since chronic inflammation, a consequence of *H.pylori* infection, can cause DNA oxidative damage in the host cells by accumulation of reactive oxygen species (7–9).

Base excision repair (BER) and nucleotide excision repair (NER) are the two DNA repair pathways believed to orchestrate the removal of oxidative lesions (10). However, recent findings suggest that the mismatch repair pathway (MMR) may also be important for the response to oxidative DNA damage (11). In addition, the enzymes from the other DNA repair pathways, such as direct reversal repair (DRR) and double-strand break repair (DSBR) could work in an integrated way (10).

Both BER and NER pathways identify and excise DNA lesions before polymerase and ligase activities fill the resultant gap. The primary role of the MMR pathway is the repair of base–base mismatches and insertion/deletion loops (11). Additionally, other DNA repair pathways are important for genome integrity. Double-strand breaks (DSB), which are common and very severe lesions, have two major routes where non-homologous DNA end-joining (NHEJ) recombination has been suggested to be the canonical route, whereas homologous recombination (HR) is believed to be the alternative route (12). However, some DNA lesions can be repaired by DRR, such as those caused by alkylating agents. The addition of methyl/ethyl groups at the O6 position of the guanine ring, catalyzed by methyl-(ethyl)-transferases, are reversed by DRR (13,14).

Although these repair systems are effective, polymorphisms in key enzymes that influence their efficiency could be relevant for maintenance of genome integrity. In GC, the association of the bacterial genotype with inefficient repair caused by repair enzyme polymorphisms can lead to genetic instability. Genomic instability can be inferred by microsatellite instability (MSI), characterized by expansions or contractions of microsatellite regions. Microsatellites are short sequences of tandem repeats, 1–4 bp in length, dispersed throughout the mammalian genome (15,16). The Bethesda Panel recommends the use of five microsatellite sequences to investigate instability. However, other studies have shown that BAT25 and BAT26 loci are the two stable loci in humans that are considered reliable markers for the identification of instability (17–20).

Identifying the host repair profile that leads to genetic instability and also the influence of the bacterial stream on this process seems to be relevant to understanding GC development. Therefore, this study aimed to determine by MSI if polymorphisms in the key repair enzyme pathways BER (*XRCC*1 G28152A), NER (*XPD* A35931C and *ERCC*1 C354T), DRR (*MGMT* A533G), HR (*RAD*51-135G>C and *XRCC*3 C18067T), and MMR (*MLH*1-93G>A) interferes with repair efficiency. In addition, the virulence of *H.pylori* strains was considered as well as histological subtype, age and gender.

Materials and methods

Patients and specimens

This study was approved by the ethics committee of the General Hospital of Fortaleza under the protocol number 071002/10. A total of 120 surgically resected adenocarcinoma specimens were obtained from three public hospitals in Fortaleza, Ceará, Brazil: Santa Casa de Misericórdia Hospital, Cesar Cals General Hospital and General Hospital of Fortaleza. The patients did not undergo prior chemotherapy or radiotherapy to ensure that there was no bias in the MSI analysis. Fragments of tumor were collected during gastrectomy and frozen at –80°C. Histological diagnosis and tumor classification were based on Lauren's criteria.

DNA extraction, *H.pylori* detection and genotyping

Genomic DNA was extracted from frozen tumor tissue samples mainly consisting of tumor cells (>80%), using the cetyltrimethyl ammonium bromide (CTAB) method adapted from Foster and Twell (21). *Helicobacter pylori* infection was detected by amplification of the *urease*C gene and virulence genes (*vac*A, *cag*A, *cag*E and *vir*B11) were identified using specific primers and conditions, as previously described by Lage, Domingo, Atherton and Sozzi (22–25). Negative (water) and positive controls were assayed in each run. PCR products were separated on 6% polyacrylamide electrophoretic gels, which were then silver stained.

DNA repair polymorphism

Single nucleotide polymorphisms (SNPs) for DNA repair genes were determined by the PCR–RFLP based method as described by Yin, Crosbie, Krupa and Chen (26–29). Negative (water) control was assayed in each run. The amplified fragments were visualized in 1% agarose gels containing ethidium bromide under UV light and were digested with appropriate restriction endonucleases: *Msp*I for *XRCC*1, *Stu*I for *MGMT*, *Pst*I for *XPD/ERCC*2, *Bsrd*I for *ERCC*1, *Mva*I for *RAD*51, *Nco*I for *XRCC*3 and *Pvu*II for *MLH*1. The fragments were resolved by 6 or 8% polyacrylamide gel electrophoresis under non-denaturing conditions and silver staining. Randomly selected samples were re-genotyped (10% of samples).

Microsatellite instability analysis

All samples were analyzed for four markers (BAT-25, BAT-26, D5S346 and D2S123). MSI analysis was performed by the HRM device 7500 fast real-time PCR System (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 µl using 10 µl MeltDoctor™ HRM Master Mix (Applied Biosystems) and 5 µM of specific primers for each gene and 20 ng/µl DNA. The amplification cycle was: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C for annealing and extension. The dissociation consisted of 10 s at 95°C, 1 min at 60°C for primer annealing, 15 s at 95°C with 1% ramp to capture the HRM and 15 s at a temperature of 60°C for reannealing primers. All reactions were performed in triplicate. The melting curve was normalized by calculating the line of best fit between the two regions standardization, before and after the fall of

fluorescence, representing the melting of the PCR product, using the 7500 software v2.0. This algorithm allows the direct comparison between samples that have different starting levels of fluorescence. Tumors were categorized in MSS (Stable) when any of the markers showed instability, MSI-L (Low) when only one locus was unstable and MSI-H (High) when two or more loci were unstable.

Statistical analysis

Data were statistically analyzed using the SPSS® 17.0 (SPSS, Chicago, IL, USA) and Epiinfo™ 7 version statistical software programs, using the χ^2, Spearman test and Fisher exact tests, and $P < 0.05$ was considered statistically significant.

Results

Sample characterization

Instability was detected up to 120 cases. For BAT25, BAT26, D5S346 and D2S123, MSI was observed in 12.93% (15/116), 13.55% (16/118), 20.33% (24/118) and 23.93% (28/117), respectively. Considering at least one marker as unstable, MSI (L+H) was observed in 47.5% (57/120) of the cases. MSI-L and MSI-H were observed in 29.16% (35/120) and 18.33% (22/120), respectively. Lauren classification could be obtained in 117 cases. The intestinal subtype was slightly more frequent than the diffuse subtype (61/117, 52.13%), and there were more males than females (77/120, 64.16%). The patients' median age was 65 years old (ranging from 23 to 92 years old). No significant differences were observed between MSI and gender, tumor stage, lymph node involvement, metastasis or age, even considering different cutoff points (50, 55 and 60 years old).

Almost all samples were positive for *H.pylori* [93.27% (111/119)]. In the tumors with MSI-H, the patients were infected with *vac*As1 strains ($P = 0.03$) (Table 1). The *H.pylori* strain was categorized considering the presence of the *vac*As1 allele and the *cag*-PAI island gene as *more virulent* (the *vac*As1 allele and at least one gene of the *cag*-PAI - *cag*A, *cag*E or *vir*B11) or as *less virulent* (*vac*As1 allele but without *cag*-PAI genes or *vac*As2 allele strains regardless of the presence of *cag*-PAI genes). Considering this classification, it was observed that the *most virulent* strains were significantly associated with MSI ($P = 0.04$), mainly with MSI-H cases ($P = 0.009$) when compared to MSS cases.

MSI and polymorphisms of DNA repair enzymes

The presence of instability was significantly associated with the *ERCC*1 polymorphic allele ($P = 0.009$) (Table 2). The logistic regression test showed the prediction of *ERCC1* polymorphic allele carriers with MSI ($P = 0.001$ OR = 4.056 Date not shown). Among the unstable cases (H versus L), the *XRCC*3 polymorphic allele (CT and CT + TT) was associated with MSI-H ($P = 0.004$ and 0.01, respectively). According to these results, these enzymes were associated. Accordingly, it was observed that the patients carrying the *ERCC*1 polymorphic allele (CT + TT) and the *XRCC*3 polymorphic allele (CT + TT) were significantly associated with instability ($P = 0.04$), especially MSI-H ($P = 0.03$).

Considering the histological subtypes, MSI was more frequent ($P = 0.02$) in the intestinal (55%; 34/61) than in the diffuse (35%; 20/56) subtype, with a prevalence of MSI-H ($P = 0.01$). Taking the repair enzyme polymorphisms in the intestinal subtype (Table 3) into account, MSI was significantly associated with *ERCC1* ($P = 0.004$ and *RAD51* (p=0.01) polymorphic allele carriers. Also, MSI-H was significantly associated with *XRCC*3 (CT) heterozygous carriers, in contrast to the wild-type carriers, which was more associated with

Table 1. MSI cases distributed according to the genes and virulence of *H.pylori N*(%)

	*ure*C-	*ure*C+	*P*	*cag*A-	*cag*A+	*P*	*vac*As2	*vac*As1	*P*	*vac*Am2	*vac*Am1	*P*	*cag*E-	*cag*E+	*P*	*vir*B11-	*vir*B11+	*P*	Less Virulents	More Virulents	*P*
S[a]	6 (8)	57 (92)		23 (40)	34 (60)		10 (17)	47 (82)		39 (69)	17 (30)		27 (47)	30 (52)		26 (45)	31 (54)		20 (35)	37 (65)	
MSI-L[a]	1 (2.8)	34 (97.2)	0.21	12 (35)	22 (65)	0.6	6 (17)	28 (82)	0.9	25 (75)	8(24)	0.53	18 (53)	16 (47)	0.6	12 (35)	22 (64)	0.3	9 (26)	25 (74)	0.39
MSI-H[a]	1 (4.7)	20 (95.3)	0.49	5 (25)	15 (75)	0.2	0 (0)	20 (100)	0.03	13 (65)	7(35)	0.7	9 (45)	11 (55)	0.8	7 (35)	13 (65)	0.4	1 (5)	19 (95)	0.009

MSI-L → low instability, MSI-H → high instability.
[a]MSS → stable.

Table 2. Genotypes of repair enzymes distributed according to MSI cases

	MSI-H[a] *N* (%)	MSI-L[a] *N* (%)	MSS[a] *N* (%)	*P* value *HxL*	*P* value *HxS*	*P* value *H+L X S*	Total
RAD51							
GG	17 (77)	25 (71)	54 (86)				96 (80)
GA	4 (18)	5 (14)	6 (10)	0.82	0.27	0.24	15 (12.5)
AA	1 (5)	5 (14)	3 (5)	0.25	0.96	0.18	9 (7.5)
GA+AA	5	10	9	0.62	0.35	0.09	120
XRCC3							
CC	8 (36)	24 (69)	32 (51)				64 (53)
CT	14 (64)	8 (23)	26 (41)	0.004	0.13	0.66	48 (40)
TT	0 (0)	3 (9)	5 (8)	0.32	0.27	0.5	8 (6.6)
CT+TT	14	11	31	0.01	0.24	0.5	120
MLH1							
GG	7 (32)	11 (31)	21(33)				39 (32.5)
GA	13 (59)	21 (60)	37 (59)	0.96	0.92	0.86	71 (59)
AA	2 (9)	3 (9)	5 (8)	0.96	0.84	0.82	10 (8.3)
GA+AA	15	24	42	0.97	0.89	0.83	120
MGMT							
AA	15 (68)	29 (83)	49 (78)				93 (77.5)
AG	5 (23)	3 (9)	10 (16)	0.12	0.42	0.82	18 (15)
GG	2 (9)	3 (9)	4 (6)	0.79	0.58	0.63	9 (7.5)
AG+GG	7	6	14	0.19	0.36	0.93	120
XRCC1							
GG	11 (50)	21 (60)	34 (54)				66 (55)
GA	7 (32)	9 (26)	22 (35)	0.52	0.97	0.52	38 (31)
AA	4 (18)	5 (14)	7 (11)	0.57	0.42	0.57	16 (13)
GA+AA	11	14	29	0.45	0.74	0.81	120
XPD							
AA	12 (55)	17 (49)	33 (52)				62 (51)
AC	6 (27)	12 (34)	23 (37)	0.58	0.55	0.77	41 (34)
CC	4 (18)	6 (17)	7 (11)	0.93	0.52	0.37	17 (14)
AC+CC	10	18	30	0.66	0.86	0.86	120
ERCC1							
CC	7 (32)	13 (37)	40 (66)				60 (50)
CT	9 (41)	13 (37)	12 (20)	0.69	0.006	0.003	34 (28)
TT	6 (27)	9 (26)	9 (15)	0.76	0.037	0.014	24 (20)
CT+TT	15	22	21	0.68	0.006	0.0009	118

MSI-L → low instability, MSI-H → high instability.
[a]MSS → stable.

MSS (P = 0.04). In the diffuse subtype, despite the low frequency of *MGMT* polymorphic genotype (GG) carriers, almost all of them (80%; 4/5) were unstable when compared to wild-type (30.2%; 13/43) (P = 0.046).

MSI, polymorphisms of DNA repair enzymes and *H.pylori*

Genomic stability status was analyzed according to *H.pylori* virulence and patient genotype (Date not shown). From these analyses, patients carrying the *ERCC1* polymorphic allele (CT + TT) and infected with the most virulent strains were statistically associated with MSI (P = 0.001; 31/44 cases, 70.4%). Additionally, among the MSI cases, patients with the heterozygous genotype *XRCC3* (CT) and infected with the most virulent strains were associated with MSI-H (P = 0.02; 12/19 63.1%).

Taking the group of patients with the intestinal subtype and infected with the most virulent strains into account, there was a tendency for an association between MSI and the *RAD51* polymorphic allele (GC + CC) (P = 0.05). MSI was also associated with the *ERCC1* polymorphic allele (CT + TT) (P = 0.005; 21/29 cases, 72.4%), highlighting the significant frequency of MSI-H when compared to MSS (P = 0.03). Correlation analysis in unstable cases showed a negative correlation between the presence of the wild-type *XPD* genotype (AA) and the *ERCC1* polymorphic allele (CT + TT) (r = –0.388; P = 0.023). Despite the small number of cases (N = 8), all of them were infected with the most virulent strains (data not shown).

No difference was observed considering the less virulent strains or the diffuse subtype, but in the correlation analysis considering only unstable cases, it was observed that the *XPD* polymorphic allele (AC + CC) was correlated with the *XRCC3* wild-type genotype (CC) (r = –0468, P = 0.038) in the diffuse subtype, but with no association with *H.pylori* virulence in these nine cases.

Discussion

No study was found associating MSI, polymorphisms in repair enzymes and presence of *H.pylori*. In fact, the relation between polymorphisms in DNA repair enzymes and MSI in cancer is restricted to *MLH1*-93G>A and colorectal cancer (30–32). Only one study from Zhu *et al.* (33) approached some of the polymorphisms in mismatch repair genes with risk of MSI in GC. Thus, the present study investigated MSI considering the genotypic profile of patients for

Table 3. Genotype frequency of repair enzymes according to histological subtype and instability level *N*(%)

	Intestinal (61)							Diffuse (56)						
	MS				*P* value			MS				*P* value		
	S	L	H	L+H	*S* versus *L*	*S* versus *H*	*S X* (L+H)	S	L	H	*L+H*	*S* versus *L*	*S* versus *H*	*S X* (L+H)
RAD51														
GG	25 (96.1)	14 (73.6)	11 (68.7)	25 (71.4)				28 (77.7)	11 (73.3)	5 (100)	16 (80)			
GC	1 (3.84)	2 (10.5)	4 (25)	6 (17.1)	0.32	0.031	0.07	5 (13.8)	3 (20)	0 (0)	3 (15)	0.44	0.47	0.62
CC	0 (0)	3 (15.7)	1 (6.25)	4 (11.4)	0.05	0.14	0.05	3 (8.3)	1 (6.6)	0 (0)	1 (5)	0.89	0.46	0.64
GC+CC	1 (3.84)	5 (26.3)	5 (31.5)	10 28.5)	0.02	0.01	0.01	8 (22.2)	4 (26.6)	0 (0)	4 (20)	0.45	0.19	0.45
Total	26	19	16	35				36	15	5	20			
XRCC3														
CC	16 (61.5)	12 (63.1)	6 (37.5)	18 (51.4)				16 (44.4)	11 (73.3)	2 (40)	13 (65)			
CT	8 (30.7)	5 (26.3)	10 (62.5)	15 (42.8)	0.79	0.04	0.35	17 (47.2)	3 (20)	3 (60)	6 (30)	0.05	0.55	0.16
TT	2 (7.6)	2 (10.5)	0 (0)	2 (5.7)	0.78	0.39	0.91	3 (8.3)	1 (6.6)	0 (0)	1 (5)	0.49	0.72	0.16
CT+TT	10 (38.4)	7 (36.8)	10 (62.5)	17 (48.5)	0.91	0.12	0.43	20 (55.5)	4 (26.6)	3 (60)	7 (35)	0.05	0.61	0.14
Total	26	19	16	35				36	15	5	20			
MLH1														
GG	12 (46.1)	12 (63.1)	10 (62.5)	22 (62.8)				2 (40)	9 (60)	24 (66.6)	33 (64.7)			
GA	13 (50)	7 (36.8)	4 (25)	11 (31.4)	0.31	0.15	0.15	3 (60)	4 (26.6)	8 (22.2)	12 (23.5)	0.27	0.14	0.15
AA	1 (3.8)	0 (0)	2 (12.5)	2 (5.7)	0.32	0.49	0.94	0 (0)	2 (13.3)	4 (11.1)	6 (11.7)	0.7	0.74	0.72
GA+AA	14 (53.8)	7 (36.8)	6 (37.5)	13 (37.1)	0.25	0.30	0.19	3 (60)	6 (40)	12 (33.3)	18 (35.2)	0.39	0.24	0.26
Total	26	19	16	35				5	15	36	51			
MGMT														
AA	18 (69.2)	17 (89.4)	12 (75)	29 (82.8)				30 (83.3)	11 (73.3)	2 (40)	13 (65)			
AG	5 (19.2)	2 (10.5)	3 (18.7)	5 (14.2)	0.29	0.89	0.49	5 (13.8)	1 (6.6)	2 (40)	3 (15)	0.51	0.14	0.48
GG	3 (11.5)	0 (0)	1 (6.25)	1 (2.8)	0.15	0.56	0.15	1 (2.7)	3 (20)	1 (20)	4 (20)	0.08	0.17	0.046
AG+GG	8 (30.7)	2 (10.5)	4 (25)	6 (17.1)	0.10	0.68	0.21	6 (16.6)	4 (26.6)	3 (60)	7 (35)	0.32	0.06	0.11
Total	26	19	16	35				36	15	5	20			
XRCC1														
GG	13 (50)	12 (63.1)	8 (50)	20 (57.1)				20 (55.5)	9 (60)	3 (60)	12 (60)			
GA	10 (38.4)	4 (21)	6 (37.5)	10 (28.5)	0.23	0.97	0.45	12 (33.3)	4 (26.6)	1 (20)	5 (25)	0.47	0.54	0.57
AA	3 (11.5)	3 (15.7)	2 (12.5)	5 (14.2)	0.64	0.93	0.92	4 (38.8)	2 (13.3)	1 (20)	3 (15)	0.62	0.56	0.55
GA+AA	13 (50)	7 (36.8)	8 (50)	15 (42.8)	0.38	1.00	0.57	16 (44.4)	6 (40)	2 (40)	8 (40)	0.77	0.61	0.74
Total	26	19	16	35				36	15	5	20			
XPD														
AA	14 (53.8)	10 (52.6)	9 (56.2)	19 (54.2)				18 (50)	6 (40)	3 (60)	9 (45)			
AC	9 (34.6)	8 (42.1)	4 (25)	12 (34.2)	0.73	0.61	0.97	14 (38.8)	4 (26.6)	2 (40)	6 (30)	0.56	0.63	0.8
CC	3 (11.5)	1 (5.2)	3 (18.7)	4 (11.4)	0.48	0.63	0.98	4 (11.1)	5 (33.3)	0 (0)	5 (25)	0.10	0.57	0.21
AC+CC	12 (46.1)	9 (47.3)	7 (43.7)	16 (45.7)	0.93	0.87	0.97	18 (50)	9 (60)	2 (40)	11 (55)	0.51	0.52	0.71
Total	26	19	16	35				36	15	5	20			
ERCC1														
CC	18 (69.2)	6 (31.5)	6 (37.5)	11 (31.4)				21 (61.7)	7 (46.6)	1 (20)	8 (40)			
CT	7 (26.9)	7 (36.8)	7 (43.7)	14 (40)	0.11	0.11	0.04	5 (14.7)	6 (40)	1 (20)	7 (35)	0.07	0.38	0.06
TT	1 (3.8)	6 (31.5)	3 (18.7)	9 (25.7)	0.006	0.04	0.004	8 (23.5)	2 (13.3)	3 (60)	5 (25)	0.55	0.09	0.48
CT+TT	8 (30.7)	13 (68.4)	10 (62.5)	23 (65.7)	0.012	0.04	0.004	13 (38.2)	8 (53.3)	4 (80)	12 (60)	0.32	0.1	0.12
Total	26	19	16	35				34	15	5	20			

seven enzymes from various repair pathways also associated with *H.pylori* virulence factors, taking the histological subtypes and age into account.

In GC, the frequency of MSI varies according to the number of markers studied. In this study, in addition to the mononucleotides BAT25 and BAT26, two more markers were analyzed, as suggested by Bethesda panel (D5S346 and D2S123) (34). The presence of MSI in 47.5% (57/120) of the cases is in accordance with the variation found in the literature, ranging from 25.2 to 58.3%, in which BAT25 and BAT26 were included in the analysis (35,36). MSI prevalence in the intestinal subtype is also reported in the literature (37). The lack of association between MSI and tumor progression and lymph node involvement found in this study was is in accordance with the studies by Hayden and Vauhkonen (38,39). In contrast to reports in the literature that there is a tendency of MSI to be more frequent in older patients (39), we found no association between MSI and age.

Besides the importance of *H.pylori* in gastric carcinogenesis, when it comes to MSI, this bacterium has been little explored. Due to its importance, the bacterial genes were explored in this study. In our analyses, only the *vac*As1 allele was associated with MSI. This allele is well established in GC risk (3,40), even in cases without the *cag*A gene (41). Its importance is probably due to its role in increasing the inflammatory response. In the carcinogenic process, the chronic inflammatory on host gastric epithelial cells leads to the DNA damage, epigenetic alterations, changes in gene expression and cell signaling pathways (42–44). Promoter hypermethylation of the *MLH1* gene is the main mechanism leading to MMR deficiency and MSI. However, our study highlights the polymorphism of repair enzymes as relevant for genomic instability as it is discussed below. Also, this finding corroborated the use of this gene for categorizing the strains as *more or less virulent*.

In the literature, MSI is associated with the MMR pathway. However, our data showed that *ERCC*1 (CT + TT) and *XRCC*3 (CT + TT) were associated with MSI and MSI-H, respectively, thus supporting the idea that the repair pathways act cooperatively and in an integrated manner. Indeed, ERCC1 is an enzyme involved preferentially in NER, but it can interact and contribute in other pathways, such as HR. The encoded protein forms a heterodimer with XPF endonuclease (also known as ERCC4), and the heterodimeric endonuclease catalyzes the 5′ incision in the process of excising the DNA lesion (45). Despite *ERCC*1 C354T being a synonymous polymorphism (Asn118Asn), the polymorphic allele has been related to risk for other cancers such as endometrial and lung cancer (26,46). Additionally, Figg (47) showed that patients in cancer treatment who carried the heterozygous or the polymorphic genotype had a longer survival time (progression-free or median overall).

On the other hand, the *XRCC*3 C18067T polymorphism (Thr241Met) stood out for the MSI-H phenotype. Despite the importance of XRCC3 in the repair of DSB through HR, little is known regarding its biochemical properties or specific function. It is known that XRCC3 directly interacts with RAD51 to form a stable complex, although the biological significance of this complex formation remains to be defined (48), using an animal model, showed that the biological activity of XRCC3 depends on its ability to bind and hydrolyze ATP. This step seems to be important for disrupting the *in vivo*.

RAD51-XRCC3 complex, whereas ATP hydrolysis by XRCC3 is required for a subsequent step in HR (49), using leukocyte DNA from healthy volunteers, showed that the presence of the *XRCC*3 C18067T polymorphism (Thr241Met) was significantly associated with higher DNA adduct levels. In addition, this polymorphism has been described as a risk factor for gastric (50).

As both enzymes were significantly associated with MSI, they were analyzed in combination. This analysis enabled us to assume that the presence of the *XRCC*3 (CT + TT) polymorphic allele was relevant for the MSI-H phenotype, even in combination with the *ERCC*1 wild-type (CC). Another study in GC (51) showed that the *XRCC*3 C18067T polymorphism was a relevant risk factor only in association with another *XRCC*3 polymorphism (26304C>T), which was not approached in the present study. In chronic gastritis, the risk was present when associated with *XRCC*1 399Gln (52). For lung cancer, the risk was present when associated with smoking (14).

Taking the presence of *H.pylori* into account, the most virulent strains were associated with MSI-H cases. This association corroborates the study by Machado (53), who showed that *H.pylori* interferes with the BER and MMR pathways, decreasing mRNA and protein expression, which could result in low activity of the MMR pathway and, consequently, the MSI phenotype. The compromise of MMR could explain the involvement of other DNA repair pathways and the association of polymorphisms of these enzymes with MSI status, observed in this study.

When the data were analyzed according to histological subtypes, it was observed that the association between *ERCC*1 and *XRCC*3 polymorphisms with MSI seen in the total samples was in fact due to the intestinal subtype. In this histological subtype, the instability associated with *RAD*51 polymorphism could explain the cases where the interaction between *ERCC*1 and *XRCC*3 polymorphisms was not present. RAD51 is a key enzyme with a crucial role in the HR pathway (54), in which it interacts with XRCC3 and cooperatively modulates replication fork progression on damaged chromosomes (28). Besides Poplawski (54), suggested that the *RAD*51-135G>C polymorphism would affect mRNA stability or translational efficiency, resulting in differential enzyme amount or function.

The intricate interplay between the repair enzymes was also observed by the correlation in MSI cases between the *XPD* wild-type and the *ERCC*1 polymorphic allele. It is known that ERCC1 forms a heterodimeric endonuclease with XPF, also involved in DNA repair of inter-strand crosslinks as well as in the repair of DSBs by HR where XPD protein with helicase function, along with other proteins, demarcates the lesion. Thus, the correlation between the *XPD* wild-type and the *ERCC*1 polymorphic allele suggests that MSI in these cases was a consequence of the DNA breaks resulting from normal *XPD* activity (wild-type) and that the next step would be compromised by the presence of polymorphisms in *ERCC*1. It was also interesting to observe that in both correlations, *ERCC*1 with *XRCC*3 and *XPD* with *ERCC*1, the presence of more virulent strains was observed, highlighting the host-bacteria interaction in the carcinogenesis process.

Unlike in the IGC, in diffuse tumors, only the *MGMT* homozygous polymorphic genotype was significantly associated with MSI but with a low frequency. Contradictory results are reported in the literature regarding this polymorphism. A study in UK (27) associated the polymorphism allele of *MGMT* Lys178Arg with a lower lung cancer risk, while others (55,56) showed in an experimental study with several other polymorphisms, that the polymorphic alleles seem to result in O-6 methylguanine (O-6MeG) repair with the same or similar efficiency as the wild type. However, they did not discard the possibility that they could affect the stability or processing of the protein or transcript in some way. Additionally, this polymorphism was associated with cervical cancer and lung cancer when combined with the polymorphism Ile143Val in the same gene (57–59). Regarding *H.pylori* strains, no association with *H.pylori*

virulence was found in this subtype. These results emphasize the apparently low influence of *H.pylori* on the diffuse pathway.

In summary, the data of this study indicated that in gastric tumors, polymorphisms in DNA repair enzymes can result in an instability phenotype, mainly in the intestinal subtype. Polymorphism of *ERCC*1 C354T and *XRCC*3 C18067T are especially relevant in the intestinal subtype, whereas in the diffuse subtype, the *MGMT* (A533G) polymorphism stood out. In addition, in the IGC, *H.pylori* infection with a more virulent strain appeared to contribute to this process, when associated with *ERCC*1 C354T and *XRCC*3 C18067T, demonstrating a bacteria-host relationship. This research has also emphasized the need for studies considering the histological subtypes to understand the etiology of GC, especially because most studies have failed to distinguish these subtypes. However, since analyzing the molecular and clinical characteristics of GC has been complicated by histological heterogeneity, another possibility was recently published from The Cancer Genome Atlas (TCGA) project. A molecular classification dividing GC into four subtypes was suggested: (i) tumors positive for Epstein–Barr virus, and the additional three subtypes that were associated with *H.pylori*: (ii) microsatellite unstable tumors, (iii) genomically stable tumors and (iv) tumors with chromosomal instability (60). This kind of classification corroborates the importance of the present study to provide a patient stratification.

Lastly, there is only one paper related to polymorphism of mismatch repair genes and MSI risk in GC recently published (33) providing evidence about this association, although the MLH1 polymorphism seemed mainly to decrease risk of MSI in GC, indicating the need to analyze the number of risk-genotypes in addition to further validation. In this context, considering that MSI in GC could be induced by several factors, when the intricate interplay between MSI and polymorphisms of DNA repair enzymes in GC *H.pylori*-associated is discussed, it is interesting to highlight that our work is a pioneer in making all of these associations, bringing valuable findings into this field.

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