Evolutionarily-Related *Helicobacter pylori* Genotypes and Gastric Intraepithelial Neoplasia in a High-Risk Area of Northern Italy

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**Abstract:** *Helicobacter pylori* (*Hp*) is the major recognized risk factor for non-cardia gastric cancer (GC), but only a fraction of infected subjects develop GC, thus GC risk might reflect other genetic/environmental cofactors and/or differences in virulence among infectious *Hp* strains. Focusing on a high GC risk area of Northern Italy (Cremona, Lombardy) and using archived paraffin-embedded biopsies, we investigated the associations between the *Hp vacA* and *cagA* genotype variants and gastric intraepithelial neoplasia (GIN, 33 cases) versus non-neoplastic gastroduodenal lesions (NNGDLs, 37 cases). The *glmM* gene and the *cagA* and *vacA* (s and m) genotypes were determined by polymerase chain reaction (PCR) and sequencing. *Hp* was confirmed in 37/37 (100%) NNGDLs and detected in 9/33 GINs (27%), consistently with the well-known *Hp* loss in GC. *CagA* was detected in 4/9 *Hp*-positive GINs and in 29/37 NNGDLs. The *vacA* s1a and m1 subtypes were more common in GINs than in NNGDLs (6/7 vs. 12/34, p=0.014, for s1a; 7/7 vs. 18/34, p=0.020 for m1), with significant *vacA* s genotype-specific variance. The GIN-associated *vacA* s1a sequences clustered together, suggesting that aggressive *Hp* strains from a unique founder contribute to GC in the high-risk area studied.

**Keywords:** *Helicobacter pylori*; virulence factors; pathogenicity; phylogenetic diversity; gastric intraepithelial neoplasia; Italy

1. Introduction

*Helicobacter pylori* (*H. pylori*, *Hp*) infection is an ascertained major risk factor for gastric and duodenal ulcer, non-cardia gastric cancer (GC), and gastric mucosa-associated lymphoid tissue lymphoma [1–3]. However, these pathologic processes depend on a number of variables, including *Hp* virulence factors, host susceptibility, and environmental cofactors [3,4]. Focus on the pathogen resulted in the identification of specific virulence markers [5,6]. The cytotoxin-associated gene (*cagA*),
not present in every \textit{Hp} strain, is a marker for a genomic pathogenicity island associated with \textit{Hp} infection severity [5,7]. The vacuolating cytotoxin A (\textit{vacA}) gene, present in all \textit{Hp} strains, encodes a protein that directly induces progressive vacuolization, mitochondrial damage, cytochrome c release, and cell death [8,9]. Two variable parts, the signal (s) and the middle (m) regions, can be distinguished within \textit{vacA}, defining specific \textit{vacA} genotypes that, in various populations, are associated with disease severity [10]. The \textit{Hp} strains possessing the \textit{vacA} s1 genotype are highly cytotoxic and have been linked to peptic ulcer and GC [5,11]. Italy is a country with high \textit{Hp} infection rates, ranging from 40\% to 71\%, and with a high burden of antibiotic-resistant \textit{Hp} strains [12–15]. However, GC incidence is higher in definite regions of Northern and Central Italy compared to Southern Italy and the Italian islands [16–18], despite the lower \textit{Hp} infection prevalence in Northern and Central Italy [19]. It is still debated whether the higher geographic risk of GC in Northern and Central Italy is due to host, environmental, or bacterial factors or to combinations among such factors [20–27]. Here, to assess the relationships between \textit{Hp} virulence factors and non-cardia gastric intraepithelial neoplasia (GIN), we analyze the \textit{Hp} genotypes found in an archived paraffin-embedded series of GINs and non-neoplastic gastro-duodenal lesions (NNGDLs) from Cremona (Lombardy), a high GC risk area in Northern Italy [16,28].

2. Materials and Methods

2.1. Cases and Histopathological Evaluation

Archived formalin-fixed, paraffin-embedded endoscopic or surgical biopsy specimens from a total of 70 patients with GIN or non-neoplastic gastro-duodenal lesions (NNGDL) were selected after a pathological review from the 1998-1999 files of the Pathology Unit, Cremona Hospital, Cremona, Lombardy (a well-known high-GC-risk region in Italy [16,28]). These included 33 biopsy samples representative of non-cardia GIN, of which 12 were from patients also diagnosed with invasive GC (mean age of patients 66 years, range 50–82 years, 11 females, 22 males) and 37 biopsy samples representative of the NNGDL (mean age of patients 52 years, range 29–75 years, 16 females, 21 males). The 37 NNGDL biopsies presented antral-predominant non-atrophic \textit{Hp}-positive gastritis that has a low risk of gastric carcinoma [29], associated with gastric erosions in 17 cases and with duodenal ulcer in 10 cases.

Histological diagnosis of GIN was restricted to the biopsies showing both altered glandular architecture and abnormalities in cytology and differentiation but lacking any (even doubtful) infiltrating features [30,31]. For diagnostic purposes, the NNGDLs were documented by two biopsies from the antrum, one from the angulus, two from the corpus, and two from the duodenal bulb. For each biopsy, four-micrometer sections stained with hematoxylin-eosin (histological examination) and with Giemsa (\textit{Hp} identification) were re-reviewed by one of us (R.A.C.) according to the Updated Sydney System [32]. Only the antral biopsies, which showed patent inflammatory changes and were \textit{Hp}-positive by Giemsa staining, were used for \textit{Hp} DNA analysis.

The study was performed in agreement with the guidelines of the Declaration of Helsinki for human medical research. The anonymized archived paraffin-embedded tissue blocks exceeded the 20 years-time limit requiring an ethics approval for research use, according to Italian regulations [33].

2.2. DNA Extraction, Polymerase Chain Reaction (PCR) and Sequence Analyses

DNA extraction from formalin-fixed, paraffin-embedded biopsies was as previously reported [34]. Amplifications were carried out using nested or semi-nested PCRs targeting short amplicons retrievable from damaged DNA (Table S1) [35]. PCR specificity, confirmed by sequencing, was tested on 3 clinical \textit{Hp} isolates and 3 \textit{Hp}-negative human DNAs (not shown). To evaluate assay sensitivity, the DNAs from the clinical isolates were quantified by UV spectrophotometry and used to prepare 10-fold serial dilutions ranging from 10 ng to 1 fg. Positive amplifications were obtained with as little as 10 fg of DNA (not shown).

GIN and NNGDL DNAs were first amplified for the human \textit{β-globin} sequence, to assess DNA quality, then the GINs, for which the \textit{Hp} status was not documented by Giemsa staining, were tested.
for the *glmM* (*ureC*) gene [35] to obtain molecular evidence of *Hp* infection. Next, the *glmM*-positive GINs and all the Giemsa-positive NNGDLs were amplified for the conserved *cagA* 5′-region and for *vacAs* and *m*. PCR products were directly sequenced using an ABI PRISM BigDye™ Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and sequence variants were confirmed on independent DNA amplifications. Sequences were deposited in the GenBank under accession numbers from EU881365 to EU881408 (*vacAs*, 43 sequences), EU881409 to EU881456 (*vacAm*, 47 sequences), and EU881457 to EU881492 (*cagA*, 35 sequences).

### 2.3. Data Analysis

Phylogenetic trees and multidimensional scaling (MDS) plots were constructed using the *Hp* genotypes detected in the present study and 55 GenBank *Hp* genotypes of various geographic origin characterized for the *vacAs* and *m* regions. These included 10 genotypes from Colombia, 4 from the USA (unspecified States), 9 from Alaska, 2 from Arizona, 3 from Kenya, 1 from Italy, 1 from the United Kingdom, 2 from Kazakhstan, 15 from Japan, 3 from Thailand, 1 from Korea, 1 from Taiwan, 1 from China, and 2 geographically undefined. Comparison of *vacA* sequences was performed for nucleotide positions 252-330 (*vacAs*, 79 bp) and 2393-2496 (*vacAm*, 104 bp), using GenBank accession number #S72494 as reference. The phylogenetic tree was constructed through the neighbour-joining method using MEGA X [36]. The Kimura 2-parameter [37], with a uniform rate of heterogeneity, was applied as a genetic distance measure to calculate the distance matrix. Significance of clusters was calculated using bootstrap percentages after replication of 10,000 trees. The genetic distances for *vacAs* and *m* across 100 *Hp* sequences (45 from this study and 55 from GenBank) were graphically represented on a two-dimensional space using MDS (SPSS for Windows, version 11.5).

The F-statistic (Fst) approach [38] was applied to verify statistical differences in genotype distributions. Analysis of molecular variance (AMOVA), as implemented in Arlequin suite v3.5 [39], was carried out to analyze the frequencies of *vacAs*, *vacAm*, and combined *vacAs* and *m* genotypes. Matrices of pairwise Fst values were plotted using the MDS procedure. Significance was assessed by 10,000 permutations.

To obtain median-joining (MJ) networks of *vacAs* sequences, the DnaSP v.6 software [40] was applied to the sequence data to identify segregating variants. Tables of variants were used in the MJ algorithm [41] option of NETWORK 5 (Fluxus Technology Ltd).

Fu’s Fs-test of selective neutrality [42], which detects whether the pattern of diversity in a population is consistent with neutrality, was performed using Arlequin suite v3.5 [39]. If the test significantly deviates from neutral expectation, it is assumable that selection and/or recent population expansion is/are responsible for the observed diversity pattern. Since Fs tends to be negative when there is an excess of recent mutations (and therefore an excess of rare genotypes), a large negative Fs value can be taken as evidence against the neutrality of mutations.

The network clade subclustering structure was verified with the Maximum Parsimony (MP) method [43] using MEGA X [36]. The bootstrap consensus tree, inferred from 1000 replicates, was taken to represent the evolutionary history of the *Hp* genotypes in the considered network clade [44]. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 [43,45], in which initial trees were generated with random addition of sequences (100 replicates). Risk analysis was assessed calculating odds ratios (OR) and confidence intervals (CI) from two-by-two tables.

To investigate the phylogenetic relationships between our isolates and previously characterized *Hp* strains, the *cagA* 5′-region sequences were aligned with CLUSTAL W [46]. A phylogenetic tree was then constructed through the neighbor-joining method using MEGA3 version 3.0. Based on the presence of *cagA*, 36/55 GenBank sequences were included in this analysis.
3. Results

3.1. GIN-Associated Hp Genotypes

The 33 GINs, whose Hp status could not be assessed by low-sensitivity Giemsa staining due to the well-known loss of Hp during gastric carcinogenesis [3], were first tested for the glmM gene sequence. The glmM-positive samples were then analyzed for the cagA and vacA genes (s and m regions) (Table S2). Overall, 9/33 GINs (27%) were glmM-positive and 4 of these were cagA-positive. The lack of cagA in some GINs is consistent with the evidence that cagA may contribute to gastric carcinogenesis but is not required for the maintenance of the neoplastic phenotype after transformation [47]. One of the 4 cagA-positive GINs (Italy 7, Table S2) yielded 2 cagA sequences differing at np 15012 (GenBank #AF282853), suggesting double infection. Both the vacA s and m regions were amplified in 6/9 glmM-positive GINs. Of the remaining 3 glmM-positive cases, one (Italy 9) was not amplifiable for both vacA regions, while the other two were not amplifiable for vacA m (Italy 2) and for vacA s (Italy 8), respectively. All the Hp strains identified in GINs presented the s1 genotype, corresponding to the s1a subtype in 6/7 cases. Notably, 4 cases shared the same vacA s and m sequences. The remaining vacA s strain (Italy 5) matched subtype s1b, except for Ala18, typical of s1a, suggesting recombination between s1a and s1b. The vacA m1 type was detected in all cases.

3.2. NNGDL-Associated Hp Genotypes

The 37 Hp-positive NNGDLs were directly analyzed for the cagA and vacA genes. Twenty-nine cases (29/37, 78%) were cagA-positive, including 7 with multiple cagA genotypes (Table S2).

The vacA s sequence could be typed in 34/37 cases, of which 29 (29/34, 85%) were s1 and 5 (5/34, 15%) were s2 (Table S2). Of the 3 cases not amplifiable for vacA s, one (Italy 23) showed the vacA m2 genotype (cagA-positive); another (Italy 24, cagA-positive) contained two different vacA m1 sequences, suggesting double infection; the third (Italy 36), undetermined for vacA s and m, harbored different cagA genotypes, also pointing to mixed infection. Of the 29 isolates with a definable vacA s1 genotype (that also included multiple- and single-strain infections), 12 (41%) were s1a, 14 (48%) s1b, and 3 (10%) were s1a and s1b recombinants (Table S2). The vacA s2 genotype was less frequent (5/34 cases, 15%) and mixed infection was found in 1/5 positive cases.

With regard to the m region, 16/34 cases (47%) had the m1 allele (including two mixed); the m2 was also found in 16/34 cases (47%, including two mixed). The remaining 3 cases (3/37, 6%) could not be subtyped for the m region.

Thus, excluding five isolates where both the signal and the middle regions were undetected, the following vacA type combinations were identified in the NNGDLs: s1a-m1 (7/32, 22%), s1a-m2 (3/32, 9%), s1b-m1 (5/32, 16%), s1b-m2 (7/32, 22%), s1b-m1/m2 (2/32, 6%), rec s1a/s1b-m1 (3/32, 9%), and s2-m2 (5/32, 16%).

3.3. Comparison between the Hp Genotypes of GINs and NNGDLs

The cagA gene was detected in 4/9 GINs and 29/37 NNGDLs, with an overall prevalence of 72% (33/46) among all the Hp-positive GINs and NNGDLs. The vacA s1a and m1 subtypes were much more frequent in the vacA-positive GINs than in the vacA-positive NNGDLs (6/7 vs. 12/34 for s1a, p=0.014; 7/7 vs. 18/34 for m1, p=0.020), while the s1b subtype, not found in the 7 vacA-positive GINs, was detected in 14/34 vacA-positive NNGDLs (0/7 vs. 14/34, p=0.036).

Most of the retrieved Hp sequences were unique, with an excess of gene diversity (H=1.0000+-0.0047) for vacA, higher for vacA s (0.9838+-0.0086) compared to vacA m (0.9212+-0.0241). A driving force that could underlie such high levels of sequence variation is population expansion under neutral evolution. To highlight the deviation of the sequence variability pattern from neutral expectations, we performed a Fu’s Fs neutrality test on all the vacA sequences and, separately, on the s and m regions. A largely negative test value (Fs=-21.28254; p<0.000001) pointed to a deviation from neutral expectation for vacA. Fu’s Fs test indicated an excess of recent mutations for the s region,
supported by a significant negative value (Fs=-7.23602; p=0.041) and selective neutrality for the m region.

To investigate the relationships between the Hp genotypes and the type of gastric lesion, we focused on 45 vacA s and m sequences from the present study (7 from GINs, 38 from NNGDLs) and on 55 worldwide GenBank sequences. The phylogenetic tree (Figure 1) and the MDS plot of the genotypes distance matrix showed that the vacA s and m sequences from the NNGDLs were distributed in 6/10 clusters (Figure 2), while almost all the GIN sequences clustered together in clade s1-m1. Phylogenetic analysis indicated a clear separation of the m1 and m2 sequences in two clades, with the vacA s subclusters within these clades. Five of the 7 vacA s and m GIN sequences clustered in the major s1a-m1a clade, although in two different subclusters. As highlighted by branch length, Italy 5, the only s1b-m1a sequence associated with GIN, differed from the other s1b sequences in the phylogenetic tree. This was consistent with the fact that Italy 5 presented a C variant typical of s1a at nucleotide position 269, suggesting an origin through recombination between s1a and s1b. The vacA s and m sequences retrieved from Italy 7 clustered in a sister branch of the m2 clade (as s1a-m2). In contrast, the vacA s and m sequences from the NNGDLs were distributed in all clades, except the s1c-m1 and s1c-m2 clusters, that included only East Asian isolates. Notably, two possible recombinants with consistent variation at vacA s (Italy 13 and Italy 19) joined a separate branch in subcluster s1b-m1a.
**Figure 1.** Phylogenetic tree of 100 vacA s and m *Helicobacter pylori* sequences. The 45 sequences obtained in this study are indicated by arrowheads; asterisks and red arrowheads highlight association with gastric intraepithelial neoplasia. Other sequences, labeled according to strain designation, are from GenBank.
Figure 2. Multidimensional scaling plot of 45 Helicobacter pylori vacA s and m sequences obtained in this study (red: gastric intraepithelial neoplasia, GIN; green: non-neoplastic gastro-duodenal lesions, NNGDLs) and of 55 worldwide Helicobacter pylori sequences from GenBank (blue), based on the vacA gene (both s and m regions). Scaled stress value=0.0208.

AMOVA, applied to all the Hp genotypes found in the GINs and in the various NNGDL subsets (non-atrophic gastritis, non-atrophic gastritis associated with gastric erosions, and non-atrophic gastritis associated with duodenal ulcer), showed that “within-groups” variance was 97.49% for both vacA s and m, and 95.33% considering only vacA s. “Among groups” variance was 2.51% (Fst: 0.02512, p<0.000001) for vacA s and m combined, and 4.67% (Fst: 0.04674, p<0.000001) when only vacA s was considered. The genetic variance of vacA m was almost completely (98.4%) “within-groups” (Fst: 0.01604, p<0.000001).

Pairwise Fst values comparison, applied to the vacA sequences, highlighted a significant variance in the genotype distribution between the Hp sequences associated with GINs and NNGDLs. When Fst was computed on vacA s and m combined, the GIN genotypes significantly differed from those found in non-atrophic gastritis alone (Fst: 0.13618, p=0.04336±0.0021) and in non-atrophic gastritis plus duodenal ulcer (Fst: 0.18218, p=0.04891±0.0020) (Figure 3A). When Fst was computed on vacA s, the GIN genotypes significantly differed from those detected in non-atrophic gastritis alone (Fst: 0.17998, p=0.01485±0.0011) and in non-atrophic gastritis plus duodenal ulcer (Fst: 0.23198, p=0.01475±0.0012) (also with a trend for those associated with non-atrophic gastritis plus gastric erosions, Fst: 0.11173, p=0.06772±0.0028) (Figure 3B). Fst analysis did not show significant divergent variance in vacA m genotype distribution (not shown).

Figure 3. Multidimensional scaling plots of pairwise Fst values representing differences in Helicobacter pylori genotype distribution between gastric intraepithelial neoplasia (GIN, red square), non-atrophic antral gastritis alone (NAG), NAG plus duodenal ulcer (NAG_DU) and NAG plus gastric erosions (NAG_GE) (black squares). A: combined vacA s and m sequences. B: vacA s sequences.
The MJ network based on vacA s, used to visualize the relationships between the Hp-associated lesions, showed that the NNGDL genotypes joined clusters s1a, s1b, and s2, while the GIN genotypes clustered in s1a (Figure 4), except one s1a/b recombinant (Italy 5, Table S2). Duodenal ulcer was associated with the s1a genotype only in cases manifesting non-atrophic gastritis with gastric erosions, i.e., a more aggressive clinical phenotype (Italy 27 and Italy 28, Table S2).

![Figure 4. Median-joining (MJ) network of vacA s genotypes. The non-neoplastic gastro-duodenal lesions cluster to s1a, s1b, and s2, while the cases of gastric intraepithelial neoplasia (GIN), except GIN5 (Italy 5, an s1a/b recombinant), join cluster s1a. Dots representing gastric lesions are color-coded as follows: GINs, red; non-atrophic gastritis (NAG) alone, green; NAG plus gastric erosions, grey; NAG plus duodenal ulcer, yellow. Dot sizes reflect the number of cases sharing the same vacA s genotype.](image)

The subclustering of the MP tree for the s1a genotypes highlights a strict evolutionary relationship between the GIN Hp genotypes (Figure 5). The vacA s1a sequences were grouped based on four evolutionary parsimony informative sites of the cluster s1a dataset, one of which split the tree into two subclades, s1aI and s1aII, with a significant 63% bootstrap value. The s1aI subclade included Italy 25 (non-atrophic gastritis plus gastric erosions), Italy 27 (non-atrophic gastritis plus duodenal ulcer), and all the GIN genotypes in the dataset. Two GINs shared Hp genotype with a case of non-atrophic gastritis plus gastric erosions (Italy 20), and with a case of non-atrophic gastritis alone (Italy 10). Subclade s1aII included sequences associated with non-atrophic gastritis alone, such as Italy 16 and 34 (same genotype) and Italy 32, and non-atrophic gastritis plus duodenal ulcer (Italy 28). Italy 21 and Italy 29 (both non-atrophic gastritis plus gastric erosions) were separated by distance from subclades s1aI and s1aII.
Figure 5. Maximum parsimony (MP) tree of the vacA s1a genotypes found in the present study. The MP method split the tree into two subclades, designated I and II (bootstrap value: 63%), highlighting a subclustering structure of the vacA s1a cluster. All the GIN-associated Helicobacter pylori sequences (red) were included in subclade I of clade s1a, suggesting strict evolutionary relationships. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3, in which initial trees are generated by random addition of sequences (100 replicates). The bootstrap consensus tree, inferred from 1000 replicates, represents nine genotypes found in the Helicobacter pylori strains retrieved from 20 gastro-duodenal lesions. Multiple infections showing the same vacA s1a sequence are indicated with “a” and “b” in the strain designation. Concomitant lesions (non-atrophic gastritis alone (NAG), NAG plus duodenal ulcer (NAG_DU), and NAG plus gastric erosions (NAG_GE) are in brackets.

The Hp vacA s1a genotype was associated with the NNGDLs in 35.3% (12/34) of the cases. In contrast, 86% (6/7) of the Hp-positive GINs had the vacA s1a genotype. Although confidence intervals were unreliable due to small numbers, the OR computed comparing GINs and NNGDLs (OR, 11; 95% CI 1.2-99.4; χ2: 6.193, p=0.012) suggests an association between the vacA s1a Hp genotype and GC risk.

Finally, the cagA 5’ sequences detected in our study were closely related to a cluster containing only Western strains (data not shown), which is consistent with evidence that this region may distinguish European from East Asian Hp genotypes [48].

4. Discussion

The present study analyzed a series of GINs and NNGDLs from a high-GC-risk area in Northern Italy [16] for genetic evidence of Hp infection and for Hp genotype characteristics in the cagA 5’ and vacA s and m regions. We found Hp sequences in only 27% of the GIN cases. This confirms that Hp can be retrieved from GINs, although it is well known that infection with this pathogen, which is the strongest known non-cardia GC risk factor, is lost during the neoplastic progression to GC [18]. Given the relatively high frequency of Hp infection in Italy [12,13], the finding of Hp in GIN does not necessarily imply that infection contributed to the pathogenesis of the lesion. However, the GIN-associated Hp strains found in this study tended to contain the cagA pathogenicity island and pertained to the vacA s1 and m1 genotypes, known to be associated with severe disease [5,11]. This suggests a specific selection of aggressive strains, which could contribute to GIN.

It has also been demonstrated that distinct Hp vacA s genotypes have preferential geographical distributions [11,49–52]. Few Italian studies reported the results of Hp vacA genotyping in gastric lesions. Two studies on dyspeptic patients from Central and Southern Italy [20,24] found a higher prevalence of vacA s1 compared to s2. Furthermore, in Northeast Italy, Zambon et al. [22] reported that the cagA-positive vacA s1 genotype was more common in patients with gastric ulcer or duodenitis than in patients with antral-predominant gastritis only. We confirmed the high prevalence of vacA s1
in the cases tested (GINs and NNGDLs) and, after characterization of the \textit{vacA} subtype, we found that 44% of the cases were s1a, 34% s1b, 10% s1a/s1b recombinants, and the remaining 12% s2.

Various studies linked the gastric disease phenotype to the \textit{vacA} genotype of the infecting \textit{Hp} strain. The \textit{vacA} s1-m1 strain is highly cytotoxic [5,10] and is often detected in patients with precancerous lesions [10,53,54]. In our study, all the GIN-associated strains showed the \textit{vacA} s1-m1 genotype, and, except for one s1a/s1b recombinant, were of s1a subtype. In contrast, the \textit{Hp} strains retrieved from the NNGDLs showed no significant difference in s1a and s1b frequencies.

The conventional Fst approach confirmed the importance of the discrepancy in \textit{vacA} s frequencies, showing that the variance of \textit{vacA} genotypes distribution in the GINs significantly differed from that in the NNGDLs, particularly non-atrophic antral gastritis and non-atrophic antral gastritis plus duodenal ulcer, and that \textit{vacA} m, despite its reportedly independent association with cytotoxic activity [10], was not related to the GINs. In fact, network analysis based on \textit{vacA} s genotypes showed that all the GIN-associated \textit{Hp} sequences, except an s1a/b recombinant, were in cluster s1a. Moreover, the MP method, used to verify the subclustering structure of cluster s1a, highlighted that the \textit{Hp} genotypes associated with the GINs were strictly evolutionarily related in the s1aI subclade. Thus, while our study design does not allow an accurate estimate of the GC risk conferred by \textit{Hp} infection, the observed OR value suggests that infection with locally-circulating \textit{Hp} \textit{vacA} s1a strains might contribute to high GC risk in the Cremona area.

5. Conclusions

\textit{Hp} is a highly heterogeneous pathogen and the \textit{Hp} strains circulating in Italy remain poorly known. The genetic heterogeneity observed in this study, focused on a high-GC-risk area, could reflect a sudden expansion of \textit{Hp} strains, as evidenced by the Fu’s Fs-test, indicating an excess of recent mutations. The GIN-associated \textit{Hp} sequences tended to cluster together, suggesting strict evolutionary relationships, consistent with the fact that the cases examined derive from a restricted geographic region [16], where GC risk could be contributed by pathogenic strains that evolved locally from a unique founder. Our study has some limitations, including small sample size, focus on a limited geographic area, and lack of documentation of other factors, such as tobacco use, that could influence GC risk. This should be clarified by additional investigations involving larger numbers of GINs/GCs and NNGDLs from geographic areas with different levels of risk in Italy and elsewhere.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1: Table S1: PCR primers used to amplify the human β-globin gene and the \textit{Helicobacter pylori} glmM, \textit{cagA}, and \textit{vacA} sequences; Table S2: Pathological characteristics of the 46 \textit{H. pylori}-positive cases studied with genotyping results.

**Author Contributions:** All authors have read and agreed to the published version of the manuscript. Conceptualization: S.T., F.V., R.M-C. Methodology: S.T., S.P., F.V., L.R. Software: F.V. Validation: S.T., F.V., S.P., L.R. Formal analysis: F.V., S.T., R.M-C., R.A.C., E.B., M.N., R.M-C. Resources: F.V., M.N., R.M-C. Data curation: S.T., F.V., R.A.C., R.M-C. Writing – original draft: S.T., F.V., R.A.C., M.N. Writing – review and editing: S.T., F.V., R.M-C. Visualization: F.V. Supervision: F.V., R.M-C.

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