

***Helicobacter pylori* infection and gastric carcinoma: Not all the strains and patients are alike**

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Abstract

Gastric carcinoma (GC) develops in only 1%-3% of *Helicobacter pylori* (*H. pylori*) infected people. The role in GC formation of the bacterial genotypes, gene polymorphisms and host's factors may therefore be important. The risk of GC is enhanced when individuals are infected by strains expressing the oncoprotein CagA, in particular if CagA has a high number of repeats containing the EPIYA sequence in its C'-terminal variable region or particular amino acid sequences flank the EPIYA motifs. *H. pylori* infection triggers an inflammatory response characterised by an increased secretion of some chemokines by immunocytes and colonised gastric epithelial cells; these molecules are especially constituted by proteins composing the interleukin-1beta (IL-1 β) group and tumour necrosis factor-alpha (TNF- α). Polymorphisms in the promoter regions of genes encoding these molecules, could account for high concentrations of IL-1 β and TNF- α in the gastric mucosa, which may cause hypochlorhydria and eventually GC. Inconsistent results have been attained with other haplotypes of inflammatory and anti-inflammatory cytokines. Genomic mechanisms of GC development are mainly based on chromosomal or microsatellite instability (MSI) and deregulation of signalling transduction pathways. *H. pylori* infection may induce DNA instability and breaks of double-strand DNA in gastric mucocytes. Different *H. pylori* strains seem to differently increase the risk of cancer development run by the host. Certain *H. pylori* genotypes (such as the *cagA* positive) induce high degrees of chronic inflammation and determine an increase of mutagenesis rate, oxidative-stress, mismatch repair mechanisms, down-regulation of base excision and genetic instability, as well as generation of reactive oxygen species that modulate apoptosis; these phenomena may end to trigger or concur to GC development.

Key words: *Helicobacter pylori* infection; CagA; CagA gene polymorphism; Haplotype; Human gene mutation;

Gene methylation; Gastric carcinoma; Inflammatory cytokine

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Core tip: CagA and the *cagA* types may play different roles in the intestinal and diffuse histotypes of gastric carcinoma (GC); The current criteria of *Helicobacter pylori* (*H. pylori*) strain classification based on their carcinogenic potential gave rise to confusion and should be unified. The possible role of inflammatory cytokine haplotypes in GC development should be reassessed taking into account some host's factors, the most important being different ethnic origin. Infection by the *cagA* positive *H. pylori* genotype may determine an increased inflammatory response and a consequent enhancement of mutagenesis rate, oxidative-stress, reactive oxygen species generation, dysfunction of DNA repair mechanisms, genetic instability and resultant high risk of GC development.

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INTRODUCTION

Gastric carcinoma (GC) is the second most frequent cause of death from cancer worldwide and the most common example of a neoplasia developing on a ground of a chronically inflamed mucosa. GC has also another record: It is the only known malignant tumour that can develop as a consequence of a chronic bacterial infection^[1]. In 1994, the International Agency for Research on Cancer classified the organism responsible for the infection, *Helicobacter pylori* (*H. pylori*) - a Gram negative, microaerophilic and spiral-shaped species that finds its *habitat* in human stomachs - as a definite carcinogen to humans (Group 1): The connection of *H. pylori* with gastric cancer was considered similar to that existing between the cigarette smoke and lung cancer^[2].

The bacterium *H. pylori*: Not all the strains are alike

It soon became clear, however, that such comparison was reductive and too simplistic, especially because the ability of these bacteria to trigger a neoplasm is not limited to the inflammatory and immune response to the infection that they cause, but it also resides in a series of bacterial factors capable of prompting and modulating the carcinogenic process^[3].

As is the case for all diseases, also GC develops from the concomitance of three factors: The etiological agent, the host and the environment. Of course, many other factors may occur; for example, the cancer histological

variant, the degree of differentiation of the neoplasia *etc.*^[4]. Regarding the etiological agent, *H. pylori*, there are many indications that not all strains are equivalent in their carcinogenic potential and that those expressing an immunodominant peptide determinant called CagA (cytotoxin associated gene A), are endowed with an increased inflammatory and carcinogenic potential^[5-9]. A first point has therefore been established: Strain genomic diversity corresponds to different ability to promote cancer. The possibility that a bacterial factor (CagA) could trigger or concur to the development of GC is one of the most important scientific achievements following the isolation of *H. pylori*.

The importance of being called CagA positive

It is worthwhile mentioning the steps that paved the way to the discovery of CagA. At the end of the 80ies, Leunk *et al.*^[10] first proposed that *H. pylori* should not be considered a clonal pathogen, as a relevant proportion of isolates produce a vacuolating toxin, which could account in part for the gastric mucosa damage observed in infected individuals. Afterward, our group suggested that infection by cytotoxic strains increased the risk of developing peptic ulceration^[11] and that virtually all cytotoxic isolates also secreted a 120 kDa highly immunogenic protein, later called CagA^[12]. In 1992, Crabtree *et al.*^[13] demonstrated, through *ex vivo* experiments, that such a protein was produced either by the bacteria isolated in culture and also by the organisms colonizing the gastric epithelium: Gastric antral explants of patients with GC and other pathologies were cultured *in vitro* for a few days; the bacteria that colonized the mucosa kept on secreting this peptide, which could lastly be detected in the culture medium by using immunological methods^[13]. In 1993, the same team established, for the first time, the existence of a relationship between infection by strains expressing the 120 kDa protein and GC development^[14]. Their observations were important also because these researchers found anti-120 kDa protein mucosal IgA antibodies even in the absence of systemic IgG to this protein and, in some patients, also in cases with urease negative biopsies (false negatives). In the same year (1993), the gene encoding for the 120 kDa protein was cloned, sequenced and called *cagA* due to the strict association of protein expression with cytotoxin production^[5]. As a result of these findings, the number of studies dealing with the characterisation of CagA and its potential carcinogenicity increased exponentially and results lead to the common conclusion that such peptide is a major factor in gastric carcinogenesis.

CagA is the product of the homonymous gene placed at the end of the so-called pathogenicity island (PAI) *cag*, a fragment of DNA encompassing an approximately 40 kb cluster of genes involved in virulence. In the field of bacteriology there are numerous examples of PAIs harboured by diverse bacterial species or their virulent variants, whether they are human (*Bordetella*

pertussis, *Escherichia coli*, *Salmonella enterica*, etc.) or plant pathogens (*Agrobacterium tumefaciens*). In some species, PAI genes cooperate to translate effectors (mainly proteins) endowed with carcinogenic potential inside colonised cells. In *H. pylori*, such determinant is CagA. Similarly, *A. tumefaciens* exploits the Type IV secretion system *vir* to translate a single-stranded form of T-region (T-strand) coated by the ssDNA-binding protein VirE2 (T-Complex) into the host's vegetal cell nuclei. Once inside the nucleus, the T-strand can be converted in a double-stranded form (T-DNA), whose expression causes an uncontrolled host cell proliferation and tumour development^[15].

Epidemiological and genomic studies suggest that the development of GC is a possible consequence of infection by strains expressing CagA^[1,8,9,14,16]. In effects, using Mongolian gerbils infected experimentally, it was shown that only CagA positive (CagA+) *H. pylori* strains were able to induce stomach tumours^[17]. In addition, a study of our group revealed that, while virtually all patients with intestinal histotype of GC had serum antibodies to CagA, the prevalence of anti-CagA antibodies in patients with the diffuse GC variety was similar to that observed in infected controls without neoplasia^[16]. These data were confirmed by the results of an epidemiological study: The overall GC risk in infected people lacking anti-CagA antibodies (CagA-) was increased, but in non-significant way; in any case, CagA- *H. pylori* infection was associated with the growth of the diffuse variety of GC (with an OR of 9.0)^[8].

It therefore seems that infections by CagA+ strains expose people to an increased risk of GC respect to infections by CagA- *H. pylori* strains, which can only be associated with the diffuse histotype. In effect, things work slightly differently. In a recent study, we examined for the presence of *cagA* up to 25 distinct, well separated colonies per patient with GC; even though only individuals with diffuse histotype GC harboured *cagA* negative (*cagA*-) organisms, in all cases patients were also infected by at least one *cagA* positive (*cagA*+) strain^[18]. These observations do not corroborate the supposed propensity of strains lacking *cag* PAI to concur to diffuse GC development and suggest that, for a better comprehension of the role played by *cagA* in the histological variety of GC, many colonies per patient should be examined genomically.

CagA phosphorylation by mucocytes: Like shooting oneself in the foot

H. pylori organisms expressing CagA differ in their carcinogenic potential. Let us have a look at the mechanisms that may influence the ability of such a protein to trigger and/or concur to GC formation. CagA, following colonisation, is translated into the gastric epithelial cells through a conjugative apparatus encoded by the *cag* PAI genes upstream *cagA*; then, a portion of intracellular CagA is phosphorylated by numerous kinases, members of the host cell Src family (such as Yes, Lyn, Fyn and

c-Src,) at the EPIYA C'-terminal site (Glu-Pro-Ile-Tyr-Ala) motif of tyrosine^[19]. Phosphorylated CagA physically interacts with the oncogenic tyrosine phosphatase SHP-2 (Src homology phosphatase 2), modifying cellular functions and altering mammalian signal transduction machineries. SHP-2, in fact, is implicated in the regulation of cell adhesion, spreading and migration. In this manner, phosphorylated CagA causes deregulation of SHP-2 and induces abnormal proliferation, as well as movement of cells of the gastric epithelial layer, activates mitogenic signalling and disturbs host-signalling routes^[20]. All these events may also predispose cells to accumulate multiple genetic and epigenetic alterations involved in gastric tumorigenesis^[21].

Unphosphorylated CagA, on the other hand, interacts with the tumour suppressor protein of p53 (ASPP2), which also exert an apoptosis-stimulating activity^[22]. In normal conditions, following genotoxic and oncogenic stimuli, ASPP2 associates with tumour suppressor p53, activates it and induces apoptosis. After interaction with CagA, cytosolic p53 is recruited by ASPP2 and subsequently is degraded by an enzyme complex that control cell-cycle and apoptosis, the proteasome. As a consequence, the apoptotic response of host cells is inhibited. In other words, unphosphorylated CagA takes control of ASPP2 and subverts the tumour suppressor pathway of apoptosis-stimulating protein p53 with consequent promotion of cell survival and cell transformation^[20-22]. The resultant abnormal proliferation of gastric epithelial cells may contribute to GC development.

Individual CagA proteins have different biological activity and tumorigenic potential: When size matters

H. pylori strains secreting CagA protein endowed with increased biological activity can be considered more virulent and even more closely associated with gastric cancer. At the end of 90's, the group of Graham discovered the ability of distinct CagA proteins to perturb cellular functions might vary in different isolates^[23]. The CagA C'-terminal region contains one or more repeats of the same amino acids in sequence (Glu-Pro-Ile-Tyr-Ala, or EPIYA)^[5]; the teleonomic significance of such phenomenon reflects the bacterial strategy to generate antigenic diversity, which may protect the organisms from the immune response. The number of EPIYA motifs correlates with the size of the *cagA* variable region. Yamaoka *et al.*^[23] basing of the amplicon sizes obtained with primers encompassing the entire *cagA* variable region, classified *H. pylori* isolates in *cagA* structural types A, B, C and D (amplicons characterising types B and D have the same size and can be differentiated by sequencing). Strains with the *cagA* structural type C have the highest number of EPIYA phosphorylation motifs and were isolated significantly more often from patients with GC^[23], confirming a previous observation that individuals with GC are infected by strains expressing CagA proteins with higher mass^[24]. The incr-

eased carcinogenic potential of the *cagA* structural type C was also confirmed by another study of the same group, which showed that patients infected by *H. pylori* with this *cagA* genotype run a higher risk of developing gastric mucosa atrophy, a precancerous condition^[25].

Now, it should be highlighted that the primers used in these studies to amplify the *cagA* variable region were designed on oriental (Japanese) strains, which may differ from western strains in the nucleotide sequence encoding the C'-terminal variable region^[26]. Probably for this reason not all surveys on the same subject have confirmed the Yamaoka *et al.*^[23,25]'s findings. In an initial study of our group, in which we used the Yamaoka's primers to amplify the *cagA* variable region of Italian strains^[27], we sometime obtained amplicons shorter than the PCR product that characterises the *cagA* structural type A; strains producing such amplicons, which presented a deletion of about 100 bp respect to *cagA* type A, were named type A(I), with (I) standing for Italy, because, as far as we know, similar *cagA* structural variety had not previously been described. In a more recent investigation, we observed similar proportions of the various *cagA* structural types in Italian *H. pylori* organisms isolated from GC cases and from controls (patients without neoplasia, with chronic gastritis only)^[28]. In addition, in the control subjects, we frequently detected strains with the *cagA* structural type A(I). The increased prevalence of such a *cagA* type in individuals without GC prompted us to hypothesise that the reduced dimensions of the encoded CagA may decrease the ability of these bacteria to trigger a neoplastic process. As a matter of fact, the capability of CagA of binding SHP-2, and therefore of disturbing the various cellular functions, is regulated by the amounts of tyrosine phosphorylation site sequences, *i.e.*, the CagA size^[20]; therefore, the shorter the protein, the less numerous are the EPIYA repeats that undergo phosphorylation and the lower is the carcinogenic potential of strains.

The employment of primers proposed by Yamaoka *et al.*^[23] to amplify the *cagA* variable region of western strains has sometimes led to results similar to those of the Japanese researchers: South African researchers, for instance, have confirmed that patients with GC have an increased prevalence of strains with type C CagA^[29]. Investigations dealing with this important subject, however, are not numerous; in addition, sometimes they attained different conclusions and have even contributed to create confusion in this subject. Malaysian authors, for instance, using the primers designed by Yamaoka *et al.*^[23], determined the presence and distribution of *cagA* variants among different ethnic groups and various gastroduodenal diseases^[30]. They obtained three types of amplicons and named the *cagA* subtypes with capital letters, A, B and C (like did Yamaoka *et al.*^[23]), but, just to complicate this topic further, they used a different criterion (respect to that of the previous study) and called subtype C the strains with the smallest amplicon

size and subtype B those with the greatest one. Specific *cagA* subtype A strains (those yielding amplicons of intermediate size) were predominantly isolated from Chinese compared to Malays and Indians patients. Since Chinese patients have the highest risk of GC disease respect to the other ethnic groups, these investigators concluded that *cagA* subtyping could be used as a clinical biomarker for severe outcome of infection. Such statement, however, was based on indirect observations because they did not examine strains from GC cases^[30].

In 2002, Higashi *et al.*^[20] observed that the ability of CagA secreted by different strains to disturb host-cell functions can be influenced by the strength of SHP-2 binding activity, which was increased in *H. pylori* strains obtained from patients living in East Asian areas (Japan, Chorea and China) respect to those isolated from patients of Western countries (Europe, America, and Australia). In addition to the number of EPIYA repeats, it was also found that polymorphism in the nucleotide sequence flanking the regions that encode EPIYA could affect the potential of different *H. pylori* strains to promote gastric carcinogenesis^[31]. According to the geographic regions in which strains are isolated, it is possible to characterise the *cagA* variable region on the basis of the number and the type of sequences. The Western *H. pylori* CagA has two segments of 32 and 40 amino acids flanking EPIYA (EPIYA-A and EPIYA-B types) and one to three 34 amino acid EPIYA-C segments (A-B-C type CagA)^[21]. Eastern CagA presents EPIYA-A and EPIYA-B segments, but none of the EPIYA-C fragment; it has instead one copy alone of a segment called EPIYA-D, which represents the main tyrosine phosphorylation site^[20,21]. In western strains, the major site of tyrosine phosphorylation of CagA is EPIYA-C; the tyrosine residues that characterize EPIYA-A and EPIYA-B segments are phosphorylated only very weakly. Such a difference in phosphorylation degrees resides in the diverse consensus high-affinity binding sequence for the SH2 domains of SHP-2. Unlike what happens for EPIYA-D type, the western EPIYA-C type of CagA differs by a single amino acid from the consensus SHP-2 binding sequence^[21].

In conclusion, the carcinogenic potential of *H. pylori* varies according to the number and the *cagA* structural types of the EPIYA flanking regions. East Asian CagA has an increased virulence and a strong ability to trigger GC, while, among western isolates, more carcinogenic are the helicobacters with two or three CagA EPIYA-C sites. This was also demonstrated by a study in which it was observed that 83.3% of GC strains possessed multiple EPIYA-C sites, vs only 5.2% of strains isolated from patients with chronic gastritis only (controls)^[32].

Often, *cagA*+ strains are also isolated from patients with duodenal ulcer (NF personal observation). This finding may create confusion, because is common knowledge that patients with duodenal ulcer are like protected from GC development; however, the results of a recent study^[33] showed that the increased virulence

of strains with CagA EPIYA-C type augmented the risk of gastric cancer and not peptic ulceration. Some studies diverge from these conclusions: Findings of an investigation carried out in Colombia suggest that polymorphic CagA proteins, based on sequences flanking the EPIYA motifs, are not clearly associated with the outcome of the infection^[34]. The absence of association between the CagA polymorphisms and pathogenesis of gastroduodenal diseases could be due to geographic factors and/or the host's genetic features and environmental determinants.

In conclusion, the results of this kind of investigations are potentially useful, but the confusion existing in this field ought to be rectified and the different researchers should use the same criteria for classification. The various groups, however, have reached a common conclusion: Not all the strains are alike in their carcinogenic potential.

NOT ALL PATIENTS ARE ALIKE: THE ROLE OF THE HOST'S INFLAMMATORY CYTOKINE HAPLOTYPES IN GC DEVELOPMENT

Background

The hypothesis that human genetic polymorphisms may affect predisposition to GC has recently been explored. GC develops in only 1%-3% of *H. pylori* infected individuals, which suggests that the host background matters in this neoplasia. Several pro-inflammatory cytokines are produced by the immune system against *H. pylori*; among them, IL-1 β is of paramount importance; a second one is tumor necrosis factor- α (TNF- α); both of them are closely related to epithelial injury and gastric hypochlorhydria^[35,36]. At low concentrations, TNF- α enhances the protective inflammatory response; at high concentrations, it can injure the gastric mucosa and cause severe pathology^[37]. IL-1 β increases the surface molecule expression on endothelial cells, causing leukocytes to adhere; IL-1 β also induces the production of macrophage chemokines leading to neutrophil activation. Recent investigations have revealed that there is a genetic regulation of the host cytokine response to inflammatory stimuli. Genomic variants of *IL-1 β* and *TNF- α* were shown to correlate with the clinical outcomes of tumors, including GC^[38,39]. The *IL-1 β* gene cluster is polymorphic, with some alleles present at relatively high frequencies. Particular *IL-1 β* haplotypes enhance the risk of GC because they induce an over expression of its product in the stomach, causing chronic hypochlorhydria, which in turn may produce gastric atrophy and, eventually and in the presence of other risk factors, GC^[40]. In addition, patients with a particular haplotype of the gene that encodes IL-1RA (receptor antagonist) have an elevated risk of developing GC. IL-1RA is an anti-inflammatory cytokine, which is a competitor for IL-1 β receptors, thus regulating the possible

harmful effects of IL-1 β receptors.

The role of the host in GC development could be important because the inflammatory response to infections varies from patient to patient due to the gene polymorphism of inflammatory and anti-inflammatory cytokines. Many studies have been performed in the last 15 years, with the scope of identifying a genetic marker that can determine whether or not people carrying the infection might be at risk of developing GC in *H. pylori* infected patients^[41-59]. Such a marker is still lacking, and we shall try to explain some of the reasons underlying this problem.

The association between chronic inflammation and cancer has been known since Virchow, in 1864, wrote that cancer would arise from sites of inflammation: "Chronic irritation which is manifested by a chronic inflammation is a key promoter of cancer" (Quoted by Balkwill and Mantovani^[60]). Individual cytokines were specifically examined, in particular the proinflammatory ones. The most well known cytokine is IL-1, along with its receptor (IL-1R) and the antagonist of this receptor (IL-1RA); all of them share the chromosomal location of the *IL-1* gene family (namely 2q13-14). IL-1 β has thus been established as an important regulator of carcinogenesis, characteristic of interactions between the host and environment^[54].

IL-1 β haplotypes

The *IL-1 β* gene displays considerable polymorphism^[54]; the presence of C to T transition was frequently found either in the promoter region at positions -511 (CT; dbSNP: rs16944), at position -31 (TC; dbSNP: rs1143627) or in the coding region at position +3954 (CT; dbSNP: rs1143634) base pairs from the origin of transcription. The two single nucleotide polymorphisms (SNPs) within promoter region are in linkage disequilibrium. The *IL-1 β* -31 TC substitution disrupts a TATA-box motif; this leads to several transcription factors having altered binding affinities, resulting in modified IL-1 β transcription. The *IL-1 β* +3954 CT substitution is a synonymous SNP. It was demonstrated *in vitro* that the C to T transition at positions -511 and +3954 correlated with elevated IL-1 β levels as a result of lipopolysaccharide (LPS)-stimulated IL-1 β protein secretion^[54].

The paradigm of all subsequent studies regarding GC with respect to different haplotypes in cytokines was the focus of the paper by El-Omar *et al.*^[38], who noted for the first time that the presence of two polymorphisms (rs16944 and rs1143627) in the promoter region of the *IL-1 β* gene, identified an increased risk of hypochlorhydria, as a result of *H. pylori* infection and GC^[38]. These polymorphisms determine an increased secretion of IL-1 β ^[61], a conclusion confirmed and generalized by later reports^[62]. The discrepancy of results reached in different papers was clarified only after several years, once taking into account the origin of the population and the type of GC. Overall, more than 90 publications dealt with this issue, over half of them from Asia, and a single one

from North America, a clear indication of the perceived relevance of this neoplasia in the different populations.

Since the single studies are extremely inconsistent and, if taken alone, contribute little to the general overview, we opted for reporting from selected meta-analyses in this paper. Meta-analyses accrued from time to time in a number of studies originating from different countries, which is one of the main causes of contradictory results, as well as from different histological variety of stomach malignancies, another cause of strong difference in results^[41-59].

The overall findings from the large amount of efforts can be summarized as follows:

(1) *IL-1 β receptor antagonist (IL-1 β RA)* polymorphism: The most credible and consistent association of peculiar genetic variation with GC was found for *IL-1 β RA* haplotypes. Four alleles, numbered 1 to 4, are widely present in the general populations. People carrying the homozygous allele 2/2 (*IL-1 β RN2*) were found to be at higher risk of developing cancer among non-Asian populations. Moreover, the analysis of GC patients altogether, without stratifying according to histological type, anatomic site or country of origin, showed that patients carrying homozygous allele 2, or *IL-1 RN2* had an increased risk of developing cancer, which was statistically significant. The risk was found both in cardia and non-cardia types of neoplasia. A possible explanation for the risk stems from the high *IL-1 β* levels circulating among *IL-RA* allele 2/2 carriers^[63].

(2) *IL-1 β -31 CT* polymorphism: A second plausible association was the decreased risk of GC in Asians carrying the haplotypes in the *IL-1 β -31 CC* promoter region. A decreased risk of GC among *IL-1 β -31C* carriers was confirmed, but solely for Asian patients.

(3) *IL-1 β -511 CT* polymorphism in populations of different ethnic origin: Sub-analysis of various populations revealed a statistically significant association of stomach cancer with the *IL-1 β* polymorphism at promoter region -511 CT in case-control studies based on populations (OR = 1.20, 95%CI: 1.00-1.43)^[54]. The association is more consistent if only Caucasian populations are analyzed. Nevertheless, if taken together, the studies failed to show the association when stratified by ethnicities; *IL-1 β -511 CT* polymorphism according to tumor site: A significant association of *IL-1 β -511 CT* promoter region polymorphism was observed for stomach cancers when the tumor site (cardia vs non-cardia) was taken into account, as well as for histology subtypes (intestinal or diffuse/mixed). The association was present both in the case of non-cardia GC (OR = 1.57, 95%CI: 1.06-2.31) as well as intestinal GC (respectively OR = 1.57, 95%CI: 1.06-2.31 and OR = 1.24, 95%CI: 1.04-1.49)^[54]; *IL-1 β +3954 CT* polymorphism: Recently, Xu *et al*^[54] performed a meta-analysis that was confirmed by that one by Xue *et al*^[49]: There is a lack of association between *IL-1 β +3954 CT* and GC risk.

(4) *IL-10* haplotypes: *IL-10* - regarded as the ma-

jor anti-inflammatory cytokine - will bind in form of homodimer its complex receptor, comprising four *IL-10* receptor molecules, namely 2 *IL-10 R1* and 2 *IL-10 R2*. The binding induces *STAT3* signaling *via* the phosphorylation of the cytoplasmic tails of *IL-10* receptor 1. *IL-10* can inhibit the synthesis of pro-inflammatory cytokines; moreover it can block the function of nuclear factor-kappa B (NF- κ B), and has other regulatory properties, *e.g.*, *JAK-STAT* signaling^[64]. The *IL-10* gene is known to possess several SNPs, some in the distal region upstream of the coding gene (-1082 A/G, -819 T/C) and a proximal one (the -592 A/C). Again, the complex signaling and polymorphism of *IL-10* can explain the contradictory results of the investigations.

(5) *IL-10 -1082 AG* polymorphism: A clear and curious dichotomy is evident, that is, when the studies were stratified according to Asian and non-Asian populations the observations reached opposite results. The Asian populations had greater risk of GC among *IL-10 -1082 G* carriers; conversely, there was a decreased risk among the non-Asian populations. Meta-analysis specific for *IL-10* confirmed for Asian population the increased risk for intestinal type of gastric neoplasia in *IL-10 -1082 GG* or *GA* haplotypes^[53]; *IL-10 -592 AC* polymorphism: The -592 AC polymorphism failed to show any association, as the odd ratios for GC were 0.93 and 0.94 for homozygous and heterozygous population^[55,65]; *IL-10 -819 TC* polymorphism: Little data is available for this polymorphism, confirming a protective effect in Asian populations. Nevertheless, it was not found to be associated with the reduced susceptibility to GC in individuals infected with *H. pylori* compared to uninfected controls. The *IL-10 -819 TT* genotype was found to be inversely correlated with the risk of the diffuse subtype, but not the intestinal subtype GC^[51].

(6) *IL-8 - 251* polymorphisms: Continuous expression of human *IL-8* in transgenic mice (whereby *IL-8* is under the control of its own regulatory elements) increased tumorigenesis. Therefore, *IL-8* may play an important role in gastrointestinal cancers. Elevated *IL-8* levels could be linked to a poor prognosis of neoplasia, henceforth its levels may be indicative of more aggressive GCs.

Early data seemed to provide a possible association in GC as well^[66]. A recent meta-analysis showed that the *IL-8 -251 AA* genotype in the Han population correlates with augmented risk of developing GC and *AA* genotype carriers appear to be more likely to develop GC in Asian populations. In addition, the *IL-8 -251 AA* genotype tended to be related to intestinal GC, but not with *H. pylori* infectious status^[52]. There was no link between *IL-8* polymorphisms and *H. pylori*-related gastric malignancies in non-Asian populations in all the meta-analyses examined^[48,67].

(7) *TNF- α* polymorphism: Experimental studies have implicated *TNF- α* in processes that are involved in cancer progression, including promotion of metastatic behaviour and cancer associated cachexia^[68,69]. The lack of *TNF- α*

in mice makes them resistant to carcinogenesis^[70]. Clearly, such observation highlighted the link between genetic haplotypes for *TNF-α* and GC.

***TNF-α* -308 AG polymorphism:** It was surprising to find a lack of association of this polymorphism with increased risk of GC, with only one exception: Non-Asian patients with distal cancer and homozygous for -308 AA alleles; the association, moreover, appeared to exist for cancer of diffuse type only. However, this association was not confirmed when only good quality studies were taken into account, according to Persson *et al.*^[48]. Opposite conclusions were obtained by Zhu *et al.*^[59]; they recently analyzed all studies and concluded that, in the Caucasian populations, *TNF-α* rs1800629 (-308 AG) polymorphism indeed posed increased risk of GC. They used several genetic comparison models, *i.e.*, A vs G, AA vs GG and AA vs GG/GA that gave OR respectively of 1.32, 1.76 and 1.62, all highly significant (A vs G: OR = 1.32, 95%CI: 1.12-1.56, *P* = 0.001; AA vs GG: OR = 1.76, 95%CI: 1.37-2.26, *P* < 0.001; AA vs GG/GA: OR = 1.62, 95%CI: 1.27-2.07, *P* < 0.001)^[59].

***TNF-α* -238 polymorphism** did not correlate with an increased cancer risk^[48,58].

***TNF-α* 857 CT polymorphism:** Reports on this topic are quite controversial. Cen *et al.*^[57] recently published his analysis of nine studies (all the reported ones); overall, they confirm that the *TNF-α* 857 CT polymorphism posed an elevated risk of GC solely among Asians; all four genetic models considered T vs C, TT vs CC, CT vs CC and TT vs CT gave consistent data, respectively with OR of 1.19, 1.44, 1.19 and 1.21 (their statistical significance being *P* = 0.002, *P* = 0.032, *P* = 0.008, *P* = 0.003 respectively).

***TNF-β* 252 AG polymorphism:** A weak association with stomach malignancy was present in Asian populations, according to Xu *et al.*^[71]. Analysis by ethnicity revealed that the *TNF-β* 252 AG polymorphism correlated with a minor risk of GC (G vs A: OR = 1.10, 95%CI: 1.02-1.19, *P* = 0.015) exclusively in Asians, not in Caucasians.

Dutch patients were analyzed for their polymorphism of IL-1β; they were found to carry lower risk of GC when heterozygous for either the IL-1B -511 and for the IL1β -31 TATA-box (genotype T/C)^[72]. The EBV status of the patients did not affect this correlation and there could therefore be an early shared molecular mechanism in the progression of EBV-positive and negative GCs^[72].

IL-6 polymorphism was not studied in relation to GC.

IL-6 knockout mice develop cancer less frequently^[73]. It is therefore plausible that high IL-6 levels will promote tumorigenesis. Today, IL-6 is considered to be a relevant tumor-promoting factor also in humans. Indeed, it was correlated with glioma, lymphoma and melanoma at first, then with solid cancers such as breast and colorectal neoplasia (also ovarian and pancreatic), prostate, renal and colorectal cancers.

IL-6 is a critical factor during chronic inflammation, since it is required for the induction of effector Th17

cells and inhibits the differentiation of regulatory T cells.

Stomach cells, however, lack IL-6 receptors; hence it cannot dimerize with the second receptor (gp 130) and the "classic signaling" is restricted to cells bearing both mIL-6R and gp130 on their surface. The latter is widely expressed, whilst mIL-6R expression is limited to some leukocytes, hepatocytes and cancer cells. It is therefore quite understandable that, as recently reported, a large meta-analysis on 105000 people established the lack of association of cancer risk with *IL-6* polymorphism in Caucasians^[74], despite the association which holds true for Africans.

Other cytokines

Gene polymorphism concerning cytokine different from those we have dealt with was recently considered in relation to the risk of developing GC. The studies are not sufficiently large so far; however it is worth reporting that a haplotypes of *IL-17*, *IL-17F* rs763780 TC, was significantly associated with GC development in Asian population^[75].

IL-11 was taken into consideration in a single study^[76]. A reduced risk for developing cancer at the gastric site was found for a polymorphism in the *IL-4* -590 CT gene in Caucasian but not in Asian populations. *H. pylori* status was not taken into consideration in these studies^[77].

Different results in different studies: The origin of the problem

IL-1β, *TNF-α* and the remaining dozens of cytokines are not the final executor of immune signaling or the resulting consequences in cancer promotion and spread. IL-1, *TNF-α*, together with bacterial antigens, LPS and several other signaling molecules bind their respective receptors on the cell membrane; a cascade of signals ensues upon receptor activation, which depends on the levels of Mg-ATP availability (in turn on Mg²⁺ concentration in cells). Numerous different proteins are involved and regulate the signaling pathway, which finally results in the activation of a large family of DNA-binding proteins, the NF-κB family^[78], which is a complex that regulates DNA transcription. NF-κB dimers are formed upon activation, stimulating the transcription of genes that encode cytokines, growth factors, chemokines, and anti-apoptotic factors^[79]. However, some NF-κB dimers act by repressing, whilst others activate specific genes.

Cytokine polymorphism and Epstein-Barr virus-associated GC

Worldwide, it was noted that Epstein-Barr virus (EBV) is present in a relevant proportion of malignant tumors of the stomach, with an incidence that is inversely proportional to that of GC. In the USA 16% to 18% of all stomach tumors were found EBV-associated (EBVaGC), in Southern China only 4.3%^[80]; a survey of 101 published papers reported that EBVaGC was evident in 7.08% of intestinal type GC, while diffuse type GC had

an incidence of 9.82%^[80]. Western and Central Asian countries had significantly more EBV positive cases than South-Eastern countries; in Europe, the frequency of EBV infection ranged from 1.7% in the United Kingdom to 40% in Poland^[80].

An *in vitro* model of EBVaGC was used to demonstrate that gastric cells, following EBV infection, have a high IL-1 β expression, compared to EBV-negative gastric tumour cells. EBV-positive clones rapidly proliferated and were shown to be anchorage-independent in colony-forming assays^[81].

Since EBV infection is highly prevalent in all populations, whilst EBVaGC is quite rare, there were attempts to identify people who run an increased risk of developing GC. Polymorphisms of proinflammatory, as well as anti-inflammatory cytokines were studied, in particular in the promoter regions of *IL-10* and *TNF- α* . For the latter, the allele -308 A (linked to high levels of *TNF- α*) had significantly higher frequency among EBVaGC individuals (23.3%) when compared to control subjects (12.0%, $P < 0.05$). The opposite was found in the case of the anti-inflammatory *IL-10*: The high-producer allele (-1082 G) was found to be less frequent in EBVaGC patients in comparison to controls (6.3% vs 3.0%, $P < 0.05$)^[39,72].

The extreme complexities of all these interactions can explain the great variability in data when investigating the possible correlation between cytokine haplotypes and GC.

Gleanings on the usefulness of characterising *H. pylori* infected individuals for inflammatory haplotype

In addition to the complexity of this subject, the expectations created by the assertion that the host's factors could contribute to the development of GC are disappointing, at least as far as the host's inflammatory response to *H. pylori* infection is concerned. Once we get into details, we realize that in fact, the only determinant that really matters is the infection. The examination of the scientific literature on the cytokine subject has led to contradictory results: For each cytokine, the observations made by studying Caucasian people cannot be applied tout course to Asians; in certain cases, we get opposite results. In the different surveys, one can find association of determined haplotypes of inflammatory cytokines with an increased risk of GC, the opposite, or nil. Even the results of meta-analyses do not agree one another, according to whether studies are carried out by Chines or researchers from other nations. What does it mean? Is it because cytokines are not the final effectors, as they principally work on the long and winding road paved by the broad NF- κ B family, which leads to GC (which means that the final response to inflammatory stimuli is far from hitting its target)? And what about the observations that people suffering from diseases far more inflammatory than chronic gastritis, such as rheumatoid arthritis, are likely protected from developing GC^[82].

These observations may suggest that, if host factors are important in GC development, they probably have to be sought outside of the genes encoding the inflammatory cytokines.

NOT ALL PATIENTS ARE ALIKE:

MOLECULAR BIOLOGY

The recent advances of molecular biological techniques allowed researchers to reach important insights into the oncogenesis mechanisms in gastric cancer. Besides the well-known pathogenic factor, *H. pylori*, several oncogenes and tumour suppressor genes, including cell cycle regulation genes involved in the growth and signal transduction pathways, have been identified^[83-85]. In particular, alterations of genes involved in signalling pathways deregulation, patterns of aberrant DNA methylation, and chromosomal imbalances have been evidenced^[86,87].

CHROMOSOMAL INSTABILITY

Chromosomal instability (CIN) represents one of the main type of genomic instability observed in several neoplasms and it has been observed in a large cohort of patients with gastric cancer^[88]. In particular, it is commonly detected in gastric malignant tumours and has been shown in up to 84% of gastrointestinal cancers^[89].

CIN is characterized by chromosomal anomalies, including gain or loss of the complete chromosome (aneuploidy) and segments of chromosomes (loss of heterozygosity, amplifications and translocations)^[90]. These abnormalities can impact on the oncogenes expression, tumour suppressor genes and other genes, as well as those involved in digestion, DNA repair, growth regulation, and control of cell cycle checkpoint^[91-93]. The genetic mechanisms leading to CIN are not entirely known; *H. pylori* infection, smoking habit and some chemical substances such as nitrates and nitrites probably have an effect on inducing CIN; anyway their influence is actually uncertain^[94]. On the other side, defects of chromosome segregation (CS), imperfect DNA damage response (DDR), anomalies in cell cycle regulators and telomere dysfunction have been identified as factors leading to numerical and structural chromosome alterations^[95,96]. These carcinogens may alter chromosomes and the cytoskeleton promoting malignant modification^[97].

CS alterations

CS represents an important cellular process inducing the gastric epithelial cells division. Alterations of CS regulating mechanisms can cause DNA alterations or mitotic failures, leading to unfixable mutations as well as chromosomal number alterations^[98]. In particular, the three recently proposed ways producing CIN are: Altered expression, polymorphisms and/or mutations of mitotic genes implicated in CS and the carcinogen activity upon

susceptible genetic background of individuals^[99,100]. Many authors showed an aberrant expression of mitotic genes in CS. Moreover, the altered expression of BUB1 protein (involved in controlling the spindle assembly checkpoint), was significantly increased in patients with diffuse type gastric adenocarcinoma, but not related to DNA ploidy^[89]. Furthermore, in another study, BubR1 and AURKB (proteins involved in the mitotic spindle assembly) expression resulted in association with a low risk of GC progression^[101-103]. Aurora kinase A (AURKA/STK15), a cell-cycle-regulated kinase with important role in microtubule formation and stabilization during CS, is often overexpressed in adenocarcinomas of the stomach, showing a suggestive new oncogenic pathway in GC^[104].

Defective DDR

The mucosa of the stomach is continually subject to several environmental and intracellular mutagens, like ROS, *H. pylori* infection, nitrates, sodium, nitrites, and other water and food contaminants, able to induce DNA damage through different mechanisms^[105,106]. Failure of the most important mechanisms of repair [nucleotide excision repair, base excision repair, mismatch repair (MMR) and recombination and/or DDR] may conduce to CIN and genetic aberrations, favouring carcinogenic process^[107,108]. Several studies revealed differential mRNA expression of genes implicated in DNA repair process: *ATM* and *HMGB1* (implicated in base excision repair), *RAD23B* (involved in nucleotide excision repair), *UBE2V2*, *MUS81* [involved in resolving Holliday junctions (a branched DNA structure that contains four double-stranded arms joined together, considered the central intermediate in homologous recombination)], *REV3L* (involved in replication post-DNA damage), *TP53*, *hHR23A* and *DDB1* (implicated in nucleotide excision repair), and *XRCC1* (implicated in single-strand breaks repair) and *MUTYH* (implicated in base excision repair)^[109-113].

H. pylori

H. pylori has been shown to be able to induce DDR and double-strand breaks in gastric cancer with a mechanism of adhesion of bacteria that takes place between Lewis epitopes of the host and BabA adhesin^[114]. Anyway, gastric mucosa cells can repair the DNA lesions induced by short-term infections. On the other side, prolonged infections induce saturation of repair mechanisms with a consequent ineffective DNA repair and malignant process begin. Moreover, continued infections lead to chronic inflammation, with resulting increase of mutagenesis rate, oxidative-stress, down-regulation of MMR mechanisms, instability of genes and modulation of apoptosis by means of ROS formation^[115-119]. Gastric inflammation represents an important host response able to induce *H. pylori*-related carcinogenesis^[120]. In fact, in infected patients with *IL-1 β* , *TNF- α* , *IL-10* and *IL-8* polymorphisms, has been observed an in-

creased risk of distal gastric cancer progression^[120,121]. Furthermore, different *H. pylori* strains seem to differently increase cancer risk by means of host genotypes^[122] as these bacteria are able to communicate with their hosts. The equilibrium is determined both by host and bacterial features and may explain the reason why some *H. pylori* strains augment the carcinogenesis risk. For example, CagA positive strains promote severe gastritis and increase the pro-inflammatory cytokines' level. This may lead to an environment favourable to the growth of other bacteria that can support inflammation and continually induce oxidative stress, increasing the risk for GC^[1].

MICROSATELLITE INSTABILITY

Microsatellite instability (MSI) represents a genomic instability commonly detected in almost half of patients with GC. It is often observed in the Lynch syndrome (hereditary non-polyposis colorectal cancer) and in several sporadic cancers^[123]. MSI phenotype is characterized by a high replication mistake rate leading to insertions and/or deletions of nucleotides within microsatellite repeats in neoplastic areas^[123]. The MMR proteins are able to detect and repair these alterations, causing the dysfunction in MMR genes (*MLH1* and *MSH2*) a MSI phenotype's establishment, with a consequently power off of cancer suppressor genes' and loss of heterozygosity^[124,125]. To this address, genes that are frequently modified induce cell cycle regulation and apoptosis (*TGF β RII*, *RIZ*, *IGFIR*, *TCF4*, *BAX*, *FAS*, *CASPASE5*, *BCL10* and *APAF1*) or are involved in the maintenance of genomic integrity (*MSH6*, *MED1*, *MSH3*, *BLM*, *RAD50*, *ATR*, and *MRE11*)^[126].

DEREGULATION OF SIGNALLING TRASDUCTION PATHWAYS

The effects of genomic destabilization consist of aneuploidy and gain or loss of the chromosome tracts involved in mRNA transcription. Genomic alterations can modify the normal cellular biology with a consequent neoplastic switch^[127]. The clearly explored pathways that probably are involved in gastric pathogenesis are Wnt/betacatenin, extracellular signal-regulated MAPK, Hedgehog, Notch, NF- κ B, TGF- β /BMP pathways, COX2/PGE₂, and tyrosine kinase signalling^[128-143].

Finally, several studies evidenced that pathway deregulation involved in systemic inflammatory response, such as IL-11/STAT1/gp130/STAT3, can induce a carcinogenic transformation too^[144,145].

CONCLUSION

GC is a multifactorial disease. The main determinant, *H. pylori* infection, can be considered a *sine qua non* for GC development; however, despite almost all individuals who get GC are currently, or have been infected, it

is neither a necessary nor a sufficient condition. The intricacy of this topic resides in the proportion of infected people that will never get GC: 97% to 99%, according to the ethnic groups and geographic areas. Other remarks are unraveling the tangle: Almost all *H. pylori* strains from Japan and East Asia, the areas with the highest incidence of GC, are *cagA*+ and can be considered highly carcinogenic; in addition, the infection by certain *cagA* genotypes in western countries increases by far the risk of GC.

At this point, one may wonder why these strains keep on infecting people. Following along with the evolution, only the characteristics that provide a selective advantage continue to be transmitted; this is a basic rule in eukaryotic and prokaryotic worlds. What is the benefit of being infected by carcinogenic strains? Why do they not disappear? People infected by strains that multiply the risk of GC by many times over, cannot be considered advantaged. Possible answers could reside in the following observations: (1) 97% to 99% of people never acquire GC; (2) the development of the sequence gastritis-metaplasia-dysplasia-cancer takes 40 years, or more, after the infection; which means that all women, as well as men, are fertile before the age at which GC occurs (many men also in old age, but they are less important, statistically speaking); hence, fertility is not affected by cancer development; and (3) women, who develop GC far less frequently than men, are the necessary genetic traits holders. Could these answers satisfy the laws of evolution (or distract them)? And how can the occurrence of GC in younger and younger ages be explained?

Apart from the complexity of this subject, the prospect created by the assertion that the host's factors, such as the way the host reacts to infectious stimuli, may be important in the development of GC is discouraging. Despite cytokines involved in the inflammatory response to infection, there are more than 30, just over half a dozen that have been examined in the relationship of *H. pylori* infection with GC and only haplotypes of *IL-1* and *TNF- α* genes were found to possibly increase the GC risk, but only if the ethnicity of patients is not considered. Pursuing this line of inquiry makes us run the risk of sounding racist.

The hypothesis that the dissection of oncogenes and tumour suppressing genes could provide us with an answer to the question whether host factors are important in GC development has only been partly proved. However, the conclusions have led us to a starting point, that is, they ended to indirectly confirm the pathogenic role of strains expressing CagA. The local and systemic levels of substances endowed with an increased mutagenic potential, ROS, generated by immunocytes, and the consequent DNA damage are far higher when the infecting organisms harbour the *cag* PAI.

In conclusion, as regards the development of GC, not all the *H. pylori* strains and patients are alike and not all share the same responsibility, but the only deter-

minant that really matters is the infection.

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