Gastric marginal zone lymphoma is associated with polymorphisms in genes involved in inflammatory response and antioxidative capacity

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Gastric marginal zone lymphoma (GMZL) is strongly associated with *Helicobacter pylori* infection, which induces a chronic inflammatory response. Inflammation can result in DNA damage related to its severity, the cellular antioxidant capacity, and the integrity of DNA repair mechanisms. *Interleukin-1* (*IL-1*) polymorphisms have been shown to be important mediators of inflammation, while *glutathione S-transferase GST T1* and *GST M1* polymorphisms are believed to affect cellular antioxidant capacity. We aimed to determine whether polymorphisms at the *IL-1* and *GST T1* and *GST M1* loci modulate the risk of developing GMZL. Blood and biopsy samples were obtained for a historical series of 66 GMZL cases, whereas blood samples were available from 163 healthy controls. Genotypes were obtained for *GST T1*, *GST M1*, *IL-1 RN*, and *IL-1B-31* using PCR-based techniques. *H pylori* infection was found in 86.0% of cases, whereas in the control population only 37.4% tested positive. The *IL-1 RN 2/2* genotype was significantly associated with risk of GMZL (odds ratio [OR], 5.51; 95% confidence interval [CI] 2.16-14.07), but not the IL-1B-31 genotype. Likewise, the *GST T1 null* genotype was strongly associated with risk of GMZL (OR, 9.51; 95% CI 4.57-19.81), but not the *GST M1* genotype. Evidence was found of effect modification between the *IL-1 RN* and *GST T1* genotypes ($P = .02$). The combination of the *IL-1 RN 2/2* and *GST T1 null* genotype was most strongly associated with risk of GMZL (OR, 32.29; 95% CI 6.92-150-63). These results support the hypothesis that the risk of developing GMZL is influenced by inter-individual variation in the cellular inflammatory immune responses to *H pylori* infection, and to antioxidative capacity. (Blood. 2003;102:1007-1011)

Introduction

The association of gastric marginal zone lymphoma (GMZL) with prior infection by *Helicobacter pylori*[1] has been reported in both epidemiologic[2] and pathologic studies.[3] The onset of GMZL is preceded by the development of mucosa-associated lymphoid tissue (MALT), acquired as a result of *H pylori* infection, which may undergo multistep progression through monoclonal proliferation to malignant lymphoma.[3] Transition to GMZL is associated with an increasing level of DNA damage within the infiltrating lymphoid tissue,[4] a putative cause of which is oxidative stress generated as a consequence of *H pylori*–induced inflammation. Reactive oxygen species (ROS) are important mediators of this effect, and a direct correlation between the level of ROS and the level of DNA damage to the gastric mucosa has been reported.[5,6] The severity of DNA damage occurring in an infected individual will depend on a number of factors, including the strain and virulence of *H pylori*, the severity of the inflammatory response, and the individual's ability to detoxify ROS. Therefore, *H pylori*–associated GMZL provides an ideal system in which to study the effects of genetic variation on important modulators of the immune response and on mediators of oxidative stress.

The immune response resulting from *H pylori* infection is primarily via Th1, a proinflammatory response, mediated by a cascade of cytokines including interleukin 18 (IL-18), IL-12, IL-1, tumor necrosis factor–α and interferon-γ. These cytokines regulate their own levels of expression by autocrine effects, but they also influence downstream cytokine expression, thereby amplifying the inflammatory response. IL-1 is one of the principal proinflammatory cytokines that mediate the Th1 immune response following *H pylori* infection, with IL-1β production being up-regulated in the presence of *H pylori*.[7,8] IL-1β is also one of the most powerful inhibitors of gastric acid secretion, the hypochlorhydria associated with high-producer variants of IL-1 governing the extent of *H pylori* infection and distribution of gastritis. As a consequence, polymorphic variants in IL-1 may affect not only the primary inflammatory response but also the level of response following amplification.

The *IL-1* gene cluster is situated on chromosome 2q and comprises 3 related genes within a 430-kb region: *IL-1A*, *IL-1B,* and *IL-1 RN,* which encode the proinflammatory cytokines IL-1α and IL-1β and their endogenous receptor antagonist IL-1ra, respectively.[9] Three biallelic polymorphisms in *IL-1B* have been described, all *C>T* base transitions found at positions −511, −31, and +3954 bp from the transcriptional start site.[10,11] Strong linkage disequilibrium between the *IL-1B-31 C* and *IL-1B-511 T* variants has been reported, with the *IL-1B-31*, *IL-1B-511 T* C haplotype occurring at a frequency of 0.38 in the white population.[10,11] The *IL-1B-31* polymorphism is situated within a TATA sequence of the *IL-1B* promoter, and has been shown to markedly affect DNA-protein interaction in vitro.[10,11] The homozygous genotype *IL-1B-31 TT*

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has been associated with increased IL-1β production,[12] but this finding remains unconfirmed.[13]

For *IL-1 RN* a penta-allelic 86-bp variable number tandem repeat (VNTR) in intron 2 has been described, with the most common allele, *IL-1 RN*1,* corresponding to 4 repeats. The *IL-1 RN*2* allele, corresponding to 2 repeats, has been reported at a frequency of 0.12 in the white population and has been associated with chronic inflammatory conditions such as ulcerative colitis and autoimmune conditions such as Sjögren syndrome.[14,15] The *IL-1 RN*2* allele has been associated with enhanced IL-1β production in vitro[16]; however, data regarding its effects on IL-1ra production are contradictory.[17-19] It has been reported that *IL-1B* and *IL-1 RN* alleles are inherited as an extended haplotype, associated with inflammatory diseases.[20,21]

Major cellular defense mechanisms against DNA damage are the glutathione S-transferase (GST) enzymes. In addition to their role in the detoxification of potential carcinogens by catalyzing conjugation to glutathione, these enzymes also have a strong antioxidant function, neutralizing free radicals. There are 4 cytosolic GST families, including GST T1 and GST M1. Independent gene deletions exist at both the *GST T1* and *GST M1* loci, resulting in a lack of active protein,[22] and the null genotypes of both loci have been associated with several malignancies.[23-27] We hypothesize that lack of GST T1 or GST M1 activity may be associated with an increased risk of developing GMZL in *H pylori*–infected individuals, and that this risk may be enhanced by interaction with the proinflammatory genotypes of *IL-1*.

Materials and methods

Samples

Sixty-six cases of GMZL (aged 38-86 years) were identified from multiple sources throughout the Yorkshire region in the north of England. Archived samples, comprising paraffin blocks of biopsies, surgical specimens, and peripheral blood smears, were obtained, as were available. Pure populations of normal cells were extracted where possible from biopsy and surgical blocks, the presence of tumor cell contamination monitored by comparison with sections stained with hematoxylin and eosin. Normal cells were present in all samples extracted, although the presence of a small amount of tumor material in some samples cannot be excluded. All cases were histologically validated according to the criteria of the Revised European-American Classification of Lymphoid Neoplasms (REAL) classification,[28] using the standard panel of markers utilized by the Hematological Malignancy Diagnostic Service at Leeds General Infirmary (CD20, 79, 10, 5, 23, 3, Ki67, and BCL-2). A reference series of 163 healthy population controls (aged 28-65 years) was collected as part of an ongoing lymphoma study carried out by the Leukemia Research Fund (LRF) Epidemiology and Genetics Unit; each control provided a blood sample. Ethical approval to establish a DNA repository for cases of hematologic malignancy and to carry out a case-control comparison of genotypes was obtained from the North and Yorkshire Multi-Centre Research Ethics Committee (MREC).

Determination of *H pylori* status

H pylori status of the reference population was determined using a commercial enzyme-linked immunosorbent assay (ELISA) system (Sigma Aldrich, Poole, United Kingdom) on frozen plasma. The *H pylori* status of the cases was determined using the above ELISA test where plasma samples were available, in conjunction with microscopic inspection of gastric biopsy sections stained with modified Giemsa by an expert pathologist to determine the presence or absence of the *H pylori* bacterium. Of the cases for which it was possible to determine *H pylori* status, 91% were determined by inspection of stained sections and 9% by ELISA. An overlap occurred for 1 case, with identical results determined by the 2 techniques.

Genotyping

Genomic DNA was extracted using the method of Jackson et al.[29] In order to check the integrity of the DNA, all samples extracted from paraffin blocks were amplified using a primer set expected to amplify a product of 600 bp, ensuring that genotypic status was not misclassified owing to the absence of a polymerase chain reaction (PCR) product resulting from poor-quality DNA. *IL-1B-31* genotypes were determined using TaqMan allelic discrimination PCR (Applied Biosystems, Foster City, CA).[10,11] *IL-1 RN* alleles were determined using PCR product sizing,[10,11] whereby products were sized relative to a 100-bp DNA ladder and were coded as follows: allele 1 = 4 repeats (442 bp), allele 2 = 2 repeats (270 bp), allele 3 = 5 repeats (528 bp), allele 4 = 3 repeats (356 bp), allele 5 = 6 repeats (614 bp). Because of their rarity, the 3, 4, and 5 alleles were grouped for statistical analysis. *GST T1* and *GST M1* genotypes were determined using PCR as previously described,[27,30] with the modification that an annealing temperature of 56°C was used. These assays do not allow for discrimination between carriers of 1 and 2 intact alleles. Therefore, heterozygous and homozygous wild-type individuals were grouped for analysis.

Statistical analysis

Within the case series and within the population-based control series, associations between age and *H pylori* status were tested using a nonparametric test for trend.[31] Associations between sex, genotype, and *H pylori* status were tested with the Pearson χ^2 test, as were associations between age, sex, *H pylori* status, and polymorphism distribution. Hardy-Weinberg equilibrium was assessed for *IL-1 RN* and *IL-IB-31* within the case and control series. Unconditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) associated with risk of GMZL, adjusted for age and sex. Statistical interactions were tested using the likelihood ratio test, by comparing the model with 2 genotype effects included as independent factors against the model where both independent effects plus an interaction term were included.[32] All analyses were conducted using Stata version 7 (Stata, College Station, TX).

Results

The median age at diagnosis of the GMZL case series was 65 years (range, 38-86 years), with 27 (40.9%) being male. The median age of the reference population was 60 years (range, 28-65 years) with 99 (60.7%) being male. *H pylori* status was determined for 50 (75.8%) of the 66 GMZL cases, with 43 (86.0%) of the 50 cases confirmed as being positive for the presence of *H pylori*. *H pylori* status was determined for all individuals in the reference population, with 61 individuals (37.4%) being *H pylori* positive. Among controls, positive *H pylori* status was significantly associated with increasing age ($P < .01$), but there was no significant difference in *H pylori* distribution between the sexes.

Distribution of *IL-1* genotypes

The distribution of *IL-1B-31* was not significantly associated with age or sex in either the case series or the reference population (data not shown). Allele distributions at *IL-1B-31* were in Hardy-Weinberg equilibrium for both the cases and reference samples (cases, $P = .17$; controls, $P = .06$). The frequency of the *IL-1B-31* homozygous genotype (*CC*) in the reference population was 14.2%, in accordance with previously published data.[10,11] Although there was an increased incidence of the *IL-1B-31 CC* genotype in the *H pylori*–negative controls (16.8%) compared with the *H pylori*–positive controls (9.8%), there was no statistically significant difference in *IL-1B-31* genotype distribution between

Table 1. Comparison of *IL-1B-31*, *IL-1 RN*, *GST T1*, and *GST M1* gene frequencies in the GMZL case series and the reference population

	Cases (n = 66)		Reference population (n = 163)			
Allele	No.	%	No.	%	OR*	95% CI
IL-1B-31						
Wt (TT)	29	46.0	74	45.7	1.00	—
Het (CT)	22	34.9	65	40.1	0.76	0.38-1.54
Hom (CC)	12	19.0	23	14.2	1.13	0.47-2.73
Not known	3	—	1	—	—	—
IL-1 RN						
1/1	21	35.6	74	45.7	1.00	—
1/2	13	22.0	66	40.7	0.66	0.29-1.53
1/345	5	8.5	9	5.6	1.87	0.48-7.31
2/2	20	33.9	13	8.0	5.51	2.16-14.07
Not known	7	—	1	—	—	—
GST T1						
H/H	28	42.4	141	86.5	1.00	—
Null	38	57.6	22	13.5	9.51	4.57-19.81
GST M1						
H/H	25	37.9	74	45.4	1.00	—
Null	41	62.1	89	54.6	1.10	0.59-2.07

H/H indicates heterozygous/homozygous wild-type; —, genotype not found.
*Adjusted for age and sex.

these 2 groups ($P = .24$). The *IL-1B-31 CC* genotype was observed in 19.0% of the GMZL cases, compared with 14.2% of the controls, with no associated risk of GMZL suggested (OR, 1.13; 95% CI, 0.47-2.73; Table 1). The *IL-1B-31 CT* genotype was observed in 34.9% of the GMZL cases, compared with 40.1% of the controls, also with no associated risk of GMZL suggested (OR, 0.76; 95% CI, 0.38-1.54).

The distribution of *IL-1 RN* was not significantly associated with age or sex in either the case series or the reference population (data not shown). Allele distributions for *IL-1 RN* were in Hardy-Weinberg equilibrium for the controls ($P = .08$), while the cases were strongly out of equilibrium ($P = .0001$). The frequency of the *IL-1 RN 2/2* genotype was 8.0% (Table 1), in accordance with previously published data.[10,11] The *IL-1 RN 2/2* genotype was present at a similar frequency in *H pylori*–negative controls (7.9%) and *H pylori*–positive controls (8.2%). The *IL-1 RN 2/2* genotype was observed in 33.9% of cases, compared with 8.0% in the control reference population, showing an association with an increased risk of GMZL (OR, 5.51; 95% CI, 2.16-14.07; Table 1). No associations were seen with GMZL risk for either the *1/2* genotype (OR, 0.66; 95% CI, 0.29-1.53) or the *1/3,4,5* genotype (OR, 1.87; 95% CI, 0.48-7.31). No evidence was found for statistical interaction between the *IL-IB-31* and *IL-1 RN* genotypes ($P = .25$).

Distribution of *GST* genotypes

The frequency of the *GST T1 null* genotype in the reference population was 13.5% (Table 1), which was comparable to previously published data.[33] The distribution of *GST T1* was not significantly associated with age or sex in either the case series or the reference population (data not shown). The *GST T1 null* genotype was present in 16.7% of the *H pylori*–negative and 8.2% of the *H pylori*–positive controls; there was no statistically significant difference in *GST T1* genotype distribution between these 2 groups ($P = .13$). The *GST T1* genotype was significantly associated with risk of GMZL: 57.6% of GMZL cases had the *GST T1* null genotype, compared with 13.5% of the reference population (OR, 9.51; 95% CI, 4.57-19.81).

The frequency of the *GST M1 null* genotype in the reference population was 54.6% (Table 1), comparable to previously published data.[33] The distribution of *GST M1* was not significantly associated with age or sex in either the case series or in the reference population (data not shown). The *GST M1 null* genotype was present in 57.8% of the *H pylori*–negative controls and 49.2% of the *H pylori*–positive controls ($P = .28$). The distribution of the *GST M1 null* genotype between cases (62.1%) and the reference population (54.6%) was similar (OR, 1.10; 95% CI, 0.59-2.07), suggesting no association with GMZL risk (Table 1). No evidence was found for statistical interaction between the *GST T1* and *GST M1* genotypes ($P = .75$).

Combination of *GST T1* and *IL-1 RN* genotypes

IL-1 RN and *GST T1* were both found to have significant associations with risk when they were analyzed together using a multiple variable logistic regression model. Evidence was found for a statistical interaction between *IL-I RN* and *GST T1* genotypes ($P = .02$), suggesting that the effect of *IL-1 RN* may be modified depending on the *GST T1* genotype and vice versa. Odds ratios, adjusted for age and sex, were calculated for each combination of the 2 genotypes (Table 1), using *GST T1 H/H* and *IL-1 RN 1/1* as the baseline genotype in the comparison. The estimated effect of *IL-1 RN 1/2* was nonsignificant in combination with the *GST T1 H/H* genotype (OR, 0.50; 95% CI, 0.18-1.36), but significant in combination with the *GST T1 null* genotype (OR, 4.82; 95% CI, 1.08-21.41). In combination with the *GST T1 H/H* genotype, no significant effect was found for the *IL-1 RN 2/2* genotype (OR, 0.90; 95% CI, 0.17-4.78), whereas in combination with the *GST T1 null* genotype, the *IL-1 RN 2/2* genotype was associated with a significant risk (OR, 32.29; 95% CI, 6.92-150.63). These results suggest that the risk associated with the *IL-1 RN 2* allele may be dependent on the additional presence of the *GST T1 null* genotype.

Discussion

Our results suggest that the development of GMZL is modulated by genetic variants at both the *IL-1* and *GST* loci. It is proposed that the genetic variants analyzed modulate the severity of the immune response to *H pylori* and determine the body's antioxidative capacity (Figure 1).

Two previous studies investigating the role of *IL-1 RN* and *IL-1B* genotypes in susceptibility to gastric cancer have been

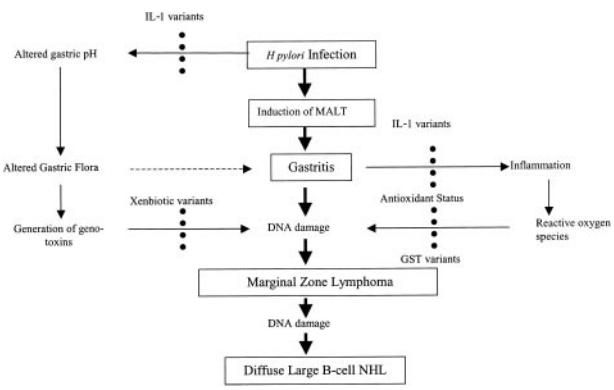

Figure 1. Model to assess genetic modification of risk for *H pylori*–induced GMZL. Black circles indicate modulated by; dashed arrows, possible link; solid arrows, established link; and bold arrows, disease progression.

carried out.[10,11,34] Both reported an increased associated risk of gastric carcinoma with inheritance of the *IL-1B-31* (*CC*)*/IL-1 RN 2/2* genotype. The authors suggest that the *IL-1B-31 C* allele in the context of *H pylori* infection is associated with a proinflammatory phenotype and therefore an increased risk of DNA damage and ultimately gastric carcinoma.[10,11,34] However, reports on the functional effects of the *IL-1B-31* polymorphism are conflicting,[12,13] and functional effects at cytokine loci are often governed by extensive haplotypes.

Our data suggest a significant association between GMZL and inheritance of the *IL-1 RN 2/2* genotype, consistent with that previously observed in gastric carcinoma. In contrast, we found no associated risk with *IL-1B-31* genotype. An explanation for this may be that changes in IL-1 proinflammatory responses associated with *IL-1B-31* may be mediated via an interaction with *IL-1 RN*; however, we found no evidence for this in our data.

IL-1 RN encodes the IL-1 receptor antagonist IL-1ra, and the *IL-1 RN*2* allele has been associated with decreased IL-1ra levels[17,19] and consequently increased IL-1β levels. We postulate that the association between the *IL-1 RN *2* allele and GMZL functions via a mechanism similar to that seen in gastric carcinoma. The high IL-1β levels associated with the *IL-1 RN *2* allele favor the proinflammatory response; at the same time, the concomitant inhibition of gastric acid facilitates widespread *H pylori* colonization of the gastric mucosa, promoting the development of MALT in response to infection. This decreased gastric acid secretion may also heighten DNA damage by permitting superinfection by enteric bacteria that enhance the production of carcinogenic N-nitroso compounds.[35] *H pylori*–induced hypochlorhydria also significantly decreases levels of vitamin C in gastric juice,[36] further facilitating the formation of N-nitroso compounds.

We describe a significant association between the *null* genotype of *GST T1* and increased risk of GMZL. No effect was observed for the *GST M1 null* genotype. Associations between the *GST T1 null* genotype and increased risks of astrocytoma, meningioma, myelodysplasia,[37] and acute myeloid leukemia[25] have been reported. *GST T1* has a strong antioxidant function in neutralizing free radicals. We propose that in GMZL, the associated risk seen with the *GST T1 null* genotype is due to compromised antioxidant capacity and not due to the metabolism of a specific xenobiotic substrate. Combinations of genetic variants that increase IL-1β levels, promoting the immune response to *H pylori* infection and the production of ROS, plus variants that decrease the levels of antioxidants would likely increase the risk of GMZL further (Figure 1). We found evidence of modulation of effect between the *IL-1 RN* and *GST T1* genotypes and GMZL risk, showing that both play important roles in the pathway of disease development.

H pylori has been shown to be an infection strongly linked to the development of GMZL. Thus, in our hypothesis the effects of *IL-1* variants would be through their modulation of the immune response to *H pylori*, and the effects of *GST* variants would be through the antioxidative capacity they provide, enabling the body to detoxify ROS produced in this response. Among cases for which *H pylori* status could be determined, 86.0% were positive for the presence of the bacterium; however, the frequency of both the *GST T1 null* and *IL 1 RN 2/2* genotypes was similar in cases determined to be *H pylori*–positive and those with unknown or negative status. However, the absence of *H pylori* in the biopsy sections examined does not preclude the presence of *H pylori* elsewhere in the gastric mucosa. The demonstrated presence of *H pylori* in the majority of cases strongly supports the link between the presence of *H pylori* and development of GMZL in our case series. Within the reference population, no associations were found between the *IL-1-31*, *IL-1 RN*, *GST T1,* or *GST M1* genotypes and *H pylori* status, providing no evidence that these genes are associated with the development of *H pylori* infection. The whole reference population serves well as a group with which to make comparisons of genotype frequencies in the cases, to determine which genotypes may modulate the development of GMZL subsequent to *H pylori* infection.

A small proportion of GMZL may not necessarily be associated with *H pylori*, for example in patients with autoimmune disease.[38] Ideally, we would have been able to classify cases as to whether or not they were associated with *H pylori* and evaluate the genotype effects within the 2 subgroups. From the archived samples it could not be definitively demonstrated that *H pylori* had not been involved. *H pylori* serology and/or presence in tissue may change with increasing pathologic progression; thus, *H pylori* may not always be evident in samples of more advanced disease. Analysis restricted to cases and controls where *H pylori* status had been demonstrated estimated effects for *GST T1* and *IL-1 RN* consistent with the full analysis. The *IL-1 RN 2/2* genotype was seen in 30.0% of the cases, compared with 8.2% in the controls (OR, 3.93; 95% CI, 1.08-14.33). The *GST T1* genotype was seen in 58.1% of the cases, compared with 8.2% of the controls (OR, 15.01; 95% CI, 4.67-48.23). Making this restriction substantially reduced the number of cases and controls, and the precision of the estimates was reduced, particularly for the more rare variants. Differential effects of the *IL-1 RN 2/2* genotype in combination with *GST T1* were not observed in this restricted analysis, as had been observed in the full analysis; however, this may have been because of limited statistical power due to small numbers. No evidence was found for a differential frequency of the *GST T1 null* and *IL-1 RN 2/2* genotypes between cases determined to be *H pylori*–positive and those with unknown or negative status.

We were unable to obtain samples from population controls of comparable ages for all the cases. Population controls aged 28 to 65 years were used as a reference population for cases aged 38 to 86 years. In a study of metabolic gene polymorphism frequencies in control populations,[33] it has been shown that the allele frequencies of metabolic genes including *GST M1* and *GST T1* do not differ significantly by age. Within our control series, age was not significantly associated with the allele frequencies of any of the genotypes used in our analyses. This would indicate that the controls could act as an appropriate reference group for the older cases. Adjusting for age and sex in our analyses of association between genotype and risk of GMZL did not alter the estimates to any substantial degree. Similarly, repeating the analyses using data restricted to cases and controls of comparable ages did not produce estimates substantially different from the estimates based on the full data (data not shown). This confirmed that the age differences between the case series and the population controls were not leading to bias in our estimates.

The limited statistical power of small studies to detect associations between polymorphic genotypes and disease predisposition is particularly important with regard to effect modification. To achieve adequate statistical power to estimate individual gene effects with good precision and further describe interaction between genes, large sample sizes may be required.[39] This study, although small, suggests associations between genotypes and susceptibility to GMZL, which would be important to investigate further in larger studies.

This study provides support for the hypothesis that the risk of developing GMZL is influenced by interindividual variation in inflammatory response and antioxidative capacity, particularly

through combination of the effects of the *IL-1 RN* genotype and the *GST T1* genotype. *H pylori* infection, the anticipated exposure, was confirmed in the majority of the cases, and it is proposed that the genetic variants analyzed modulate the effects of this exposure.

The model we describe is common to the development of gastric carcinoma, and it would be valuable to determine whether there are also parallels, in genetically determined risk, with the development of other MALT lymphomas, such as the marginal zone lymphomas occurring at the site of inflammation in Hashimoto thyroiditis and Sjögren disease.[40] Excesses in risk of the non-Hodgkin lymphomas have been described for patients with various conditions involving substantial immune dysfunction, particularly conditions where chronic antigenic stimulation is present.[41] The consideration of polymorphisms involved in the immune response, in combination with those involved with the prevention of DNA damage (and further, those involved in DNA repair), could allow the mechanisms underlying these associations to be explored further.

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