RESEARCH REPORT

Case–control study of the association between select *HLA* genes and anti-erythropoietin antibody-positive pure red-cell aplasia

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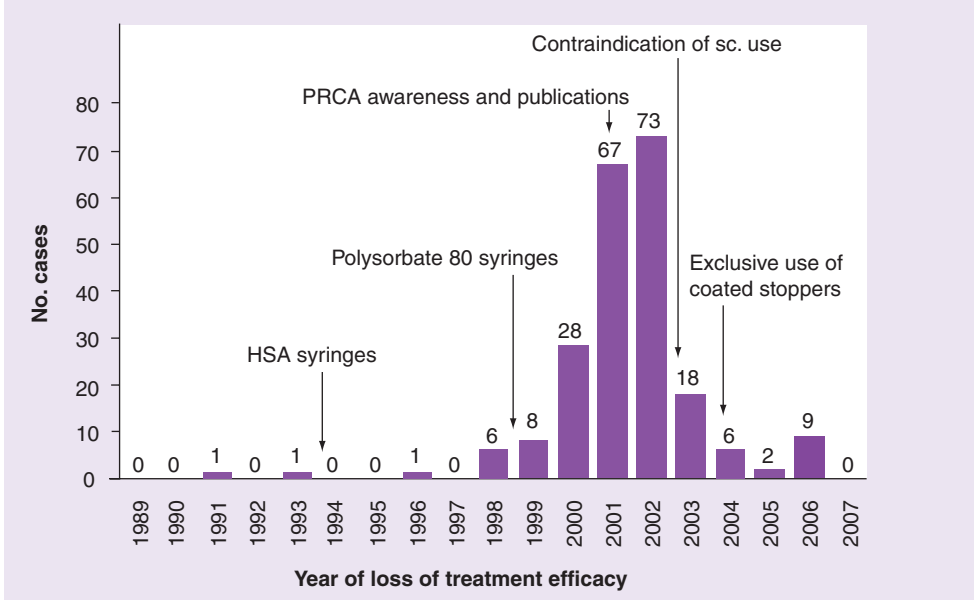
Aims: Antibody (Ab)-positive pure red-cell aplasia (PRCA) is a very rare but serious adverse event associated with recombinant human erythropoietin treatment (4.1 reports per 100,000 patient-years) in which patients produce antibodies to recombinant and endogenous erythropoietin, halting red blood cell production. In a previous case series, four Thai subjects with chronic kidney disease and Ab-positive PRCA were reported to have the *HLA-DRB1*9* allele. To confirm a possible association of *HLA-DRB1*9* and Ab-positive PRCA, we performed a pharmacogenomic analysis using subjects from an earlier case–control study of risk factors associated with Ab-positive PRCA, which had been performed using subjects from Europe or Canada. The primary goal of the analysis was to test the association between *HLA-DRB1*9* and Ab-positive PRCA. A secondary goal was to perform an exploratory analysis in order to identify additional *HLA* alleles potentially associated with Ab-positive PRCA. **Patients & Methods:** Subjects were taken from a case–control study of Ab-positive PRCA in chronic kidney disease patients treated in Europe or Canada. Ab-positive PRCA cases (n = 24) were matched to controls (n = 81) by timing of treatment exposure and, when possible, by location. **Results:** The allele frequency of *HLA-DRB1*9* was 12.5% in cases vs 1.2% in controls (p = 0.002). The frequency of the *HLA-DRB1*9*/other genotype was 25.0% in cases vs 2.5% in controls (p = 0.004; OR: 10.8 [95% CI: 2.2–53.7]). Within the exploratory analysis, six additional *HLA* alleles (*HLA-A*25*, *HLA-B*53*, *HLA-C*12*, *HLA-DQB1*3*, *HLA-DQB1*6* and *HLA-DRB1*4*) were also found to be associated with Ab-positive PRCA. **Conclusion:** This study confirmed that *HLA-DRB1*9* occurs at a significantly higher frequency in Ab-positive PRCA cases than in controls; however, within this sample set, carrying the **9* allele was neither necessary nor sufficient to cause Ab-positive PRCA.

Recombinant versions of erythropoietin (EPO), a growth factor that controls the production of red blood cells, have been used to treat anemia associated with chronic kidney disease (CKD) since 1988. During the first 10 years of use, millions of patients were treated with recombinant human EPO (rHuEPO) with only three cases of EPO antibody (Ab)-positive pure red-cell aplasia (PRCA) being described [1–3]. Ab-positive PRCA is a severe hematological disorder characterized by the production of antibodies that inhibit the activity of endogenous and rHuEPO [4–7]. Patients with Ab-positive PRCA initially respond well to rHuEPO, then suddenly experience a loss of efficacy (LOE) of treatment with simultaneous decreases in hemoglobin and circulating reticulocyte levels. Bone marrow samples from these patients show an absence of erythroid precursor cells and the maturational arrest of erythroid cells, with almost no production of red blood cells [8–11].

Starting in 1999, the frequency of reported Ab-positive PRCA cases in CKD patients began to increase, with approximately 250 cases associated with subcutaneous use of EPREX® [6,8]. Investigations concluded that organic compounds were being leached from the rubber plunger in EPREX prefilled syringes by polysorbate 80, which had replaced human serum albumin as a stabilizer in 1998 in response to concerns over potential contamination by HIV and Creutzfeldt–Jacob disease-causing prions [12–14]. It was hypothesized that the organic compounds in conjunction with the increased immunogenicity of the subcutaneous route of administration [15] induced an immune response to rHuEPO and EPO in some subjects. After withdrawal of the rubber stopper product formulation and introduction of a new FluroTec®-coated stopper formulation, the worldwide reporting rate of Ab-positive PRCA associated with EPREX returned to background rates of other rHuEPO (Figure 1) in the order of 4.1 cases per 100,000 person-years [16].

In Thailand, the clinical picture with respect to Ab-positive PRCA differs from the rest of the world, since the introduction of FluroTec-coated stoppers did not appear to reduce the

Figure 1. Cases of antibody-positive pure red-cell aplasia in chronic renal failure patients reported to Johnson & Johnson Pharmaceutical R&D by year of loss of recombinant human erythropoietin treatment efficacy*.



*These numbers have been updated from [16]. For the year 2007, 0 is the number of Ab-positive PRCA cases reported from 1 January, 2007 to 18 July, 2007.
HSA: Human serum albumin; PRCA: Pure red-cell aplasia; sc.: Subcutaneous.

incidence rate of Ab-positive PRCA (e.g., 206/100,00 patient-years of treatment and 126/100,000 patient-years of treatment for the FluroTec-coated and uncoated stoppers, respectively). After the introduction of FluroTec-coated stoppers, 12 additional cases of Ab-positive PRCA were reported worldwide by the end of 2006. Of these 12 cases, nine occurred in Thailand, which accounts for only 5.6% of the global subcutaneous EPREX use in CKD patients. Clearly, the clustering of Ab-positive PRCA case reports in Thailand warrants further investigation and suggests that other factors besides those described above impact Ab-positive PRCA rates in Thailand.

In 2005, Praditpornsilpa *et al.* published a case report of four Thai subjects with Ab-positive PRCA who had been treated with either EPREX or RECORMON® [17]. All four patients carried *HLA-DRB1*9*. The initial case series was expanded to include 16 Thai subjects with Ab-positive PRCA; 62.5% (10 out of 16) of these subjects carried *HLA-DRB1*9* [18]. This allele is rare in Caucasians (<1%), but much more common in Thailand (8.4–12.5%), with roughly 16–23% of the Thai population expected to

Box 1. Definition of recombinant human erythropoietin treatment-associated antibody-positive pure red-cell aplasia.

Normochromic, normocytic anemia unresponsive to recombinant human erythropoietin (rHuEPO) treatment at the time antibody-positive pure red-cell aplasia (PRCA) was first suspected.

Evidence of antibody-positive PRCA associated with rHuEPO treatment defined as:

- Receiving a therapeutically effective dose of EPREX® for at least 30 days followed by a sudden decrease (≥2 g/dl within 30 days) in a previously stable hemoglobin level, without causality, and one of the following:
  - Bone marrow testing showing isolated erythroblastopenia (<5% erythroblasts in bone marrow) and/or pathologic diagnosis of antibody-positive PRCA with or without anti-EPO antibody
  - Bone marrow testing showing hypocellularity or nondiagnostic findings in association with reticulocytopenia (<20 × 10^9/l) and presence of anti-EPO antibody
  - Reticulocytopenia (<20 × 10^9/l) and the presence of anti-EPO antibody in cases where bone marrow testing was not performed

Table 1. Demographic characteristics of matched subjects and disease history.

Demographic characteristics	Cases	Controls
n	24	81
Age (years), mean (±SD)	59.5 (±18.6)	56.8 (±16.0)
Sex, frequency (%)		
Male	17 (71)	48 (59)
Female	7 (29)	33 (41)
Race, frequency (%)		
Asian	1 (4)	4 (5)
Black	0 (0)	2 (2)
Other	0 (0)	1 (1)
Caucasian	23 (96)	74 (91)
Country, frequency (%)		
Canada	3 (13)	12 (15)
France	8 (33)	9 (11)
Germany	1 (4)	0 (0)
UK	7 (29)	44 (54)
Italy	1 (4)	3 (4)
Netherlands	0 (0)	4 (5)
Spain	2 (8)	0 (0)
Sweden	2 (8)	9 (11)
Disease history, frequency (%)*		
Glomerulonephritis	7 (47)	19 (28)
Diabetic nephropathy	1 (7)	14 (20)
Renal vascular disease	6 (40)	8 (12)
Interstitial nephritis	0 (0)	4 (6)
Poly/multicystic kidney disease	2 (13)	4 (6)
Obstructive/reflux nephropathy	0 (0)	4 (6)
Congenital renal hypoplasia/dysplasia	0 (0)	4 (6)
Renal neoplasms	0 (0)	1 (1)
Renal failure metabolic disease	1 (7)	2 (3)
Toxic nephropathy	0 (0)	1 (1)
Paraproteinemia	0 (0)	1 (1)
Other	1 (7)	16 (23)
n	0 (0)	5 (7)

*Disease history was known for 15 cases and 69 controls.

express *HLA-DRB1*9* [19]. Since a second set of Thai Ab-positive PRCA subjects was not available to confirm this observation owing to the rarity of Ab-positive PRCA, we performed a pharmacogenomic analysis using subjects from an earlier case–control study of risk factors associated with Ab-positive PRCA [101]. Subjects in the case–control study had been recruited from Europe or Canada while Ab-positive PRCA rates were temporarily elevated because of the increased immunogenicity of the discontinued EPREX formulation, discussed above. The primary goal of the analysis was to test the association between *HLA-DRB1*9* and Ab-positive PRCA. A secondary goal was to perform an exploratory analysis in order to identify additional HLA alleles potentially associated with Ab-positive PRCA, since variation in other *HLA* genes has been shown to influence immune response in relation to drug treatment [20–27].

Patients & methods

Patients

Subjects were taken from a retrospective case–control study designed to identify potential risk factors for EPO Ab-positive PRCA [101]. Ab-positive PRCA was defined as described in Box 1, and all cases were confirmed as having anti-EPO Ab. Ab-positive PRCA cases were identified from spontaneous postmarketing reports from Europe or Canada, or from serious adverse events reported in clinical studies at Johnson & Johnson Pharmaceutical Research and Development or its affiliated companies from January 1, 1998 to March 31, 2004, the time period during which the uncoated stopper formulation of EPREX temporarily increased Ab-positive PRCA rates. Up to four controls were matched to cases by study site and timing of EPREX exposure (e.g., control subjects received EPREX for a minimum of 2 months within ±3 months of the LOE of their matched case). Cases and controls who became unmatched for the genetic analysis owing to lack of consent for DNA testing were rematched to each other by timing of EPREX exposure (e.g., for controls, the LOE of their originally matched case had to be within ±4 months of the LOE of their newly matched case) and, if possible, by study site. All subjects had CKD and had received EPREX subcutaneously.

The Independent Ethics Committee/Independent Review Board of each participating site approved the study, which was conducted according to the Declaration of Helsinki, the International Conference on Harmonization, and the Guidelines for Good Clinical Practice. All patients gave written informed consent.

Laboratory methods

The presence of anti-EPO Ab were assayed in serum as described in Tracey *et al.* [28] and Kelley *et al.* [29]. Genomic DNA was isolated from leukocytes in whole-blood samples with AUTOPURE LS™ technology using Puregene reagents (Qiagen, Inc., CA, USA). Isolated DNA was sent to Laboratory Corporation of America (NJ, USA). Genotyping to detect the

Table 2. Frequency and percent of *HLA-DRB1*9* and 'other' alleles in all matched subjects and stratified by race.

Race	Cases			Controls			p-value‡
	n	**9*	*Other*	*n*	**9*	*Other*	
All matched subjects	48	6 (12.5%)	42 (87.5%)	162	2 (1.2%)	160 (98.9%)	0.002
Asian	2	1 (50.0%)	1 (50.0%)	8	1 (12.5%)	7 (87.5%)	0.378
Black	0	NA	NA	4	0 (0.0%)	4 (100.0%)	NA
Other	0	NA	NA	2	0 (0.0%)	2 (100.0%)	NA
Caucasian	46	5 (10.9%)	41 (89.1%)	148	1 (0.7%)	147 (99.3%)	0.003

‡*One-sided p-value calculated used Fisher's exact test.*
NA: Not available.

HLA-A, *HLA-B*, *HLA-C*, *HLA-DQB1*, *HLA-DRB1*, *HLA-DRB3*, *HLA-DRB4* and *HLA-DRB5* alleles was completed according to good laboratory practices by previously described PCR-sequence specific oligonucleotides (SSOP) methodology [30–34] with utilization of appropriate *HLA* locus probes. Genotyping was performed at intermediate resolution and all available alleles for each gene were tested. Standard alphanumeric codes were provided for subject HLA assignments [35].

Table 3. Frequency of *HLA-DRB1*9* genotypes and odds ratios for all matched subjects and stratified by race.

Race	Frequency (%)		OR‡	95% CI‡
	Cases	*Controls*		
All subjects				
n	24	81		
**9/*9*	0 (0.0%)	0 (0.0%)	NA	NA
**9*/other	6 (25.0%)	2 (2.5%)	10.8	(2.2–53.7)§
Other/other	18 (75.0%)	79 (97.5%)	1.0	–
Asian				
n	1	4		
**9*/other	1 (100.0%)	1 (25.0%)	NA	NA
Other/other	0 (0.0%)	3 (75.0%)	NA	NA
Black				
n	0	2		
**9*/other	NA	0 (0.0%)	NA	NA
Other/other	NA	2 (100.0%)	NA	NA
Other				
n	0	1		
**9*/other	NA	0 (0.0%)	NA	NA
Other/other	NA	1 (100.0%)	NA	NA
Caucasian				
n	23	74		
**9*/other	5 (21.7%)	1 (1.3%)	20.3	(2.2–184.5)¶
Other/other	18 (78.3%)	73 (98.7%)	1.0	–

‡*ORs and 95% CIs for all matched subjects were calculated within a matched logistic regression framework. ORs and 95% CIs stratified by race were calculated within an unmatched framework.*
§*p = 0.0038.*
¶*p = 0.0026.*
NA: Not available.

Statistical methods

To evaluate whether *HLA-DRB1*9* was associated with Ab-positive PRCA, *HLA-DRB1* alleles were classified as '**9*' or 'other'. One-sided Fisher's exact test was used to test whether the allele frequency of *HLA-DRB1*9* was elevated in cases compared with controls using an α of 0.05. Odds ratios (ORs) by *HLA-DRB1*9* genotype were calculated for all subjects using matched logistic regression and after stratifying by ethnic group using unmatched logistic regression.

In the exploratory analysis of the other *HLA* alleles only Caucasian subjects were used regardless of their matching status to avoid potential population stratification. For each gene, each allele was categorized as allele 'x' versus all 'other' alleles, and the frequency of allele x (*HLA-A*, HLA-B, *HLA-C*, *HLA-DQB1* and *HLA-DRB1*) or the frequency of pooled genotype categories (*HLA-DRB3*, *HLA-DRB4* and *HLA-DRB5*) were compared in cases and controls using Fisher's exact test. Since this analysis was hypothesis-generating, alleles or pooled genotype categories were classified as potentially associated if they produced a p-value of 0.100 or less from two-sided testing. This liberal criterion (for example, an α-level of 0.100) was selected since the goal of this exploratory analysis was to narrow the list of alleles to be examined in future studies rather than provide definitive conclusions with respect to the non-*HLA-DRB1*9* alleles examined here and, in addition, to compensate for the small sample size and its consequent lack of power. No adjustment for multiple comparisons was made. For alleles or pooled genotype categories classified as

Table 4. Frequency of *HLA-A* alleles in cases and controls, and p-values from comparing allele frequencies.

Alleles	Cases (2*n = 52)	Controls (2*n = 148)	p-value
**1*	10 (19.2%)	24 (16.2%)	0.669
**2*	13 (25.0%)	53 (35.8%)	0.173
**3*	7 (13.5%)	17 (11.5%)	0.804
**11*	3 (5.8%)	5 (3.4%)	0.431
**23*	1 (1.9%)	3 (2.0%)	1.000
**24*	4 (7.7%)	11 (7.4%)	1.000
**25*	4 (7.7%)	1 (0.7%)	0.017
**26*	2 (3.8%)	4 (2.7%)	0.651
**29*	2 (3.8%)	8 (5.4%)	1.000
**30*	1 (1.9%)	2 (1.4%)	1.000
**31*	2 (3.8%)	2 (1.4%)	0.278
**32*	1 (1.9%)	9 (6.1%)	0.459
**33*	1 (1.9%)	1 (0.7%)	0.453
**66*	0 (0.0%)	2 (1.4%)	1.000
**68*	1 (1.9%)	6 (4.1%)	0.679

Table 5. Frequency of *HLA-B* alleles in cases and controls, and p-values from comparing allele frequencies.

Alleles	Cases (2*n = 52)	Controls (2*n = 148)	p-value
**7*	3 (5.8%)	12 (8.1%)	0.764
**8*	7 (13.5%)	19 (12.8%)	1.000
**13*	0 (0.0%)	2 (1.4%)	1.000
**14*	1 (1.9%)	3 (2.0%)	1.000
**15*	5 (9.6%)	9 (6.1%)	0.363
**18*	4 (7.7%)	12 (8.1%)	1.000
**27*	0 (0.0%)	8 (5.4%)	0.115
**35*	7 (13.5%)	15 (10.1%)	0.606
**37*	0 (0.0%)	2 (1.4%)	1.000
**38*	1 (1.9%)	3 (2.0%)	1.000
**39*	2 (3.8%)	3 (2.0%)	0.606
**40*	3 (5.8%)	9 (6.1%)	1.000
**41*	0 (0.0%)	1 (0.7%)	1.000
**44*	5 (9.6%)	21 (14.2%)	0.479
**45*	0 (0.0%)	1 (0.7%)	1.000
**47*	0 (0.0%)	1 (0.7%)	1.000
**49*	2 (3.8%)	5 (3.4%)	1.000
**50*	1 (1.9%)	2 (1.4%)	1.000
**51*	2 (3.8%)	10 (6.8%)	0.735
**52*	1 (1.9%)	0 (0.0%)	0.260
**53*	2 (3.8%)	0 (0.0%)	0.067
**55*	3 (5.8%)	4 (2.7%)	0.379
**56*	1 (1.9%)	0 (0.0%)	0.260
**57*	2 (3.8%)	6 (4.1%)	1.000

potentially associated, ORs and 90% confidence intervals (CIs) were calculated using unmatched logistic regression.

Results

HLA-DRB1*9

Table 1 presents the demographic characteristics of the matched subjects used in the analysis of *HLA-DRB1*9* and includes the underlying disease history of the CKD subjects. All cases included within this case–control study had a history of anemia due to CKD and evidence of Ab-positive PRCA. Data was collected on history of kidney dialysis and kidney transplantation; however, owing to the small sample size, valid conclusions regarding the risk of developing PRCA associated with these underlying factors could not be made. A total of 24 Ab-positive PRCA cases and 81 controls who consented to DNA testing could be matched to each other. The ages of the cases and controls were similar: cases had a mean age of 59.5 ± 18.6 years and controls had a mean age of 56.8 ± 16.0 years. A higher proportion of the cases were male (71% [17/24]) than were the controls (59% [48/81]). Most subjects were Caucasian. The distribution of cases and controls across countries differed; however, this was owing to the fact that matching could not be performed by study site for all subjects.

The frequency of the **9* allele in controls within each ethnic group (Table 2) appears to agree with the observed population frequencies seen in Asians (8.4–12.5%), American blacks (1.7–2.8%) and Caucasian Europeans or Canadians (<1%) [19], although the accuracy of the **9* allele frequency estimated in Asians and blacks is questionable owing to the limited number of Asian and black control subjects in the sample. The frequency of **9* in Ab-positive PRCA cases could only be measured in Caucasians and Asians (10.9 [5/46] and 50.0% [1/2], respectively). The frequency of the *HLA-DRB1*9* allele differed between Caucasian cases and controls (p = 0.003). The frequency of the *HLA-DRB1*9* allele did not appear to differ significantly between Asian cases and controls (p = 0.378); however, the four Asian controls and one Asian case provided very little power to compare allele frequencies. When all matched subjects were pooled, the frequency of the *HLA-DRB1*9* allele was significantly higher in cases (12.5% [6/48]) than in controls (1.2% [2/162]; p = 0.002).

Table 6. Frequency of *HLA-C* alleles in cases and controls, and p-values from comparing allele frequencies.

Alleles	Cases (2*n = 52)	Controls (2*n = 148)	p-value
*1	2 (3.8%)	4 (2.7%)	0.651
*2	2 (3.8%)	7 (4.7%)	1.000
*3	10 (19.2%)	18 (12.2%)	0.246
*4	10 (19.2%)	20 (13.5%)	0.367
*5	2 (3.8%)	15 (10.1%)	0.248
*6	3 (5.8%)	14 (9.5%)	0.567
*7	13 (25.0%)	48 (32.4%)	0.383
*8	1 (1.9%)	3 (2.0%)	1.000
*12	7 (13.5%)	5 (3.4%)	0.015
*14	0 (0.0%)	3 (2.0%)	0.569
*15	0 (0.0%)	3 (2.0%)	0.569
*16	2 (3.8%)	7 (4.7%)	1.000
*17	0 (0.0%)	1 (0.7%)	1.000

Table 7. Frequency of *HLA-DQB1* alleles in cases and controls, and p-values from comparing allele frequencies.

Alleles	Cases (2*n = 52)	Controls (2*n = 148)	p-value
*2	14 (26.9%)	31 (20.9%)	0.440
*3	28 (53.8%)	57 (38.5%)	0.072
*4	0 (0.0%)	4 (2.7%)	0.574
*5	5 (9.6%)	26 (17.6%)	0.264
*6	5 (9.6%)	30 (20.3%)	0.093

Table 8. Frequency of *HLA-DRB1* alleles in cases and controls, and p-values from comparing allele frequencies.

Alleles	Cases (2*n = 52)	Controls (2*n = 148)	p-value
*1	4 (7.7%)	19 (12.8%)	0.450
*3	9 (17.3%)	18 (12.2%)	0.353
*4	15 (28.8%)	22 (14.9%)	0.037
*7	5 (9.6%)	22 (14.9%)	0.480
*8	1 (1.9%)	4 (2.7%)	1.000
*9	5 (9.6%)	1 (0.7%)	0.005
*10	0 (0.0%)	1 (0.7%)	1.000
*11	3 (5.8%)	20 (13.5%)	0.205
*12	3 (5.8%)	5 (3.4%)	0.431
*13	4 (7.7%)	15 (10.1%)	0.786
*14	1 (1.9%)	3 (2.0%)	1.000
*15	2 (3.8%)	16 (10.8%)	0.166
*16	0 (0.0%)	2 (1.4%)	1.000

The frequencies of the **9/*9*, **9*/other and other/other genotypes were 0.0 (0/24), 25.0 (6/24) and 75% (18/24), respectively, in all matched cases and 0.0 (0/81), 2.5 (2/81) 97.5% (79/81), respectively, in all matched controls (Table 3). In all matched subjects, the OR of the **9*/other genotype was 10.8 (95% CI: 2.2–53.7; p = 0.004) when compared with the other/other genotype. The limited number of Asian, black and other subjects made it impossible to calculate an OR for the **9*/other genotype for these races. In Caucasian subjects, the frequencies of the **9*/other and other/other genotypes were 21.7 (5/23) and 78.3% (18/23), respectively, in cases and 1.3 (1/74) and 98.7% (73/74), respectively, in controls, producing an OR of 20.3 (95% CI: 2.2–184.5; p = 0.003) for the **9*/other genotype.

Exploratory analysis of other HLA *alleles*

A total of 15 *HLA-A* alleles, 24 *HLA-B* alleles, 13 *HLA-C* alleles, five *HLA-DQB1* and 13 *HLA-DRB1* alleles occurred in this sample. Allele frequencies for *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DQB1* and *HLA-DRB1* in cases and controls, and p-values for testing whether the frequencies differ, are listed in Tables 4–8, respectively. Six additional alleles were identified as being potentially associated with Ab-positive PRCA:

- Allele frequency of *HLA-A*25* was 7.7% (4/52) in cases and 0.7% (1/148) in controls (p = 0.017) (Table 4);
- Allele frequency of *HLA-B*53* was 3.8% (2/52) in cases and 0.0% (0/148) in controls (p = 0.067) (Table 5);
- Allele frequency of *HLA-C*12* was 13.5% (7/52) in cases and 3.4% (5/148) in controls (p = 0.015) (Table 6);
- Allele frequency of *HLA-DQB1*3* was 53.8% (28/52) in cases and 38.5% (57/148) in controls (p = 0.072) (Table 7);
- Allele frequency of *HLA-DQB1*6* was 9.6% (5/52) in cases and 20.3% (30/148) in controls (p = 0.093) (Table 7);
- Allele frequency of *HLA-DRB1*4* was 28.8% (15/52) in cases and 14.9% (22/148) in controls (p = 0.037) (Table 8).

The genotype frequencies of these alleles in cases and controls, and genotypic ORs, are presented in Table 9. Most alleles appear to potentially increase susceptibility to Ab-positive PRCA. For example, the OR for the *HLA-A*25* allele in **25*/other subjects compared with other/other subjects is 13.3 (90% CI: 2.0–87.1) (Table 9). One allele appeared to be potentially protective: the OR for the *HLA-DQB1*6* allele in **6/*6* and **6*/other subjects, when compared with other/other subjects, is 0.3 (90% CI: 0.1–0.8) (Table 9). For many of these

Table 9. Genotypic frequencies and odds ratios for *HLA* alleles identified as potentially associated with antibody-positive pure red-cell aplasia.

Genotype	Cases (n = 26)	Controls (n = 74)	OR	90% CI
HLA-A*25				
**25/*25*	0 (0.0%)	0 (0.0%)	NA	NA
**25*/other	4 (15.4%)	1 (1.3%)	13.3	(2.02–87.1)
Other/other	22 (84.6%)	73 (98.7%)	1.0	(1.5–2.2)
HLA-B*53				
**53/*53*	0 (0.0%)	0 (0.0%)	NA	NA
**53*/other	2 (7.7%)	0 (0.0%)	NA	NA
Other/other	24 (92.3%)	74 (100.0%)	1.0	
HLA-C*12				
**12/*12*	0 (0.0%)	0 (0.0%)	NA	NA
**12*/other	7 (26.9%)	5 (6.8%)	5.1	(1.8–14.6)
Other/other	19 (73.1%)	69 (93.2%)	1.0	
HLA-DQB1*3				
**3/*3*	8 (30.8%)	15 (20.3%)	2.8	(1.0–7.9)
**3*/other	12 (46.1%)	27 (36.5%)	2.4	(0.9–6.0)
Other/other	6 (23.1%)	32 (43.2%)	1.0	–
HLA-DQB1*6‡				
**6/*6*	1 (3.9%)	2 (2.7%)	1.0	(0.1–8.2)
**6*/other	3 (11.5%)	26 (35.1%)	0.2	(0.1- 0.7)
Other/other	22 (84.6%)	46 (62.2%)	1.0	
HLA-DRB1*4				
**4/*4*	3 (11.5%)	1 (1.4%)	11.3	(1.6–80.8)
**4*/other	9 (34.6%)	20 (27.0%)	1.7	(0.7–3.9)
Other/other	14 (53.9%)	53 (71.6%)	1.0	

*‡If *6/*6 and *6/other subjects are pooled, their frequency becomes 15.4% (4/26) in cases and 37.8% (28/74) in controls, producing an OR of 0.3 (90% CI: 0.1–0.8).*
NA: Not available.

alleles, the impact of being homozygous could not be assessed since no homozygotes for these alleles occurred in the sample (e.g., *HLA-A*25*, *HLA-B*53* and *HLA-C*12*).

A total of seven *HLA-DRB3*, two *HLA-DRB4* and three *HLA-DRB5* pooled genotype categories were found in this sample. The frequencies of the pooled genotype categories in cases and controls, and p-values for testing whether the frequencies differ, are listed in Table 10. Only one pooled genotype category was identified as potentially associated with Ab-positive PRCA: the frequency of subjects who had no *HLA-DRB4* alleles was 26.9% (7/26) in cases and 48.6% (36/74) in controls (p = 0.067) (Table 10), producing an OR of 2.6 (90% CI: 1.1–5.8) for subjects who were either **1/*1* or **1*/absent when compared with subjects who had no *HLA-DRB4* alleles.

Discussion

Presented here are the results of a pharmacogenetic case–control study of the association between HLA alleles and Ab-positive PRCA. The results of this study agree with Praditpornsilpa *et al.*'s observation that the *HLA-DRB1*9* allele occurs at an elevated frequency in subjects with Ab-positive PRCA [17]. In this study, the odds of developing Ab-positive PRCA for patients with the *HLA-DRB1*9* allele was approximately 11-times higher than for subjects who did not have the allele. However, the presence of the *HLA-DRB1*9* allele is neither necessary nor sufficient to cause Ab-positive PRCA: most of the Ab-positive PRCA cases in the study did not have the **9* allele and not all subjects with the **9* allele developed Ab-positive PRCA.

The exact mechanism by which HLA-DRB1*9 might increase susceptibility to PRCA is currently unknown. However, the HLA

Table 10. Frequencies of pooled genotype categories in cases and controls, and p-values from comparing pooled genotype categories.

Pooled genotype category	Cases (n = 26)	Controls (n = 74)	p-value
HLA-DRB3			
**1/*1* or **1*/absent	7 (26.9%)	14 (18.9%)	0.410
**1/*2*	0 (0.0%)	1 (1.4%)	1.000
**1/*3*	0 (0.0%)	2 (2.7%)	1.000
**2/*2* or **2*/absent	6 (23.1%)	29 (39.2%)	0.159
**2/*3*	2 (7.7%)	1 (1.4%)	0.165
**3/*3* or **3*/absent	2 (7.7%)	2 (2.7%)	0.277
Absent/absent	9 (34.6%)	25 (33.8%)	1.000
***HLA-DRB4*‡**			
**1/*1* or **1*/absent	19 (73.1%)	38 (51.4%)	0.067
Absent/absent	7 (26.9%)	36 (48.6%)	0.067
HLA-DRB5			
**1/*1* or **1*/absent	2 (7.7%)	15 (20.3%)	0.225
**2/*2* or **2*/absent	0 (0.0%)	2 (2.7%)	1.000
Absent/absent	24 (92.3%)	57 (77.0%)	0.144

‡*The OR for subjects who were* *1/*1 *or* *1*/absent for* HLA-DRB4*, when compared with subjects who had no* HLA-DRB4 *alleles, was 2.6 (90% CI: 1.1–5.8).*

system plays a central role in immune response by presenting peptides derived from extracellular proteins for T-cell activation. The scientific literature to date suggests that all exogenous proteins have the potential to elicit an immune response, particularly when administered via the subcutaneous route [15]. Prior studies of HLA association and drug hypersensitivity have been recently reviewed [36] and current hypotheses suggest that drug-induced hypersensitivity involves the interaction of specific MHC molecules (e.g., HLA-DRB1*9), the T-cell receptor and the drug itself. Since most cases have been confined to a single country (Thailand), additional factors (genetic, environmental) are clearly contributing to the development of Ab-positive PRCA. Although it seems reasonable to hypothesize that the higher incidence of Ab-positive PRCA in the Thai population may, in part, be explained by the higher frequency of the HLA-DRB1*9 allele, the clinical relevance of this finding is unclear. First, owing to the extremely low incidence of Ab-positive PRCA, the number of cases was quite small. As a result, the CI for the OR is quite wide, reflecting a great deal of uncertainty in the estimate. Second, most subjects in this analysis were European or Canadian Caucasians, and other genetic and nongenetic differences between these subjects and Thai Asians, which were unaccounted for in this study, impact our ability to make inferences from one population to the other. Third, these subjects received a formulation of EPREX that has since been withdrawn from the market owing to its demonstrated immunogenic potential. Fourth, other Asian populations also have relatively high frequencies of HLA-DRB1*9, such as the Chinese Hong Kong/Singapore population (15.6%) and American Asians (11.1%) [19], yet elevated rates of Ab-positive PRCA are not observed. This suggests that other unknown factors unique to Thailand are contributing to the elevated Ab-positive PRCA incidence rate in that country.

Besides confirming the association between *HLA-DRB1*9* and Ab-positive PRCA, an additional six *HLA* alleles were identified as potentially associated with Ab-positive PRCA. Two of these alleles, *HLA-DRB4*1* and *HLA-DQB1*3*, are known to be in strong linkage disequilibrium with *HLA-DRB1*9* so it is not surprising that these alleles were identified as potentially associated with Ab-positive PRCA [37]. Given the large number of alleles examined here and the liberal criteria used to classify alleles as potentially associated, undoubtedly the association between Ab-positive PRCA and many of the additional alleles will not replicate in future studies. However, since many *HLA* genes have large numbers of alleles (for example, *HLA-B* has over

850 alleles [102]) it is important to provide data that will allow researchers to more narrowly focus future HLA-related studies of Ab-positive PRCA, since Ab-positive PRCA is an extremely rare event that limits the number and size of future studies.

Conclusion

In conclusion, we have presented results from a pharmacogenomic case–control study showing an association between *HLA-DRB1*9* and Ab-positive PRCA. Our results confirm the observation from a previous case series and identify additional putative genetic associations for future research.

Future perspective

Although this study indicates an association of PRCA with *HLA-DRB1*9*, the limitations of the study, the modest magnitude of the odds ratio and the rarity of PRCA suggest that monitoring for *HLA-DRB1*9* would not be clinically useful. Future work may further clarify the causes of the persistently high PRCA rate observed uniquely in Thailand.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Incidence of antibody-positive pure red-cell aplasia following epoetin treatment

- Antibody (Ab)-positive pure red-cell aplasia (PRCA) is a severe hematological disorder characterized by the production of antibodies that inhibit the activity of endogenous and recombinant human erythropoietin.
- The incidence of reported Ab-positive PRCA cases in chronic kidney disease patients receiving subcutaneous EPREX® increased in the period between 1999 and 2004. Except in Thailand, this incidence decreased to baseline when FluroTec®-coated stoppers were introduced.

Genetic association of* HLA *alleles with antibody-positive PRCA

- The primary goal of this analysis was to test the reported association between *HLA-DRB1*9* and Ab-positive PRCA. A secondary goal was to perform an exploratory analysis in order to identify additional HLA alleles potentially associated with Ab-positive PRCA.
- Subjects were taken from a retrospective case–control study designed to identify potential risk factors for Ab-positive PRCA.
- Genotyping to detect the *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DQB1*, *HLA-DRB1*, *HLA-DRB3*, *HLA-DRB4* and *HLA-DRB5* alleles was completed and statistical analysis was performed utilizing Fisher's exact test to compare cases and controls to identify genetic associations with Ab-positive PRCA.

HLA *alleles associated with antibody-mediated PRCA*

- In Caucasian subjects, the frequencies of the *HLA-DRB1*9*/other and other/other genotypes were 21.7 and 78.3%, respectively, in cases and 1.3 and 98.7%, respectively, in controls, producing an OR of 20.3 (95% CI: 2.2–184.5; p = 0.003) for the **9*/other genotype.
- Six additional alleles were identified as being potentially associated with Ab-positive PRCA.

Conclusion

- In this study, the odds of developing Ab-positive PRCA for patients with the *HLA-DRB1*9* allele was approximately 11-times higher than for subjects who did not have the allele.
- However, the presence of the *HLA-DRB1*9* allele is neither necessary nor sufficient to cause Ab-positive PRCA and other unknown factors unique to Thailand are likely to be contributing to the elevated Ab-positive PRCA incidence seen in that country.
- Our results confirm results from a previous case series and identify additional putative associations for future research.

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