Genetic screening analysis of patients with hereditary diffuse gastric cancer from northern and northeastern Brazil

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Abstract

Background: Hereditary diffuse gastric cancer (HDGC) is a hereditary autosomal inherited syndrome associated with CDH1 germline mutations. In Brazil, gastrointestinal tumors are among the most prevalent tumor types and constitute a serious public health problem, especially in the northern and northeastern regions. This study aimed to investigate germline mutations, methylation pattern and genomic rearrangements in the CDH1 gene and quantitative changes in the DNA of HDGC patients in northern and northeastern Brazil.

Methods: Twenty-seven DNA samples from the members of four families affected by HDGC were analyzed using array comparative genomic hybridization (aCGH), DNA sequencing and methylation pattern.

Results: No evidence of gain and loss events or any rearrangements were found in any of the samples tested using aCGH. No promoter region hypermethylation was observed either. Two of the four families presented different types of germline mutations. The 185G > T and 1018A > G germline mutations detected in this study have been described in Asian and European families, respectively. The ancestors of the two families carrying these mutations had originated from those continents.

Conclusion: This is the first study to evaluate CDH1 gene germline mutations in Brazilian families with HDGC. In our study, 50% of the families showed no CDH1 gene alterations, and it is possible that in regions with a high incidence of gastric cancer, such as northern and northeastern Brazil, environmental factors might have induced the different genetic alterations analyzed in this study.

Keywords: Array comparative genomic hybridization, aCGH, Hereditary diffuse gastric cancer, CDH1, HDGC

Introduction

In northern and northeastern Brazil, gastric cancer (GC) is the second most common type of cancer and is considered a serious public health problem because it is usually diagnosed at an advanced stage [1]. In addition to its sporadic manifestation, GC is also associated with various syndromes that predispose the carrier to cancer. Among the familial forms of GC, hereditary diffuse gastric cancer (HDGC) is the only form with a well-defined genetic cause [2,3].

In order for a family to qualify for a diagnosis of HDGC, the following criteria must be met: two or more documented cases of diffuse gastric cancer in first- or second-degree relatives with at least one diagnosed before the age of 50 years, or three or more cases of documented diffuse gastric cancer independent of the age of onset [4,5].

The correlation between an E-cadherin gene germline mutation (CDH1 inactivation) and the predisposition to diffuse gastric cancer was first identified in a large family in New Zealand [6]. Based on this finding, HDGC was...
characterized and other occurrences were described in patients with different ethnic backgrounds [7,8].

The CDH1 gene, located on the 16q22.1 chromosome, encodes the E-cadherin intercellular adhesion protein; this protein acts as a tumor suppressor and plays an important role in maintenance of the epithelial tissue architecture [9]. Mutations in this gene primarily affect both the intracellular and extracellular domains of the protein and thus affect the integrity of the protein, leading to disturbances in epithelial tissue cell–cell adhesion, increased cell motility and an enhanced infiltrative capacity and tumor metastasis [10,11]. As HDGC is an infiltrative tumor, endoscopy and biopsy-based diagnostic strategies are inefficient; furthermore, given the high penetrance of CDH1 mutations, prophylactic gastrectomy is recommended for affected patients [11,12].

Over one hundred germline mutations have been described for the CDH1 gene. Although several of these mutations have been detected in different families, to date, no hotspot has been characterized [3,13]. Although the CDH1 gene is the predominantly affected gene in HDGC, other inactivation mechanisms should be investigated. Additionally, large deletions might also be responsible for CDH1 inactivation [8,14-20]. For this reason, an array comparative genomic hybridization (aCGH) analysis is essential to the identification of quantitative alterations in the CDH1 gene or other genome regions in families affected by HDGC [21].

The objective of this study was to identify germline mutations in the CDH1 gene and/or quantitative genomic alterations in four families with HDGC in northern and northeastern Brazil.

Methodology

Patients

The samples evaluated in this study were obtained from patients who fulfilled the clinical criteria according to the latest consensus of the International Gastric Cancer Linkage Consortium [5]. The patients had been treated at referral hospitals in northern and northeastern Brazil. The samples were processed in their states of origin and then sent to the Federal University of Pará (Universidade Federal do Pará; UFPA) for genetic analysis.

A retrospective study of the family members was also conducted to identify previous generations with DGC. All genetically analyzed patients (or their guardians) signed consent forms that had been approved by the Research Ethics Committee of University Hospital João de Barros Barreto (Hospital Universitário João de Barros Barreto; HUJBB) under protocol number 274/12. Assurance was provided that the use of biological materials and participation in the study would not cause any harm to nor have any negative influence on patient treatment.

All biological materials except one sample (that was Formaldehyde Fixed-Paraffin Embedded tissue) was blood DNA. And all genetic screening analysis - mutation, deletion and methylation - were all looking for germline changes, not somatic.

DNA extraction

Peripheral blood samples were collected in EDTA-containing tubes and extracted with the QIAamp® DNA Blood Mini Kit (Qiagen N.V., Venlo, The Netherlands) according to the manufacturer’s instructions. Paraffin block sample was obtained from one patient, and extracted with the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer’s instructions.

Genotypic analysis

The obtained DNA was used to perform molecular screening of the CDH1 gene. Gene fragments containing the analyzed polymorphisms were amplified by polymerase chain reaction (PCR) using gene-specific reaction conditions and primers (Table 1) according to Brooks-Wilson et al. [22].

The result of each reaction was subjected to direct sequencing on an ABI 3130 capillary sequencing platform (Applied Biosystems/Life Technologies, Carlsbad, CA, USA). The sequences were obtained as electropherograms and analyzed with the software package provided with the equipment. The generated sequences were analyzed with the BIQ Analyzer software package [23].

aCGH

High-density probe microarray analyses were performed to determine the copy number variation (CNV). The complete genomes of all patients were evaluated with the goal of identifying genes related to tumor development.

The Affymetrix® CytoScan™ HD Array (Affymetrix, Inc., Santa Clara, CA, USA) was used; this system features a total of approximately 1.9 million probes for detecting CNV and 750,000 SNP molecular markers. The standard protocol incorporates the following eight procedures before scanning the chip: genomic DNA digestion, NSP adapter ligation, fragment amplification by PCR (Polymerase Chain Reaction), PCR product purification, PCR product fragmentation, end-labeling, hybridization and washing.

The Chromosome Analysis Suite software v1.2.1 (Affymetrix®) was used for the chip analysis.

Methylation status

For methylation analyses, the samples were subjected to DNA modification using sodium bisulfite [24]. A fragment with 22 CpGs of the CDH1 promoter region was amplified using a nested PCR strategy [25]. Fragments obtained were purified using PCR Purification Kit (Invitrogen/Life
Technologies, Carlsbad, CA, USA) and sequenced using an ABI3130 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were aligned with BioEdit v7.0.5 [26]. Methylation analyses were run in BiQ Analyzer [23] software.

Immunohistochemistry
An anti-cadherin (Abcam PLC, Cambridge, UK) commercial primary antibody was used to detect the protein product of the \( \text{CDH1} \) gene (HDGC). Streptavidin-biotin-peroxidase staining as described by Hsu et al. [27] was adopted as the immunohistochemical method. The normality parameter was defined with samples from normal (non-tumor) formalin-fixed and paraffin-embedded tissues that had been obtained from routine samples. The World Health Organization histopathological classifications for each tumor were used as well as the Lauren classification for gastric cancer [28].

Results
The detailed patient data are shown in Table 2. A total of 27 patient samples were collected for genetic analysis from four unrelated families with histories of DGC in northern and northeastern Brazil. All biological samples collected were peripheral blood, except for the patient AM05, whose sample was Paraffin-embedded tumor. The sample comprised 18 men and nine women with a mean age of 42 years and an age range of 20–75 years (24–56 years for women and 20–75 years for men).

Of these 27 patients, nine (33.4%) had been previously diagnosed with diffuse gastric cancer, whereas the remaining patients did not have any type of gastric tumor. Among the patients diagnosed with DGC, the age at diagnosis ranged from 27–67 years.

After the retrospective study and using information collected from the analyzed patients, it was possible to identify relatives with a history of DGC and other types of tumors (Figure 1).

Mutations in the \( \text{CDH1} \) gene
Among the four families of patients, two families exhibited germline mutations in the \( \text{CDH1} \) gene, namely family A from northern Brazil and family B from northeastern Brazil (Table 3). In the other two families, which originated from northeastern Brazil, no mutations were found in the evaluated \( \text{CDH1} \) exons.

Array comparative genomic hybridization (aCGH)
The aCGH sample analysis showed no gains or losses of DNA in the four tested families (27 samples).

Immunoreactivity in HDGC
CDH1 protein detection was performed only for patient AM05, a carrier of the germline mutation 185 G > T and member of family A. This patient died before the start of the study, and a genetic analysis was performed on a paraffin-embedded tumor tissue. The other study participants with this syndrome donated peripheral blood for genetic analysis, and consequently, there was no need to request paraffin blocks of the resected tumor tissues. The tumor cells of patient AM05, revealed negative immunoreactivity to the E-cadherin protein.
We analyzed the methylation pattern of a CDH1 gene promoter region fragment in one CpG island containing 22 CpG dinucleotides, to find possible heritable epimutations. No promoter region hypermethylation was observed in all 27 tested individuals, from the four families, and also in 10 healthy controls.

Discussion
In addition to sporadic manifestation, gastric cancer can be associated with various syndromes that predispose the carrier to cancer. Among the familial forms of gastric cancer, HDGC is the only syndrome with a well-defined genetic cause [2,3].

The relationship between a genetic germline mutation in the E-cadherin-encoding gene (CDH1 inactivation) and the predisposition to HDGC was first identified in a family in New Zealand [6]. Based on this finding, the HDGC syndrome was characterized, and other occurrences were described in patients with different ethnic backgrounds [29].

Mutations in CDH1 gene affect protein integrity, thus causing disturbances in cell-cell adhesion in epithelial tissues, increasing cell motility and enhancing infiltrative behavior of the tumor and metastasis development [10,11].

This genetic study of four families that carried the HDGC syndrome allowed us to evaluate a previously unaddressed issue within the Brazilian population. CDH1 gene germline mutations are observed in 30–40% of cases that meet the clinical criteria for HDGC [30,31]. In our study, 50% of the families exhibited CDH1 gene mutations. This information is useful in clinical evaluations of patients with family histories of HDGC because in addition to prophylactic measures, the information facilitates monitoring aimed at identifying the occurrence of other tumors such as lobular carcinomas of the breast; these types of cancers had also been detected in members of the families analyzed in this study [13,20,32].

The cases described in the literature demonstrate that the most common CDH1 gene alterations associated with HDGC are point mutations and small changes in the reading frame; these occur in approximately 93% of families with CDH1 gene mutations [13,20,33].

In this study, two individuals in family A, a father and son (Figure 1A), were carriers of a familial CDH1 gene mutation at nucleotide position 185 (185G > T); this mutation causes a change in the protein structure from the amino acid glycine to valine. This missense mutation was first described by Shinmura et al. [34] in a family of Japanese origin. Our results suggest the hypothesis that this 185G > T mutation was introduced into family A by the grandfather of the proband (first generation pedigree), who was of Japanese origin, and suggest that this mutation might result from this ethnic background.

A CDH1 gene germline mutation was also identified in two brothers from family B who had developed an early form of gastric cancer (before 45 years of age) [35]. The mutation was identified at nucleotide position 1018 (1018A > G) and resulted in a change from the amino acid threonine to alanine. The 1018A > G mutation was previously described in a family of European origin and in a Chinese family [36,37]. Because the proband of this family was of Portuguese descent, it is possible that their ancestors brought this mutation to Brazil upon immigration. On the other hand, another alternative to

| Table 2 Identification of the patients analyzed in this study |
|-------------------|-----------------|-----------------|
| Patient | Gender | Age at diagnosis | Type of cancer |
| (Family A) | | | |
| ^AM05 | male | 36 | *DGC |
| AM06 | female | 56 | none |
| AM07 | male | 31 | *DGC |
| AM08 | female | 26 | none |
| (Family B) | | | |
| P05 | male | 29 | *DGC |
| P06 | male | 27 | *DGC |
| P07 | male | 23 | none |
| P08 | male | 20 | none |
| (Family C) | | | |
| C05 | male | 75 | Prostate |
| C14 | male | 52 | *DGC |
| C15 | male | 50 | *DGC |
| C16 | female | 49 | *DGC |
| C17 | female | 48 | none |
| C18 | female | 45 | none |
| C19 | female | 47 | none |
| C20 | male | 25 | none |
| C21 | male | 23 | none |
| C22 | female | 42 | none |
| C23 | female | 24 | none |
| C24 | male | 56 | none |
| C25 | male | 35 | none |
| C26 | male | 31 | none |
| C27 | male | 58 | none |
| C28 | female | 31 | none |
| (Family D) | | | |
| M08 | female | 67 | *DGC |
| M09 | male | 62 | *DGC |
| M10 | male | 64 | none |

^ Paraffin-embedded tumor sample.
* - Diffuse Gastric Cancer.

Methylation status in HDGC
We analyzed the methylation pattern of a CDH1 gene promoter region fragment in one CpG island containing 22 CpG dinucleotides, to find possible heritable epimutations. No promoter region hypermethylation was observed in all 27 tested individuals, from the four families, and also in 10 healthy controls.
ancestral mutations is an increased susceptibility of different CDH1 locations to mutations.

A literature review revealed the occurrence of a total of 122 germline mutations in the CDH1 gene in HDGC [38]. Although several types of mutations have been detected in different families, no hotspot has been characterized to date [3,13]; this led us to sequence all 16 exons of the gene in the 30 members of the four analyzed families.

Of the mutations reported in the literature, approximately 15% are shared by many families worldwide, suggesting that CDH1 mutation-associated HDGC might share a common ancestry, as was suggested for families A and B in this study [33,39].

In the other two families (C and D), in which the evaluated subjects carried no CDH1 gene mutations, there were considerable numbers of HDGC-affected members in different generations (Figure 1). Pinheiro et al. [20] have proposed that many HDGC families carry some mutations in non-exonic regulatory regions of CDH1 (or in upstream regulators). Based on this, it may be possible in the future to test families C and D for allele-specific loss on CDH1.

In other populations in which the incidence of gastric cancer is high, there are also reports of HDGC-carrier families with large numbers of affected individuals who do not carry CDH1 gene germline mutations. This finding might be consequent to exposure to environmental risk.

**Figure 1** Pedigrees of hereditary diffuse gastric cancer families. (A), (B), (C) and (D) represents the four families presented in this study. The numbers present under the symbols represent the age at diagnosis. The solid symbols represent the affected members with confirmed diffuse gastric cancer diagnoses. Upper left arrows indicate the probands.

| Family ID | Patient ID | Age at onset/sex | Exon | Base changes | Amino acid change | Mutation consequence |
|-----------|------------|------------------|------|--------------|------------------|---------------------|
| A         | AM05       | 36/male          | 3    | 185 G > T   | Gly → Val        | Missense            |
|           | AM07       | 31/male          |      |              |                  |                     |
| B         | P05        | 29/male          | 8    | 1018 A > G  | Thr → Ala        | Missense            |
|           | P06        | 27/male          |      |              |                  |                     |

Table 3 Germline CDH1 mutations identified in hereditary diffuse gastric cancer families
factors or the susceptibility of individuals to genetic alterations in low-penetrance genes [40, 41].

In a study conducted in southeastern Brazil on 88 patients with early gastric cancer, 16 of whom had familial histories of gastric cancer, it was possible to detect changes in the E-cadherin protein expression in 41 individuals via immunohistochemical analysis. Although CDH1 gene sequencing was not conducted to identify possible mutations, the immunohistochemical analysis revealed the involvement of CDH1 in the early development of gastric cancer in a Brazilian population [42].

Because the CDH1 gene acts as a tumor suppressor, other gene inactivation mechanisms of this gene besides germline mutations in one allele should be investigated to identify changes to the second allele in somatic cells (second event of the Knudson hypothesis [43]), which was not able to be done in the present work. Mechanisms such as CDH1 gene deletion [33] and promoter region hypermethylation [44], were investigated in germline from those analyzed patients, because are also considered potentially responsible for its inactivation.

Cytosine hypermethylation in the CpG dinucleotides present within the CDH1 promoter region induces transcriptional silencing [45] and might thus explain the lack of E-cadherin immunoreactivity. However, analyses to confirm the presence of CDH1 gene promoter region hypermethylation in patients with HDGC have not been conclusive. For example, studies by Li et al. [46] and Concolino et al. [47] demonstrated CDH1 promoter hypermethylation-mediated gene silencing in 53–57% of patients with HDGC. Conversely, Wu et al. [48] reported an absence of this phenomenon in 140 Chinese gastric cancer patients with a familial history of HDGC.

The analyses conducted in the present study did not detect CDH1 gene promoter region hypermethylation in the families with HDGC syndrome. Our results reinforce the theory put forth by Yamada et al. [19] who, after analyzing 22 Japanese patients with early gastric cancer, suggested that germline CDH1 promoter hypermethylation was not a predisposing factor of gastric cancer.

In addition to epigenetic alterations in the promoter region, the CDH1 gene might also suffer deletions even in the absence of point mutations in the germlines of patients with HDGC [49]. Kim et al. [50] used multiplex ligation-dependent probe amplification (MLPA) to analyze 23 patients with HDGC and, similarly to our study, found no deletions or duplications in these genes.

Oliveira et al. [33] genetically analyzed 160 patients with HDGC from different geographic regions and found that CDH1 gene deletions occurred in the peripheral blood in approximately 4% of families affected by this syndrome. The same authors also observed that all families with CDH1 deletions originated from countries with low gastric cancer incidence rates. A large number of patients with HDGC, in northern and northeastern from Brazil, needs to be analyzed to verify if the absence of CDH1 gene deletions and other quantitative genomic alterations, as found in patients of this work, corroborate this observation because these regions have high gastric cancer incidence rates.

It is widely known that the most common forms of cancer develop as a result of interactions between endogenous and environmental factors such as the diet [51]. Environmental factors might be related to the high incidence of these neoplasms in the northern and northeastern regions, or for some reason the HDGC frequency is higher than elsewhere in the world, where sporadic gastric cancer is also endemic, such as Japan and Korea. In particular, Helicobacter pylori (H. pylori) infection during childhood as well as the high consumption of salt-preserved foods, infrequent use of refrigeration and low consumption rates of cereals, fresh fruits and vegetables are considered risk factors for gastrointestinal tumors [52-55].

Conclusions
This is the first study to evaluate CDH1 gene germline mutations and quantitative gene alterations in Brazilian families with HDGC. CDH1 gene germline mutations were found in 50% of the families evaluated. The detected mutations appeared to originate from Asia and Europe. This migratory flow does not rule out the possibility that environmental factors might have caused these same mutations in the affected members of the analyzed families. No quantitative changes were observed in the genomes of any of the analyzed families. It is possible that in regions with high gastric cancer incidence rates such as northern and northeastern Brazil, environmental factors or other molecular mechanisms might induce different genetic alterations from those analyzed in this study.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Conceived and designed the experiments: CFAMN, MBLB, BNB, LML, RMRB. Performed the experiments: CFAMN, MBLB, BNB, LML, ABB. Analyzed the data: CFAMN MBLB, BNB, HFR, RMRB. Wrote the paper: CFAMN, PPA, JAR, GRP, RMRB. All authors read and approved the final manuscript.

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