Chapter 4

Molecular Epidemiology of *Helicobacter pylori* in Brazilian Patients with Early Gastric Cancer and a Review to Understand the Prognosis of the Disease

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1. Introduction

Helicobacter pylori (*H. pylori*) is an universally distributed bacterium that affects more than half of the world population and is considered an important public health problem. Although colonization with *H. pylori* is not actually a disease, it is a condition that affects the relative risk of developing various clinical disorders of the upper gastrointestinal tract, as chronic gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT lymphoma) and gastric adenocarcinoma, and, possibly, extradigestive diseases.

Colonization with *H. pylori* virtually leads to infiltration of the gastric mucosa in both antrum and corpus with neutrophilic and mononuclear cells. Gastritis can be classified as an acute or chronic gastritis and it can involve all parts of the stomach or just the fundus, corpus or antrum. The chronic active gastritis is the primary condition related to *H. pylori* colonization, and other *H. pylori*-associated disorders, in particular, resulting from this chronic inflammatory process, as atrophic gastritis, causing an elevated risk of gastric cancer. Considering this association, in 1994 the bacterium was classified as a group I carcinogen by the International Agency for Research on Cancer, World Health Organization.

Molecular techniques have revealed that *H. pylori* possesses a remarkable degree of genetic diversity, which could be responsible for its adaptation in the host stomach and for its pathological characteristics, in addition to the clinical outcome of the infection, although this aspect remains unclear.

In Brazil, it is estimated that only about 10 to 15% of the gastric cancer cases are diagnosed at an early stage, aspect that directly impact the prognosis of the disease, which presents low

survival rates. Unlike patients with advanced gastric cancer, that ones diagnosed at the early stage of the disease present an excellent prognostic, in which a five-year survival rate is more than 90%.

Many early gastric cancers are believed to go through a life cycle consisting of ulcerations, followed by healing, then reulceration, and some tumors remain at this early stage for years even without treatment. Nevertheless, some of these tumors rapidly advanced, perhaps one of the principal questions concerning the gastric carcinogenesis.

Consequently, some questions can be considered about it. Is the *H. pylori* presence important for the evolution of the early lesions in advanced ones? To what extent *H. pylori* eradication could prevent the progression of lesions from one to another stage? Are the genetic characteristics of *H. pylori* strain that is colonizing the patient with early gastric cancer important for the progression of the disease in a faster or in a slower way?

The principal aim of our book chapter is to identify the genetic characteristics of *H. pylori* strains in Brazilian patients diagnosed with early distal type intestinal gastric adenocarcinoma, trying to determine the genotypic pattern of bacterium in our population through molecular techniques. Besides, other aim of our study is to discuss the principal aspects of the *H. pylori* infection and then correlate them with the development of the precancerous lesions and the development of the early gastric cancer properly, trying to understand to what extent the microorganism eradication treatment could be important to preventing the disease progression.

2. *Helicobacter pylori*

2.1. General characteristics

Helicobacter pylori (H. pylori) is a spiral-shaped Gram-negative flagellate bacterium that colonizes the human stomach and can establish a long-term infection of the gastric mucosa [1]. In gastric biopsy specimens, *H. pylori* organisms are 2.5 to 5.0 μm long and 0.5 to 1.0 μm wide, with four to six unipolar sheated flagella, which are essential for bacterial motility. When cultured on solid medium, the bacteria assume a rod-like shape and spiral shapes are infrequent or absent [2]; after prolonged culture on solid or liquid medium, coccoid forms, that are metabolically active, tipically predominate [3].

Gastric colonization with *H. pylori* affects at least half the world´s population, and, while the infection is on a fast decline in most of the western countries, mainly due to the success of therapeutic regimens and improved personal and community hygiene that prevents reinfection, the situation is exactly opposite in many of the developing countries due to failure of treatment and emergence of drug resistance [4,5]. Most studies suggest that males and females are infected at approximately the same rates [6] and, probably, the infection occurs in the childhood. In developed countries, persons of higher socioeconomic status have lower infection rates, although among certain ethnic minorities, high rates persist despite economic advancement [7].

The routes of transmission of *H. pylori* still remain unclear. Person-to-person transmission and intrafamilial spread seem to be the main route, based on the intrafamilial clustering observed in some studies [8,9]. Children are often infected by a strain which a genetic fingerprint identical to that of their parents, and they maintain this genotype even after moving to a different environment [10]. Animals harbor organisms that resemble *H. pylori,* but with the exception of nonhuman primates [11] and, under particular circumstances, perhaps cats [12] and houseflies (*Musca domestica*) [13], none harbor *H. pylori.* In the same way, food-borne transmission has not been substantiated [14]. Nevertheless, the waterborne infection remains possible [15,16].

H. pylori remains one of the most common worldwide human infections and its isolation by Marshall and Warren (1984) [17] has markedly improved our understanding about the nature of chronic gastritis and other important upper gastrointestinal disorders, such as peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma [18]. In 1994, the bacterium was classified as a group I carcinogen by the International Agency for Research on Cancer and is regarded as a primary factor for gastric cancer development [19]. For their revolutionary discovery, Marshall and Warren received the Nobel Prize in Physiology or Medicine in 2005.

In addition, in the last years, *H. pylori* infection has also been associated with some extradigestive diseases, such as iron-deficiency anemia [20], idiopathic thrombocytopenic purpura [21,22], cardiovascular diseases [23,24], hepatobiliary diseases [25,26], and diabetes mellitus [27], among others.

2.2. Virulence factors of *H. Pylori*

H. pylori populations are highly diverse and constantly change their genome, which can be an important factor in its adaptation to the host stomach and also for the clinical outcome of the infection. The changes in its genome occur mainly due to point mutations, substitutions, insertions, and/or deletions of their genome. Moreover, mixed infections are frequent and lead to exchange of DNA fragments between different *H. pylori* strains in a single host [28,29]. Experience with other bacterial pathogens suggests that *H. pylori*-specific factors may exist that influence the microorganism pathogenicity, and, these factors, together with the host genetic characteristics and the external environment, can contribute to the clinical outcome of the infection. Among the most studied virulence factors of *H. pylori* are the urease gene, the vacuolating cytotoxin gene (vacA), the cytotoxin associated gene-Pathogenicity Island (cagPAI) and the duodenal ulcer promoting gene (dupA).

Urease is an important enzyme which is produced by *H. pylori* to counteract the acidic environment of the stomach. Urease causes damage to the epithelium through the production of ammonia, that, in conjunction with neutrophil metabolites, forms carcinogenic agents that might participate in the development of gastric malignances [30,31]. Ammonia is capable to cause different cell alterations, including swelling of intracellular acidic compartments, alterations of vesicular membrane transport, repression of protein synthesis and ATP production, and cell-cycle arrest [32]. Urease might also help to the recruitment of neutrophils and monocytes in the mucosa and to the production of proinflammatory cytokines

[33]. It has been demonstrated that this enzyme plays and important hole in the *H. pylori* colonization, being observed that urease-defective bacteria mutants are not able to colonize the gastric environment [32].

VacA is a cytotoxin secreted from *H. pylori* as a large 140kd polypeptide and latter trimmed at both ends to finally deliver it in an active form to host cells, where it exerts its activity [34]. The gene encoding VacA is present in all *H. pylori* strains and displays allelic diversity in three main regions: s (signal), i (intermediate) and m (middle); consequently, the activity of the toxin varies between strains [35]. Different combinations of two major alleles of each region (s1, s2, i1, i2, m1, m2) may exist, which results in VacA toxins with distinct capability of inducing vacuolating in epithelial cells [36]. VacA induces multiple cellular activities, including the alteration in the endosomal maturation which consequently leads to vacuolating of epithelial cells, the induction of membrane-channel formation, the cytochrome c releasing from mitochondria and the binding to cell-membrane receptors activating a proinflammatory response [35].

The cagPAI is a 40kb region of chromosomal DNA encoding approximately 31 genes that forms a type IV secretion system that forms a pilus that delivers CagA, an oncoprotein, into the cytosol of gastric epithelial cells through a rigid needle structure covered by CagY, a VirB10-homologous protein and CagT, a VirB7-homologous protein, at the base [37,38]. cagA is a polymorphic gene that presents different numbers of repeat sequences located in its 3′region and each repeat region of the CagA protein contains Glu-Pro-IIe-Tyr-Ala (EPIYA) motifs, including a tyrosine phosphorylation site [39]. Upon delivery into host cells, CagA undergoes Src-dependent tyrosine phosphorylation and activates an eukaryotic phosphatase (SHP-2), leading to dephosphorylation of host cell proteins and cellular morphologic changes [40]. CagA has also been shown to dysregulate β-catenin signaling [41] and apical-junctional complexes [42], events that have been linked to increased cell motility and oncogenic transformation in a variety of models [43]. In addition, some studies have been reported that the cagPAI appears to be involved in the induction of gastric interleukin-8 (IL-8) production, a potent neutrophil-activating chemokine [44]. Consequently, the presence of the cagA gene has been associated with higher grades of inflammation, which may lead to the development of the most severe gastrointestinal diseases, such as peptic ulcer disease and gastric cancer [45-48].

H. pylori duodenal ulcer promoting gene (dupA), located in the plasticity region of bacterium genome, has been initially described as a risk marker for duodenal ulcer development and a protective factor against gastric cancer [49]. It was the first putative specific marker whose association was described using strains obtained from in both Asian (Japan and Korea) and Western (Colombia) regions and it is though to be a *vir*B4 homologue [49, 50]. dupA gene encompasses two continuous sequences, jhp0917 and jhp0918, as described in strain J99. The jhp0917 gene encodes a protein of 475 amino acids, but lacks a region homologous to the C-terminus of *vir*B4, while jhp0918 gene encodes a product of 140 amino acids that is homologous to the missing *vir*B4 region [51]. Since its discovery, dupA gene has been studied by various authors, and the results of their researches suggest that, in some places, dupA gene is not associated with an specific disease [52], or it is suggested that it can be associated with gastric cancer development [53-55], or it is directly associated with the development of duodenal ulcer

disease [50,56]. Considering all these results, it is suggested that there must be diversity in gene content that can contribute to bacterial adaptation to genetically different ethnic groups that make up de human population [57].

3. Gastric cancer

3.1. Epidemiology

Cancer is a worldwide full-scale problem as it will affect one in three men and one in four women during their lifetime [58]. Nowadays, this disease represents one in eight deaths around the world. The global cancer rate has doubled in the last 30 years of the 20th century, and will almost triple by 2030, a year in which it is foreseen that 20.3 million people will be diagnosed cancer and 13.2 million will die as a result of this disease [59].

World Health Organization estimates that 43% of cancer deaths are due tobacco, diet and infection. One-fifth of cancers worldwide are due to chronic infections, mainly from hepatitis viruses (liver), papillomaviruses (cervix), *H. pylori* (stomach), schistosomes (bladder), the liver fluke (bile duct), and human immunodeficiency virus (Kaposi sarcoma and lymphoma) [60].

Gastric cancer continues to be a major global health problem [61] and, despite the decreasing incidence and mortality rates observed worldwide over the last 50 years, it still ranks as a leading cause of cancer-related deaths in many parts of the world [62]. As symptoms are often absent or nonspecific in patients with the early stages of the disease, gastric cancer is usually diagnosed in an advanced stage, when curative options are limited. With exceptions in countries that have developed screening programs for early diagnoses, as example Japan, most patients reach treatment with cancers already in advanced stages [63]. Consequently, gastric cancer carries a poor prognosis, with an overall five-year survival rate of less than 20% [64].

Besides, one study assessed the survival of gastric cancer in population-based registries obtained in cities from four continents and concluded that the large differences observed among these areas were exclusively due to the different types of stomach cancer, highlighting the importance of the stage of the disease as an indicator of the effect of delayed diagnosis on the prognosis of these patients [65].

In Brazil, in 2005, the highest incidence rates, adjusted by age, were found in São Paulo (male, 38,8/100.000; female, 15,0/100.000) and the Federal District (male, 32,7/100.000; female 14,7/100.000) [66]. The National Institute of Cancer in Brazil estimates that, in 2014, there will occur 580.000 new cases of cancer. The most frequent cancers in Brazilian population will be non-melanoma skin (182.000), prostate (69.000), breast (57.000), colon and rectum (33.000), lung (27.000) and stomach (20.000). Considering Brazilian regions and gender, gastric cancer will be the fourth most common cancer in Brazil. In male gender, it is the second most common tumour in the North (11/100.000 cases) and Northeast (10/100.000 cases) regions; it is the fourth most common tumour in the Midwest (11/100.000 cases) and in the South (16/100.000) regions and the fifth most common in the Southeast region (15/100.000 cases). Concerning the female gender, gastric cancer is the fifth most common cancer in Brazil, the third most frequent in the

North region (6/100.000 cases), and the fifth most frequent in the Northeast (6/100.000 cases) and Southeast (8/100.000 cases) regions [67].

The chances of surviving the onset of some common cancers depend largely on how early they are detected and how well they are treated. Early detection is based on the observation that treatment is more effective when cancer is detected early. It includes awareness of early signs and symptoms of cancer and screening, which is the mass testing of people who appear to be healthy. In many developing countries, where these are not feasible, several other low technology approaches are being studied and look promising. The success of public health programmes in detecting cancer early depends on the allocation of resources, availability of qualified specialists and access to follow-up treatment [60].

3.2. Classification of gastric cancer

The vast majority of gastric cancers are adenocarcinomas. Two histologically distinct variants of gastric adenocarcinoma have been described, each with different pathophysiological features: the diffuse type and the intestinal type [68], which corresponds, respectively, to the undifferentiated or poorly-differentiated type and to the well-differentiated type, in the Japanese classification [69].

Diffuse type gastric adenocarcinoma is often associated with familial distribution and more commonly affects younger people. It consists of individually infiltrating neoplastic cells that do not form glandular structures and arises closer to the advancing border of inflammation but without any identifiable histological precursor lesion [62,70,71].

Gastric adenocarcinoma of the intestinal type is preceded by a prolonged precancerous process. In 1975, Correa and colleagues proposed a model of gastric carcinogenesis, postulating that the intestinal type of gastric cancer was the end result of progressive changes in the gastric mucosa, starting with chronic gastritis, followed by multifocal atrophic gastritis and intestinal metaplasia [72]. This model was updated in 1988 and 1992 [73,74] and the following steps were recognized: normal gastric mucosa → superficial gastritis (later renamed non-atrophic gastritis) → multifocal atrophic gastritis without intestinal metaplasia → intestinal metaplasia of the complete (small intestine) type → intestinal metaplasia of the incomplete (colonic) type → low-grade dysplasia (low-grade noninvasive dysplasia) → high-grade dysplasia (high-grade noninvasive dysplasia) → invasive adenocarcinoma [75]. These lesions are well-characterized histopathologically and represent a continuum of changes depicting multiple events that increase in intensity and extension with time [76].

Both diffuse and intestinal types are associated with *H. pylori* infection, which plays an initiating role in the pathogenesis of gastric cancer by changing many important factors, including antioxidant agents, reactive oxygen metabolites, and the balance between epithelial cell proliferation and apoptosis [77]. *H. pylori* infection induces cell apoptosis, stimulates cell proliferation in the gastric epithelium, and causes alterations or mutations of apoptosis/proliferation-related genes [78].

3.3. *Helicobacter pylori* and gastric cancer — The precancerous cascade

Exposure of gastric epithelial cells to *H. pylori* results in an inflammatory reaction with the production of reactive oxygen species and nitric oxide that, in turn, deaminates DNA causing mutations [62]. The complex interplay among *H. pylori* strain, inflammation and host characteristics, besides the external environment, may directly promote diffuse type gastric cancer or induce the cascade of morphological events that leads to intestinal type gastric cancer.

Specifically regarding to intestinal type gastric adenocarcinoma, evidence that *H. pylori* increases the risk of gastric cancer development via the sequence of atrophy and metaplasia originates from various studies, in which it was shown that *H. pylori* positive subjects develop these conditions more often than do uninfected controls [79]. Consequently, since *H. pylori* isolation, many investigators have emphasized its role in gastric carcinogenesis [80,81]. Epidemiological studies have determined that the attributable risk for gastric cancer conferred by *H. pylori* is approximately 75% [82]. Besides, in respect to localization in the stomach, premalignant lesions are most frequently localized in the antrum in the transitional zone between the antrum and corpus [83].

As explained before, Correa and colleagues (1975) proposed a model of gastric carcinogenesis, considering that the intestinal type gastric cancer probably is the result of histological continuum changes that occur especially due to *H. pylori* infection: chronic active nonatrophic gastritis, multifocal atrophic gastritis, intestinal metaplasia, dysplasia, and, finally, invasive adenocarcinoma. Consequently, the principal characteristics of each one of these stages are report below.

3.3.1. Chronic active non-atrophic gastritis

Gastritis is characterized by increased infiltration of the lamina propria with mononuclear leukocytes (chronic inflammation) and polymorphonuclear neutrophils (acute inflammation). Additionally, scattered eosinophils and mast cells can be observed. The gastritis is called "active" when polymorphonuclear neutrophils are found, representing acute inflammation. This phase of the precancerous process does not show loss of glands (atrophy) and is called "nonatrophic gastritis" in the updated Sydney classification of gastritis, adopted by most pathologists (Figure 1) [84].

The most frequent cause of gastritis is *H. pylori* infection and the severity of inflammation may vary according to the infected *H. pylori* strain [75].

3.3.2. Multifocal atrophic gastritis

Loss of normal glandular tissue is the first specific recognizable step in the precancerous cascade. Usually it is the result of a prolonged inflammatory process and tends to be multifocal, giving rise to the so-called multifocal atrophic gastritis (Figure 2). The foci of atrophy are present in the mucosa of gastric antrum and body, and their extension progresses with time [75]. More virulent bacterial strains and a permissive host immune response are strongly associated with atrophy and progression to severe disease [84].

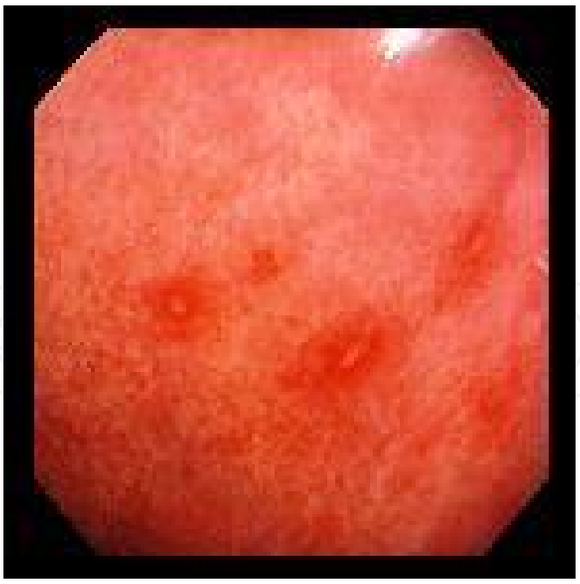

Figure 1. Chronic gastritis

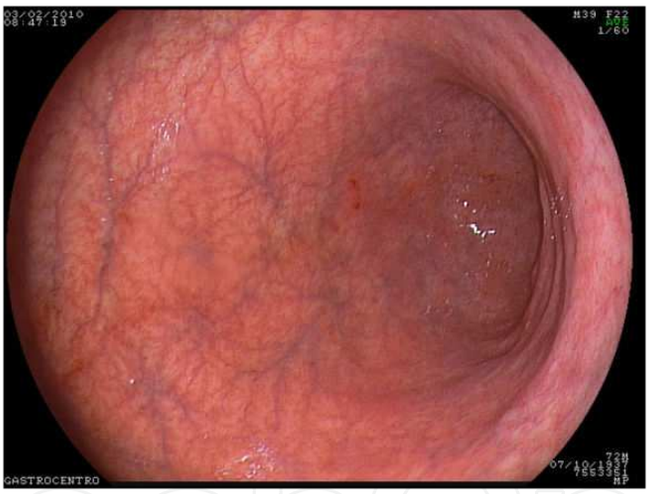

Figure 2. Atrophic gastritis

3.3.3. Intestinal metaplasia

At this stage of the gastric precancerous process, the original glands and the foveolar epithelium are replaced by cells with intestinal phenotype [84]. Intestinal metaplasia (Figure 3) is considered to be an advanced stage of atrophy because the metaplastic glands replace the original glands and chronologically the metaplastic glands appear after the gastric glands are lost. Intestinal metaplasia has been classified on the basis of morphology and enzyme histochemistry in two main types: the small intestine or complete type, and the colonic or incomplete type [75].

Up to this point in the cascade, the epithelium in the atrophic and metaplastic lesions remains well differentiated, with normal nuclear-cytoplasmic ratio, normal nuclear morphology, and normal tissue architecture. The dynamics of the precancerous process to this point shows a gradual phenotypic transformation from normal epithelium to metaplastic cells with small intestinal morphology and then to cells resembling colonic mucosa, additionally expressing gastric and colonic mucins. This process usually takes decades and is progressive, supporting the notion that although environmental alterations (bacterial factors and cytokine environment, loss of cell signaling) may have initially driven differentiation decisions, with time, permanent changes in the stem cell compartment have occurred. In some patients with incomplete metaplasia, a mild degree of nuclear atypia and architectural distortion is observed, leading some investigators to consider incomplete metaplasia as a mild form of dysplasia [84,85].

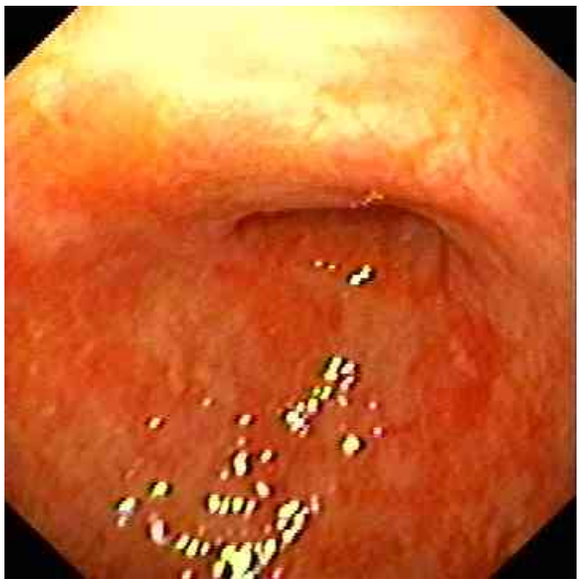

Figure 3. Intestinal metaplasia (antrum)

3.3.4. Dysplasia

Also called intraepithelial neoplasia or noninvasive neoplasia, dysplasia is characterized by a neoplastic phenotype, both in terms of cell morphology and architectural organization [75]. The nuclei of the dysplastic epithelium are enlarged, hyperchromatic, irregular in shape, and devoid of polarity [84]. The Padova classification is focused on gastric dysplasia and was developed by an international group of experienced gastrointestinal pathologists and recognizes five categories of lesions, utilizing mostly western nomenclature and grouping them numerically following the prevailing Japanese system: 1. negative for dysplasia; 2. indefinite for dysplasia; 3. noninvasive neoplasia (sub-classified in low grade or high grade); 4. suspicious for invasive carcinoma; and 5. invasive carcinoma [86].

The management of low-grade dysplasia is not well defined and there is a recommendation of annual endoscopic monitoring with rebiopsy [87]. Nevertheless, patients with high-grade dysplasia confirmed at least two gastrointestinal pathologists should undergo surgical or endoscopic resection because of the high probability of coexisting or metachronous invasive carcinoma [88].

3.3.5. Invasive adenocarcinoma

Invasive adenocarcinoma is the next stage in the cascade and requires the penetration of neoplastic cells into the surrounding stroma (Figure 4). Recent evidence suggests that this step demands that neoplastic cells acquire the capability of degrading the stromal matrix surrounding the neoplastic cells [75.

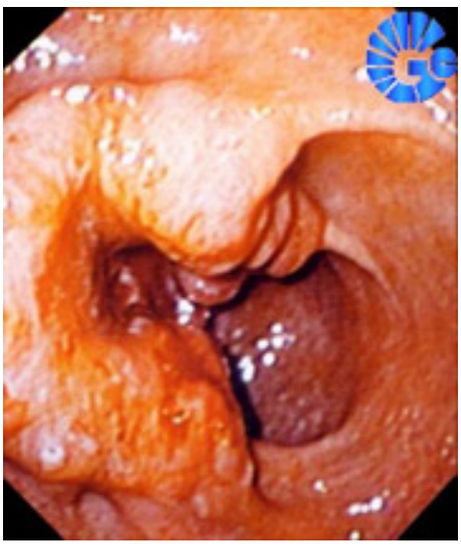

Figure 4. Gastric adenocarcinoma

3.4. Early and advanced lesions

Follow-up of patients with precursor lesions in populations at high gastric cancer risk has thrown light on the dynamics of the process. The progression of the precursor lesions described before follows a pattern of steady state, with episodes of progression to more advanced lesions and episodes of regression to less advanced lesions.

Unlike patients with advanced gastric cancer, patients diagnosed in an early stage of the disease present an excellent prognostic, in which a five-year survival rate is more than 90%. Early gastric cancer lesions are defined as the adenocarcinoma that is confined to the mucosa or submucosa, irrespective of lymphonode invasion (Figure 5). Many early gastric cancers are believed to go through a life cycle consisting of ulcerations, followed by healing, then reulceration, and some lesions remain at this early stage for years even without treatment [89]. Nevertheless, some early tumours rapidly became advanced and it is one of the principal

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questions concerning the gastric carcinogenesis. Are *H. pylori* virulence factors important to influence these alterations? To what extent *H. pylori* eradication treatment would be important to prevent the continued progression of the disease? To what extent *H. pylori* eradication in the early stage of cancer would be important to prevent the appearance of new lesions?

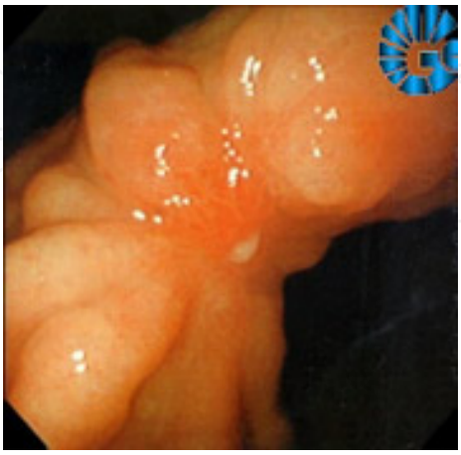

Figure 5. Early gastric adenocarcinoma

Considering these important questions, our chapter is divided into two sections: 1. the identification of the principal genetic characteristics of *H. pylori* strains in Brazilian patients with early distal type intestinal gastric adenocarcinoma, in order to determine the genotypic pattern of bacterium in our population through molecular techniques; 2. the discussion of the principal studies concerning *H. pylori* eradication and its importance to prevent the progression of precancerous lesions and the importance of *H. pylori* eradication in the early gastric cancer to prevent the development of new cancerous lesions.

4. Materials and methods

4.1. Determination of principal genotypes of *H. Pylori* in Brazilian patients with early gastric adenocarcinoma

4.1.1. Clinical samples

Clinical isolates of *H. pylori* analyzed in this study were obtained from the Laboratory of Pathology of the Center of Diagnosis of Digestive Diseases, Faculty of Medical Sciences, State University of Campinas (Campinas, São Paulo, Brazil). Third one paraffin wax-embedded specimens of gastric tissue were analyzed from a total of 31 patients with early distal type intestinal gastric adenocarcinoma. All the gastric tissue samples were obtained from endoscopic biopsy and were positive for *H. pylori* by histological analysis. Samples from gastric tissue obtained from endoscopic biopsy of patients with chronic gastritis and peptic ulcer

disease and positive for *H. pylori* were used as positive controls for all the reactions carried out in this study. The study was approved by the Ethics Committee of the Faculty of Medical Sciences, State University of Campinas.

4.1.2. Methods

Paraffin wax-embedded tissue DNA extraction was carried out with xylene and ethanol washes for paraffin removal; successive steps using proteinase K, phenol, chloroform, and isoamyl alcohol were carried out in order to isolate and purify the DNA [90]. Quantification of the obtained product and polymerase chain reaction (PCR) for human betaglobin gene [91] were carried out to guarantee the quality of all the results.

After DNA extraction, PCR for ureaseC [92], vacA (s and m) [93,94,95], cagA [96], cagT [97] and dupA (jhp0917 and jhp0918) [49] genes were performed. Primers pairs for all the genes as well as the length of the fragments are described in Table 1. PCR for ureaseC gene was carried out to confirm the positivity for *H. pylori* in all the samples.

After amplification, each PCR product was analyzed by eletrophoresis on a 2% agarose gel stained with ethidium bromide with a 0.5 X tris-acetate-EDTA buffer. A 100-bp ladder was used as standard.

Gene	Strand	Primer sequence (5´ - 3´)	Length (bp)
betaglobin	+	ACAAACTGTGTTCACTAGC	110
	-	CAACTTCATCCACGTTTCACC	
ureaseC	+	AAGCTTTAGGGGTGTTAGGGGTTT	294
	-	AAGCTTACTTTCTAACACTAACGC	
vacA (s1/s2)	+	ATGGAAATACAACAAACACAC	s1: 259
	-	CTGCTTGAATGCGCCAAAC	s2: 286
vacA m1	+	GGTCAAAATGCGGTCATGG	290
	-	CCATTGGTACCTGTAGAAAC	
vacA m2	+	ATGCTTTAATATCGTTGAGA	198
	-	GAACATGTTTTAGTGAAAGC	
cagA	+	GATAACAGGCAAGCTTTTGAGG	349
	-	CTGCAAAAGATTGTTTGGCAGA	
cagT	+	CCATGTTTATACGCCTGTGT	301
	-	CATCACCACACCCTTTTGAT	
dupA (jhp0917)	+	TGGTTTCTACTGACAGAGCGC	307
	-	AACACGCTGACAGGACAATCTCCC	
dupA (jhp0918)	+	CCTATATCGCTAACGCGCGCTC	276
	-	AAGCTGAAGCGTTTGTAACG	

Table 1. Sequence of synthetic oligonucleotide primers used to characterization of *H. pylori* strains

Then, for each specific reaction, products obtained were classified in vacA s1m1, s2m1, s1m2 or s2m2; cagA positive or negative; cagT positive or negative; and dupA positive or negative. dupA gene was considered positive when its two regions (jhp0917 and jhp0918) were positive simultaneously.

After all amplifications, a table with absolute frequencies (n) and percentages (%) was made in order to determine genotypes combinations.

4.2. Analysis of the principal manuscript references concerning the study of eradication treatment of *H. pylori* in precancerous lesions and early gastric cancer

After determination of the principal genotype presented in Brazilian patients with early gastric cancer, a review concerning the importance of eradication of *H. pylori* in precancerous lesions and in early gastric adenocacinoma was done.

5. Results

5.1. Determination of principal genotypes of *H. Pylori* in Brazilian patients with early gastric adenocarcinoma

PCR for ureaseC gene of *H. pylori* was positive for all 31 samples obtained from patients with early distal type intestinal gastric adenocarcinoma. As regards to vacA gene region s, of 31 samples, 71.0% (22 cases) were s1 and 29.0% (9 cases) were s2. Related to the vacA region m, all the samples were m1. Following this analysis, samples were classified in s1m1 or s2m1. So, 71.0% (22 cases) were s1m1 and 29.0% (9 cases) were s2m1.

Classification	
UreaseC	31 (100.0%)
vacA s1	22 (71.0%)
vacA s2	9 (29.0%)
vacA m1	31 (100.0%)
vacA m2	0 (0.0%)
vacA s1m1	22 (71.0%)
vacA s2m1	9 (29.0%)
cagA positive	19 (61.3%)
cagA negative	12 (38.7%)
cagT positive	17 (54.8%)
cagT negative	14 (45.2%)
dupA (jhp0917/jhp0918) positive	11 (35.5%)
dupA (jhp0917/jhp0918) negative	20 (64.5%)
Total	31 (100.0%)

Table 2. Frequencies and percentages of the principal genes of *H. pylori* studied in samples of early gastric cancer

As regards to gene cagA, 61.3% (19 cases) were cagA positive and, for cagT gene, 54.8% (17 cases) were positive. For dupA (jhp0917/jhp0918) gene, there were 35.5% (11 cases) of positivity. All these results can be seen in Table 2.

Genotypes combinations where then analyzed and the most prevalent genotype for gastric samples obtained from Brazilian patients with early distal type intestinal gastric adenocarcinoma was vacA s1m1, cagA positive, cagT positive and dupA negative (Table 3).

Genotype combinations: vacA s/m cagA cagT dupA (jhp0917/jhp0918)	**Early gastric cancer**
s1m1 neg neg neg	4 (12.90)
s1m1 neg neg pos	1 (3.23)
s2m1 neg neg neg	1 (3.23)
s2m1 neg neg pos	0 (0.00)
s1m1 neg pos neg	2 (6.45)
s1m1 neg pos pos	1 (3.23)
s2m1 neg pos neg	2 (6.45)
s2m1 neg pos pos	1 (3.23)
s1m1 pos neg neg	3 (9.68)
s1m1 pos neg pos	2 (6.45)
s2m1 pos neg neg	2 (6.45)
s2m1 pos neg pos	1 (3.23)
s1m1 pos pos neg	5 (16.13)
s1m1 pos pos pos	4 (12.90)
s2m1 pos pos neg	2 (6.45)
s2m1 pos pos pos	0 (0.00)
Total	**31**

Table 3. Genotype combinations for early gastric adenocarcinoma samples

5.2. Analysis of the principal manuscript references concerning the study of eradication treatment of *H. pylori* in precancerous lesions and early gastric cancer

Epidemiological studies have established a strong causal relationship between *H. pylori* infection and gastric cancer. *H. pylori* eradication is therefore likely to be one of the most promising approaches to gastric cancer prevention. Animal studies have shown that eradication of *H. pylori* infection, especially at the early stage, is effective in preventing *H. pylori*-related gastric carcinogenesis. However, the available data from human studies show that *H. pylori* eradication does not completely prevent gastric cancer and that it might be useful in patients without atrophic gastritis or intestinal metaplasia at baseline [77].

Considering these important issues, the present chapter book analized and discussed some studies that assessed the possible relationship between the eradication of *H. pylori* infection and the prevention of gastric cancer even if the precancerous cascade has started. Obviously,

there should be a point which there is no return in this process and the presence of *H. pylori* could not be more decisive to continue the progression of the precancerous lesions. With the aim to analyze these topics, we discussed some important questions concerning *H. pylori* infection, precancerous cascade and *H. pylori* eradication treatment, both in the precancerous lesions (atrophic gastritis, metaplasia intestinal and dysplasia) and in the early gastric cancer stage.

6. Discussion

Molecular techniques may be applied to the measurement of host or agent factors and of exposures. Molecular techniques help to stratify and to refine data by providing more sensitive and specific measurements, which facilitate epidemiologic activities, including disease surveillance, outbreak investigations, identifying transmission patterns and risk factors among apparently disparate cases, characterizing host-pathogen interactions, detecting uncultivatable organisms, providing clues for possible infectious causes of cancer and other chronic diseases, and providing better understanding of disease pathogenesis at the molecular level [98].

Our study determined the principal genotype of *H. pylori* strains in Brazilian patients with early distal type intestinal gastric adenocarcinoma, which is vacA s1m1, cagA positive, cagT positive and dupA negative. It provides to the scientific community important information concerning the epidemiology of gastric cancer in Brazil, as regards to the infecting strains. This principal *H. pylori* genotype was also the principal found in advanced gastric cancer samples, but, when considering the cagA gene in an isolate evaluation, it was more incident in patients with advanced gastric cancer [48].

Many factors, including a high-salt diet [99], genetic abnormality [100] and autoimmune gastritis [101], among others, have been reported concerning gastric carcinogenesis; however, it is clear that *H. pylori* infection is the most important gastric carcinogen [60, 102].

Currently recommended anti-*H. pylori* infection therapies achieve eradication rates of up to 90% [103]. Several studies have indicated that *H. pylori* screening and eradication is a cost-effective strategy for the prevention of gastric cancer in middle-aged adults, even if the treatment prevents only 20%-30% of *H. pylori*-associated cancers, and that the strategy is particularly beneficial in high-risk populations and in the long term [77,104,105,106], although the feasibility, safety and appropriated timing of this strategy for cancer prevention in the general population remains to be determined [77].

Some studies focused on patients with gastric precancerous lesions such as gastric atrophy, intestinal metaplasia and dysplasia and evaluated the effect of eradicating *H. pylori* on the intermediate lesions in the carcinogenic cascade rather than using gastric cancer as the primary end points [107].

Conflicting results have been reported on whether or not these precancerous lesions were reversible following successful eradication of *H. pylori* infection.

The investigation of Uemura et al. (2001) [108] is considered the first study providing some evidence that *H. pylori* eradication has an impact in gastric cancer. In this study, none of the 253 treated patients developed cancer, whilst there were 36 gastric cancer cases among 993 untreated patients. However, it is of note that the mean duration of follow-up after eradication was significantly shorter than the mean duration for patients who were not treated (4,8 vs. 8,5 years; $p < 0.001$). Therefore, the risk of gastric cancer development could have been understated in the treated group [107].

In a randomized placebo controlled study in China, gastritis (acute and chronic) decreased in both the antrum and the corpus at one year after *H. pylori* eradication and a slight regression of intestinal metaplasia was observed [109]. With similar results, a follow-up study developed by Zhou et al. (2003) [110] demonstrated that *H. pylori* eradication significantly reduced the severity and activity of chronic gastritis. Besides, while the proportion of intestinal metaplasia in the *H. pylori* positive group increased significantly, intestinal metaplasia in the antrum either regressed or had no progression in the *H. pylori* negative group.

In Colombia, a randomized, controlled chemoprevention trial with patients with confirmed multifocal nonmetaplastic atrophy and/or intestinal metaplasia demonstrated that, after *H. pylori* eradication therapy and/or dietary supplementation with ascorbic acid or beta-carotene, or their placebos, it was observed a significant increase in the rate of regression of the precursos lesions [111].

Other follow-up studies also identified that *H. pylori* eradication in patients with intestinal metaplasia could be important in the regression of the lesions [112-118]. In the same way, other randomized studies identified that *H. pylori* eradication in intestinal metaplasia could be important in the regression of this precancerous lesion [76,109-111,119,120]. Oppositely, other trials, both follow-up and randomized control, had not identified regression of intestinal metaplasia when *H. pylori* eradication was administrated [121-129].

Concerning to patients with gastric atrophy, the most part of the studies have identified that this lesion presents a regression when *H. pylori* eradication occurs [76, 109, 111, 119, 120, 128-130]. In Japan, where there is a significant ability in diagnostic, with a detection of 94% of early gastric cancers, one important multicenter randomized controlled trial by the Japan Gast Study Group enrolled patients undergoing previous endoscopic therapy for gastric cancer and demonstrated that eradication therapy significantly reduced the prevalence of secondary gastric cancer in a 3-year follow-up period [131]. However, this study does not demonstrate that eradication therapy can prevent newly developed gastric cancer even in secondary cancer, because the follow-up period was too short.

As well as other authors mentioned before, Ito (2009) [132] considered that theoretically *H. pylori* eradication therapy should be beneficial for cancer prevention. However, attention should be drawn to the fact that the gastric cancer risk is not similar between noninfected and eradicated people. Until now, many human studies have demonstrated that some patients develop gastric cancer even if they have undergone successful eradication therapy [133]. Considering it, we can conclude that eradication therapy may have an effect on cancer prevention if the therapy is administrated before a single cancer cell has transformed or before

cancer tissue shows invasive growth. It is likely that eradication therapy has no effect if the cancer has progressed to an advanced stage.

Wong et al. (2004) [134], with the aim to determine whether eradication of *H. pylori* infection reduces the incidence of gastric cancer, carried out a randomized, placebo controlled trial with 1630 *H. pylori* infected patients in a high-risk region of China. Their results indicated that *H. pylori* eradication can reduce the incidence of gastric cancer in patients without precancerous lesions at entry. Besides, the eradication therapy had no statistically significant effect on the incidence of gastric cancer in patients with precancerous lesions on presentation. It appears that there is a point of no return for patients with precancerous lesions and a chemoprevention strategy may work only in a subset of *H. pylori* infected subjects [135].

As regards to animal models, they are useful because they represent tractable systems that permit insights into the effects of host, pathogen and environmental factors on gastric carcinogenesis [136]. Nevertheless, the use of animals does not completely reflect *H. pylori*-induced cancer in humans. Romero-Gallo et al. (2008) [137], using a population of gerbils that received antibiotics for *H. pylori* treatment, demonstrated that the timing of intervention influences the magnitude of suppression of pro-inflammatory cytokine expression, inflammation, pre-malignant, and neoplastic lesions. These findings have demonstrated that treatment of *H. pylori* decreases the incidence and the severity of pro-inflammatory cytokine expression, as well as premalignant and malignant lesions. However, the effectiveness of eradication is dependent upon the timing of intervention [137].

A 5-year study in Japanese monkeys (*Macaca fustata*) demonstrated that *H. pylori* infection can cause gastric atrophy, increased cell proliferation, and mutation of *p53* in gastric epithelial cells [138]. In other study, with Mongolian gerbils, it was demonstrated that the resulting pathological changes in gastric mucosa are similar to those in humans [139]. Some studies demonstrated that when the animals were infected with *H. pylori* together with a carcinogen (N-methyl-N-nitrosourea or N-methyl-N-nitro-N-nitrosoguanidine), they developed gastric cancer (both diffuse and intestinal types) that were at significantly higher frequencies than animals receiving either *H. pylori* or the carcinogen alone [140-142]. Gastric cancer incidence was reduced at 75 weeks to 6.7%, 27.3%, and 38.2% in Mongolian gerbils receiving eradication treatment at early (15 weeks), middle (35 weeks) and late (55 weeks) stages, respectively. These results suggest that eradication at an early stage might be effective in preventing carcinogenesis. Other study demonstrated that application of antimicrobial therapy at 8 weeks postinoculation of bacterium attenuated inflammation, but did not completely prevent the development of premalignant and malignant lesions, indicating that the *H. pylori* eradication therapy is effective when administered at an early stage after infection [143]. Nevertheless, it is important to remember that the carcinogenic potential of *H. pylori* is strain dependent and some results of these studies could be caused by the use of an *H. pylori* strain that lacked carcinogenic potential.

In the mouse model, both *H. pylori* infection and gastric atrophy increase the serum concentration of polypeptide hormone gastrin, a hormone that controls secretion of gastric acid by the stomach´s parietal cells and hypergastrinemia is regarded to play a role in the development.

The results obtained indicated that the timing of antimicrobial eradication therapy is very important as early application can prevent the progression of gastric cancer [144].

Finally, for cancers detected early, endoscopic mucosal resection can conserve the noncancerous gastric mucosa, but it can not eliminate the recurrence of metachronous gastric cancer [145]. Fukase et al. (2008) [131], in a randomized control trial, studied a group of patients submitted to *H. pylori* eradication therapy following endoscopic resection of early gastric cancer. These patients were monitored at different time intervals: at 3 years, metachronous gastric cancer had developed in 9 of 225 patients in the eradication group compared with 24 of 250 patients in the control group, suggesting that prophylactic eradication of *H. pylori* in a high-risk population can substantially reduce gastric cancer rates.

Briefly, the most part of the studies suggests that *H. pylori* eradication is able to induce regression of precancerous lesions in most of the treated subjects, and particularly in those with baseline, early and non-severe lesions. However, it also seems that a proportion of treated subjects will still show progression of preneoplastic lesions. So, we can consider that they really are other factors that contribute to the progression of these lesions. So, *H. pylori* eradication is an effective strategy in reducing the risk of gastric cancer; however, it is not efficient enough to eradicate gastric cancer. Prevention of the infection, *H. pylori* immunization, *H. pylori* eradication in the youth, selection of the high-risk population, and alternative chemopreventive measures may be essential for optimal management of malignancy of the stomach.

7. Conclusions

All the available evidence suggests that *H. pylori* eradication might represent a primary chemopreventive strategy in a subset of subjects. However, *H. pylori* eradication in those patients who have already developed advanced preneoplastic lesions does not prevent gastric cancer development, and endoscopic follow-up should always be performed.

More research is needed to elucidate mechanisms underlying the *H. pylori*-induced gastric carcinogenesis. As the infection usually depends on the characteristics of the infecting strain, studies that determine the genotypes in gastric cancer, as we presented in our study, are necessary. Besides, more trials concerning the interaction among the infecting strain, the host characteristics and the external environment are also needed to explaining the complex gastric carcinogenesis. An understanding of biochemical, genetic and epigenetic changes following eradication therapy would be helpful to develop strategies to identify high-risk individuals, thereby contributing to effective management in gastric cancer prevention.

Finally, it is important to gain more insight into the pathogenesis of H. pylori-induced gastric adenocarcinoma, not only to develop more effective treatments for this cancer, but also because it might serve as a paradigm for the role of chronic inflammation in the genesis of other malignancies.

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