Toxigenic *Helicobacter pylori* Infection Precedes Gastric Hypochlorhydria in Cancer Relatives, and *H. pylori* Virulence Evolves in These Families

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Abstract

Purpose: *Helicobacter pylori* infection by virulent strains is associated with gastric adenocarcinoma. We aimed to determine whether infection with virulent *H. pylori* preceeded precancerous gastric hypochlorhydria and atrophy in gastric cancer relatives and quantify the extent of virulence factor evolution.

Experimental Design: *H. pylori* strains from 51 Scottish gastric cancer relatives were characterized by genetic fingerprinting and typing the vacuolating cytotoxin gene (*vacA*), the cytotoxin-associated gene (*cagA*), and housekeeping genes. We phenotyped strains by coculture with gastric epithelial cells and assessing vacuolation (microscopy), CagA tyrosine phosphorylation (immunoblot), and interleukin-8 secretion (ELISA).

Results: Toxigenic (*vacA* type s1/m1) *H. pylori* was associated with precancerous gastric hypochlorhydria ($P < 0.01$). Adult family members with this type of *H. pylori* had the same strain as currently noncohabiting adult family members in 68% cases, implying acquisition during childhood from each other or a common source. We analyzed different isolates of the same strain within families and showed that *H. pylori* commonly microevolved to change virulence: this occurred in 22% individuals and a striking 44% cases where the strain was shared within families. Microevolution in *vacA* occurred by extragenomic recombination and in *cagA* by this or duplication/deletion. Microevolution led to phenotypic changes in virulence. Passage of microevolved strains could be tracked within families.

Conclusions: Toxigenic *H. pylori* infection precedes and so likely causes gastric hypochlorhydria, suggesting that virulent *H. pylori* increases cancer risk by causing this condition. Microevolution of virulence genes is common within families of gastric cancer patients and changes *H. pylori* virulence.

Helicobacter pylori infection is a major risk factor for the development of distal gastric adenocarcinoma (1), but who develops this condition depends on host genetic susceptibility, bacterial virulence, and environmental cofactors. The best characterized host genetic factors are polymorphisms leading to increased interleukin 1β (IL-1β) production in response to infection (2, 3). These polymorphisms are even more strongly associated with gastric hypochlorhydria and atrophy (2). This supports the contention that hypochlorhydria and atrophy are precursor conditions of gastric cancer (4) and suggests that cytokine polymorphisms predispose to gastric cancer through predisposing to these precursor conditions.

H. pylori virulence factors associated with gastric cancer risk include toxic s1/m1 forms of the vacuolating cytotoxin (VacA; refs. 5–7), possession of the cytotoxin-associated gene (*cagA*; refs. 8, 9), and possession of a *cagA* allele encoding four or more tyrosine phosphorylation motifs (TPM; refs. 10–12). The *vacA* gene is polymorphic; it encodes VacA that can be either type s1 or s2 in the signal region and either type m1 or m2 in the midregion (13). Each strain has one *vacA* allele, and this can be type s1/m1, s1/m2, s2/m1 (rarely), or s2/m2 (13). Type s2 alleles encode a nontoxic form of VacA, and s1/m1 alleles encode a form which binds to and vacuolates a wider range of epithelial cells than s1/m2 (14). The *cag* pathogenicity island (PaI) encodes a type IV secretion system (15) that can penetrate epithelial cells and deliver the effector molecule CagA into the cytosol where it becomes tyrosine phosphorylated (16, 17). CagA phosphorylation is dependent upon the number of TPMs within the COOH terminal variable region (VR) of the protein (18). CagA with greater numbers of TPMs becomes more

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phosphorylated and increases the extent of SHP-2–dependent (19) cytoskeletal alterations and cellular elongations (18, 20, 21). *H. pylori* strains that possess CagA with greater numbers of TPMs are more likely to be associated with gastric cancer than those possessing CagA with fewer TPMs (10–12).

We hypothesized that if VacA and CagA increased cancer risk through predisposing to hypochlorhydria and atrophy, they would be even more strongly associated with these conditions than they are with cancer itself, as cancer does not inevitably arise in the atrophic stomach. We therefore studied these associations in a group of first-degree relatives of patients with gastric cancer that had previously been used to study the association of IL-1β polymorphisms with gastric hypochlorhydria and atrophy (2). Studying family groups of adults who had not cohabited since childhood also gave us the opportunity to assess whether family members had an identical strain of *H. pylori*, implying childhood acquisition and so that infection predated hypochlorhydria/atrophy. Whilst doing this work, we noticed that virulence factors very frequently evolved in members of these families. This phenomenon is potentially important for the gastric cancer risk of individuals, and so we characterized and quantified this finding.

H. pylori are genetically diverse, and this diversity has arisen largely through intergenomic and intragenomic recombination (22, 23). Microevolution describes minor genetic change within a strain that leads to a stable change in phenotype. Recently, microevolution of *H. pylori* virulence genes *vacA* (24, 25) and *cagA* (26) occurring in real time have been characterized on a case report basis. These changes lead to a changed virulence phenotype (24, 26). Kersulyte et al. showed an example of a strain that deleted the whole *cag* PaI through recombination (27). Other studies have used PCR to show that strains with similar genetic fingerprints may have different *vacA* or *cagA* types (28–32) or to show deletion of *cagA* or of other genes in the *cag* PaI (33–35). However, these studies do not show whether phenotype (and so potentially virulence) is changed.

The collection of *H. pylori* strains gave us the opportunity to perform, for the first time, a detailed quantitative study of microevolution of *vacA* and *cagA*. We show that microevolution of virulence is common and extensive within these cancer relatives, not only within individual stomachs, but also between family members, occurring in about half of strain transfer events within families. It occurs through recombination and duplication/deletion and alters the phenotype of the strains.

Materials and Methods

Population. *H. pylori* strains were isolated as sweeps (see below) from relatives (siblings or offspring) of gastric cancer patients from western Scotland (2, 36). We now studied 51 of these relatives who had *H. pylori* isolates available for further analysis. Twenty-seven (53%) of the relatives were female, and the median age was 42 y (range 29-71 y). Gastric acid secretion data were available (37), and severe hypochlorhydria was defined as acid secretion at ≤10 mmol/h. Inflammation in the antrum and corpus was classified by the modified Sydney system (36). Twenty (39%) of the relatives had histologically defined gastric atrophy. *H. pylori* sweeps (combination of all colonies of *H. pylori* grown from a single biopsy sample on a blood-agar plate at 37°C in a microaerobic environment) were initially genotyped for *vacA* and *cagA* VR. Sweeps that exhibited different *vacA* types or size variations in *cagA* were plated to single colonies, and single colonies of each type were isolated and used for further genotypic and phenotypic characterization.

PCR typing of* H. pylori *strains. Strains were cultured on horse blood agar plates (Oxoid) for a maximum of five passages. *H. pylori* genomic DNA was extracted as described (38). Random-amplified polymorphic DNA (RAPD)–PCR was carried out using one or more of primers 1254, 1281, 1283, or 1290 (39). The primers used for PCR amplification and nucleotide sequencing are listed in the Supplementary Table S1. Nucleotide sequencing of the entire *cagA* gene was carried out after PCR amplification of overlapping fragments using *cag* primer pairs A21F-A22R, A22F-A23R, A23F-A24R, A24F-A28R, and A28F-A27R and cloning into pGemT-Easy (Promega).

Vacuolation assay. VacA-induced vacuolation of AGS and RK13 epithelial cells was determined microscopically after coculture with *H. pylori* strains for 16 to 24 h, as described (40).

CagA phosphorylation assay and IL-8 ELISA. The extent of CagA phosphorylation was determined by coculturing *H. pylori* strains with AGS cells for 6 h as described (20). Determination of CagA-induced cytoskeletal change (16) and IL-8 secretion by ELISA was carried out as described (20).

Statistical analysis. We used Mann-Whitney *U* tests, χ^2 tests, and Fisher exact tests, as appropriate.

Results

Association of* H. pylori *virulence factors with gastric hypochlorhydria and atrophy. Of the 51 gastric cancer relatives we studied, 20 had gastric atrophy and 11 of these had severe hypochlorhydria (acid secretion, ≤10 mmol/h). Initial typing of *vacA* and *cagA* from 51 sweeps then single colonies (see Materials and Methods) showed that 4 of 51 subjects had *H. pylori* isolates of more than one *vacA* type and 9 of 51 had *H. pylori* isolates of more than one *cagA* size; these subjects were omitted from the following analyses.

First, we studied associations between the most toxigenic form of *vacA* (type s1/m1) and precancerous conditions. We found a significant association between *vacA* s1/m1 type and low gastric acid secretion. Of the nine people with severe hypochlorhydria, eight (89%) were infected with *H. pylori* with s1/m1 *vacA*, whereas only 34% of the remaining 38 subjects were colonized by an s1/m1 *vacA* strain ($P = 0.004$; Fisher exact test). Gastric atrophy was associated with infection with s1/m1 *vacA* strains, but this did not reach formal significance ($P = 0.07$; χ^2 test). However, people with gastric atrophy had significantly less stomach acid secretion if they were infected with s1/m1 *vacA* strains than s1/m2 *vacA* strains ($P = 0.009$; Mann-Whitney *U* test). This suggests that toxigenic *H. pylori* may increase cancer risk through increasing the risk of these precursor conditions, particularly gastric hypochlorhydria.

Next, we turned our attention to the *cag* PaI and *cagA*. Most (44 of 51) people in this Scottish population were infected with *H. pylori* strains possessing the *cag* PaI—the other seven had strains confirmed as *cag* PaI negative genotypically and phenotypically. Thus, our study was not powered to show any association of *cag* PaI with hypochlorhydria or atrophy, and indeed, no such associations were identified. More surprisingly, we also found no association between the number of CagA TPMs and acid secretion: Of the eight people with severe hypochlorhydria, six were infected with *H. pylori* possessing CagA with three TPMs (75%), one lacked the *cag* PaI, and only one possessed CagA with four TPMs. Of the remaining 34 people, 22 (65%) had CagA with three TPMs or

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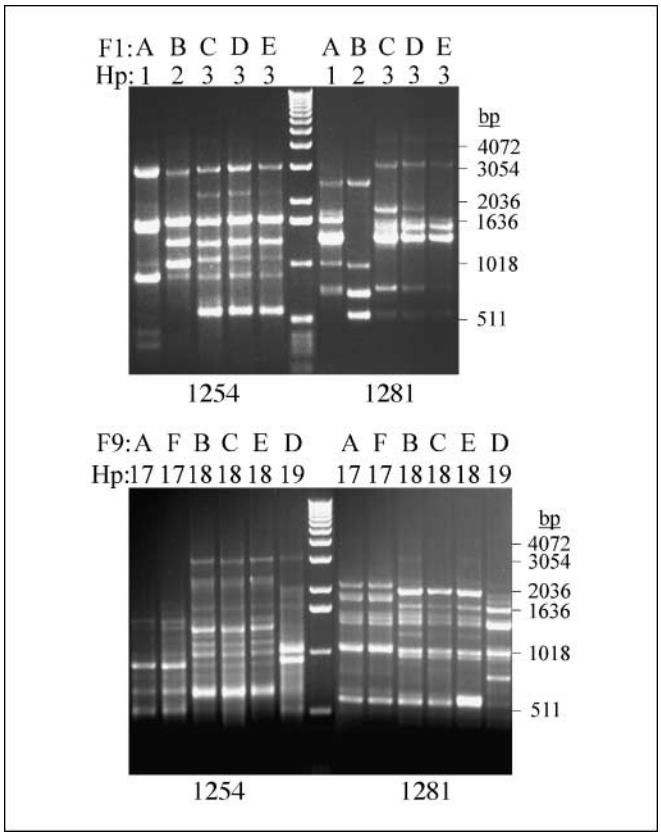

Fig. 1. Strains are shared between family members. RAPD-PCR fingerprinting of *H. pylori* strains (*Hp*) isolated from the five members of family F1 (A-E, top) and the six subjects in family F9 (A-F, bottom) using RAPD primers 1254 and 1281 showed that subjects within the same family were infected with the same strain. In family F1, strains isolated from three subjects (C, D, and E) had identical RAPD patterns (strain 3), whereas strains 1 and 2 from subjects A and B were unique. In family F9, subjects A and F were infected with strain 17, subjects B, C, and E were infected with strain 18, whereas subject D was infected with the unique strain 19.

fewer and 6 (18%) had strains lacking the *cag* PaI ($P > 0.25$). This raises the possibility that the increased cancer risk associated with higher numbers of CagA TPMs (10–12) may not be through increased risk of hypochlorhydria and atrophy, although the subject numbers studied here were fairly small so a type 2 error is possible.

Shared strains between noncohabiting adults implies childhood acquisition. The association between s1/m1 *vacA* strains and gastric hypochlorhydria could be due to the strains predisposing to these conditions or to them preferentially colonizing the hypochlorhydric stomach. One of our reasons for conducting this study in families of adult siblings was to address this issue. *H. pylori* strains from different individuals only have the same genetic fingerprints when they are passed between these individuals or acquired from a common source. Genetic fingerprinting methods have been used to show that strains are usually acquired from within the family in childhood (41–44). We argued that if noncohabiting adult siblings shared the same strain, this would be good evidence that they had acquired it in childhood. Of the gastric cancer relatives used in this study, 42 came from 12 families that provided from two–six members to the study; the other nine had no other family members in the study and are excluded from the following analysis. Family members F2-B and F12-D were infected with two distinct *H. pylori* strains by RAPD-PCR analysis, so there were 44 strains present within the 12 families. RAPD-PCR analysis (Fig. 1) showed that 29 of 44 (66%) *H. pylori* strains were shared with at least one other family member in 9 of 12 families (Table 1). Fifteen of 22 subjects with *vacA* s1/m1 strains shared that strain with at least one other family member, indicating that at least 68% of s1/m1 strains were likely acquired in childhood. Similarly, 66% of all strains were shared, implying childhood acquisition. Thus, acquisition of s1/m1 *vacA* strains likely preceded gastric hypochlorhydria.

H. pylori *microevolution is common within individual stomachs.* PCR-based genotyping of sweeps from gastric biopsies from our 51 subjects had shown that *cagA* of more than one size was amplified in nine subjects, and in two subjects, both the m1 and m2 *vacA* midregions were amplified. The most obvious explanation of this would be mixed strain infection. However, recent publications by us and others have shown occasional individuals to be infected by separate *H. pylori* isolates with different *cagA* or *vacA* genotypes, but have shown these isolates to be the same strain by various genetic fingerprinting techniques, most usually RAPD-PCR (24, 26, 31). This phenomenon has been termed microevolution. Because this phenomenon seemed fairly common among these cancer relatives, and because it has important implications for cancer risk, we explored it further. We plated out *H. pylori* samples with multiple *vacA* or *cagA* types to single isolates which had, as expected, only one type of *cagA* or *vacA*, and showed that all isolates from these 11 individuals had similar RAPD profiles (Table 1), indicating microevolution within these genes. To confirm this and to study the mechanism, we PCR amplified and did nucleotide sequencing on all or part of *cagA* or *vacA* from paired isolates, which were similar by RAPD-PCR and did phenotypic assays (VacA-induced vacuolation of AGS and RK13 cells, CagA phosphorylation, and CagA-induced hummingbird formation of AGS cells) to assess whether the isolates had changed their virulence.

Microevolution in* cagA *within individuals. Microevolution of *cagA* seemed to have occurred in 9 of 51 (18%) individuals (Table 1). Nucleotide sequencing of the *cagA* VRs showed that variation was always due to duplication or deletion of a 102-bp sequence containing the coding region for TPM type C, and in the case of strain 9, variation was due to single and double duplication/deletion of this region. The sequence surrounding the repeat region was identical between the paired isolates of seven strains and differed by only 1 and 3 bp, respectively, in the other two. Comparisons of this region of *cagA* between unrelated strains in this population showed variation in 18 to 38 bp. This confirmed the recent clonal origin of the paired isolates and suggested that microevolution had occurred by intragenomic recombination—either deletion or duplication of this region. To determine whether differences in the number of CagA TPMs altered the phenotypes of the strains, AGS cells were cocultured with the two isolates from strain 10 (3 and 4 TPMs). As expected, the CagA with four TPMs became more phosphorylated and induced longer cellular protrusions than the CagA with only three TPMs (Fig. 2). Further examples are discussed later.

Microevolution in* vacA *within individuals. Among the 42 subjects in the 12 families, only two had single colony isolates of *H. pylori* with different *vacA* types (s1/m1 and s1/m2). These subjects belonged to the same family and were siblings

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Table 1. Family groups from western Scotland and characterization of *H. pylori* strains

Family	Relative	Age	Sex	Pathology	Strain/RAPD*	*vacA*	*cagA* VR (bp) †	CagA TPMs ‡	CagA§
F1	F1-A	34	F	No atrophy	1	s1/m2	None	n.a.	N
	F1-B	36	M	No atrophy	2	s1/m2	550	ABC	Y
	F1-C	42	M	Atrophy	3	s1/m2	650	ABCC	Y
	F1-D	45	F	Atrophy	3	s1/m2	650	ABCC	Y
	F1-E	49	M	Atrophy	3	s1/m2	650	ABCC	Y
F2	F2-A	35	F	Atrophy	4	s1/m1	550	ABC	Y
	F2-B	42	M	No atrophy	4	s1/m2	550	ABC	Y
					5	s1/m1	550	ABC	Y
	F2-C	47	F	Atrophy	6	s1/m1	550, 650	ABC, ABCC	Y
F3	F3-A	38	M	No atrophy	7	s1/m1	650	ABCC	Y
	F3-B	41	F	No atrophy	8	s1/m1	650, 750	ABCC, ABCCC	Y
	F3-C	44	F	No atrophy	8	s1/m1	550	ABC	Y
	F3-D	46	F	No atrophy	8	s1/m1	650	ABCC	Y
F4	F4-A	34	F	No atrophy	9	s1/m2	550, 650, 750	ABC, ABCC, ABCCC	Y
	F4-B	38	M	No atrophy	10	s1/m2	550, 650	ABC, ABCC	Y
F5	F5-A	57	M	Atrophy	11	s1/m1	550	ABC	Y
	F5-B	58	M	Atrophy	11	s1/m1	550	ABC	Y
	F5-C	61	M	No atrophy	11	s1/m1	550	ABC	Y
F6	F6-A	32	F	No atrophy	12	s1/m2	550	ABC	Y
	F6-B	40	M	No atrophy	12	s1/m2	550, 650	ABC, ABCC	Y
F7	F7-A	46	F	Atrophy	13	s1/m2	550	n.d.	N
	F7-B	48	M	No atrophy	13	s1/m2	550	n.d.	N
	F7-C	51	F	Atrophy	14	s1/m1, s1/m2	550	ABC	N
	F7-D	52	F	No atrophy	14	s1/m1, s1/m2	550	ABC	N
	F7-E	53	M	No atrophy	13	s1/m2	550	n.d.	N
	F7-F	68	F	Atrophy	15	s1/m2	550	n.d.	Y
F8	F8-A	37	M	Atrophy	16	s1/m2	550	n.d.	Y
	F8-B	41	F	No atrophy	16	s1/m2	550	n.d.	Y
F9	F9-A	36	F	Atrophy	17	s1/m1	440	AB	Y
	F9-B	39	F	No atrophy	18	s1/m2	550	ABC	Y
	F9-C	40	F	Atrophy	18	s1/m2	550	ABC	Y
	F9-D	40	F	Atrophy	19	s1/m2	550	ABC	Y
	F9-E	41	M	No atrophy	18	s1/m2	550	ABC	Y
	F9-F	65	F	Atrophy	17	s1/m1	440	AB	Y
F10	F10-A	29	F	No atrophy	20	s1/m1	550, 650	ABC, ABCC	Y
	F10-B	31	M	No atrophy	21	s1/m2	None	n.a.	N
	F10-C	35	M	No atrophy	22	s1/m1	550	ABC	Y
F11	F11-A	40	M	No atrophy	23	s1/m2	550	ABC	Y
	F11-B	64	M	No atrophy	24	s1/m1	550	ABC	Y
F12	F12-A	51	F	Atrophy	25	s1/m1	650	ABCC	Y
	F12-B	58	M	Atrophy	25	s1/m1	None	n.a.	N
	F12-C	60	M	Atrophy	25	s1/m1	650, 750	ABCC, ABCCC	Y
	F12-D	68	F	Atrophy	25	s1/m1	650, 750	ABCC, ABCCC	Y
					26	s1/m2	550	ABC	Y
F13	F13-A	48	F	No atrophy	27	s1/m2	550, 650	ABC, ABCC	Y
F14	F14-A	39	M	No atrophy	28	s1/m2	None	n.a.	N
F15	F15-A	64	F	No atrophy	29	s1/m2	None	n.a.	N
F16	F16-A	40	M	No atrophy	30	s1/m2	None	n.a.	N
F17	F17-A	42	F	No atrophy	31	s1/m2	550	n.d.	Y
F18	F18-A	36	F	No atrophy	32	s2/m2	None	n.a.	N
F19	F19-A	47	M	No atrophy	33	s1/m1	650	n.d.	Y
F20	F20-A	54	F	No atrophy	34	s1/m2	500	n.d.	Y
F21	F21-A	71	M	Atrophy	35	s1/m1	550	n.d.	Y

**H. pylori* strains isolated from different members of the same family, which were identical or nearly identical by RAPD-PCR fingerprinting, were considered to be the same strain and, therefore, have the same strain number.
† Approximate size of the *cagA* VR by amplification with primers cag2 and cag4 (see Supplementary Table S1).
‡ Types of CagA EPIYA TPMs: A, EPIYA(K/Q)VNKKK(T/A)GQ; B, EPIY(A/T)QVAKKV; C, EPIYATIDDL; n.a., not applicable; n.d., not determined.
§ CagA expression determined by Western blotting: Y, CagA expressed and phosphorylated within AGS cells; N, no CagA expression.

(F7-C and F7-D) who, unlike other family members in our study, lived with each other as adults. All four isolates were found to be the same strain (strain 14) by RAPD-PCR analysis. PCR amplification of *vacA* from single colony isolates of strain 14 from both family members showed that both individuals had isolates with both s1/m1 and s1/m2 *vacA*. Nucleotide sequencing of the entire *vacA* gene of both isolates from both siblings revealed that, whereas the s1/m1 alleles in isolates from both siblings were identical, the s1/m2 forms were not (Fig. 3). Both s1/m2 forms of *vacA* were found to possess stop codons close to the 5′ end that prevented VacA expression (as confirmed by Western blot). As expected, isolates possessing

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these forms of *vacA* did not induce vacuolation of RK13 epithelial cells, whereas isolates possessing s1/m1 *vacA* did induce vacuolation, confirming that microevolution lead to a changed phenotype.

To obtain further evidence that we were dealing with a single parental strain, we did nucleotide sequencing of the housekeeping genes *mutY* and *yphC* of both s1/m1 and both s1/m2 isolates. *mutY* was identical between all four isolates, as was the region 5′ to *yphC* confirming parental strain identity. However, the *yphC* allele in the s1/m1 *vacA* isolate from subject C differed extensively to that of the other three isolates of strain 14, suggesting that, like *vacA*, it had been acquired horizontally from another strain. Taken together, these data suggest that the s1/m2 *vacA* isolates (which had developed minor genetic differences in *vacA*) were passed between these individuals many years previously, perhaps in childhood. In contrast, the s1/m1 *vacA* allele was likely acquired horizontally by the strain in one individual, and this isolate was passed to the other individual much later in life (because there were no nucleotide substitutions in s1/m1 *vacA* alleles between subjects). Other genes were also likely acquired horizontally by this strain, as analysis of two randomly selected housekeeping genes showed that one had undergone partial allelic replacement in one isolate.

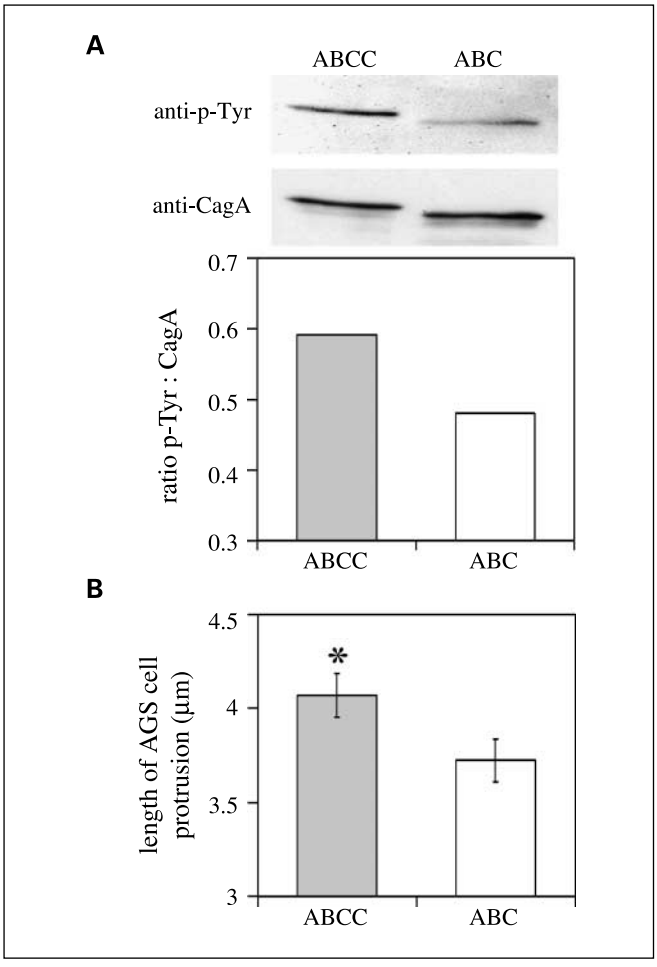

Fig. 2. Duplication of *cagA* VR TPMs in an individual alters the phenotype. The two *H. pylori* strain 10 isolates that were identical by RAPD-PCR but encoded CagA with TPMs in an ABC or an ABCC pattern were cocultured with AGS cells to determine the degree of CagA phosphorylation by Western blot analysis (*A*) and the average length of AGS cell protrusions (*B*). Densitometric analysis of the ratio of phosphorylated CagA to total CagA revealed that, as expected, duplication of the TPM-C motif increased the extent of phosphorylation (*A*). The data shown are representative of those from three separate experiments. Measurement of the lengths of 250 AGS cell protrusions showed that CagA with four TPMs induced the formation of longer protrusions than CagA with three TPMs (*B*). *, $P < 0.001$.

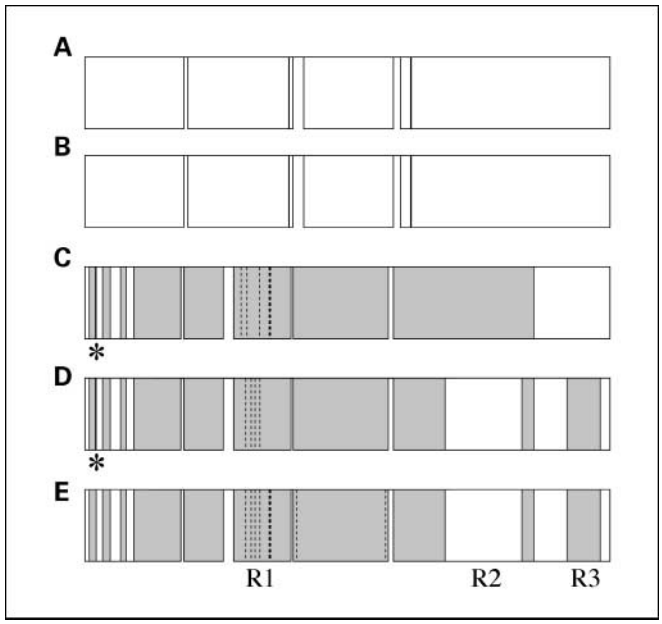

Fig. 3. Schematic representation of the *vacA* gene in *H. pylori* isolates from members of family F7. Regions of homology between *vacA* genes are represented by regions of white and gray. *A* and *B,* identical s1/m1 *vacA* alleles in *H. pylori* strain 14 from subjects F7-C and F7-D. *C* and *D,* nonidentical s1/m2 *vacA* alleles in strain 14 from subjects F7-C and F7-D, respectively. There were 80 nucleotide substitutions between the two different s1/m2 *vacA* alleles, 78 of which occurred within three regions (R1, R2, and R3) of 289, 544, and 270 bp (11, 36, and 31 substitutions, respectively). The asterisks represent stop codons located close to the 5′ end of the gene that terminates VacA translation. *E*, s1/m2 *vacA* allele in strain 13 from subject F7-B: there were only 7 of 3,915 bp differences to the s1/m2 *vacA* allele in subject F7-D above, but one of these meant there was no early stop codon and full-length VacA was translated. The dashed lines in R1 (*C-E*) represent single-nucleotide substitutions between alleles and the thick dashed lines represent four consecutive nucleotide substitutions. There were two additional single nucleotide substitutions in *vacA* from subject F7-B after R1.

Strains circulating within families have frequently microevolved to change virulence. Our analysis of strains passed between family members showed that, whereas family members had the same strains (by RAPD-PCR analysis), the isolates from different family members frequently varied in either *vacA* type or *cagA* type. We now confirmed and quantified this novel and potentially important phenomenon of virulence factor microevolution within families of gastric cancer patients. Among the family members we studied, RAPD-PCR analysis showed that strains were passed from one person to another in 18 cases, of which eight (44%) displayed microevolution; this occurred in the *cagA* VR in six cases and the *vacA* midregion in two cases (Table 1).

Turning first to the *cagA* microevolution, nucleotide sequence analysis of the *cagA* VR from the six cases showed that *cagA* had evolved by single or double duplication of a 102-bp sequence encoding a TPM. Phenotyping confirmed that this resulted in a changed phenotype (Fig. 4). Nucleotide sequencing of *mutY* and *yphC* confirmed the RAPD-PCR results that microevolved isolates were the same strain.

For *vacA*, nucleotide sequencing of s1/m1 and s1/m2 *vacA* isolates from strain 4 in members of family F2 showed that the

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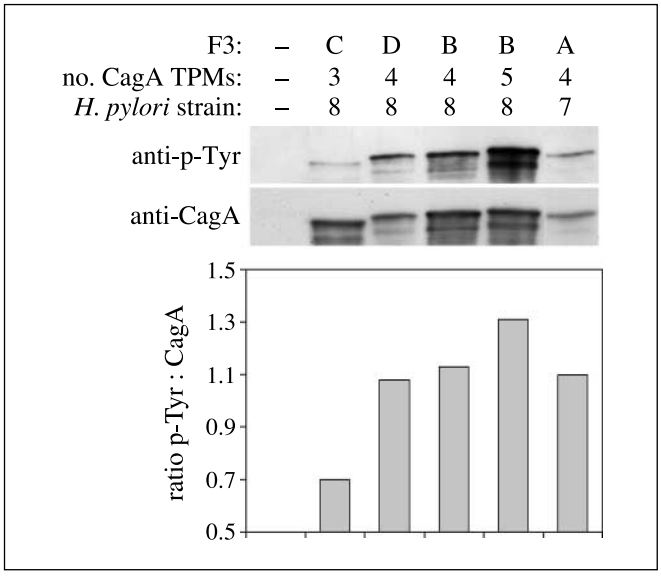

Fig. 4. Duplication of *cagA* VR TPMs in *H. pylori* strains from subjects in the same family (F3) alters the phenotype. *H. pylori* strain 8 was found to possess CagA with either three (subject C), four (subjects D and B), or five (subject B) TPMs. Coculture of the strain from different subjects with AGS cells followed by Western blot analysis and densitometry showed that increasing the number of CagA TPMs increased the degree of CagA tyrosine phosphorylation. *H. pylori* strain 7 from subject A had CagA phosphorylated to a similar extent to strain 8 from subject D. The data shown are representative of those from three separate experiments.

vacA alleles differed between these isolates. There were, excluding the regions of no homology (comprising 429 nucleotides), 403 nucleotide substitutions (10.9% of the gene) scattered throughout *vacA*. VacA was expressed by both the s1/m1 isolate from subject F2-A and the s1/m2 isolate from subject F2-B. However, we could not show a changed phenotype in this case, as both isolates induced vacuolation in RK13 cells during coculture experiments but neither isolate caused vacuolation of AGS cells. Nucleotide sequencing of *mutY* and *yphC* from different isolates of strain 4 from subjects F2-A and F2-B showed that the *mutY* fragments and the majority of the *yphC* gene were identical, confirming that these isolates were the same "strain." However, we identified another horizontally acquired DNA fragment of 600 bp which comprised the 5′ 210 nucleotides of *yphC*, the upstream intergenic region (82 nucleotides), and the first 308 nucleotides of the neighboring gene *hp0833*; 25 nucleotides in this region differed between the two isolates. This showed that *yphC*, as well as *vacA*, had been a target for partial allelic replacement by homologous recombination.

Virulence genes may be donated to unrelated strains through homologous recombination. As virulence genes can be acquired horizontally from unrelated strains, we next asked whether we could show donation of virulence genes to unrelated strains. In family F3, subjects B, C, and D had *H. pylori* isolates which were the same strain (strain 8) by RAPD-PCR, whereas subject A carried a different strain (by RAPD), strain 7. Strain 8 isolates from subjects C and D possessed *cagA* encoding 3 and 4 TPMs, respectively, whereas isolates of strain 8 from subject B (which showed microevolution within an individual) encoded *cagA* with either four or five TPMs. Unrelated strain 7 possessed *cagA* with four TPMs that was almost identical (one base change) to the *cagA* VR of strain 8 from subject D, suggesting that strain 7 may have acquired *cagA* from strain 8 in subject D. Phenotypic analysis revealed that CagA from strain 7 was phosphorylated to a similar extent after coculture with AGS cells to CagA from subjects B and D (Fig. 4).

In family F7 described previously, nucleotide sequencing of the entire *vacA* gene from strain 13 from family member F7-B revealed that this sequence was nearly identical to the s1/m2 *vacA* of strain 14 in subject D: only 7 of 3,915 nucleotides were different, but one of these substitutions meant that there was no 5′ stop codon in the *vacA* allele in strain 13 (Fig. 3). As expected, VacA was expressed in strain 13 and induced vacuolation in RK13 cells. Thus, it seems that *vacA* from strain 13 in subject B was acquired by strain 14 in subject D, where it was mutationally inactivated, and this isolate was passed to subject C. This likely occurred in childhood, as a small number of nucleotide substitutions are now present between *vacA* alleles in these three isolates.

A third example is discussed in more detail below and shown in Fig. 5.

Studying microevolution of virulence factors allows passage of strains within families to be mapped (Fig. 5). In family F12, *H. pylori* strain 25 was isolated from all four members of the family, and these isolates were shown to be the same strain by RAPD-PCR and by sequencing of *mutY* and *yphC* (Table 1). Strain 26 from subject D (who was also infected with strain 25) was unrelated (Table 1). Single-colony isolates of *H. pylori* strain 25 from subjects C and D both showed intrastrain evolution of *cagA* by duplication/deletion within the *cagA* VR and had identical nucleotide sequences between subjects; it is

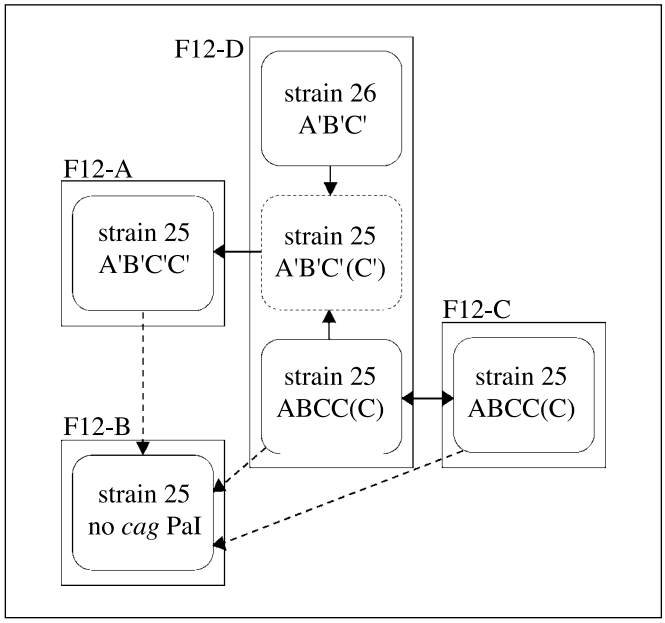

Fig. 5. Passage of *H. pylori* strains between family members in family F12. All four subjects in family F12 were infected with *H. pylori* strain 25 and subject D was also infected with strain 26. Subjects C and D both have strain 25 with *cagA* with four and five VR TPMs. These are identical between these subjects, and so have been passed between them. Strain 25 in subject A possesses *cagA* with a VR that is more closely related to *cagA* from strain 26 than from strain 25 in subjects C and D. This implies that strains 25 and 26 have recombined in subject D such that strain 25 has acquired the *cagA* VR from strain 26. This hybrid strain has then been passed to subject A. Duplication of the TPM-C motif may have occurred within subject D or after transfer to subject A. Strain 25 from subject B lacks the entire *cag* PaI, so it may have evolved from strain 25 from any of the other family members.

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possible that the strain had microevolved in one family member, and then both isolates had been passed to the other. The *cagA* VR in strain 25 from subject A had 19 nucleotide substitutions, implying that it was acquired horizontally from another strain. Comparison with the *cagA* VR of strain 26 showed that this strain was the likely donor, in that the nucleotide sequence exhibited only 2-bp differences, although the strain 25 isolate in subject A had also undergone a 102-bp duplication of the third TPM of strain 26. Nucleotide sequencing of the entire *cagA* alleles from *H. pylori* strain 25 from subject A and strain 26 from subject D, and the shorter *cagA* allele (encoding four TPMs) of strain 25 from subject D showed that there had been extensive recombination throughout *cagA*. The *cagA* alleles from these three isolates varied in 119 positions with 56 of these changes occurring within 966 bp of the 3′ end of the gene, which includes the VR. Taken together this suggests that strains 25 and 26 had recombined in subject D, such that *cagA* from strain 26 had recombined extensively with *cagA* from strain 25, and this included donation of its VR. This recombined strain 25 would then have been passed to subject A where duplication of the third TPM may have occurred (Fig. 5). The strain 25 *H. pylori* isolate from subject B was found to lack the entire *cag* PaI. This isolate may have evolved from any of the otherwise identical strains from the other members of the family (Fig. 5). Phenotypic analysis revealed the isolate of strain 25 from subject B, lacking the *cag* PaI, failed to induce IL-8 from cells, whereas *cag* PaI–positive isolates of strain 25 and strain 26 from other members of the family did induce IL-8 secretion, as expected, and had CagA translocated into cells and phosphorylated to an extent proportional to the number of CagA TPMs. Thus, this family showed genetic and phenotypic evolution of virulence factors in a strain which was passed between family members, and this allowed tracking of the passage of this strain within the family.

Discussion

H. pylori strains possessing type s1/m1 *vacA* were strongly associated with gastric hypochlorhydria in this population of first-degree relatives of gastric cancer patients in Scotland. Intestinal-type distal gastric cancer arises in a stepwise manner with simple *H. pylori*–associated gastritis developing to atrophy and hypochlorhydria, metaplasia, dysplasia, and cancer (4). Human genetic polymorphisms leading to high-level IL-1β secretion increase gastric cancer risk, but increase the risk of gastric hypochlorhydria even more markedly (2), implying that they convey their cancer risk through increasing the risk of this precursor condition. In this study, we show that *vacA* type s1/m1 strains of *H. pylori*, which several studies have shown are associated with increased gastric cancer risk (5–7), also increase the risk of hypochlorhydria, suggesting that they too increase cancer risk by increasing the risk of this precursor condition.

Studying *H. pylori* strains within families showed that most strains (66%) were shared between family members. As siblings rarely cohabit as adults and strains are rarely passed between spouses (43, 44), this suggests that strains were acquired during childhood, as has been shown for other populations (41, 42). This therefore implies that *H. pylori* strains possessing type s1/m1 *vacA* were acquired before the development of gastric atrophy and hypochlorhydria and suggests that the association between toxigenic strains and these conditions is likely to be causal.

Within the population studied here, we found extensive microevolution in *cagA* and significant microevolution in *vacA* in strains within individuals and in strains passed between family members. The finding in individuals is not novel: our studies follow previous case reports of microevolution in *vacA* and *cagA* leading to changes in phenotype (23, 24), although we show that this occurs more commonly than suspected, at least in these gastric cancer relatives. Our finding of *H. pylori* microevolution in these cancer families and its high frequency is novel and has important biological and clinical implications.

Turning first to *H. pylori cagA* and *vacA* microevolution within individuals, we found this in 11 of 51 (22%) subjects, which is likely to be a vast underestimate of the true frequency. Inevitably, our sampling represents a snapshot in time and also involves only a tiny area of the stomach—that contained in a single biopsy specimen. Also our study gives no clue as to how long cocolonization of phenotypically different isolates in the same stomach has persisted and will persist. This will depend on whether the phenotypic change confers a selective advantage and the extent of any such advantage. Given these limitations, it is interesting that our study gives similar frequencies to those recently reported in an Irish population. Like us, these workers screened for differences in the size of the *cagA* VR by PCR in strains from individual stomachs, although they did not check results or study likely mechanisms by performing nucleotide sequence analysis and nor did they check for phenotype (31). Nevertheless, their PCR analysis suggested that 3 of 19 (16%) individuals examined may have had isolates of the same strain with different *cagA* sizes coexisting in their stomach. This proportion is similar to that we report.

The 12 family groups in our population allowed us to study the transmission of strains between relatives of cancer patients and for the first time to show and quantify microevolution of *vacA* and *cagA* in strains passed between members of the same family. Besides its biological and clinical relevance, determining microevolution within families is a much better indicator of the extent of microevolution than looking in individuals. The main advantage is that, in effect, it is the result of sampling over a longer time period; family studies will pick up microevolution which has occurred at any stage because the parental strain was passed between two individuals, most usually in childhood. Sampling isolates from separate stomachs also avoids the potential criticism that microevolution may be an *in vitro* phenomenon due to recombination between subclones during initial culture; in the family scenario, these subclones are kept entirely apart. The extent of microevolution we describe in strains shared between family members (44%) is both important and entirely unexpected. Owen and Xerry (30) studied 16 individuals in five families, and although they did not focus on *cagA* or *vacA*, they described one example of a strain shared between husband and wife which differed for *vacA* midregion by PCR typing, indicating that microevolution may have taken place in that strain. Carroll et al. studied one sibling pair from a family and found different *vacA* signal region types, providing an indication that this part of *vacA* may have microevolved (31).

The frequency with which microevolution of virulence factors occurs within families has important implications for bacterial persistence. That it occurs at all implies that there must

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be selective advantages to *H. pylori* of having different *vacA* types or different numbers of *cagA* TPMs in different hosts. The environmental drivers of this are unknown. For *vacA*, they are not likely to be differences in acid production or presence or absence of atrophy, as our results suggest that these changes follow rather than precede colonization by *vacA* s1/m1 strains. Equally, for *cagA*, atrophic hypochlorhydric subjects in our study were not preferentially colonized by strains with higher or lower numbers of CagA TPMs. We are now performing further studies *in vitro* and *in vivo* to elucidate the selective advantages different virulence factor polymorphisms offer to individual strains under different conditions.

Microevolution in *vacA* and *cagA* has important implications for *H. pylori* pathogenesis. Changing *vacA* or *cagA* type may provide a partial explanation of why studies examining the relationship between bacterial virulence and disease do not show tighter associations. Indeed, if *H. pylori* microevolution occurs throughout the life of a human host, close associations of current virulence factor genotypes with disease would be unexpected. We already know that multiple strain infection is common, especially in developing countries (45–47), and knowing also that patients can be infected by the same strain with different virulence levels further complicates this.

Finally, and perhaps most importantly, our work has important clinical implications for families of gastric cancer patients. Although we did not have strains from cancer patients themselves available for study, our investigation of their families shows that strains are usually shared. However, this does not mean that different individuals have *H. pylori* with the same virulence; indeed, in about half the cases where strains are shared, this is not the case. Thus, if our clinical practice changes (as we believe it should), such that we screen young relatives of cancer patients for *H. pylori*, the safest option would be to treat all who are positive. We still do not know for sure whether this would change their cancer risk, but subgroup analysis of a large randomized treatment trial suggests that it might do so in patients who have not yet developed gastric atrophy (48).

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