

## Unravelling an HLA-DR Association in Childhood Acute Lymphoblastic Leukemia

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Genetic and environmental factors play an interactive role in the development of childhood acute lymphoblastic leukemia (ALL). Since the demonstration of a major histocompatibility complex (MHC) influence on mouse leukemia in 1964, an HLA association has been considered as a possible genetic risk factor. Despite extensive efforts, however, no strong evidence comparable to the H-2<sup>k</sup> influence on mouse leukemia has been shown. The number of negative serological studies resulted in a loss of interest and consequently, no molecular HLA-DR association study has been published to date. We reconsidered the HLA-DR association in childhood ALL in 114 patients from a single center and 325 local newborn controls by polymerase chain reaction (PCR) analysis of the HLA-DRB1/3/4/5 loci. With conventional analysis, there was a moderate allelic association with the most common allele in the HLA-DR53 group, HLA-DRB1\*04, in the whole group that was stronger in males ( $P = .0005$ , odds ratio = 2.9). When the other expressed HLA-DRB loci were

examined, homozygosity for HLA-DRB4\*01, encoding the HLA-DR53 specificity, was increased in patients (21.1% v 8.3%; odds ratio = 2.9,  $P = .0005$ ). Consideration of gender showed that all of these associations were reflections of a male-specific increase in homozygosity for HLA-DRB4\*01 (32.8% v 4.0%; odds ratio = 11.7, 95% confidence interval [CI] = 4.9 to 28.0;  $P = 3 \times 10^{-8}$ ). This highly significant result provided the long-suspected evidence for the HLA-DR influence on the development of childhood ALL while confirming the recessive nature of the MHC influence on human leukemogenesis as in experimental models. The cross-reactivity between HLA-DR53 and H-2E<sup>k</sup>, extensive mimicry of the immunodominant epitope of HLA-DR53 by several carcinogenic viruses, and the extra amount of DNA in the vicinity of the HLA-DRB4 gene argue for the case that HLA-DRB4\*01 may be one of the genetic risk factors for childhood ALL.

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**A**CUTE LYMPHOBLASTIC leukemia (ALL) is the most common cancer in children.<sup>1</sup> Large epidemiological studies have suggested some of the factors involved in susceptibility,<sup>2-4</sup> with most consistent ones being an association with a history of maternal fetal loss<sup>5-7</sup> and a putative infection in the etiology.<sup>8-10</sup> It is now generally agreed that both genetic and environmental factors play an interactive role in the development of childhood ALL, particularly in the common ALL (cALL) subtype.<sup>4</sup>

The involvement of the HLA system has also been examined in childhood ALL following the demonstration of a major histocompatibility complex (MHC) influence on the development of mouse leukemia.<sup>11</sup> In 1964, Lilly et al<sup>11</sup> reported an increased risk of spontaneous and virus-induced leukemia in congenic mice homozygous for the H-2<sup>k</sup> haplotype. Although, human studies in leukemia have not shown a consistent association, they strongly suggested a recessive influence as in the experimental models.<sup>12</sup> The serological studies in childhood ALL point to an association with HLA-A2.<sup>13</sup> Despite the excessive number of serological studies on the HLA-DR locus, no polymerase chain reaction (PCR)-based investigation of this

locus in childhood ALL has been published. The only HLA-DR/DQ association was inferred as a male-specific homozygosity for the HLA class II supertype DR53 from HLA-DQA restriction fragment length polymorphism (RFLP) analysis of 63 patients and an adult control group.<sup>14</sup> It is important to examine the HLA-associated susceptibility to childhood ALL, as this may provide clues to leukemogenesis in general and to the role of other risk factors. For example, it has been repeatedly suggested that an HLA class II association with childhood leukemia would provide support for a viral link in the etiology.<sup>4,15</sup> On the other hand, we pointed out that an HLA association may be the common immunogenetic basis for leukemia susceptibility and increased reproductive failure rate in leukemic families.<sup>12</sup> Indeed, both childhood leukemia<sup>16</sup> and spontaneous recurrent miscarriages<sup>17,18</sup> are associated with increased parental HLA-DR compatibility.

The supertypic specificity HLA-DR53 is encoded by the HLA-DRB4 locus in the HLA class II region. HLA-DRB4 is one of the expressed HLA loci, which exists only on haplotypes possessing HLA-DRB1\*04, DRB1\*07, and DRB1\*09. The expression level of HLA-DRB4 is variable,<sup>19</sup> and it is not expressed only by the HLA-B57,DR7,Dw11 haplotype.<sup>20</sup> The HLA-DRB4 gene or its protein product HLA-DR53 have been associated with increased risk for all major types of leukemia.<sup>14,21-24</sup> Most HLA-DR53 haplotypes carry HLA-A2, which is the other risk factor.<sup>13</sup>

In the present study, we have investigated all expressed HLA-DRB loci in a large group of patients using local newborns as controls by directly typing the HLA-DRB1/3/4/5 loci by PCR analysis. The data, therefore, can be used to estimate the relative risk of developing childhood ALL for a newborn baby. The results showed a highly significant association of a homozygous HLA-DR genotype in childhood ALL with a strong gender effect. This association was not correlated with the age groups or disease subtypes.

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## MATERIALS AND METHODS

**Controls.** Random, anonymous umbilical cord blood samples were obtained from babies born in the University Hospital of Wales and Llandough Hospital in Cardiff over a period of 12 months. It was not practically possible to obtain samples from each and every newborn over this period, but no newborn baby was excluded on the basis of any selection criteria. The samples were collected until the number in each sex group exceeded 150. In the final group of 325 newborns, there were 150 boys and 175 girls.

**Patients.** The patient group consisted of 114 patients (61 boys and 53 girls;  $\leq 14$  years) with childhood ALL consecutively diagnosed in Cardiff since 1988. Cross-checking with the Wales Leukemia Registry showed that five samples were missing in the study group. Non-Caucasoid patients were not excluded ( $n = 4$ ). The number of patients with cALL was 77 (40 boys and 37 girls). The study was performed in accordance with the guidelines set up by the local ethics committee.

**DNA extraction.** DNA from patients diagnosed before 1996 was extracted by the salting-out method.<sup>25</sup> DNA from more recent leukemia samples and all newborn samples were prepared from peripheral blood using the QIAamp Midi-Blood kit (Qiagen, West Sussex, UK).

**HLA-DRB typing.** All patients and newborn controls were typed using the Biotest DRB-ELPHA (enzyme-linked probe hybridization assay) kit (Biotest, West Midlands, UK) according to the manufacturer's instructions. This kit allows low-resolution DNA-typing of all expressed HLA-DRB loci (DRB1/3/4/5). No further subtyping of any allele was attempted.

**Determination of homozygosity for HLA-DR supertypes.** HLA class II supertypes are not allelic with each other and some haplotypes even lack a supertype. Because of this, to determine homozygosity for HLA class II supertypes (homozygosity for HLA-DRB3, -DRB4, or -DRB5), HLA-DRB1 typing was necessary. A sample was assigned as homozygous for HLA-DRB4\*01 (HLA-DR53) when no other supertype was detected and the HLA-DRB1 type consisted of any combination of HLA-DRB1\*04, \*07 or \*09. Because no further allelic subtypic was performed, this meant having a double dose of the HLA-DRB4 gene. A sample was assigned as HLA-DR52 homozygous if the HLA-DRB1 type consisted of only HLA-DRB1\*03, \*11, \*12, \*13, \*14. Those typed as having only HLA-DRB1\*15 and/or \*16 were HLA-DR51 homozygous.

**HLA-Bw4/6 typing.** The supertypes of the HLA-B locus in the class I region were typed by PCR analysis using allele-specific primers.<sup>26</sup> PCR products were visualized by ethidium bromide on 2% agarose gels.

**HSP70-2 typing.** Patients and controls were typed by PCR analysis for a biallelic (183 bp and 188 bp) polymorphism arising from a pentanucleotide duplication linked to 3' untranslated region (UTR) of the HSP70-2 gene.<sup>27</sup> The presence of the duplication (188 bp) associates most commonly with the presence of the *Pst*I site in HSP70-2 (ie, the 8.5-kb *Pst*I/RFLP allele).<sup>27</sup> The primers and the reaction conditions were as described by Dressel and Gunther.<sup>27</sup> PCR products were analyzed on a 2.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet (UV) illumination.

**Determination of sex.** Documented gender was checked on the HLA-DRB4\*01 homozygous samples using a Y-chromosome-specific PCR assay.<sup>28</sup> The primer sequences were:

Y1.1 5'-tccactttattccagcctcttc

Y1.2 5'-ttgaatggaatggaacgaatgg.

Amplification reactions were set up in 50  $\mu$ L volume containing 1.5 mmol/L magnesium chloride. For amplification, samples were first heated at 95°C for 7 minutes and then a two-step cycle consisting of 1.5 minutes at 92°C and 1.5 minutes at 63°C was used for 25 cycles. Five microliters of the reaction mixture was used for direct visualization by ethidium bromide on agarose gel.

**Statistical analysis.** Comparisons between two observed frequencies were made using the  $\chi^2$  test (with Yates' correction when a cell frequency was less than 20) unless any expected frequency was less than 5, in which case, Fisher's exact test was used. All *P* values are two-tailed for one degree of freedom. The odds ratio (OR) was calculated as  $OR = ad/bc$  where a,b,c,d are the entries to the  $2 \times 2$  table. The 95% confidence interval (CI) of the OR was calculated as  $OR^{(1 \pm 1.96/X)}$  (*X* is the square-root of the *X*<sup>2</sup>), the 95% CI of a single proportion was calculated as  $p \pm 1.96 \cdot \sqrt{p(1-p)/n}$ .<sup>29</sup> The deviation of an observed homozygosity rate from the Hardy-Weinberg expectation (*p*<sup>2</sup>) was tested by the two-tailed Z-test for a single proportion.

Although the statistical analysis included multiple and subgroup comparisons, the conventional statistical safeguards were not applied because of the magnitude of the *P* value for the main association and because the same association has been noted before.

## RESULTS

**Conventional analysis.** This included the comparison of frequencies for HLA-DRB1 alleles in patients and controls. The allele frequencies (proportion of subjects possessing the relevant allele) instead of gene frequencies were examined as recommended for HLA-disease association studies.<sup>30</sup> When this was done for the 13 classical HLA-DRB1 alleles and three supertypes (Tables 1 and 2), a weak association was found for HLA-DRB1\*04 (-DR4) between the patients and controls (*P* = .02; OR = 1.7; 95% CI = 1.1 to 2.6). The increase in the allele frequency was restricted to males (*P* = .0005; OR = 2.9; 95% CI = 1.6 to 5.4). Homozygosity for HLA-DRB1\*04 was more markedly increased in male patients compared with the male newborns (*P* = .003; OR = 9.6; 95% CI = 2.1 to 43.5). As shown below, these were not independent associations.

**Analysis of supertypes.** Despite no significant change in supertypic allele frequencies between patients and controls (*P* = .42), there was an increase in homozygosity for HLA-DRB4\*01 (-DR53) in patients (Table 2). The increase in the whole group yielded an OR of 2.9 (*P* = .0003; 95% CI = 1.7 to 5.2). The difference was again mainly due to a high homozygosity rate in male patients (OR = 11.7, 95% CI = 4.9 to 28.0, *P* =  $3 \times 10^{-8}$ ). No significant change was observed for girls with the same genotype (7.5% v 12.0%; *P* = .51). None of the four non-Caucasoid patients was homozygous for HLA-DRB4\*01 (they were not excluded from the analysis). Because HLA-DRB1\*04 is the most common member of the DR53 supertypic group, its association was thought to be secondary to the association of HLA-DRB4\*01.

There was no correlation between the age and the homozygosity rate. In particular, it was not significantly different in the childhood ALL peak age (24 to 60 months; *n* = 61) compared with the rest of the group (16.4% v 26.4%; *P* = .28). The same comparison only for boys (30% v 35.5%; *P* = .85) did not show any difference either.

To see whether a sampling error in the control group has caused this association, the homozygosity rate in boys with ALL was compared with that of the RFLP-defined healthy adult males (*n* = 449) of our previous study. The result was again statistically significant (32.8% v 14.5%, *P* = .0006). The comparison was also made to the female-specific newborn homozygosity rate (32.8% v 12.0%, *P* = .0002). Thus, it was clear that the association was due to an increased homozygosity

**Table 1. Allele Frequencies and Homozygosity Rates (%) in the HLA-DRB1 Locus**

|       | Newborns<br>n = 325 | Males<br>n = 150 | Females<br>n = 175 | Patients<br>n = 114 | Males<br>n = 61 | Females<br>n = 53 |
|-------|---------------------|------------------|--------------------|---------------------|-----------------|-------------------|
| DR1   |                     |                  |                    |                     |                 |                   |
| AF    | 20.6                | 19.3             | 21.7               | 12.0                | 11.5            | 26.4              |
| HR    | 0.3                 | 0.0              | 0.6                | 1.8                 | 0.0             | 3.8               |
| DR2*  |                     |                  |                    |                     |                 |                   |
| AF    | 30.5                | 15.7             | 27.4               | 36.0                | 29.5            | 43.4              |
| HR    | 4.3                 | 6.0              | 2.9                | 2.6                 | 3.3             | 1.9               |
| DR3†  |                     |                  |                    |                     |                 |                   |
| AF    | 28.3                | 28.7             | 28.0               | 23.7                | 24.6            | 24.5              |
| HR    | 3.4                 | 2.0              | 4.6                | 0.9                 | 0.0             | 1.9               |
| DR4   |                     |                  |                    |                     |                 |                   |
| AF    | 29.5                | 27.3             | 31.4               | 41.2                | 52.5            | 28.3              |
| HR    | 1.5                 | 1.3              | 1.7                | 7.9                 | 11.5            | 3.8               |
| DR5*‡ |                     |                  |                    |                     |                 |                   |
| AF    | 16.0                | 19.3             | 13.1               | 11.4                | 9.8             | 13.3              |
| HR    | 4.6                 | 4.7              | 4.6                | 4.4                 | 3.3             | 5.7               |
| DR6*‡ |                     |                  |                    |                     |                 |                   |
| AF    | 27.4                | 29.3             | 25.7               | 17.5                | 16.4            | 18.9              |
| HR    | 4.0                 | 2.7              | 5.1                | 3.5                 | 4.9             | 1.9               |
| DR7   |                     |                  |                    |                     |                 |                   |
| AF    | 26.8                | 23.3             | 29.7               | 25.4                | 29.5            | 20.8              |
| HR    | 1.2                 | 0.0              | 2.3                | 1.8                 | 3.3             | 0.0               |
| DR8   |                     |                  |                    |                     |                 |                   |
| AF    | 3.4                 | 2.7              | 4.0                | 6.1                 | 3.3             | 9.4               |
| DR9   |                     |                  |                    |                     |                 |                   |
| AF    | 2.8                 | 3.3              | 2.3                | 2.6                 | 3.3             | 1.9               |
| DR10  |                     |                  |                    |                     |                 |                   |
| AF    | 2.5                 | 2.6              | 2.3                | 1.8                 | 1.6             | 1.9               |

Homozogosity rates were not shown when there was no more than one homozogotes in any group.

Abbreviations: AF, allele frequency; HR, homozogosity rate.

\*Because of low frequencies of DRB1\*12, \*14 and \*16, the alleles of the classical DR types DR2, DR5 and DR6 were grouped together.

†The typing system used cannot distinguish between DRB1\*17 and DRB1\*18.

‡DRB1\*11, \*12 in the DR5 group, and DRB1\*13, \*14 in the DR6 group did not show any significant change separately. For *P* values, see text.

rate in male patients, but not the low homozogosity rate in newborn boys.

The previous RFLP-based study comprised the first 63 patients of the current group. In the additional 51 patients diagnosed since then, there were 25 boys. The homozogosity rate for HLA-DRB4\*01 in this group was 40.0%. This was significantly higher from the newborn controls as a separate group ( $P = 3 \times 10^{-6}$ ). Therefore, if the new patients were treated as an independent group, the results of the two separate groups were in agreement.

Another significant deviation between patients and controls was found in the analysis of supertypes. The allele frequency of HLA-DR52 (the frequency of possessing the HLA-DRB3 gene regardless of its alleles) was decreased in the whole group of patients ( $P = .003$ ), but mainly in males ( $P = .0009$ , OR = 0.4, 95% CI = 0.2 to 0.7) and not in females. These deviations were not due to changes in homozogosity rates for HLA-DR52, as the decrease in homozogosity was not statistically significant. These findings suggested that a dominant protection was conferred by HLA-DR52 in line with our previous finding in

chronic lymphocytic leukemia (CLL).<sup>24</sup> Although significant, this negative association at the allelic level should be interpreted with caution, as it may be the mirror image of the positive association with a homozogous genotype.

**Homozogosity rate for HLA-DRB4\*01 and gender.** As shown in Table 2, the distribution of homozogosity for HLA-DRB4\*01 between genders was different for patients and newborns. In the patients' group, 20 of 24 homozogotes were male (sex-ratio = 0.83), whereas the proportion of males in newborns was 6 of 27 giving a sex-ratio of 0.22 ( $P = .00002$ ). This skewed sex-ratio for homozogotes resulted in significant differences in gender-specific homozogosity rates for the leukemia susceptibility genotype in patients ( $P = .001$ ) and in controls ( $P = .016$ ). The decreased homozogosity rate for HLA-DRB4\*01 in newborn boys was tested against the expected frequency calculated from the gene frequency in newborns (30.9%). The male-specific homozogosity rate in newborns (4.0%) was significantly lower than the expected rate (9.6%, 95% CI = 6.4 to 12.8;  $P = .02$ ).

**Homozogosity for HLA-DRB4\*01 in the cALL group.** Because of the possible immunologic and genetic differences in the etiology of cALL, this group was analyzed separately for the main HLA association. In the whole group, the homozogosity rate for HLA-DRB4\*01 was 23.4% (8.3% in controls,  $P = .0004$ , OR = 3.4; 95% CI = 1.7 to 6.6). Again, this was due to an increased homozogosity rate in boys with cALL (37.5% v 4.0% in newborn boys,  $P = 2 \times 10^{-6}$  [Fisher], OR = 14.4, 95% CI = 5.8 to 35.9). Homozogosity in female patients with cALL was not significantly different from female newborns (8.1% v 12.0%,  $P = .58$ ).

**Homozogosity for HLA-DRB4\*01 in the non-cALL group.** In the group of 37 patients who had non-cALL, the homozogosity rate was 16.2% ( $P = .12$  for comparison with newborns); and the male-specific homozogosity rate for patients with non-cALL ( $n = 21$ ) was 23.8% ( $P = .005$ ; OR = 7.5 for comparison with newborn boys; 95% CI = 2.0 to 28.1). Thus, the association seemed to be stronger for cALL, but the number of patients with non-cALL is too small to reach a firm conclusion.

**Allele and genotype frequencies for HLA-B supertypes and HSP70-2 alleles.** The only slight differences concerned the HSP70 alleles (Table 3). These changes were interpreted as

**Table 2. Allele Frequencies and Homozygosity Rates (%) in the HLA-DRB3/4/5 Loci**

|      | Newborns<br>n = 325 | Males<br>n = 150 | Females<br>n = 175 | Patients<br>n = 114 | Males<br>n = 61 | Females<br>n = 53 |
|------|---------------------|------------------|--------------------|---------------------|-----------------|-------------------|
| DR51 |                     |                  |                    |                     |                 |                   |
| AF   | 30.5                | 34.0             | 27.4               | 36.0                | 29.5            | 43.4              |
| HR   | 4.3                 | 6.0              | 2.9                | 2.6                 | 2.3             | 3.8               |
| DR52 |                     |                  |                    |                     |                 |                   |
| AF   | 60.1                | 67.3             | 55.4               | 44.7                | 42.6            | 47.2              |
| HR   | 15.7                | 12.7             | 18.3               | 8.8                 | 6.6             | 11.3              |
| DR53 |                     |                  |                    |                     |                 |                   |
| AF   | 53.6                | 51.3             | 55.4               | 57.9                | 67.2            | 47.2              |
| HR   | 8.3                 | 4.0              | 12.0               | 21.1                | 32.8            | 7.5               |

Homozogosity for HLA-DR51, -DR52, -DR53 correspond to double dose of HLA-DRB5, -DRB3, -DRB4, respectively (see Materials and Methods); for *P* values, see Results.

Abbreviations: AF, allele frequency; HR, homozogosity rate.

**Table 3. Allele Frequencies (%) and Homozygosity Rates (%) for HLA-Bw4/6 and HSP183/188 Alleles**

|                | Newborns<br>n = 325 | Males<br>n = 150 | Females<br>n = 175 | Patients<br>n = 114 | Males<br>n = 61 | Females<br>n = 53 |
|----------------|---------------------|------------------|--------------------|---------------------|-----------------|-------------------|
| <b>Bw4</b>     |                     |                  |                    |                     |                 |                   |
| AF             | 56.6                | 54.7             | 46.9               | 59.6                | 63.9            | 54.7              |
| HR             | 10.8                | 9.3              | 12.0               | 9.6                 | 13.1            | 5.7               |
| <b>Bw6</b>     |                     |                  |                    |                     |                 |                   |
| AF             | 89.5                | 90.7             | 88.6               | 90.4                | 86.9            | 94.3              |
| HR             | 43.7                | 45.3             | 42.3               | 40.4                | 36.1            | 45.3              |
| <b>HSP-188</b> |                     |                  |                    |                     |                 |                   |
| AF             | 59.4                | 59.3             | 59.4               | 50.0                | 49.2            | 50.9              |
| HR             | 15.7                | 19.3*            | 12.6               | 8.8                 | 6.6             | 11.3              |
| <b>HSP-183</b> |                     |                  |                    |                     |                 |                   |
| AF             | 84.0                | 80.0             | 87.4               | 91.2                | 93.4†           | 88.7              |
| HR             | 40.3                | 40.6             | 40.6               | 50.0                | 50.8            | 49.1              |

Abbreviations: AF, allele frequency; HR, homozygosity rate.

\*Higher than the homozygosity rate in male patients ( $P = .02$ ).

†Higher than the allele frequency in male-newborns ( $P = .03$ ). None of the homozygosity rates in newborns was significantly different from the expected homozygosity rates (11.4% for Bw4, 44.0% for Bw6, 14.3% for HSP-188, and 38.7% for HSP-183).

secondary to the linkage disequilibrium between HSP188 and HLA-DR52 and HSP-183 and HLA-DR53 (data not shown). More importantly, comparisons between the observed homozygosity rates in newborns and expected homozygosity rates did not show any violation of the Hardy-Weinberg equilibrium in these loci (unlike the HLA-DRB4 locus).

#### DISCUSSION

This is the first PCR-based HLA-DR association study in childhood ALL, which analyzed more than 100 patients and controls from a single center using the same technique. It showed that the odds ratio to develop childhood ALL is 11.7 for a newborn boy having a double dose of the HLA-DRB4\*01 gene with a negligible probability of error. We unravelled this highly significant association by taking into account the supertypes, homozygosity, and gender. While one third of boys with ALL had this genotype, in newborns, the same genotype had a decreased frequency in boys.

The lack of strong allelic associations in the DRB1 locus partly explains why previous studies did not find the same association. Since the demonstration of the MHC influence on the development of mouse leukemia in 1964,<sup>11</sup> a number of studies have investigated HLA associations in childhood and adult leukemias, some of which included the analysis of HLA-DR/DQ antigens or genes.<sup>14-16,21-24,31-41</sup> With a few exceptions, these studies did not report an HLA-DR association as strong as the present study has found. This may have been due to the technical difficulties to detect supertypes and their subsequent exclusion from the analysis.

Among the above-mentioned studies, when HLA-DR53 was investigated, an association or a hint of it was usually found. The earliest HLA-DR studies in childhood ALL showed an increase for HLA-DR7.<sup>31-33</sup> In one study, adult ALL showed a weak association with HLA-DR4<sup>37</sup> and in another, CLL was associated with HLA-DR53.<sup>22</sup> The most convincing result on the relevance of HLA-DRB4\*01/DR53 as a risk factor for leukemia was presented by Seremetis et al.<sup>21</sup> They used a

monoclonal antibody specific for the hypervariable region 3 (HVR3) epitope of HLA-DR53 and found a relative risk of 7.9 ( $P < .000005$ ) for acute myeloblastic leukemia (AML). Also, in our other molecular studies in ALL, CML, and CLL, the homozygous genotype for HLA-DR53 had an increased frequency in patients either by RFLP<sup>14,23</sup> or PCR analysis.<sup>24</sup>

Although no other molecular study has investigated the same locus in childhood leukemia, a parallel between our results and mouse models can be postulated. The first study on the influence of the MHC on the development of virus-induced and spontaneous leukemia in congenic mice found a strong influence of a homozygous MHC genotype.<sup>11</sup> Since then, the influence of homozygosity for the H-2<sup>k</sup> haplotype has been confirmed in many other studies,<sup>42-44</sup> and one of the susceptibility loci has been mapped to the class II region of this haplotype.<sup>44,45</sup> Similar to the lack of any increase in the allele frequency of HLA-DRB4\*01 in childhood leukemia, heterozygosity for the H-2<sup>k</sup> haplotype has no effect on mouse leukemogenesis. Similarities extend further by the observation that a monoclonal antibody specific for the class II supertype of the H-2<sup>k</sup> haplotype is cross-reactive with the human HLA-DR53 specificity.<sup>46</sup>

In a study of a polymorphic system, chance deviations in the genotype frequencies may result in spurious associations. This possibility was considered in the analysis of the data. The high homozygosity rate for HLA-DRB4\*01 in boys with ALL is in accord with our earlier finding inferred from HLA-DQA1 RFLP analysis in a smaller group. Although the sample size of 61 for boys with ALL is still relatively small, this includes all patients diagnosed in the last 10 years and could not be increased in a single-center study. Suffice it to say that if there were to be no more homozygotes in the next 61 male patients with ALL, the association would still be significant (hypothetical  $P$  value = .001). Another finding suggesting the male-specificity of the association with HLA-DRB4\*01 is that the genotypes of the other two loci in the class I and class III regions did not show any change in patients or any sex-specific difference in allele or genotype frequencies. It was not possible to have an age- and sex-matched control group, which would have been ideal. Although, the association seemed to be primarily due to an excess of homozygotes for HLA-DRB4\*01 in male patients, a possible age-related change in the frequency of this genotype in healthy children may have contributed to the magnitude of this association and should be considered in the interpretation of the results.

The gender effect we observed has not been reported in HLA association studies in leukemia before. There is, however, a similar observation in rheumatoid arthritis (RA), which is also associated with HLA-DRB4\*01 and, the main member of this supertypic family, HLA-DRB1\*04. The strongest susceptibility genotype for RA is compound heterozygosity for HLA-DR4 (HLA-DRB1\*0401/\*0404), which is a homozygous genotype for HLA-DRB4\*01. In a disease, which predominantly affects females, this particular genotype occurs preferentially in young males in two different populations including Britain.<sup>47,48</sup> In another study, homozygosity for HLA-DR53, detected by RFLP analysis, showed a male-specific decline with age.<sup>49</sup> It appears that this homozygous genotype is associated with deleterious effects only in young males, the reasons for which are, at present, unknown.

The decreased frequency of the homozygous leukemia susceptibility genotype in newborn boys suggested an association of reproductive failure with this genotype. If this is the case, prenatal selection against a homozygous HLA genotype is not surprising, as parental sharing of HLA-DR alleles is a known risk factor for both reproductive failure<sup>17,18,50,51</sup> and childhood ALL.<sup>16</sup> To date, no particular allele has been shown to be responsible for this effect. What the present study adds to our knowledge is a possible gender effect in the influence of HLA-DR homozygosity on reproductive failure. If our finding is confirmed by larger studies, this would favor the genetic hypothesis (presence of HLA-linked recessive lethal genes) without arguing against the immunological hypothesis (deleterious effect of fetomaternal HLA compatibility) of the immunogenetic background of reproductive failure.

The genetic hypothesis proposes that HLA-linked recessive lethal genes cause pregnancy failure in the case of homozygosity for them. The typical example of this is the lethal alleles of the mouse *t*-complex. In mice bearing two *t*-haplotypes from different complementation groups, some fetus may survive, whereas all fetuses homozygous for the same lethal *t*-haplotype die. In the group of *t6/tw5* heterozygotes bearing a double dose of a recessive lethal allele, sex affects the lethality and a deficit of males among live births has been noted in independent studies.<sup>52,53</sup> Interestingly, the MHCs embedded into *tw5* and *t6*-haplotypes share a high level of homology detected at the DNA level.<sup>54</sup> The male-specificity of fetal loss due to recessive lethals in or around MHC is not then unprecedented, but our finding remains to be confirmed in human studies.

Because miscarriages and childhood leukemia tend to occur in the same families<sup>5-7</sup> and parental HLA sharing is a risk factor for each condition separately, it is plausible that the same HLA genotype may be a risk factor for both conditions. It is particularly important that the ongoing US Childrens Cancer Group case-control study has so far identified only the maternal history of fetal loss as a risk factor for childhood ALL.<sup>3,7</sup> This connection is further supported by the reports that survivors of threatened abortions are at a higher risk to develop childhood leukemia.<sup>5,6</sup> In the most recent study, mothers had a history of at least one fetal loss almost in one third of childhood ALL cases.<sup>7</sup> Another line of support is the well-known association of HLA-DR53 or its members (HLA-DR4 and -DR7) with antiphospholipid antibody syndrome.<sup>55</sup> This antibody is present in 15% of women with a recurrent miscarriage history and usually causes early (first trimester) miscarriages in 90% of them.<sup>56</sup> All of these findings provide circumstantial evidence that the leukemia susceptibility genotype may be responsible for the increased miscarriage incidence in leukemic families. The very low sex-ratio in newborns homozygous for HLA-DRB4\*01 coupled with a very high ratio in ALL suggested the association of this genotype with reproductive failure and an increased risk of ALL among those who survived the prenatal challenge. This scenario is in agreement with the model proposing that childhood malignancies and reproductive failure share the same genetic background.<sup>12,57,58</sup>

Although the human MHC has been extensively examined, it may still be possible that an unknown gene linked to HLA-DRB4 may be responsible for the deleterious effects of the susceptibility genotype. The 110-kb to 160-kb extra amount of

DNA in the class II region exclusive to the DR53 haplotypes may harbor a recessive lethal/deleterious gene.<sup>59-61</sup> To the best of our knowledge, this area on this haplotype has not been sequenced as part of the human genome project. Our results, together with those of the other studies,<sup>21,23,24,49</sup> indicate a need for the inclusion of a DR53 haplotype in the chromosome 6 sequencing project.

Compelling epidemiological evidence suggests that childhood cALL develops as a result of a rare response to a common viral infection.<sup>4,8,9</sup> It has been also emphasized that an HLA association would implicate a viral involvement.<sup>4,8</sup> The present study found an OR of 14.4 for a newborn boy to develop childhood cALL, therefore, homozygosity for HLA-DRB4\*01 is most likely to be the HLA genotype responsible for the putative abnormal immune response to a childhood viral infection. This genotype occurred in a third of boys with cALL. Although there is no direct evidence for any virus to be the long-suspected link, it has been previously suggested that the HLA-DR53 homozygous genotype may cooperate with adenovirus in promotion of preleukemic clones that have arisen spontaneously.<sup>10</sup> The putative role of adenovirus in this model is a nonmutagenic one and involves an interaction with HLA-DR53 in the evasion of an immune response towards the transformed preleukemic clone. In this context, the very high degree of molecular mimicry between HLA-DR53 epitope and an adenoviral protein may be pertinent.<sup>62</sup> The large tegument protein of Epstein-Barr virus (EBV) also shows a seven consecutive amino acid mimicry with the same HLA-DR53 epitope as well as five amino acids of the *BamHI*-M rightward reading frame 2 (BMFR2) early nuclear protein.<sup>62</sup> A recent case control study found an increased prevalence of antibodies to EBV in leukemic children under 6-years old<sup>63</sup> (adenovirus was not considered in that study). This suggests that the HLA-DR53 association may also point to EBV as a candidate virus involved in the development of childhood ALL.

In summary, the results of the present study suggest that homozygosity for HLA-DRB4\*01 (-DR53) is a genetic risk factor for childhood ALL in boys. DRB4\*01 homozygosity may also be the common immunogenetic basis for a proportion of recurrent spontaneous miscarriages observed in leukemic families. We hope that these results will be used as a guide for further functional studies and be considered in the ongoing National Children's Cancer Studies in the US and UK.

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