Abstract

Objectives: To characterise the subtypes of HLA-B27 in disease associated patients in the North Island of New Zealand.

Methods: The subjects were 194 patients from the North Island of New Zealand. These had previously been positively tissue-typed for HLA-B27 owing to its associations with spondyloarthopathies and anterior uveitis. The subjects gave informed consent to further testing of their samples, which were HLA-B27 subtyped using DNA sequence based typing. Statistical analysis of the data was performed using the chi square test with Yates' correction and Pearson's chi square test.

Results: Nine different subtypes were described in this study. HLA-B*2701, B*2702, B*2704, B*2705, B*2706, B*2707, B*2709, B*2710 and B*2715. Within the study the prevalence of B*2704 was significant in Asians (p<0.001) and the prevalence of B*2705 was significant in Europeans (p<0.001). Of the three major subtypes identified (B*2702, B*2704 and B*2705), none showed a statistical association to disease category. HLA-B27 homozygosity was not significantly different to the healthy population (p=0.918).

Conclusions: HLA-B27 subtypes were analysed in disease associated patients of the New Zealand North Island population. The subtypes found reflected the HLA-B27 subtypes found in previous studies of the different ethnicities involved.

Key words: HLA-B27, subtypes, disease association, spondyloarthopathies, uveitis.

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Introduction

The class I human leukocyte antigen (HLA) system is part of the adaptive immune response and is involved in presentation of peptides to cytotoxic T lymphocytes (CTL)(1). HLA antigens are encoded for by the major histocompatibility complex (MHC) region of genes on the short arm of chromosome 6 (1). HLA-B27 is a serological specificity that represents a family of 53 allelic subtypes (2). The spondyloarthopathies (SpA) comprise a family of chronic inflammatory diseases affecting the spine, joints, eyes and skin. HLA-B27 is strongly associated with ankylosing spondylitis (AS), related SpA and anterior uveitis (AU) (3-5). The New Zealand Tissue Typing Laboratory currently ascertains the presence or absence of HLA-B27 in referred samples by DNA polymerase chain reaction (PCR) with sequence specific primers (SSP) technique, using the AllSet™ Gold SSP HLA-B27 Low Res Kit (Invitrogen™ Carlsbad, CA, USA). This technique does not specify the allelic subtype of HLA-B27 in any given sample. Epidemiological studies have implicated B*2701 to B*2704, B*2713, B*2714, B*2715 and B*2724 with AS (6-26). In certain populations however, B*2706, B*2707, B*2708 and B*2709 appear to have a protective role (14,16,19,21,24,26-29). B*2705, and B*2704 to a lesser degree, have been shown to be associated with AU (30,31). There is little information on disease association with the other HLA-B27 subtypes.

The advent of DNA typing to the sequence level and the discovery that some HLA-B27 subtypes confer protection against SpA (14,16,19,21,24,26-29) provides the rationale for research in this area. An understanding of HLA-B27 subtypes positively and negatively associated with SpA may give insights into the pathogenesis of these disorders and could pave the way to future treatments. The objective of this study was to characterise the HLA-B27 subtypes in patients from the NZ North Island population whose samples have been referred for HLA-B27 testing due to clinical symptoms characteristic of SpA and uveitis.

Materials and methods

Subjects

Between April 2006 and April 2007 the NZ tissue typing laboratory tested 1894 patients from the North Island of NZ for HLA-B27 due to symptoms of associated disease. This tissue type was demonstrated in 338 patients. Of these, 194 gave informed consent for their sample to be further subtyped and were included in the study. Participants in the study declared their own ethnic group. Clinical symptoms were taken from the request forms of the original samples and classified as SpA, uveitis or no clinical information available. The study received multi-region ethical approval through the Health Research Council of NZ (application: MEC/07/11/146).

DNA sequencing

DNA sequence based typing (SBT) was performed based on the method described by Dunn et al (32). Briefly: a heterozygous amplicon of the entire HLA-B locus was produced from each sample following manufacturer's instructions for the Expand High Fidelity PCR System (Roche, Basel, Switzerland) and using primers for the 5' and 3' untranslated regions of the gene (Table 1). The PCR product was compared to DNA Molecular Weight Marker VII (0.081-8.57 kbp) (Roche, Basel, Switzerland) by agarose gel electrophoresis on a 0.8%-1.2% agarose gel electrophoresed for 1 hour at 85v. PCR product purification was performed using Agencourt® AMPure® beads (Beckman Coulter Fullerton, CA, USA), following manufacturer's instructions. The purified PCR product concentration and purity were checked on a NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA). Big Dye chemistry was used to perform cycle sequencing using ABI Prism® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Exons 2 and 3 of amplified HLA-B were sequenced in both directions using 4 different HLA-B specific primers (Table 1). Agencourt® CleanSEQ® beads (Beckman Coulter Fullerton, CA, USA) were used, following manufacturer's instructions, to remove unincorporated dye terminators. DNA sequenced fragments were separated and analysed by capillary electrophoresis on a Hitachi 3130xl/ Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

DNA sequences were compiled and analysed against a database of HLA alleles using SBTengine software version 2.8.0.0 (Genome Diagnostics B.V., Utrecht, The Netherlands) (see Figure 1). The

H}_{\text{B27}} \text{ polymorphism associated with disease in a New Zealand population}

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software assigned allele type was recorded.

**Statistical analysis**

The differences in allele frequencies in the different population groups were analysed using the chi-square test (Yates’ correction). This test was also used to analyse the association of the disease groups with the different subtypes. Pearson’s chi-square test was used to calculate the significance of B*27 homozygosity in this study.

A population of 1,703 blood donors, who had been tissue typed, was used to calculate the gene frequency of HLA-B*27 (the proportion of HLA-B*27 alleles out of all the HLA-B genes in the population (33)) and phenotype frequency of HLA-B27 (the proportion of the population with the HLA-B*27 allele, regardless of whether it occurs in a heterozygous or homozygous state (33) in the healthy population.

**Results**

Out of the 194 patients tested, 158 (81.44%) were European, 16 (8.24%) were Asian, 12 (6.19%) were Māori or European/Māori. The remaining eight (4.12%) were from other population groups.

Ninety six (49.48%) subjects were male and 98 (50.52%) were female. Eleven (11) males and 12 females had symptoms of uveitis. Fifty seven (57) males and 59 females had symptoms of SpA. There was no significant difference between males and females with symptoms of disease.

One hundred and sixteen (116) patients had symptoms of SpA on their sample request forms and 23 had symptoms of uveitis. Two of these patients had symptoms of both SpA and uveitis. No clinical information was available for 57 patients. The distribution of the disease categories across the different ethnic groups is represented in Table 2.

A total of 204 HLA-B*27 subtypes were identified in this study. One hundred and eighty four (184) heterozygous patients each had one HLA-B*27 subtype. Four patients were heterozygous for HLA-B27 subtypes: two had B*2702, B*2705, one had B*2701, B*2705, and one had B*2704, B*2706. Five patients were homozygous for the B*2705 subtype and one patient was homozygous for the B*2704 subtype. Homozygosity of HLA-B27 in disease associated patients was not significantly different to the general population (p=0.918).

Of the 204 total, nine different subtypes were identified: B*2701, B*2702, B*2704, B*2705, B*2706, B*2707, B*2709, B*2710 and B*2715 occurred once each. The distribution of HLA-B*27 alleles across the different population groups is represented in Table 3. The association of the prevalence of the major alleles, B*2702, B*2704 and B*2705, were compared for each population group. Of significance were B*2705 in Europeans (p<0.001) and B*2704 in Asians (p<0.001).

The significance of B*2702, B*2704 and B*2705 to each disease category was calculated for those patients for whom clinical information was available. There was no significance of any of these alleles to any disease category.

A new variant of B*2704 was discovered during this project. A nucleotide change in Exon 3 at position 435 (AAG-AAA) resulted in a silent mutation in a lysine residue.

The HLA-B*27 gene frequency in the healthy population was calculated as 0.046. The HLA-B27 phenotype frequency was calculated as 0.089.

### Table 1. Amplification and sequencing primers used

<table>
<thead>
<tr>
<th>Amplification Primers</th>
<th>Primer</th>
<th>Direction</th>
<th>Location</th>
<th>Sequence (5’→3’)</th>
</tr>
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<tr>
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<td>forward</td>
<td>-320 to ATG start</td>
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<tr>
<td>3B38</td>
<td>reverse</td>
<td>+175 after term</td>
<td>CTggggAggAAACACAggTCAgCATgggAAC</td>
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<table>
<thead>
<tr>
<th>Sequencing Primers</th>
<th>Primer</th>
<th>Exon</th>
<th>Direction</th>
<th>Location</th>
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<td>Intron 1, 121-140</td>
<td>ggACcAgACcAgTCcAgCATgggCKCA</td>
<td></td>
</tr>
<tr>
<td>3B44</td>
<td>Exon 2</td>
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<td>Intron 2, 496-514</td>
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<td></td>
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<tr>
<td>5Bln2</td>
<td>Exon 3</td>
<td>forward</td>
<td>Intron 2, 226-243</td>
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<tr>
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<td>Exon 3</td>
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### Table 2. Disease categories and ethnicity

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<th>Disease</th>
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</thead>
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<td>European</td>
<td>Asian</td>
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<tr>
<td>SpA</td>
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<td>9</td>
</tr>
<tr>
<td>uveitis</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>unknown</td>
<td>47</td>
<td>4</td>
</tr>
</tbody>
</table>

* Two patients had symptoms of both SpA and uveitis, thus the total occurrences of disease categories exceeds the total number of patients in the study.

### Table 3. HLA-B*27 allele frequencies across the entire group.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Population Group</th>
<th>Total*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>European</td>
<td>Asian</td>
</tr>
<tr>
<td>B*2701</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>B*2702</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>B*2704</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>B*2705</td>
<td>152</td>
<td>4</td>
</tr>
<tr>
<td>B*2706</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>B*2707</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
The mechanisms of HLA-B27 association with SpA

The strong association of HLA-B27 to SpA and uveitis has been known since 1973 (3,4). The mechanism of the association between HLA-B27 and the SpA remain uncertain. The current hypotheses have been reviewed and suggest three main lines of thought (34-38):

1. Molecular mimicry may occur through HLA-B27 presenting peptide epitope from gram negative bacteria such as Klebsiella to CTL. Some of the CTL may cross-react with self-ligand, eliciting an autoimmune inflammatory response (39).

2. Before being expressed on the cell surface, the HLA-B27 heavy chain proteins fold slowly and they can misfold and accumulate in the endoplasmic reticulum (ER). The resultant ER stress may activate unfolded protein and subsequently activate nuclear factor-κB, inducing pro-inflammatory cytokines (40,41).

3. HLA-B27 heavy chain proteins can form covalent homodimers which are expressed on the cell surface. Leukocyte receptors recognise these with a specificity pattern that is distinct from, yet overlaps that of the recognition of normal HLA-B27 heterodimers. The interaction with homodimers may alter normal immunomodulatory mechanisms and potentially cause excess pro-inflammatory cytokine release (42).

Other genetic factors and SpA

An assessment of the genetic risk for AS has identified that genes linked to the MHC contribute only 31% of the total genetic risk for AS (43). Other HLA types shown to have an association with AS are HLA-B60, HLA-DRB1 (44,45) and HLA-B*1403 (46). Research has identified some killer cell immunoglobulin like receptors also have an association with AS in conjunction with HLA-B27 (47). A 270kb region of the MHC class III region of chromosome 6, containing 23 genes outside of the HLA-B27 region has been identified as conferring susceptibility to AS (45). These considerations need to be taken into account when considering HLA-B27 subtypes’ association to SpA.

Uveitis

Uveitis is a common complication of AS in some patients (5,30,31,48). HLA-B27 associated uveitis however, occurs regardless of AS and is considered a separate clinical entity (31). A recent study of HLA-B27 in uveitis in a NZ population has identified 36% of bilateral uveitis patients and 47% of unilateral uveitis patients as being HLA-B27 positive (48). Of these patients, 41% had AS. In the present study two of patients with uveitis symptoms also had SpA symptoms; however, the numbers were too small to draw reliable conclusions.

HLA-B27 typing techniques

HLA-B27 has been typed using various techniques (49). Initial studies used microcytotoxicity methods to serologically determine the presence or absence of HLA-B27 on the surface of lymphocytes (3). Flow cytometry techniques make use of monospecific antisera and fluorescent labelling to determine HLA-B27 positivity (12,50). PCR-based DNA technology for tissue typing has allowed the presence of HLA-B*27 to be determined at the genetic level. PCR-SSP technique uses DNA primers to directly amplify nucleotide sequences of interest, which can then be detected by gel electrophoresis (49). Low to high resolution DNA typing is possible with this technique. A PCR melting assay has also been described (50). Sequence specific oligonucleotide (SSO) technique involves all allelic sequences from the locus of interest being amplified by PCR. PCR products are then hybridised to oligonucleotide probes specific to the DNA sequence of interest. Biological labelling allows the sequences of interest to be distinguished. This technique can be used for low or high resolution HLA typing (49). DNA sequencing gives high resolution typing with few ambiguities (32,49,51). Different groups have described SBT techniques for HLA typing as well as ways to reduce ambiguities (32,51). A limitation of the sequencing done in this project was the occurrence of ambiguities. In this circumstance the most likely combination of alleles was chosen. Further sequencing with more specific primers or of regions outside the exon 2 and 3 regions could have helped resolve this (52).

HLA-B27 subtype and epidemiology

According to 2006 census data, the ethnic groups of the NZ North Island population are 72.31% European or other ethnicity (including New Zealander), 17.08% Māori, 11.25% Asian, 8.86% Pacific Peoples, and 1.03% Middle Eastern/Latin American/African (53). This compares with 81.44% European, 8.24% Asian, 6.19% Māori or European/Māori and 4.12% from other population groups in this study. The apparent overrepresentation of Europeans and underrepresentation of other ethnicities in this study is intriguing. Participants in this study do not, however, represent a random selection of the NZ North Island population. Sources of bias for the representation of population groups here may include differences in bringing symptoms to the attention of a doctor and different levels of response to the invitation to participate in the study. There were similar numbers of males and females in this study: 49.48% of subjects were male and 50.52% of subjects were female. This compares favourably with the 48.88% males and 51.12% females in the NZ North Island population in 2006 (53). The ratio of males to females was similar in the SpA and uveitis disease groups and amongst the different ethnicities.

Europeans

The largest population group represented in this study was European (81.44%), and B*2705 was the most common subtype found. The association of B*2705 with Europeans was significant (p<0.001). B*2705 is the predominant subtype amongst Europeans (8,16,17,26,42). It is thought that the other HLA-B27 subtypes have
evolved from B*2705 (54,55). B*2701 and B*2702 were found exclusively in Europeans in this study; however, the numbers of these subtypes were not high enough to be significant. Previous research has noted B*2702 as a major HLA-B27 subtype in European populations (8,16,17,26,41). B*2702 is thought to have entered the European population from the Middle East (16). B*2702 is the most common subtype in Ashkenazi Jews with AS in Israel (7) and shows a high incidence in Turkish populations (10,22).

Asians

Asians comprised 8.24% of the study. The frequency of HLA-B27 subtypes has been studied in various populations in Asia. Studies of the Han Chinese (12,20), Taiwaneese (12,28,56), Malays (57), Indians (21) and research of the Asian population have found B*2704 to be the most common subtype (6,19). This allele has also been described in ethnic groups from Western India at various frequencies (25,58). Indeed, B*2704 is thought to be almost completely restricted to Asians and their descendants (6). This was reflected in the present study where B*2704 was the most common subtype in the Asian ethnic group and the association was reflected in the present study where B*2704 was the most common subtype in the same populations and its most closely associated subtype in the same population. One worldwide study of HLA-B27 subtypes involving 12 samples of Māori also found only B*2704 and B*2705 subtypes (16). The small numbers of Māori and Māor/ Europeans in this study prevent reliable conclusions being drawn from the data for this group. The prevalence of HLA-B27, Au and SpA in Māori are unknown and could be an additional source of bias that influenced the representation of Māori in this study. A recent study of human genetic diversity in the Pacific estimated European admixture in Māori to be approximately 12% (59). Admixture between Europeans and Māori may have altered the prevalence of HLA-B27, its subtypes and associated disease in the Māori population.

HLA-B27 subtypes of interest

B*2706

Some HLA-B27 subtypes have been shown to confer protection against SpA in different populations. B*2706 has been previously shown to have a negative association to AS in Thais (16,19), Singapore Chinese (27) and Indonesians (21) and was absent in a Taiwanese population of AS patients (28). All participants in the present study were originally HLA-B27 typed to aid diagnosis of HLA-B27 associated disease. B*2706 was found in one patient in this study who was heterozygous for HLA-B*27, also having a B*2704 allele. This patient was Filipino and had symptoms of SpA. B*2704, B*2706 heterozygosity has been previously described in AS patients in Indonesia (16,60). One study of two mixed Chinese/Indonesian families suggested that the existence of B*2704, B*2706 heterozygotes with SpA symptoms is evidence that although B*2706 is not associated with SpA, it does not confer protection either (60). Two patients with AS in China have been identified as carrying B*2706 (16). Examinations of the low association of B*2706 with AS compare it with B*2704, the common disease associated subtype in the same populations and its most closely related allele (61,62). B*2704 and B*2706 differ in the β-pleated sheets of the peptide binding groove of the molecule. An amino acid difference at position 114 results in histidine (His) and aspartic acid (Asp) respectively in pocket D and a difference in position 116 results in Asp and tyrosine (Tyr) respectively in pocket F (2,61,62). It has been proposed that differences in peptide binding between B*2704 and B*2706 are responsible for their differential association with AS(27,61,62).

B*2709

B*2709 was demonstrated in one patient in the study of European origin. Unfortunately, no clinical details were available for this patient. B*2709 is thought to have a protective role against AS in Sardinians (16,24,29); however, this subtype has been previously described in an Italian patient with AS (23). Furthermore, B*2705 and B*2709 are on distinct HLA haplotypes in the Sardinian population which allows for involvement of other genetic factors on the HLA haplotype in the protection from, or susceptibility to AS (63,64). The differences between B*2709 and the common disease associated subtype B*2705 have been studied (65-68). These two alleles differ by one amino acid at position 116 resulting in His and Asp respectively in the peptide C-terminus accommodating F pocket (2,67). Differences between these subtypes do not result in a difference in the accumulation of heavy chain proteins in the ER (67). B*2705 is more flexible than B*2709 in the peptide groove and the C-terminal peptide anchor can become partially detached. These structural differences may influence HLA-B27 interactions with CTL (66).

B*2707

Our study found B*2707 in one patient who was Lebanese. Unfortunately, no clinical details were available on this patient. B*2707 has been described as having a possible protective role against AS in the Greek Cypriot population (26). A study of an Israeli population (7) and another of the Han Chinese (20) found B*2707 only in healthy controls; however, only two and three respective examples of this allele were identified. Several other studies have, however, demonstrated this allele in patients with AS (9,16,17,19,21,22,24). Interestingly, B*2707 lacks Asp116 as do B*2706 and B*2709 (69). The association of B*2707 with AS is not explained by the misfolding of heavy chains (70).

B27 homozgyosity

Five European subjects in this study were homozygous for B*2705 and one Asian patient was homozygous for B*2704. The levels of HLA-B27 homozygosity were not significantly different to the general population (p=0.918). HLA-B27 homozygosity has been shown to influence the severity of AS (71). Whether HLA-B27 homozygosity confers an increased risk of AS or not is contested (71,72). AS patients that are HLA-B27 homozgyotes are also more likely to develop other SpA and uveitis (71,73). The B*2704 homozygote identified in this study had symptoms of uveitis.

Further work in this field could include epidemiological studies of HLA-B27 subtypes in a healthy NZ population. A comparison of HLA-B27 subtypes in between the disease associated and healthy populations could be used to calculate the relative risk of disease for different ethnic groups in NZ. A limitation of this study was the lack of clinical symptoms available on historic sample request forms. Seeking more complete clinical information from physicians would be helpful in future studies.

In conclusion, this project analysed HLA-B27 subtypes in 194 disease associated patients in the North Island of NZ. Nine subtypes were described: B*2701, B*2702, B*2704, B*2705, B*2706, B*2707, B*2709, B*2710 and B*2715. Within the study B*2704 was significant to the Asian ethnic group (p<0.001) and B*2705 was significant to the European ethnic group (p<0.001). The three major subtypes identified were B*2702, B*2704 and B*2705. None of these showed a statistical association to disease category. HLA-B27 homozgyosity was not significantly different to the healthy population (p=0.918). The subtypes found reflected the HLA-B27 subtypes found in previous studies of the different ethnicities involved.

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Author contributions

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