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Genetic polymorphisms in the Toll-like receptor signalling pathway in *Helicobacter pylori* infection and related gastric cancer

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ABSTRACT

Background: Gastric cancer (GC) is a progressive process initiated by *Helicobacter pylori*-induced inflammation. Initial recognition of *H. pylori* involves Toll-like receptors (TLRs), central molecules in the host inflammatory response. Here, we investigated the association between novel polymorphisms in genes involved in the TLR signalling pathway, including *TLR2*, *TLR4*, *LBP*, *MD-2*, *CD14* and *TIRAP*, and risk of *H. pylori* infection and related GC.

Methods: A case-control study comprising 310 ethnic Chinese individuals (87 non-cardia GC cases and 223 controls with functional dyspepsia) was conducted. Twenty-five polymorphisms were detected by MALDI-TOF mass spectrometry, PCR, PCR–RFLP and real-time PCR.

Results: Seven polymorphisms showed significant associations with GC (*TLR4* rs11536889, *TLR4* rs10759931, *TLR4* rs1927911, *TLR4* rs10116253, *TLR4* rs10759932, *TLR4* rs2149356 and *CD14* −260 C/T). In multivariate analyses, *TLR4* rs11536889 remained a risk factor for GC (OR: 3.58, 95% CI: 1.20–10.65). *TLR4* rs10759932 decreased the risk of *H. pylori* infection (OR: 0.59, 95% CI: 0.41–0.86). Statistical analyses assessing the joint effect of *H. pylori* infection and the selected polymorphisms revealed strong associations with GC (*TLR2*, *TLR4*, *MD-2*, *LBP* and *TIRAP* polymorphisms).

Conclusions: Novel polymorphisms in *TLR2*, *TLR4*, *MD-2, LBP*, *CD14* and *TIRAP*, genes encoding important molecules of the TLR signalling pathway, showed clear associations with *H. pylori*-related GC in Chinese.

1. Introduction

Gastric cancer (GC) is the fifth most common type of cancer and the third leading cause of cancer-related death worldwide, with high incidence rates being reported in East Asia, Eastern Europe, and parts of Central and South America [1]. Although *Helicobacter pylori* is an essential aetiological factor for GC, the pathogenesis of GC involves the combined effects of bacterial, host and environmental factors [2].

Currently, it is well established that most cancer cell genotypes are the manifestation of 7 essential alterations in cell physiology that collectively dictate malignant growth: (1) self sufficiency in growth signals, (2) insensitivity to growth-inhibitory signals, (3) evasion of programmed-cell death (apoptosis), (4) limitless replicative potential, (5) sustained angiogenesis, (6) tissue invasion/metastasis, and (7) inflammation [3,4]. GC can be

Abbreviations: CTLs, C-type lectin receptors; CI, 95% confidence intervals; DAMPs, damage-associated molecular patterns; ELISA, enzyme-linked immunosorbent assay; EM, Expectation-Maximization; FOXP3, forkhead box protein P3; FD, functional dyspepsia; GC, gastric cancer ; HWE, Hardy–Weinberg equilibrium; HREC, Human Ethics Committee; LD, linkage disequilibrium; LPS, lipopolysaccharide; LR, logistic regression; MALDI-TOF, matrix assisted laser desorption ionisation time-of-flight ; NCBI, National Center for Biotechnology Information; NHMRC, National Health and Medical Research Council; NSAIDs, non-steroidal anti-inflammatory drugs; NLRs, nucleotide-binding oligomerization domain-like receptors; OR, odds ratios; PAMPs, pathogen-associated molecular patterns; PCR, polymerase chain reaction; PCR–RFLP, polymerase chain reaction–restriction fragment length polymorphism; PRRs, pattern-recognition receptors; RLRs, retinoic acid-inducible gene-I-like receptors; Treg, natural regulatory T; TLRs, Toll-like receptors; UTR, untranslated region; χ^2, chi-square goodness-of-fit test.

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subdivided into two histological categories according to the Lauren classification: intestinal-type and diffuse-type GC. The morphological changes in intestinal-type GC, described for the first time by Correa in a human model of gastric carcinogenesis, are part of a continuum and include chronic inflammation, atrophy, cell type conversion and loss of cellular differentiation [5]. In this case, chronic inflammation is the result of *H. pylori* infection of the gastric mucosa.

In the human gastric mucosa, the first four physical-chemical barriers encountered by *H. pylori* are the mucus layer, gastric epithelial cells and pattern recognitions receptors (mainly the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)).

TLRs on epithelial cells recognize diverse pathogen-associated molecular patterns (PAMPs) such as flagella (TLR5), peptidoglycan (TLR2), unmethylated CpG motifs (TLR9), and lipopolysaccharide (LPS) (TLR4) [6]. Although most studies conclude that TLR4 is the first innate immune response against *H. pylori*, others argue that TLR2 is the initial barrier against infection [7,8]. The most likely scenario is that both receptors are involved in the early immune response against *H. pylori*, which has been recently demonstrated in a study conducted by Yokota et al. [9]. These authors not only showed that *H. pylori* LPS was initially targeted by TLR2 as described by others, but, for the first time, showed that this TLR2 activation leads to increased proliferation of gastric epithelial cells and TLR4 expression via the MEK1/2-ERK1/2 pathway [9].

Further, TLRs not only recognize PAMPs, but are also involved in the detection of endogenous ligands known as damage-associated molecular patterns (DAMPs). Release of DAMPs, which are especially targeted by TLR2 and TLR4 during cancer progression [10], may cause chronic inflammation through T-cell and NK cell dysfunction, a phenomenon observed in some malignancies including cervical and pancreatic cancer [11,12]. Given this, TLRs might play a role in both gastric carcinogenesis mediated by *H. pylori* infection and in gastric cancer perpetuation associated with immunosuppression. In the latter case, lower TLR activity should minimize the effects of chronic inflammation and the likelihood of tumor development.

Genetic polymorphisms have emerged in recent years as determinants of disease susceptibility and severity, which is particularly true in gastrointestinal malignancy [13]. Therefore, polymorphisms within genes involved in the inflammatory response during *H. pylori* infection might directly influence gastric immunopathology and determine the manifestation of *H. pylori*-related complications including GC. Recent studies, conducted in diverse human populations including Caucasians, Asians and Latin Americans, have shown associations between the polymorphisms *TLR1* rs5743618 (Ile602Ser) [14], *TLR2* −196 to −174del [15], *TLR2* rs3804099 [16], *TLR4* rs4986790 (Asp299Gly) [15], *TLR4* rs4986791 (Thr399Ile) [17], *TLR4* rs11536889 (+3725 G/C) [15], *TLR5* rs5744174 [18], *TLR9* rs187084 (−1486 T/C) [19] and *CD14* rs2569190 (−260 C/T) [15], and risk of GC development.

Given that the TLR signalling pathway, especially TLR2, TLR4 and associated molecules (LBP, MD-2, CD14 and TIRAP), are key components during *H. pylori* infection, in the current study, we comprehensively examined the association between 25 recently identified polymorphisms involved in the TLR signalling pathway (*TLR2*, *TLR4*, *LBP*, *MD-2, CD14* and *TIRAP*) and risk of *H. pylori* infection and related GC, in a high risk ethnic Chinese population.

2. Methods

2.1. Subjects

Subjects were ethnic Chinese individuals presenting for upper gastrointestinal endoscopy at The Changi General Hospital (Singapore) and the University Hospital of Malaysia (Kuala Lumpur). Exclusion criteria included infection with the Human Immunodeficiency Virus, any condition associated with immunosuppression, and the use of prescribed non-steroidal anti-inflammatory drugs (NSAIDs), anti-microbial agents or acid suppressants in the two-month period prior to recruitment.

Patients were recruited during the period January 2004 to April 2007. Eighty-seven patients, histologically diagnosed with primary non-cardia GC (International Classification of Diseases, 9th revision, code 151), were included in the current study. The control group comprised 223 individuals diagnosed with functional dyspepsia (FD), which was defined as pain or discomfort centred in the upper abdomen without any identifiable organic disease (including at upper endoscopy), in accordance with the Rome II classification system [20].

The current study was approved by the Human Ethics Committee (HREC) of the University of New South Wales (HREC 08115 and HREC 02144). Written informed consent was obtained from each individual at recruitment.

2.2. H. pylori detection

H. pylori status was determined by means of an in-house enzyme-linked immunosorbent assay (ELISA) [21] and immunoblot (MPD Helico Blot 2.1, MP Biomedicals, Australia) according to the manufacturer's instructions. These results have been partially published elsewhere [22].

2.3. Polymorphisms

Electronic databases (PUBMED, Scopus, Science Direct, Ovid, Biosis Previews, Scirus databases, CINAHL, IMBIOMED, Scielo and LILACS) were used to search for polymorphisms involved in the TLR signalling pathway that were associated with cancer, infectious disease or were functionally relevant. Twenty-five polymorphisms in six genes, which were reported to have a minor allele frequency >1% in the National Center for Biotechnology Information (NCBI) dbSNP, were selected for analysis (Table 1).

2.4. Genotyping

From each individual included in the study, genomic DNA was extracted from peripheral whole blood samples using the QIAamp® Blood DNA Mini Kit as described by the manufacturer (Qiagen; Hilden, Germany). DNA was rehydrated in sterile water and normalised to 10 ng/μl for customised SNP genotyping of 20 polymorphisms in *TLR2*, *TLR4*, *LBP*, *CD14*, *MD-2* and *TIRAP* through the application of matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry, the Sequenom MassARRAY iPLEX™ assay (San Diego, CA, USA) [23,24], at the Australian Genome Research Facility Ltd., St. Lucia, University of Queensland, Australia.

Two polymorphisms could not be included in the Sequenom MassARRAY iPLEX™ assay, and therefore, were genotyped by means of polymerase chain reaction (PCR)–restriction fragment length polymorphism (PCR–RFLP) and real-time PCR. Genotyping of *TIRAP* rs8177374 (S180L) was performed using PCR–RFLP. PCR products were digested with the restriction endonuclease *Bst*XI (New England Biolabs; Arundel, Australia) and subjected to 2% agarose gel electrophoresis. Ten percent of the study sample was randomly selected and sequenced to validate the results obtained with PCR–RFLP. Genotyping of *TIRAP* rs8177400 (D96N) was performed using real time-PCR. Hairpin primers were designed based on the methodology published by Hazbon et al. [25] and Chan et al. [26]. Experiments were performed using the Rotor Gene 6000 real-time PCR cycler (Corbett Life Sciences; Doncaster, Australia). The