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HLA-DQA1 genotyping by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and restriction endonuclease digestion in Papua New Guinea

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SUMMARY

We have used PCR-SSCP, a technique based on the conformation of single-stranded DNA, to characterize the HLA-DQA1 gene in four geographically diverse population groups in Papua New Guinea. Among the 294 individuals that were studied from Goroka, north coast of Madang, Kimbe and Wanigela, we detected 5 of the 20 known variants of this gene locus. These included alleles 0101, 0102, 0103, 0301 and 0501. Furthermore, variable mobility shifts observed for alleles 0301 and 0501 from Madang suggested a further 3 variants. All 15 combinations of the 5 confirmed alleles were detected and their respective gene frequencies found to be consistent with the groups' ethnic and linguistic diversity. In respect to their frequencies and the observed overall allelic heterozygosity, the distribution in Kimbe showed some similarity to that in the north coast of Madang while Madang and Goroka were the most different. The distribution of alleles 0102 and 0501 was observed to be similar for Goroka and Wanigela as was 0301 for Madang and Wanigela. Our results, confirmed by endonuclease digestion, show PCR-SSCP to be a highly sensitive technique that can be used to characterize HLA-DQ antigens. In addition, the simplicity of the method provides an opportunity for large-scale typing of HLA antigens.

Introduction

The human leukocyte antigens (HLA) are cell-surface heterodimeric glycoproteins, encoded by genes located on the short arm of chromosome 6. Three types of these molecules, referred to as class I, class II and class III antigens, are expressed by this gene complex. The class I and II antigens have been identified as dominant determinants of immune responses in humans, while class III make up components of the complement system where they induce cell lysis. Class I antigens have been well characterized using serology and biochemical assays (1,2) and, more recently, by nucleotide detection methods (3). They are widely distributed in tissues and are found on the surfaces of most cells. On the other hand, class II antigens, including HLA-DP, HLA-DQ and HLA-DR, are confined to certain cell types, namely those involved in the immune response such as B cells, activated T cells and some antigen-presenting macrophages (4). They consist of two integral membrane proteins, the α and β chains, that are characterized by an extensive degree of allelic polymorphism. Unlike DP and DR antigens, which possess non-polymorphic α chains, both the α and β chains of DQ antigens are polymorphic (5-7), with 20 variants of HLA-DQA1 gene and 39 of HLA-DQβ gene having been detected to date (8). The array of polymorphism expressed by this gene is vital in regulating host immune responses whereby foreign antigens can be specifically bound and presented to the T helper cells for neutralization. Although HLA class I antigens have traditionally been associated with susceptibility to some autoimmune diseases, recent evidence indicates that class II antigens, including HLA-DQα, also have an important role (9,10). This may be because these antigens form the first line in

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immunological defence and elicit the initial immune response. Moreover, being highly polymorphic, this gene provides a useful genetic marker used in population or individual identification studies.

Studies involving these antigens in Papua New Guinea (PNG) have been limited due to the logistic difficulties associated with the obtaining of viable B lymphocytes for mixed lymphocyte cultures (MLC). Nevertheless, use of MLC and also serology has led to indeterminate classifications partly due to high linkage association shown by alleles of this gene with those of the adjacent loci, in particular the DR gene. For instance, most of these gene products were initially associated with DR specificities using serology, but have since been reclassified (4,10). Additionally, both MLC and serology have limitations because they are directed towards detecting previously defined antigens. They are also cumbersome and require a large panel of serologically defined homozygous antigens and test cells.

The establishment of the molecular basis for characterization of these antigens through cloning and sequencing has now paved the way for the incorporation of a number of nucleotide detection methods (11-13). Following on from the use of restriction fragment length polymorphism (RFLP) (14), PCR-based detection methods have been introduced and are now used to detect specific HLA alleles. In this approach, nucleotide sequences are amplified using specific primers that flank the target DNA segment and the product analyzed with specific nucleotide probes (7,15,16). Alternatively, the amplified product can be analyzed by cleaving with specific restriction endonucleases (17,18). Although these methods have been used successfully to type HLA antigens in the past, they are limited only to discriminating the already known polymorphisms. This means that previously unidentified polymorphisms and novel alleles that are not recognized by the panel of restriction enzymes or nucleotide probes being used will go undetected. In addition to the requirement for radioisotopes, cross-hybridization among genetically similar sequences can result during oligonucleotide probing and heterozygotes can be difficult to distinguish following restriction digestion.

The advent of single-strand conformation polymorphism (SSCP) now provides yet another option by which HLA genes can be characterized. This method involves denaturation of DNA into single strands that are subsequently separated electrophoretically in a gel matrix. The rationale of this method is that under non-denaturing conditions, single-strand DNA segments form sequence-specific conformations, which cause shifts in electrophoretic mobility. Its potential to detect even single-base changes ensures that two very similar alleles can be distinguished (19,20). To circumvent problems posed by linkage disequilibrium and the limitations of other methods, we have used PCR and SSCP in conjunction with endonuclease digestion to amplify a 242 base pair (bp) DNA fragment from exon 2 of the DQA1 gene to detect variants of this gene in four population groups in PNG.

Materials and Methods

Study population

Following informed consent, 5 ml of heparinized blood was collected from each of 294 individuals from Papua New Guinea, including 90 villagers from the north coast villages in Madang (Madang Province), 89 from Kimbe (West New Britain Province), 24 from Wanigela (Central Province) and 91 from Goroka (Eastern Highlands Province). DNA was extracted from the buffy coats for analysis using standard methods.

Polymerase chain reaction

A 242 bp segment in exon 2 of HLA-DQA1 was amplified from approximately 1 μg of extracted DNA using sense primer GH26, 5'-GTG CTG CAG GTG TAA ACT TGT ACC AG- 3' and antisense primer GH27, 5'-CAC GGA TCC GGT AGC AGC GGT AGA GTT G-3' (17). The PCR reaction mixture consisted of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 μM of each primer and 0.025 units of Taq DNA polymerase in a final volume of 100 μl. Amplification was performed by denaturation at 94°C for 1 minute, followed by annealing at 56°C for 1.5 minutes and extension at 72°C for 1 minute for 30 cycles.

Restriction endonuclease digestion

After amplification, an 8 µl aliquot of each PCR product was separated on 1.4% agarose gel to check for the target 242 bp DNA segment. All positively amplified products were then digested simultaneously with *Rsa* I and *Hae* III restriction enzymes. Digestion was achieved by incubating the mixtures at 68°C overnight in appropriate incubation buffers; 10 µl of each digest was resolved on 12% polyacrylamide gel. The restricted DNA fragments were detected by staining with ethidium bromide and visualization under ultraviolet light.

Single-strand conformation polymorphism

SSCP was performed by mixing 5 µl of each PCR product with 10 µl of 88% formamide dye (10 mM EDTA, pH 8.0 and 0.01% bromophenol blue) and the mixture heated at 96°C for 5 minutes before snap quenching on ice for 5 minutes. 10 µl aliquots were then applied on a pre-heated 20% polyacrylamide gel that contained 49:1 parts of acrylamide:bis-acrylamide and separated at 10 volts/cm for 6 hours at room temperature. The separated single strands were detected by silver staining.

Data analysis

All generated data were stored on Foxpro. The theoretical gene frequencies were calculated according to the Hardy-Weinberg distribution. The observed genotypes were compared to the theoretical distribution by the χ^2 goodness-of-fit test; the allelic heterozygosity (h), which measures the overall allelic diversity for a population, was calculated according to $[1 - \sum (X_i)^2] [n / (n-1)]$, where X_i is the respective allele frequency and n is the sample size, in this case being the number of alleles (21).

Results

HLA-DQA1 variants were determined for 294 individuals from four geographically diverse locations in PNG. From these individuals, we were able to detect 5 of the 20 known alleles including alleles 0101, 0102, 0103, 0301 and 0501 (Figure 1). Except for alleles 0101 and 0102, the digests clearly showed allele-specific restriction patterns when resolved in 12% polyacrylamide gels. The amplified gene fragments of 0101 and 0102 differ by a single nucleotide that is not detectable by *Rsa* I and *Hae* III (17). Although these alleles could have been distinguished using *Mn* I digestion, this was not done in the study.

Similarly, the single-stranded DNA obtained following SSCP showed mobility shifts that were characteristic for the respective alleles. In this case, the independent migration of each strand of denatured double-strand DNA ensured ready detection of the alleles that were present in the 4 population groups. Moreover, with this technique even alleles 0101 and 0102, which are inseparable by endonuclease digestion with *Rsa* I and *Hae* III, showed characteristic DNA banding patterns. Likewise, allele 0103, which differed from 0102 by two nucleotides, was also distinguishable. Additionally, the different migrating patterns observed in DNA bands for 0301 and 0501 from individuals in Madang suggest that these alleles could be divided further into 2 and 3 subtypes respectively. Moreover, heterozygotes of these alleles were also easily detected. This was achieved through interpreting both the resolved patterns of single-stranded DNA and the banding patterns formed by heteroduplexes. The heteroduplexes were particularly useful in the cases where single-stranded DNA showed variable number of bands and intensity. In all cases, putative heterozygotes were confirmed

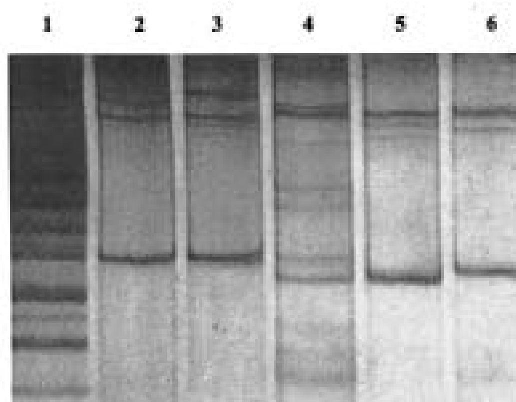

Figure 1. Lane 1 is 1-kilobase DNA ladder, lane 2 allele 0101, lane 3 allele 0102, lane 4 heterozygous allele 0103/0501, lane 5 allele 0501 and lane 6 allele 0301.

TABLE 1

ALLELE FREQUENCIES DETERMINED IN THE STUDY FOR THE 4 POPULATION GROUPS

Allele	Goroka	Madang	Kimbe	Wanigela
DQA1*0101	0.33	0.21	0.27	0.13
DQA1*0102	0.32	0.16	0.11	0.31
DQA1*0103	0.11	0.03	0.13	0.21
DQA1*0301	0.15	0.26	0.14	0.27
DQA1*0501	0.08	0.34	0.35	0.08
Allelic heterozygosity h =	0.76	0.75	0.76	0.80

by pooling of the respective homozygotes that produced the same hybrid patterns after separation. Except for the inability of the endonuclease digestion to distinguish between alleles 0101 and 0102, the distributions of the HLA-DQA1 variants detected by both methods were comparable and the combined allele frequencies of 0101 and 0102 obtained for comparisons were seen to be similar in both cases (results not shown).

Distribution of the respective alleles and the overall allelic heterozygosity for the four population groups are presented in Table 1. Allele 0501 reached the highest frequency of 0.35 in Kimbe, followed by 0101 (0.33 in

TABLE 2

DISTRIBUTIONS OF HLA-DQA1 GENOTYPES IN GOROKA

Genotype	Observed (O)	Expected (E)	$(O-E)^2/E$
0101/0101	1	9.91	8.01
0101/0102	42	19.22	27.00
0101/0103	5	6.61	0.39
0101/0301	6	9.01	1.01
0101/0501	5	4.80	0.01
0102/0102	2	9.32	5.75
0102/0103	4	6.41	0.91
0102/0301	4	8.74	2.57
0102/0501	5	4.66	0.02
0103/0103	0	1.10	1.10
0103/0301	10	3.00	16.33
0103/0501	1	0.60	0.23
0301/0301	3	2.05	0.44
0301/0501	2	2.18	0.01
0501/0501	1	0.58	0.30

$\chi^2 = 64.08$; df 10; $p<0.001$
Gene frequency was calculated assuming Hardy-Weinberg equilibrium and 10 degrees of freedom obtained by subtracting the number of alleles from the total possible combinations

Goroka), 0102 (0.32 in Goroka), 0301 (0.27 in Wanigela) and 0103 (0.21 in Wanigela). However, the distribution of these alleles within the four population groups was variable. Allele 0501 appears to be the common type in the coastal groups in the north of PNG (Kimbe and the north coast of Madang) and uncommon in the highlands (Goroka) and the south coast (Wanigela). In Goroka, alleles 0101 and 0102 were more common, although increased frequencies were also observed for 0101 in Madang and Kimbe and for 0102 in Wanigela. Similar frequencies for alleles 0103 and 0301 were observed in Kimbe and Goroka.

Using the obtained allelic frequencies, we also determined genotype distribution according to Hardy-Weinberg and compared this to those observed in the study. Various combinations of these alleles as determined by SSCP are presented in Tables 2, 3, 4 and 5 for all population groups. Overall, all 15 possible combinations of these alleles were detected, although this was not observed for each individual group. Nondetection of some combinations in each individual population group is expected owing to the small number typed. With the exception of a few genotypes that varied considerably from the expected frequency, most of the genotypes had a frequency distribution that was consistent with the theoretical expectation. Marked deviations from the expected genotype frequencies were observed for alleles 0101, 0102 and 0103, all of which contributed to significant deviation ($p<0.05$) from that expected under Hardy-Weinberg equilibrium in all four population groups. In Goroka, the prevalence of 0101/0102 heterozygosity was particularly high followed by that of 0103/0301. These genotypes were also observed to be more common than expected in Kimbe and Wanigela and so was 0103 homozygous in Madang. On the contrary, 0101 homozygotes were observed to be less common than expected in Goroka and completely absent in Kimbe.

TABLE 3

DISTRIBUTIONS OF HLA-DQA1 GENOTYPES IN NORTH COAST VILLAGERS OF MADANG

Genotype	Observed (O)	Expected (E)	$(O-E)^2/E$
0101/0101	3	3.97	0.24
0101/0102	11	6.05	4.05
0101/0103	0	1.13	1.13
0101/0301	11	9.83	0.14
0101/0501	10	12.85	0.63
0102/0102	0	2.30	2.30
0102/0103	0	0.86	0.86
0102/0301	9	7.49	0.30
0102/0501	9	9.79	0.06
0103/0103	1	0.08	10.58
0103/0301	1	1.40	0.11
0103/0501	3	1.84	0.73
0301/0301	5	6.08	0.19
0301/0501	15	15.91	0.05
0501/0501	12	10.40	0.25

$\chi^2 = 21.62$; df 10; $p<0.025$
Gene frequency was calculated assuming Hardy-Weinberg equilibrium and 10 degrees of freedom obtained by subtracting the number of alleles from the total possible combinations

TABLE 4

DISTRIBUTIONS OF HLA-DQA1 GENOTYPES IN KIMBE

Genotype	Observed (O)	Expected (E)	$(O-E)^2/E$
0101/0101	0	6.45	6.45
0101/0102	12	5.29	8.51
0101/0103	8	6.25	0.49
0101/0301	3	6.73	2.07
0101/0501	25	16.82	3.98
0102/0102	0	1.08	1.08
0102/0103	3	2.55	0.08
0102/0301	2	2.74	0.20
0102/0501	2	6.85	3.43
0103/0103	0	1.50	1.50
0103/0301	5	3.24	0.96
0103/0501	8	8.10	0.00
0301/0301	2	1.74	0.04
0301/0501	11	8.72	0.60
0501/0501	8	10.90	0.77

$\chi^2 = 30.16$; df 10; $p<0.001$
Gene frequency was calculated assuming Hardy-Weinberg equilibrium and 10 degrees of freedom obtained by subtracting the number of alleles from the total possible combinations

Discussion

A previous study of HLA-DR haplotypes in Oceania has shown DQ antigens associated with DR2, DR5 and DRw8 to be genetically diverse (22). Although no specific DQA and DQB RFLPs for the region have been detected at these loci, DQ RFLPs detected in PNG highlanders differed from the usual DRw8, DQw1 associated pattern (23), suggesting that inter-population variation can be expected at this locus. Moreover, the distribution of these alleles can also be population-specific as indicated by the association of DRB3 genes with DRw6 specificity in Polynesians as opposed to that of DQ genes with DRw8 in Melanesians. Indeed in as far as PNG is concerned previous genetic studies based on serum protein isoelectric focusing (24), HLA class I antigen specificity (23) and HLA class II RFLPs (4,25) have demonstrated marked contrast in the distribution of the respective antigens among highland and coastal Melanesians. This variation in frequency was also reflected in the segregation of HLA-DQA1 alleles detected in our study population.

Our population groups representing the four geographical regions in Papua New Guinea showed contrasting distributions of alleles 0101, 0102, 0103, 0301 and 0501 for this gene locus. In most cases, the distribution of these alleles was found to correlate with the groups' ethnic and linguistic diversity. Alleles 0101, 0102 and 0103 which were detected frequently in Goroka were noted to be less common in Madang, with near absence of 0103. Lower frequencies for 0101 and 0102 were equally observed in Kimbe, which indicates that these alleles are uncommon in these northern coastal Papua New Guinean groups compared to the highlands. The greatest difference was seen in the distribution of allele 0501 which, previously and also in the current study, was observed to be rare in the highlands, but more prevalent in north coastal Melanesians (4). Despite this observation, the similar distribution of alleles 0103 and 0301 shown for

TABLE 5

DISTRIBUTIONS OF HLA-DQA1 GENOTYPES IN WANIGELA

Genotype	Observed (O)	Expected (E)	$(O\text{-}E)^2/E$
0101/0101	0	0.41	0.41
0101/0102	6	1.93	8.58
0101/0103	0	1.31	1.31
0101/0301	0	1.68	1.68
0101/0501	0	0.50	0.50
0102/0102	0	2.31	2.31
0102/0103	4	3.12	0.25
0102/0301	3	4.02	0.25
0102/0501	2	1.19	0.55
0103/0103	0	1.06	1.06
0103/0301	5	2.72	1.91
0103/0501	1	0.81	0.04
0301/0301	2	1.75	0.04
0301/0501	1	1.04	0.00
0501/0501	0	0.15	0.15

$\chi^2 = 19.04$; df 10; $p<0.05$
Gene frequency was calculated assuming Hardy-Weinberg equilibrium and 10 degrees of freedom obtained by subtracting the number of alleles from the total possible combinations

Goroka and Kimbe suggests that individuals in these two groups have some similarities. This was also reflected in the overall allelic heterozygosity. The distribution of these alleles showed a markedly different pattern in Wanigela and Goroka from that in Madang and Kimbe. Similar prevalence rates noted for alleles 0102 and 0501 in Wanigela and Goroka suggest an influence of independent but similar selective pressure or its absence in the case of 0501. The increased frequency observed for 0101/0102 heterozygotes in both these groups suggests that this genotype may be selected due to heterozygous advantage; this is also true for Kimbe and, to a lesser extent, Madang. It is possible, however, that these differences may have occurred by chance.

There were, however, some notable differences between our results and that of Gao and coworkers (4). This was especially in the higher frequency of allele 0301 from Madang, which was 26% in our series in comparison with 5% that had been established earlier. This allele showed a similar distribution in Wanigela in this study and was almost twice as common as in either Kimbe or Goroka. Since previous studies have shown some north coast villagers in Madang to be Melanesians of Austronesian stock (26), it is not unlikely that certain genetic entities found in Austronesian groups such as Wanigela would be common here as well. This has for instance been reported for the predisposition to glucose intolerance and susceptibility to non-insulin-dependent diabetes in the neighbouring Takia of Karkar Island and in the Wanigelas (27,28). The presence of two possible variants of allele 0301 shown in the current study for Madang suggests that the oligoprobes used in the previous study may have been limited to detecting only one of the variants, hence contributing to the low frequency. However, although the 2% frequency determined for the less common of the two variants of 0301 that was detected in this study is comparable to 5%

as determined in the previous study, its distribution in the highlands would have been similarly affected if this was the case. Therefore on the basis of the strong linkage association shown by this allele with HLA-DRB1 0403, 0405 and 0410 and a low frequency noted for the haplotype in Madang and other coastal areas (4), we suggest that the distribution of this polymorphism be further investigated.

Our results for alleles 0101 and 0102 also show distributions which differ from those established for the highlands and Madang in the previous study. This difference is more marked in Madang where 21% and 16% of the respective alleles were described in contrast to 3% and 39% determined previously (4). The difference in the methods used could account for this especially when taking into account the small sample sizes that were investigated in both studies. Despite this, similar distributions observed for these alleles in Rabaul in the previous study show our observation to be consistent with the shared Austronesian admixture (29).

Increased frequencies observed for a few genotypes including 0101/0102 in this study suggest that these differences could have equally been due to nondetection of rare polymorphisms. In particular, under suboptimal conditions, related alleles will show similar resolutions resulting in their segregating together and hence increasing the observed frequencies. Although selective pressure suggested earlier may be a plausible explanation for these increases, it is more likely that nondetection of closely related alleles may be responsible, since the migration of secondary structures assumed by single-stranded DNAs can be influenced by slight changes in the gel temperature and composition.

Alternatively, newly introduced genes may cause a rise in genotype frequencies. This is especially so in situations where the concerned gene has only recently been introduced and requires time for it to reach equilibrium. However, this would be inconsistent with the traditional population structure, demographic attributes and the longstanding practice of preferential village endogamy observed in PNG. In view of these and the established theoretical improbability of deviations from the Hardy-Weinberg expectations in such settings (30), we conclude that the apparent departures observed for HLA-DQA1 alleles may have resulted from the inability of SSCP to detect alterations in the mobility of these alleles. In addition, the conditions used in this study may not have been optimal for clearly distinguishing between alleles with similar conformations as would be anticipated for subvariants of 0101 and 0102 identified recently (8,16).

Despite these reservations, our results, as also confirmed by endonuclease digestion, show PCR-SSCP to be a highly sensitive method with the capacity to detect specific HLA-DQA1 alleles. By using this method we further confirmed the presence of the 5 alleles determined previously for this gene in our population and showed that their distribution in the four population groups was variable. The overrepresentation of a few of these alleles, however, is likely to be due to the failure of resolution by this method rather than being caused by gene selective pressure. Running SSCP gels under controlled temperature to maintain reproducibility, adjusting gel composition and running buffer concentration, and addition of glycerol have been shown to improve resolution (31,32). In addition to the 5 confirmed alleles, possible variants of alleles 0301 and 0501 detected in the study suggest that a limitation in the detection methods used previously could account for the apparent restricted polymorphism reported for this gene locus. The observed sensitivity of this method combined with its capacity for improvement offers a less labour-intensive means of characterizing HLA-DQA1 alleles than using serology and MLC. Additionally, its simplicity further provides an opportunity for large-scale typing of HLA.

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