ORIGINAL ARTICLE

IL-1B-511 ALLELE T AND IL-1RN-L/L PLAY A PATHOLOGICAL ROLE IN *HELICOBACTER PYLORI* (*H. PYLORI*) DISEASE OUTCOME IN THE AFRICAN POPULATION

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ABSTRACT

BACKGROUND: Many of the pathogenic effects of Helicobacter pylori infection are related to chronic active inflammation, which is controlled and maintained by the complex interplay of pro-inflammatory and anti-inflammatory mediators. Pro-inflammatory genetic polymorphisms tend to increase the risk of development of gastric cancer. In Africa, the data are scarce regarding the effects of these polymorphisms on gastric pathology. The objective of this study is therefore to investigate the pro-inflammatory genetic polymorphisms and their role in H. pylori-related gastric disorders in a select African population.

METHODS: This cross-sectional prospective study recruited six hundred and ninety six adult subjects with a history of uninvestigated dyspepsia. The H. pylori status was determined by tissue Giemsa staining, Rapid Urease Test (RUT), H. pylori stool antigen test (HpSAT), and PCR using the 16s-rRNA gene. The polymorphisms in IL-1B (511 C/T), TNF-A (_308 G/A) and IL-1RN were assessed by the PCR-restricted fragment length polymorphism (RFLP).

RESULTS: H. pylori was significantly associated with gastric pathologies investigated (P = 0.0000). Heterozygous allele TC of IL-1 β -511 was significantly associated with H. pylori infection (p = 0.003815). Similarly, allele IL-1 RN*2/2 and allele IL-1 RN-L/L were associated with H. pylori infection (p = 0.0025 and p = 0.0203). Allele T of IL-1 β -511 and IL-1 RN-L/L are more frequent in H. pylori associated gastric pathologies in this series.

CONCLUSION: Allele T of IL-1 β -511 and long allele IL-1 RN-L/L play a role in H. pylori disease in this population.

KEY WORDS: Pro-inflammatory cytokines, Helicobacter pylori, Pathologies, Africa

INTRODUCTION

Since the discovery of *Helicobacter pylori* (*H. pylori*) (1) and its subsequent association with upper gastrointestinal (UGI) pathologies (2, 3, 4), a ripple effect has been observed which has resulted in new insights into the impact of the bacterium on the pathology and physiology of the stomach (5). Of particular concern to Africa is the perception that *H. pylori* infections in Africa are at odds in several aspects with those in the west (6, 7).

The level of gastric acid secretion and the presence of a pro-inflammatory response contribute significantly to the development of either duodenal ulcer disease or atrophic gastritis. Importantly is the interleukin-1 (IL-1) cytokine which is encoded by a gene cluster that contains the polymorphic IL-1B (encoding the IL-1ß cytokine) and IL-RN (encoding the IL-1 receptor antagonist) genes. IL-1ß is a potent pro-inflammatory cytokine and the most potent known inhibitor of acid secretion (8). The IL-1 gene cluster contains several polymorphisms, such as IL-1B-31C, IL-1B-511T, and IL-1RN*2/2, which lead to high-level

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expression of IL-1ß. This subsequently leads to reduced acid output, which is associated with corpus-predominant colonization by *H. pylori*, resulting in pangastritis, formation of atrophic gastritis, and increased risk of gastric cancer (9, 8, 10, 11, 12, 13).

Similar effects have been observed for genes encoding tumor necrosis factor alpha (TNF-α) and IL-10. The TNF-A-308A genotype is associated with increased TNF-α production, which, together with IL-1, influences gastrin production and thus acid production by gastric parietal cells (14). The TNF-A-308A genotype is therefore associated with *H. pylori* infection and increased risk of gastric cancer (10, 15, 16, 17, 18). Similarly, expression of the anti-inflammatory cytokine IL-10 is affected by the haplotypes described for the IL-10 gene. The GCC haplotype is associated with a higher expression level of IL-10 and hence favors an anti-inflammatory response, whereas the ATA haplotype results in lowered IL-10 levels and a shift toward a proinflammatory response (10, 19, 16, 18). The GCC haplotype is associated with colonization with more-virulent *H. pylori* strains (16), whereas the ATA haplotype is associated with increased risk of gastric cancer (10, 19, 18). Interestingly, several independent studies have shown that while single polymorphisms may increase the risk of development of gastric cancer only two- to threefold, the presence of multiple proinflammatory genotypes increases this risk substantially (10, 11, 12, 16, 18).

The interaction between the different pro- and anti-inflammatory polymorphisms, the immune status of the host, and the characteristics of the colonizing *H. pylori* strain jointly determine disease outcome (13). In Africa, the data are scarce regarding the effects of these polymorphisms on gastric pathology. This study is therefore conducted to investigate the pro-inflammatory genetic polymorphisms and their role in *H. pylori*-related gastric disorders in a select African population.

MATERIALS AND METHODS

Study Population: Six hundred and ninety six patients presenting with dyspepsia at the gastroenterology clinic of the Aga Khan University Hospital, Nairobi and who had been referred for endoscopy were recruited. Approval was obtained from the ethical board of the university, and the study conducted according to the ethical guidelines of the declaration of Helsinki as revised in 2000 (20). All subjects provided written informed consent before entering the study. All of the above patients were screened for *H. pylori*. Two hundred and ninety (41.6%) were randomly selected for pro-inflammatory cytokine studies.

Sample collection: For each of the patient, 6 gastric biopsy specimens were obtained for histological evaluation, DNA extraction, and *H. pylori* detection. Four biopsies were obtained from incisura angularis and two from the pre-pyloric region. Biopsies for histological evaluation were transported to the laboratory submerged in formalin, in the laboratory, they were fixed and paraffin embedded. An experienced pathologist examined and reported the findings from all the histological slides. Those intended for DNA extraction were immediately submerged in 5% Brain Heart Infusion (BHI) agar enriched with 7% Fetal Bovine Serum (FBS) upon extraction and stored in deep freezer (-80^{0}C) till processing

***H. pylori* status:** The *H. pylori* status of the study population was determined by tissue Giemsa staining, the Rapid Urease Test (RUT), *H. pylori* stool antigen test (HpSAT), and by PCR using the C97-20 and H3A-20 primers for the 16s-rRNA gene (F-GGCTATGACGGGTATCCGGC, R-GCCGTGCAGCACCTGTTTTC). PCR conditions were 95°C for 3 min and 34 cycles of 95°C for 30 seconds, 63°C for 1 min, 72°C for 1min, and a final extension at 72°C for 10 min. This gave a product size of 765bp). A positive score was considered on agreement by two diagnostic tools. Where only one tool was positive, the score was uncertain.

Pro-inflammatory Cytokines Typing: The single nucleotide polymorphisms in IL-1B (511 C/T) and TNF-A (_308 G/A), as well as the variable number of tandem repeats (VNTR) polymorphism of IL-1RN were assessed. The genomic human DNA used for genotyping was extracted from gastric biopsies using the QIAgen DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. 200µl DNA was extracted from each sample, 50µl was aliquoted and kept at 4^{O}C for routine use and the remaining 150µl stored at -20^{O}C. From the 50ul aliquot, a

1.10 dilution was made and all PCR experiments were performed on the dilution. Restriction endonucleases were used to digest the PCR products which allowed for the recognition (after electrophoresis) of the sequence variation between individuals at the recognition site. PCR-RFLP was set up in a 20 µl reaction which included 5 units of endonuclease and 15 µl of amplicon. This was incubated at 37^0C for 3h. The products of RFLP were visualized by agarose gel electrophoresis. Five µl of PCR product plus 3 µl of gel loading buffer type was loaded into a 1% agarose in TBE 1X buffer stained with ethidium bromide to visualize the PCR products. For visualizing enzyme digestion products 7 µl of enzyme digestion product plus 3µl gel loading buffer was used. The appropriate marker was loaded into an additional well. Electrophoresis was performed at 70 V for 1h. Gels were photographed using the Gel Doc system (Bio-Rad).

Genotyping of the _511 (C/T) polymorphism in the IL-1B gene: The polymorphism at position _511 of the IL-1B gene was determined by the PCR-restricted fragment length polymorphism (RFLP). The primer sequence used was; Forward (5'-TGGCATTGATCTGGTTCATC-3') and reverse (5'GTTTAGGAATCTTCCCACTT-3'). Amplifications were carried out in a final volume of 30µl. The thermocycling conditions were: 95°C for 15 min; then 35 cycles of 95.5°C, 35°C and 55°C for 1 min each, followed by 72°C for 45sec and a final 72°C for 10 min. The digestion of PCR products was carried out at 37°C with AvaI. The resulting DNA fragments were resolved by electrophoresis on a 2.7% agarose gel stained with ethidium bromide. Based on restriction product sizes, three alleles were observed, homozygous allele T/T showed an intact product (304bp), whereas allele C/C showed products that were split into two, 190 and 114 bp. Heterozygous allele T/C had both C and T alleles.

Genotyping of the _308 (G/A) polymorphism in the TNF-A gene: The polymorphism at position _308 of the TNF-A gene was determined. Initially, a PCR was carried out with the primers (TNF-308_RFLP1<5'-GAGGCAATAGGTTTTGAGGGCCAT-3'>, and TNF-308_RFLP2<5'-GGGACACACAAGCATCAAG-3'>). The PCR conditions were: 95°C for 15 min and 29 cycles of 95.5°C for 35 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The digestion of PCR products was carried out at 37°C with E. NCO 1. The resulting DNA fragments were resolved by electrophoresis on a 2.7% agarose gel stained with ethidium bromide. Interpretation was based on band sizes, a single band of 147 bp designated as homozygous allele TNF-α A/A, whereas a single band of 126 bp was allele TNF-α G/G. If the product was split into two fragments 147 bp and 128 bp, it was designated heterozygous TNF-α G/A.

Genotyping of the Polymorphism in the VNTR of the IL-1RN Gene: The polymorphic region within intron 2 of the IL-1RN gene was amplified using the following PCR conditions; 95°C for 15 min and 34 cycles of 95.5°C for 35 sec, 60°C for 30 sec, 72°C for 1min, and a final extension at 72°C for 10 min and (IL1RN1 <5'-CCCCTCAGCAACACTCC-3'>, IL1RN2 <5'-GGTCAGAAGGGCAGAGA-3'>) primer sequences. The size of the amplified product was determined by electrophoresis on a 2.7 % agarose gel stained with ethidium bromide. Alleles were sized relative to a 50 bp DNA ladder. Allele 1 (IL-1RN*1,4) was 440 bp, allele 2 (IL-1RN*2,2) was 270 bp, allele 3 (IL-1RN*3,5) was 530 bp, allele 4 (IL-1RN*4,3) was 356 bp, whereas allele 5 (IL-1RN*5,6) was 616 bp. All alleles with more than two repeats were designated long (L/L).

RESULTS

H. pylori was detected with high frequency in patients with Gastritis, PUD and GERD implying a significant risk to the development of these pathologies (p =0.0000, p = 0.0000, p = 0.0000, respectively) (Table 1).

Analysis of polymorphisms distribution in relation to *H. pylori* status (Table 2) showed that heterozygous allele TC of IL-1 β -511 is significantly associated with *H. pylori* infection (p = 0.003815). The short IL-1 RN*2/2 and the long allele IL-1 RN-L/L are both significantly associated with *H. pylori* infection (p = 0.0025 and p = 0.0203 respectively). Homozygous allele G and heterozygous allele G of TNFα_308 is not associated with *H. pylori* infection in this series (p = 0.2051).

Table 1: Prevalence of *H. pylori* in different disease profiles

Diagnosis (n)	Positive (%)	Uncertain (%)	Negative (%)	P- Values
Gastritis (211)	115 (54.5)	6 (2.8)	90 (42.6)	0.0000
PUD (31)	23 (74.2)	0 (0)	8 (25.8)	0.0000
GERD (36)	12 (33.3)	0 (0)	24 (66.6)	0.0000
NAM (10)	1 (10)	1 (10)	8 (80)	

**PUD-peptic ulcer disease, GERD-gastrointestinal reflux disease, NAM- normal appearing mucosa*

Table 2: Interleukin 1 Polymorphisms in Relation to *H. pylori* Status

		H. pylori Status		
		Positive (151)	**Negative (119)**	**Uncertain (N=20)**
IL-1 β -511	TT	63(41.7)	45(37.8)	0(0)
	TC		49(41.2)	0(0)
	CC		25(21)	20(100)
IL-1RN	S/S	38(25.2)	13(10.9)	0(0)
	S/L	3(2.0)	6(5.0)	0(0)
	L/L	110(72.8)	100(84)	20(100)
TNFα	GG	54(35.8)	36(30.3)	0(0)
	GA	97(64.2)	81(68.1)	7(35.0)
	AA	0(0)	2(1.7)	13(65.0)

A profile of pro-inflammatory cytokines in relation to gastric pathologies (Table 3) reveals that provocative allele T of IL-1 β -511 and IL-1 RN-L/L are more frequent in these patient groups. As shown in Table 3, both the homozygous IL-1B-511T/T genotype and the heterozygous IL-1B-511T/C genotype showed a significant association with gastric pathologies risk if all patients were included ($p < 0.05$). Equally IL-1 RN-L/L was significantly associated with gastric pathologies (Table 3).

Table 3: Interleukin 1Polymorphisms in Different Disease Pathologies

Polymorphisms		**Gastritis(208)**	**GERD(n=39)**	**GU (n=10)**	**DU (16)**	**GU/DU (5)**	**Normal(10)**	**CA(2)**
IL-1 β -511	TT	73(35.1)	17(43.6)	5(50.0)	9(56.3)	1(20.0)	3(30.0)	
	TC	99(47.6)	19(48.7)	5(50.0)	4(25.0)	3(60.0)	6(60.0)	1(50.0)
	CC	36(17.3)	3(7.7)		3(18.8)	1(20.0)	1(10.0)	1(50.0)
IL-1RN	S/S	40(19.2)	3(7.7)		4(25.0)	1(20.0)	1(10.0)	
	S/L	7(3.4)	2(5.1)					2(100.0)
	L/L	161(77.4)	34(87.2)	10(100.0)	12(75)	4(80.0)	9(90.0)	
TNFα	GG	68(32.7)	9(23.1)	4(40.0)	3(18.8)	2(40.0)	3(30.0)	1(50.0)
	GA	129(62.0)	28(71.8)	6(6.0)	11(68.8)	3(60.0)	7(70.0)	1(50.0)
	AA	11(5.3)	2(5.1)		2(12.5)			

Legend: GERD (Gastrointestinal Reflux Disease), GU (Gastric Ulcer), DU (Duodenal Ulcer)

Haplotype frequency showed that haplotype T of IL-1 β -511 and haplotype G of TNFα_308 are more prevalent in the study population. Even though haplotype T of IL-1 β -511 is more provocative, its occurrence with the less aggressive haplotype G of TNFα_308 waters

down its pathological activities (Table 4). As shown in Table 4, haplotype analyses support the aetiologic role of IL-1B-511T alleles in gastric pathologies associated with *H. pylori* infection.

Table 4: Frequency of IL – 1 haplotypes in Different Disease Pathologies

Polymorphisms	Gastritis(416)	GERD(n=78)	GU (n=20)	DU (32)	GU/DU (10)	Normal(20)	CA(4)
IL-1 β-511T	245(58.9)	53(67.9)	15(75)	22(68.8)	5(50.0)	12(60)	1(25)
IL-1 β-511C	141(41.1)	25(32.1)	5(25)	10(31.3)	5(50.0)	8(40)	3(75)
Polymorphisms	**Gastritis(416)**	**GERD(n=78)**	**GU (n=20)**	**DU (32)**	**GU/DU (10)**	**Nor(20)**	**CA(4)**
TNF-α/G	265(63.7)	46(59)	14(70)	17(53.1)	7(70)	13(65)	3(75)
TNF-α/A	151(36.3)	32(41.0)	6(30)	15(46.9)	3(30)	7(35)	1(25)

Legend: GERD (Gastrointestinal Reflux Disease), GU (Gastric Ulcer), DU (Duodenal Ulcer)

DISCUSSION

H. pylori was detected with high frequency in patients with Gastritis, PUD and GERD implying a significant risk to the development of these pathologies (p =0.0000, p = 0.0000, p = 0.0000, respectively). Analysis of polymorphisms showed that heterozygous allele TC of IL-1 β -511 was significantly associated with *H. pylori* infection (p = 0.003815). Similarly, the short IL-1 RN*2/2 and the long allele IL-1 RN-L/L were both significantly associated with *H. pylori* infection (p = 0.0025 and p = 0.0203 respectively). Homozygous allele G and heterozygous allele G of TNFα_308 is not associated with *H. pylori* infection in this series (p = 0.2051). *IL1B-511T* and the short *IL1RN*2* have been found to be associated with gastric cancer risk in Caucasians but not in Asians (28-30). In this study *IL1B-511T* association with *H. pylori* gastric pathologies was null. However, association as established between gastric pathologies and heterozygous allele TC of IL-1 β -511. Elsewhere, Noncardia cancers have shown stronger associations with IL-1B -511 CT (31), this implies that the African population is likely to develop this type of cancer if infected with *H. pylori*.

A profile of pro-inflammatory cytokines in relation to gastric pathologies revealed that provocative allele T of IL-1 β -511 and IL-1 RN-L/L were more frequent in *H. pylori* associated gastric pathologies. The L-C-T haplotype, including the wild-type alleles IL-1RNL and IL-1B-511C, has always been associated more frequently with controls than intestinal gastric cancer cases (32). Although epidemiological studies suggest that interleukin-1 (IL-1) genetic polymorphisms are involved in *H. pylori*-related gastric carcinogenesis (21, 32), in Africa; the data are scarce regarding the effects of these polymorphisms on gastric pathology. IL-1 RN-L/L may supply reliable disease marker estimates in this population given their high frequencies in this subgroup analysis.

A recent study by Kimang'a et al. (22) compared the gene expression of the Th1-specific pro-inflammatory cytokines IL-1α and IL-18 in Caucasian and African population in two distinct geographical regions. The Kimang'a study found no elevation of these cytokines in both patient groups, a finding that is different from other studies showing an induction of IL-18 or IL-1b in *H. pylori*-induced gastritis (23, 24, 25). Taking into consideration that IL-33 is closely related with respect to origin; receptor and signaling pathways to IL-1 and IL-18, the study investigated this cytokine in relation to *H. pylori* infection and did not find a differential expression of this cytokine in *H. pylori*-infected patients. Despite its genetic relatedness to IL-1 and IL-18, IL-33 acts predominately as the inducer of Th2 response (26). Although several studies suggest that Africans develop a stronger Th2 response towards *H. pylori* than other ethnicities (27), the expression levels of IL-33 were not different between *H. pylori*-infected versus *H. pylori*-

negative individuals in both study groups implying that a Th2-dominating profile does not play a major role in both Kenyan and German patients.

Carriers of both the IL-1B-511 allele and IL-1RN*2/2 have been reported to be at increased risk of hypochlorhydria and gastric cancer (8, 28). IL-1 genetic polymorphisms also influences *H. pylori*-related gastric mucosal IL-1beta levels and are related to gastric inflammation and atrophy, factors thought to be important in gastric carcinogenesis (29). Tumor necrosis factor α (*TNFa*) polymorphisms have not been demonstrated to affect gastric mucosal levels of TNFα (16), but they have been shown to affect gastric inflammation and cancer risk in some (12). Additionally, in this study, no association was found between TNFα and *H. pylori* associated pathologies.

In conclusion, Allele T of IL-1 β -511 and long allele IL-1 RN-L/L play a role in *H. pylori* disease in this population. IL-1 RN-L/L may supply reliable disease marker estimates in this population given its high frequencies in this subgroup analysis.

ACKNOWLEDGEMENTS

DAAD - Deutscher Akademischer Austausch Dienst, Aga Khan University Hospital, Nairobi, Jomo Kenya University of Agriculture and Technology, Prof. Dr. med . Peter Malfertheiner and PD Dr. Thomas Wex, Otto von Guericke University, Magdeburg, Germany

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