Genetic factors associated with intestinal metaplasia in a high risk Singapore-Chinese population: a cohort study

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Abstract

Background: Intestinal metaplasia (IM) is an important precursor lesion in the development of gastric cancer (GC). The aim of this study was to investigate genetic factors previously linked to GC risk for their possible association with IM. A total of 18 polymorphisms in 14 candidate genes were evaluated in a Singapore-Chinese population at high risk of developing GC.

Methods: Genotype frequencies were compared between individuals presenting with (n = 128) or without (n = 246) IM by both univariate and multivariate analysis.

Results: Carriers of the NQO1 609 T allele showed an association with IM in individuals who were seropositive for Helicobacter pylori (HP+; OR = 2.61, 95% CI: 1.18-5.80, P = .018). The IL-10 819 C allele was also associated with IM in HP+ individuals (OR = 2.32, 95% CI: 1.21-4.43, P = .011), while the PTPN11 A allele was associated with IM in HP- individuals (OR = 2.51, 95% CI: 1.16-5.40, P = .019), but showed an inverse association in HP+ subjects (OR = 0.46, 95% CI: 0.21-0.99, P = .048).

Conclusion: Polymorphisms in NQO1, IL-10 and PTPN11, in combination with HP status, could be used to identify individuals who are more likely to develop IM and therefore GC.
assessment of *Helicobacter pylori* (HP) infection can help to identify high risk individuals since this is a proven risk factor for GC[3,4]. Various genetic factors have also been associated with an increased risk for the development of GC [5-8]. These polymorphisms could be used in conjunction with HP status and together with dietary and environmental factors to target screening programs towards individuals deemed to be at high risk.

GC is thought to arise via a multi-step pathway that involves intestinal metaplasia (IM) as a precursor lesion[9]. It has been estimated that 0.25-1.1% of IM lesions will progress to GC annually, representing an 18-78-fold increased lifetime risk of developing this disease in comparison to the general population[10,11]. In the present study, we have investigated a panel of 18 polymorphisms in 14 candidate genes for their association with IM precursor lesions in a Singapore-Chinese population considered to be at increased risk of GC because of age greater than 50 years. These polymorphisms were chosen for study because previous research has shown them to be risk factors for GC. They included SNPs in genes involved in the immune response (*IL-1β, IL-10, PTPN11*) [12-14], folate metabolism (*FR-α, MTHFR*) [15,16], cell growth (*EGF, HER2*) [17-19], cell survival (*STCH*) [20], cell invasion (*MMP2*) [21] and DNA damage or repair (*NQO1, SULT1A1, TP53, ADPRT*) [22-26].

**Methods**

**Subjects**

Subjects were recruited from the Gastric Cancer Epidemiology and Molecular Genetics Program (GCEP). This project is a prospective cohort study aiming to enroll 4,000 Singapore-Chinese subjects aged more than 50 years from four major public hospitals in Singapore (National University Hospital, Tan Tock Seng Hospital, Singapore General Hospital, Changi General Hospital). It offers screening by endoscopy and systematic follow-up for a minimum of 5 years [27]. Chinese subjects older than 50 years of age who met the following criteria were eligible to enroll in the study: (i) symptoms of dyspepsia (ie. bloating, distension, nausea, stomach pain etc), (ii) family history of gastric cancer, or (iii) a medical condition that required them to undergo gastroscopy. They must also be able to attend all study visits assigned to them. Subjects who could not undergo gastroscopy, had a history of stomach cancer or surgery, had a disabling illness, or were unable to provide informed consent were ineligible for the study. Clinical information including demographics, medical history and family history were obtained. Informed consent was obtained from all subjects and the study was approved by the institutional review boards of all hospitals involved. Blood samples from 374 individual subjects collected between April 2004 and December 2006 were used for genotyping in the present study.

Three biopsies from the antrum, body and cardia were collected for histopathological examination during each endoscopic surveillance episode. IM was diagnosed from mucosal biopsies in three locations (antrum, body and cardia) for each subject and by consensus amongst three pathologists according to the updated Sydney System for the classification and grading of gastritis [28]. In cases where H. pylori was identified in biopsies, eradication therapy was administered according to standard clinical guidelines. For 339/374 (91%) individuals, the HP status was determined using the Helicoblot2.1 serology test (Genelabs Diagnostics, Singapore). In individuals where this test was not performed, the HP status was determined from histological examination of biopsies from the antrum, body and cardia, as well as from past medical history. Blood samples (8 mls) were collected into Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ) and the mononuclear cells isolated and stored at -80°C prior to DNA extraction using Tri-Reagent (MRC Inc, Cincinnati, OH).

**Helicoblot2.1 serology test**

This serological assay uses a Western Blot nitrocellulose strip containing electrophoretically separated proteins from a bacterial lysate of an ulcer-causing type strain of H. pylori and a recombinant antigen of H. pylori (Genelabs Diagnostics, Singapore). When incubated with diluted serum/plasma, specific antibodies to the various antigens, if present, will bind to the H. pylori antigens on the strip. These bound antibodies appear as dark bands upon reaction with goat anti-human IgG conjugated with alkaline phosphatase and a 5-bromo-4-chloro-2-indolyl-phosphate/nitroblue tetrazolium substrate solution. In order to identify the various bands present, the strip is compared with reference strips of non-reactive (negative) and reactive (positive) controls run concurrently. Determination of H. pylori seropositivity was based on criteria recommended by the kit manufacturer. They consist of (1), 116 kD (CagA) positive band present with one or more of the following bands: 89 kD (VacA), 37 kD, 35 kD, 30 kD (UreA) and 19.5 kD together, or with the current infection marker, (2) the presence of any one band at 89 kD (VacA), 37 kD or 35 kD, with or without current infection marker, or (3) the presence of both 30 kD and 19.5 kD with or without current infection marker.

**Selection of gene polymorphism panel**

A systematic literature search in PubMed was carried out using the terms "gastric cancer" and "polymorphism". From a total of 78 candidate polymorphisms identified, 18 were found to be significantly associated with the risk
of GC and were therefore included in the current investigation of IM.

**Genotyping**

Table 1 shows the PCR primers, annealing temperatures and product sizes for 17 SNPs investigated in this study by pyrosequencing. The 86-bp variable number of tandem repeats (VNTR) polymorphism in ILRN was genotyped using PCR followed by size analysis using gel electrophoresis. The primers and PCR conditions were the same as previously reported[29]. Polymorphisms were recorded in their most commonly used notation for easy cross-referencing. For PCR, 50 ng DNA was amplified in a 25 μl reaction containing 1 × FastStart Reaction Buffer, 2 mM magnesium chloride, 10 μM deoxynucleotide mix, 500 nM each of the forward and reverse primers and 1 unit FastStart Taq Polymerase (Roche Diagnostics, Mannheim, Germany). PCR cycling comprised of 4 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at the appropriate annealing temperature and 30 seconds at 72°C, before conclusion with 7 minutes at 72°C.

Pyrosequencing was performed by incubating the PCR products with 3 μl of streptavidin magnetic beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and 1× binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20) and mixing for 10 minutes at 37°C. The product mix was then denatured by 5 seconds incubation in 0.2 M NaOH solution and washed in annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate) for 10 seconds. The single-stranded products were transferred to an annealing buffer containing 15 pmol of the sequencing primer (Table 1) and incubated for 2 minutes at 80°C in a Hybrid Maxi 14 hybridization oven (Thermo Electron, USA). Pyrosequencing was then performed on a PSQ96MA pyrosequencer instrument (Biotage AB, Uppsala, Sweden). Samples that failed to give a genotype result after the first analysis were repeated up to two times.

| Locus          | Forward Primer | Reverse Primer | °C | bp | Sequencing Primer | Dispensation |
|----------------|----------------|----------------|----|----|-------------------|--------------|
| IL10 -- 1082 A/G | CTCATCAAAAGATCCCACC | AGGCTTGATAGGATGTCCT | 60 | 253 | ACCTCTAACAGGTCCTTCTTA | gagcagta     |
| IL10 -- 819 T/C | GAGCATAATAACGAAGGGC | AAGTTCTTCTGCTGGTGA | 60 | 207 | CTTCTTGATAGGATGTCCTTCA | gctgacctc   |
| IL-1B -- 511 T/C | CATAGAATTGCTAGGCC | GCCCTCCTCCTGCTGGTGA | 60 | 230 | AAACCTGACAGGAGGCTCT | atctgacga   |
| MMP2 -- 1306 T/C | AACTGACATCCAGGC | TGAAGGTTCTTCTGGAC | 60 | 265 | AACCCTTGCAGCATC | gctgacctc   |
| EGF +61 A/G     | GTCATGCCCTCTTCTTGGA | CAGAGCAAGGAAAGGCTCTTA | 60 | 266 | CCAATCAGGAAAGGCTCTTA | gagctgacg   |
| PTPN11 (int1) A/G | TGGAGAAGGCTGCAAGGC | TGGTCCTTCTAACGACTTGT | 60 | 182 | TCTGTCCTTAAAGGACTCTA |tgactgactc   |
| NQO1 C609T     | AACTGCGATGCCAAGG | TGGTCTCTCATCACAATAT | 60 | 191 | GCTGCTTCCAGTCTTTA | cactgctgca  |
| STCH rs2242661 | AACTGCAATCTGGACCT | GACTTTATAATACAA | 60 | 203 | GCCGAAAAGGAAAGGCTCTTGA | gagcagctc   |
| STCH rs1882881 | CTATGGAGGCTGGCAGGA | ACTCCAGCTACAGGCAA | 65 | 213 | CAGGCTTTTTCATCACTTG | gagctgacctc |
| STCH rs12479   | CTTGGAAGGCTGTTGTAAT | GCAAAAGGCTCTCGGATACCAAA | 60 | 312 | ATGTTTCAAGCACCACATT | gctggttggtc |
| STCH rs9982492 | TGGGTACCCCTTTTGA | AGTATGACCTCGCCATCTTAACTTTAA | 60 | 193 | CCATCTGCTCTTTACCTGATC | actcgtgtc   |
| SULT1A1 G638A  | GCAGATCGCCCTGCTGAGG | TGAGGAGCTGGTGATGTA | 65 | 233 | CTTGAGATTTGCTGGG | tgacgactc   |
| ADPRT T2285C   | GATAACCTAAGTGCGGGGC | ACAACGCTTCCAGGAGGCTCT | 65 | 262 | TGGTCTCCAGGCGGCA | cactgctgctc |
| HER2 +17ex17 A/G | GTCTCCCTCAACCACACACA | CTCACGCTGCTGGATGAGT | 65 | 145 | CCGCCCTGCTGCTGGTGA | cgtgctgtc   |
| TP53 C215G     | TCCAAGAGATGATGAGG | AAGCCCGAGAGGAAAAAGCTGAG | 60 | 230 | CAGAGCTGCTCCGCACTTGA | tgcagctgctc |
| FR-a A1314G    | AAGTTGGAAGACTGACGCCC | TGGCAGCCTGCTCCACACACCA | 60 | 183 | GCTGGGCTGCTCAAA | cgtgctgata   |
| MTHFR C677T    | ACTGTCATCCCTATTGGG | TCGTGTAGCCTTTCACA | 60 | 168 | GAGGTTGCTCGGGGA | cgtgctgata   |
The genotyping success rate varied from 85-99% for the 18 polymorphisms.

**Statistics**

Univariate analyses were carried out by Pearson’s chi-square or the Fisher’s exact test to examine for associations between genotype distributions, IM status and clinical factors. As there were more than one polymorphism investigated in IL10 and STCH, the haplotypes were also considered in the analyses. Variables found significantly associated with IM in the univariate analyses for all cases, and HP+ and HP- subgroups were entered in respective multivariate logistic regression models. The analyses were based on the assumption of a dominant genetic model. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL) software at the 5% significance level. The Woolf test was used to test for homogeneity of OR between two strata. As each polymorphism was tested for association with IM independently, it was not necessary to control for the family-wise error rate. Thus, no adjustment was made for multiple testing.

**Results**

The characteristics of 374 subjects evaluated in this study are shown in Table 2. A total of 128 were diagnosed with IM and 246 without IM. No significant differences between IM+ and IM- groups were apparent for sex, family history of GC (including 1st degree and 2nd degree relatives), alcohol consumption (at least one unit of wine, beer or liquor per week) or smoking status (at least one cigarette per day for a minimum of one year). IM+ subjects showed a significantly higher incidence of HP infection and were also older ($P < 0.05$).

Genotype frequencies for the 18 polymorphisms investigated for association with IM are presented in Table 3. All polymorphisms were in Hardy-Weinberg equilibrium ($P > 0.05$), with the exception of IL10 -819T/C, NQO1 609C/T and TP53 Arg72Pro. By univariate analysis, the NQO1 609 T allele was the only variant in the overall cohort that was significantly associated with IM (OR = 1.82, 95%CI: 1.05-3.15, $P = 0.032$). In HP- individuals, only the PTPN11 rs2301756 A allele was significantly associated with IM (OR = 2.51, 95%CI: 1.16-5.40, $P = 0.019$). Three polymorphisms in HP+ individuals were associated with IM in univariate analysis: the IL-10 819 C allele (OR = 2.32, 95%CI: 1.21-4.43, $P = 0.011$), NQO1 609 T allele (OR = 2.61, 95%CI: 1.18-5.80, $P = 0.018$) and PTPN11 A allele (OR = 0.46, 95%CI: 0.21-0.99, $P = 0.048$). The haplotypes in IL10 and STCH were not significantly associated with IM in overall cohort, HP-, as well as HP+ groups.

In multivariate analysis that included all cases, HP status and age were significantly associated with IM, while the NQO1 T allele showing borderline association (Table 4). In HP- individuals, the PTPN11 A allele was the only factor associated with IM. However, in HP+ individuals the factors of older age and the NQO1 609 T allele, IL-10 819 C allele and PTPN11 A allele were all significantly associated with IM. These results suggest that HP status is an effect modifier of the association between IM and the PTPN11 A allele ($P = 0.002$). As it is possible that IM+/HP- cases in this study had prior unrecorded HP infection[30], subgroup analysis on cases with a "revised HP+" status (either HP+/IM-, HP+/IM+ or HP-/IM+) was also performed. Age (OR = 2.10, 95%CI: 1.24-3.56, $P = 0.006$) and IL-10 -819 C allele (OR = 1.82, 95%CI: 1.07-3.08, $P = 0.027$) were the only significant variables in this subgroup.

**Discussion**

In this study, 18 polymorphisms that were previously linked to GC were investigated for possible associations with IM in a Singapore-Chinese population. The assumption was made that IM represents a precursor lesion for the development of GC and hence should have similar genetic risk factors. The cohort evaluated here was considered to be at elevated risk for GC because of the selection of individuals aged >50 years[27]. As expected, older individuals and those demonstrating seropositivity for HP showed a doubling in the frequency of IM (Table 4).

Following univariate analysis, 3 genotypes were found to be associated with IM. The NQO1 609 T allele was associated with IM, particularly in HP+ individuals. The IL-10 -819 C allele was also significantly associated with IM in HP+ cases. Interestingly, the PTPN11 A allele in intron 3 (rs2301756) was associated with increased incidence of IM in HP- individuals but a decreased incidence in HP+ cases. In multivariate analysis, all 3 polymorphisms remained significantly associated with IM, with the excep-

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**Table 2: Characteristics of study subjects in relation to the presence of IM.**

| Characteristic | Total (%) | IM+ (%) | IM- (%) |
|---------------|-----------|---------|---------|
| Subjects      | 374       | 128     | 246     |
| Mean age ± SD (range) | 60.5 ± 7.8 | 62.9 ± 7.8 | 59.2 ± 7.5 |
| Age 50-59 yrs | 190 (51)  | 48 (38)* | 142 (58)* |
| Age 60-69 yrs | 133 (36)  | 55 (43)  | 78 (32)  |
| Age ≥70 yrs   | 51 (13)   | 25 (19)  | 26 (10)  |
| Male          | 207 (55)  | 72 (56)  | 135 (55) |
| Family history of GC | 66 (18)     | 23 (18)  | 43 (17)  |
| HP infection  | 191 (51)  | 84 (66)* | 107 (43)* |
| Drinker       | 66 (18)   | 22 (17)  | 44 (19)  |
| Smoker        | 90 (24)   | 30 (23)  | 60 (24)  |
| Chronic gastritis | 290 (78)   | 115 (90) | 175 (71) |
| Atrophy gastritis | 194 (52)   | 97 (76)  | 97 (39)  |

* $P < 0.05$
Table 3: Distribution of genotype frequencies according to IM and HP infection status

| Gene polymorphism (rs number) | Genotype | IM- | IM+ | HP- | HP+ |
|------------------------------|----------|-----|-----|-----|-----|
| ADPRT Val762Ala (rs1136410) | TT       | 71  | 31  | 33  | 9   |
|                              | TC       | 117 | 60  | 64  | 24  |
|                              | CC       | 33  | 16  | 13  | 6   |
| EGF +61A/G (rs4444903)       | AA       | 22  | 5   | 13  | 1   |
|                              | AG       | 103 | 55  | 54  | 20  |
|                              | GG       | 110 | 58  | 50  | 18  |
| FR-α 1314A/G (none)          | GG       | 164 | 95  | 74  | 30  |
|                              | GA       | 74  | 31  | 43  | 12  |
|                              | AA       | 6   | 1   | 5   | 0   |
| HER2 Ile/Val (rs1801200)     | AA       | 174 | 92  | 87  | 32  |
|                              | AG       | 60  | 30  | 29  | 8   |
|                              | GG       | 1   | 1   | 1   | 0   |
| IL1RN 86-bp VNTR (none)      | 44       | 212 | 101 | 112 | 33  |
|                              | 24       | 28  | 18  | 9   | 4   |
|                              | 34       | 1   | 2   | 0   | 2   |
|                              | 54       | 0   | 1   | 0   | 0   |
|                              | 22       | 2   | 2   | 0   | 1   |
| IL-1β -511C/T (rs16944)      | CC       | 64  | 35  | 33  | 10  |
|                              | CT       | 119 | 62  | 63  | 21  |
|                              | TT       | 48  | 23  | 20  | 10  |
| IL-10 -819T/C (rs1800871)    | TT       | 131 | 55  | 57  | 21  |
|                              | TC       | 78  | 46  | 39  | 15  |
|                              | CC       | 22  | 16  | 17  | 3   |
| IL-10 -1082A/G (rs1800896)   | AA       | 207 | 100 | 98  | 37  |
|                              | AG       | 21  | 14  | 13  | 3   |
|                              | GG       | 2   | 0   | 2   | 0   |
| MMP2 -1306C/T (rs243865)     | CC       | 178 | 79  | 85  | 28  |
|                              | CT       | 46  | 22  | 26  | 8   |
|                              | TT       | 3   | 2   | 2   | 0   |
| MTHFR 667C/T (rs1801133)     | CC       | 132 | 77  | 64  | 23  |
|                              | CT       | 98  | 42  | 50  | 16  |
|                              | TT       | 14  | 7   | 8   | 2   |
| NQO1 609C/T (rs1800566)      | CC       | 64  | 21  | 27  | 10  |
|                              | CT       | 143 | 80  | 78  | 25  |
|                              | TT       | 28  | 22  | 13  | 4   |
The NQO1 609 T allele which was associated with borderline significance in the overall cohort (P = 0.056).

Previous data lends support to our observations. NQO1 (NAD(P)H: quinine oxidoreductase 1) codes for a cytosolic enzyme that protects cells from oxidative damage by preventing the generation of semiquinone free radicals and reactive oxygen species[31]. The C to T substitution at nucleotide 609 in exon 6 results in a change of amino acid from Pro to Ser at codon 187[32]. Whereas the CC homozygous wildtype genotype (Pro/Pro) has full enzymatic activity, the TT genotype (Ser/Ser) completely lacks activity. The NQO1 609 TT genotype has been associated with an increased risk for various tumour types including gastrointestinal and urological cancers[33-36]. An increased risk of GC in patients with a family history of upper gastrointestinal cancers was also reported for the NQO1 609 TT genotype in a study on Chinese subjects[22]. Our observation of increased prevalence of IM in carriers of the NQO1 609 T allele concurs with earlier reports on its association with various cancers and can be explained by a decreased activity for the detoxification of environmental and dietary carcinogens.
The NQO1 C609T polymorphism was previously associated with seropositivity to HP in a Japanese study[37], thus raising the possibility that it is an indirect risk factor for IM via association with HP infection. However, we found no association between the NQO1 C609T polymorphism and HP infection in the present cohort (results not shown).

Carriers of the IL-10 -819 C allele express higher mucosal levels of IL-10 (interleukin 10) mRNA and experience colonization with more virulent HP strains[38]. Similar to NQO1 C609T, no association was observed here between the IL-10 T-819C polymorphism and HP infection. The current result showing the IL-10 -819 C allele is associated with IM is at odds with an Italian study that reported the TT genotype was associated with increased risk of IM[29]. However, two studies in Chinese and German populations found no associations between IL-10 T-819C and IM[38,39].

Other common polymorphisms in the IL-1β and TNF-α cytokine genes have been proposed to influence the host response to HP and therefore the risk of developing GC[13,29,38-42]. The IL-1β C-511T and IL-10 A-1082G polymorphisms were investigated in this cohort, but no significant associations were found with seropositivity to HP or with the presence of IM (Table 3). Previous studies reported the IL-1β -511 T allele increased the risk of IM in some[38,39], but not all populations[12]. One study found an association between the IL-10 A-1082G polymorphism and IM[12,43], but 3 other studies did not[12,29,39].

PTPN11 (protein tyrosine phosphatase, non-receptor type 11) encodes for SHP-2, a protein tyrosine phosphatase thought to play a key role in intracellular signaling elicited by growth factors and cytokines[44]. Interactions between the HP cagA protein and SHP-2 in gastric epithelial cells are believed to contribute to the development of GC[45]. The PTPN11 AA genotype was associated with reduced risk of gastric atrophy in a Japanese population of HP seropositive individuals[14,30]. In those studies, the assessment of gastric atrophy was done with serology test (pepsinogen levels). The present results on IM in HP seropositive Singapore-Chinese support these earlier observations, although the number of AA genotype individuals (n = 6) did not allow separate evaluation of this group. The diagnosis of IM was based on histology examination. The PTPN11 intron 3 G/A SNP may be in linkage disequilibrium with a coding marker that influences the interaction of SHP-2 with cagA and subsequent downstream signaling. However, its association with increased frequency of IM in HP negative individuals suggests it may play a role independently of this factor.

Conclusion
In summary, we found 3 polymorphisms associated with IM in a Singapore-Chinese population that was at high risk for GC because of older age and seropositivity for HP. The value of these SNPs in facilitating more cost-effective surveillance programs awaits further validation in large, independent cohorts.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
FZ participated in the design of the study and its coordination, performed the statistical analysis and drafted the manuscript. ML performed the literature review/statistical analysis and drafted the manuscript. JH, KWL, MST and KGY provided clinical and biological insights for the study. SL and KXK carried out the genotyping of the samples. BI drafted the manuscript. RS participated in its design and coordination, supervised the study and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This work was funded in part by the National Medical Research Council of Singapore (NMRC/TCR001-NUS/2007), Biomedical Research Council of Singapore (BMRC 04/11/21/19/312) and the Singapore Cancer Syndicate (SCS#BUS1, SCS#GN015). The authors would like to thank contributors from the Singapore Gastric Cancer Consortium that include Khek Yu HO, Yoshiaki ITO, Christopher JL KHOW, Andrea RAJNAKOVA, Kwong Ming FOCK, Choon Jin OOI, Chung King CHIA, Wee Chian LIM, Wei Keong WONG, Andrew WONG, Ming TEO, Nilesh SHAH, Robert HEWITT, Bow HO, Kee Seng CHIA, Yoon Pin LIM, Jimmy JB SO, Lynette PHAY.

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Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-230X/9/76/prepub