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Low Virulence of *Helicobacter Pylori* and Gastric Cancer: the Contribution of Polymorphisms of *iNOS* and DNA Repair Enzymes

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Abstract

This study aimed to investigate the interaction between the *iNOS* C >T polymorphism, the genotype of *H. pylori* strains and polymorphisms in DNA repair genes (*OGG-1, APE-1 and PARP-1*) in a set of gastric cancer patients. In our methods, polymorphism was assessed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) and *H. pylori* detection/genotyping by PCR. A significant result shows this *iNOS* polymorphism more frequent among young gastric cancer patients than older patients (p= 0.020). Host genetic features, such as DNA repair enzymes (*OGG-1, APE-1, PARP-1*), are also important in preventing the malignancy process. Polymorphisms present in these enzymes associated with iNOS activity could lead to gastric cancer even in the presence of low virulent *H. pylori* strains. Within our results, *iNOS* homozygous wild-type (CC) genotype and *APE-1* polymorphic allele (TG+GG) group were more infected by *H. pylori* low-virulent strains (p=0.021). In Conclusion, our study indicates the importance of *H. pylori* and host DNA repair enzymes genotypes in gastric carcinogenesis in interection with this specific *iNOS* polymorphism.

Keywords: Gastric cancer; *Helicobacter pylori*; *PARP-1; APE-1; OGG-1*

Introduction

Gastric cancer is the fifth most common cancer and the second leading cause of cancer-related mortality in the world [1]. In Brazil, it is an important cause of cancer-related death in patients, with a high prevalence in the Northeast region [2]. According to Lauren's classification, the histological subtypes, intestinal and diffuse, show distinct histological and epidemiological features, as well as a different prognosis [3,4]. Furthermore, this tumor can be located in the proximal stomach (cardia), or distal (antrum, non-cardia). Differences between tumors located in the cardia or non-cardia region, as well as intestinal or diffuse subtypes, suggests that they represent distinct diseases with different etiologies. Gastric carcinogenesis is a multifactorial process, and *Helicobacter pylori is* the main initiator of inflammation and atrophic changes in the gastric mucosa [5]. The association between chronic *H. pylori* infection and the development of gastric cancer is well established [6]. It is known that both bacterial virulence and host genetic susceptibility are associated with cancer risk [7].

H. pylori has a great genetic diversity, and virulence factors play important roles in mucosal injury, especially the genes *cag*A (*cytotoxin associated gene A)* and *vacA*, (*vacuolating cytotoxin A),* more specifically *vacA*s1m1. *cag*A is involved in many host cell alterations and tightly associated with gastric cancer risk [8,9]. The *vac*A gene is present in essentially all *H. pylori* strains. VacA is a potent toxin, where it induces the formation of vacuoles in host cells. Additionally, the *cag*E and *vir*B11 genes have been found at a relevant frequency in gastric cancer patients [10]. The chronic inflammatory process in the presence of *H. pylor*i increases the expression of iNOS and can generate large amounts of reactive oxygen species (ROS), which could lead to cell injury, with large amounts of NO leading to DNA lesions [11,12]. The DNA damage caused by ROS can lead to gene alterations and, therefore, requires continuous DNA repair.

Polymorphism in the *iNOS* gene that leads to increased expression or altered function of the enzyme could affect the level of the DNA lesions and therefore increase DNA damage. In this context, Daff et al. [13], using a bacterial culture, found a deletion located six amino acids from the currently studied C>T polymorphism in exon 16. The proximity between this deletion and C>T polymorphism has been suggested by Jing Shen et al. [14] and Jesper Johannesen et al. [15] to account for the increase in iNOS activity. The increased activity is associated with several types of diseases, such as bladder cancer [16,*17],* and diabetes [18], besides gastric cancer [14].

Recently, host genetic susceptibility to cancer related to polymorphisms in DNA repair enzymes has been investigated [19,20]. In gastric cancer, our team observed and reported before [21] that polymorphisms in some enzymes of the base excision repair (BER) system, responsible for recognizing and removing the damaged base, should be investigated such as the following: OGG-1 Ser326Cys [22,23], associated with reduced DNA repair capacity [24]; APE-1 Asn148Glu [25,26], associated with increased sensitivity to ionizing radiation in homozygosis [26]; and PARP-1 Val762Ala, associated with gastric cancer risk besides *cagA(+) H. pylori* infection [27].

Therefore, this study aimed to investigate the interaction between the *iNOS* C >T polymorphism, the genotype of *H. pylori* strains and polymorphisms in DNA repair genes (*OGG-1, APE-1* and *PARP-1*) in a set of gastric cancer patients.

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Materials and Methods

Patients and specimens

This study was approved by the ethics committee of the Federal University of Ceará. A total of 109 adenocarcinoma specimens, surgically resected, were obtained from three public hospitals in Fortaleza, Ceará State, Brazil: Walter Cantideo Hospital at Federal University of Ceará, Santa Casa de Misericórdia Hospital and Cesar Cals General Hospital. Fragments of tumor were collected during gastrectomy and frozen at -80^{O}C. Histological diagnosis and tumor classification was based on Lauren's criteria.

DNA extraction, *H. pylori detection* and genotyping

Genomic DNA was extracted from frozen tumor tissue samples consisting mainly of tumor cells (>80%), using the cetyltrimethyl ammonium bromide (CTAB) method adapted from Foster and Twell [28]. *H. pylori* infection was detected by amplification of the *urease* C gene, and virulence genes were identified using specific primers and conditions, as previously described by Lage et al. [29] and Domingo et al. [30], Atherton et al. [31] and Sozzi et al. [32]. 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 a PCR–RFLP based method as described by Vodicka et al. [33] and Shen et al. [14]. Negative (water) and positive (DNA containing known DNA repair genes) controls were assayed in each run. The amplified fragments were visualized in 2% agarose gels containing ethidium bromide under UV light and were digested with appropriate restriction endonucleases. The fragments were resolved by 8% polyacrylamide gel electrophoresis under non-denaturing conditions and silver staining. Randomly selected samples were re-genotyped (10% of samples).

Statistical analysis

All statistical analyses were conducted with the SPSS® 15.0 version statistical software program (SPSS, Chicago, IL, USA), using the χ^2 and Fisher exact tests, and $p<0.05$ was considered statistically significant.

Results

In this study, the intestinal type was slightly more frequent than the diffuse (60/109 or 55.0% versus 49/109 or 45.0%), and most of the tumors were located in the non-cardia region of stomach (75.2%; 82/109). The genotype distributions were as follows: *iNOS* 78.0% CC (85/109), 21.1% CT (23/109) and 0.9% TT (1/109); *APE-1* 38.5% TT (42/109), 47.7% TG (52/109) and 13.8% GG (15/109); *PARP-1* 69.7% AA (76/109), 26.6% AG (29/109) and 3.7% GG (4/109); *OGG-1* 56% CC (61/109), 39.4% CG (43/109) and 4.6% GG (5/109).

With regard to the *iNOS* genotype distribution, no statistical difference was observed between histological characteristics or tumor localization (Table 1). However, considering 55 years old as the patients' age cutoff, the *iNOS* wild-type (CC) was significantly (p= 0.020) more frequent in patients aged ≥ 55 years than those <55 years old (83.1% versus 16.8%, respectively), as shown in Table 1.

Among the samples, 92.7% (101/109) were *H. pylori* positive; of these, 65.3% (66/101) were *cag*A(+), 50.5% (51/101) were *cag*E(+), 60.4% (61/101) were *vir*B11(+), 72.3% (73/101) were *vac*A *s*1*m*1, 17.8% (18/101) were *vac*A *s*1*m*2, 3.9% (4/101) were *vac*A *s*2*m*1 and 7.9% (8/101) were *vac*A *s*2*m*2.

Table 1: *iNOS* genotype frequency considering tumor's stomach location, histopathological characteristics and patients' age.

	CC (n=85)	CT or TT (n=24)	*p-value*
Cardia	21 (24.7%)	6 (25.0%)	0.81
Non-cardia	64 (75.3%)	18 (75.0%)	
Diffuse	38 (44.7%)	11 (45.8%)	0.89
Intestinal	47 (55.3%)	13 (54.2%)	
< 55 years	16 (61.5%)	10 (38.4%)	**0.02***
≥ 55 years	69 (83.1%)	14 (16.8%)	

The *H. pylori* strains were placed in group A, B or C, according to the vacA genotype as suggested by Lima et al. [10]. Group A was *s1/m1*, B was *s1/m2* or *s2/m1* and C was *s2/m2*. The number that comes with the specific *vacA* group letter indicates the combination of *cagA*, *cagE* and *virB11* genes. Number 1 indicates triple-positive strains and number 4 means triple-negative. Considering *H. pylori* as high (A1->B2) or low virulence (B3->C4) strains, a similar distribution of *iNOS* genotypes (p=0.086) was observed between these groups.

Considering the context of *H. pylori* virulence and polymorphism of iNOS and repair enzyme genes, the *iNOS* genotypes were combined with the genotypes of each repair enzyme as shown in Table 2. In this table, it is seen that patients carrying the *iNOS* homozygous wild-type genotype (CC) + *APE-1* polymorphic allele (TG+GG) where were statically more frequently infected by low-virulent strains (B3->C4 [88.2% (15/17); p=0.021] compared with carriers of both wild-type genotypes (*iNOS*-CC + *APE*-1-TT) (11.8%; 2/17). The opposite was observed considering the association with *PARP-1*, where patients carrying both homozygous wild-type genotypes (CC+AA, iNOS and PARP-1, respectively) were more frequently infected by low virulent strains compared with patients carrying CC+AG\GG genotypes (88.2% versus 11.8%, respectively). No difference was observed considering the association with *OGG-1* genotypes or the polymorphic allele of *iNOS* (CT+TT) with the repair enzyme genotypes. Those data are shown in Table 2.

Discussion

It is known that the inflammatory response with high NO production contributes to tissue damage, suggesting it as a possible role in the carcinogenesis process [14]. Additionally, a deficiency in DNA repair also contributes to the malignant changes in gastric cells [34]. In this way, polymorphisms that foster increased DNA damage may contribute to gastric cancer development, such as the *iNOS* Ser608Leu polymorphism and DNA repair enzyme polymorphism (OGG-1 Ser326Cys, *APE-1* Asp148Glu and *PARP-1* Val762Ala) [14,34,35]. Because polymorphism can detected from any biological sample, including peripheral blood, they are promising predictive molecular markers for disease susceptibility which make them a tool for clinic use. However polymorphism susceptibility depend on the multiple allelic variation, as well as environment factors. Since in gastric cancer the inflammatory process depends on *H. pylori* virulence, the bacterial genotype must be considered.

Table 2: *H. pylori*´s strains vs *APE-1*, *OGG-1*, *PARP-1* in genotyped patients for *iNOS*.

Repair enzymes genotypes		*iNOS* genotype	A1 -> B2	B3 -> C4	*p-value*
APE-1	TT	CC	26 (41.9%)	2 (11.8%)	**0.021***
	TG or GG		36 (58.1%)	15 (88.2%)	
	TT	CT or TT	7 (41.2%)	2 (40.0%)	0.68
	TG or GG		10 (58.8%)	3 (60.0%)	
OGG-1	CC	CC	36 (58.1%)	10 (58.8%)	0.82
	CG or GG		26 (41.9%)	7 (41.2%)	
	CC	CT or TT	8 (47.0%)	4 (80.0%)	0.21
	CG or GG		9 (53.0%)	1 (20.0%)	
PARP-1	AA	CC	39 (63.0%)	15 (88.2%)	**0.043***
	AG or GG		23 (37.0%)	2 (11.8%)	
	AA	CT or TT	11 (64.7%)	4 (80.0%)	0.47
	AG or GG		6 (35.3%)	1 (20.0%)	

Definitions: the *H. pylori* strains were grouped in A, B or C, according to *vac* A genotypes as suggested by *Lima et al.* 2011. The group A means *s*1/*m*1, B is *s*1/*m*2 or *s*2/*m*1 and C is *s*2/*m*2. The number that comes with the specific *vac* A group letter indicates the combination of *cag* A, *cag* E and *vir*B11 genes. Number 1 indicates triple-positive strains and number 4 means triple-negative. Considering *H. pylori* as high (A1->B2) or low virulence (B3->C4) strains.

Concerning the frequency of the *iNOS* genotypes, the present study found a high polymorphic allele frequency, as found by Shen et al. [36] in a study of a Chinese population with gastric cancer (24.4%). The low frequency of the *iNOS homozygous genotype (TT) identified in the present study, was similar to the value of* 3.4% reported by Fermin-Mearin et al. [37] *in a* study conducted in Spain with esophageal achalasia.

The association observed in the present study between *iNOS* wild-type genotype and older patients could be explained when *iNOS* activity is taken into account. It is interesting to note that Escames et al. [38], using an animal model, showed a natural increase in *iNOS* activity and NO production in the aging process. When the gene polymorphisms of the repair enzymes were associated with the *iNOS* polymorphism, our data indicated that the high *iNOS* activity produced by the Ser608Leu polymorphism of *iNOS* is probably connected with the early development of gastric cancer, since the frequency of patients carrying the *iNOS* polymorphic allele (CT+TT) was higher in patients <55 years old than ≥ 55 years (38.4% versus 16.8%, respectively). On the other hand, since there is a natural increase in *iNOS* activity with aging, this polymorphism may not have an influence in older patients [38].

In this study, a high percentage of the tumor samples were *H. pylori* positive, which is in line with the finding of a high rate of infection with *H. pylori* in the Brazilian population [39].

Several polymorphisms in DNA repair enzymes, including *OGG-1*, *APE-1* and *PARP-1*, have been studied in many types of cancer, because of their role in maintaining genome integrity [19,22,40]. OGG1-mediated removal of 8-oxoguanine from DNA, which is a major base damage produced by ROS, may be affected by the presence of the Ser326Cys polymorphism. This polymorphism may decrease *OGG-1* substrate specificity and capacity to excise 8-oxoguanine due to remodeling of its phosphorylation status and cellular localization [34]. The frequency of the *OGG-1* polymorphic genotype (GG) was similar to that found by Hanaoka et al. [41] in studying Brazilian patients with gastric cancer. Despite the large number of studies showing *OGG-1* as an important DNA-repair enzyme, no substantial results were obtained in the present study. Thus, there is a need for further studies analyzing the polymorphism of *OGG-1* associated with those of other enzymes of the BER pathway to find a possible relevant association.

In the analysis involving *iNOS* and the repair enzyme genotypes, the patients carrying the *iNOS* wild-type genotype (CC) who had the polymorphic allele (TG/GG) of *APE-1* as well as the *PARP-1* wild-type allele, were more infected by low-virulence *H. pylori* strains. It is known that APE-1 is a multifunctional enzyme, which is responsible for DNA repair of apurinic/apyrimidinic sites. However, the polymorphism Asp148Glu described in this gene can affect repair efficiency, as observed by Hu et al. [42] using cell culture. Additionally, it was shown that this polymorphism leads to greater sensitivity to ionizing radiation. Thus, our data suggest that *APE-1* polymorphism contributes to gastric carcinogenesis associated with low-virulence strains, possibly due to the inability of the polymorphic enzyme to communicate with other repair enzymes [25]. Concerning *PARP-1* wild-type association with these strains, it is important to note that 15 of the 19 samples showing *PARP-1* (AA) associated with low-virulence *H. pylori* strains (78.9%) were carriers of the *APE-1* polymorphic allele, highlighting the relevance of the *APE-1* polymorphism. Another explanation for this apparently controversial result is that PARP-1 normal activity consumes a large amount of NAD+ and, therefore, cell energy. Even though *PARP-1* has an important role in DNA repair, its activation may cause necrosis and inflammation due to this energy depletion contributing to the carcinogenic process [43].

The results of this study of the polymorphic allele of *iNOS* considering 55 years as a cutoff, suggest the involvement of the Ser608Leu polymorphism in the process of gastric carcinogenesis among young patients. Moreover, low-virulence strains of *H. pylori* appear to act in gastric carcinogenesis when associated with the presence of the polymorphic allele of *APE*-1 or wild-type of *PARP*-1. These data of this study point these polymorphisms as potential marker for future use in clinical screening considering the age and *H. pylori* virulence.

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