HLA complex-linked heat shock protein genes and childhood acute lymphoblastic leukemia susceptibility

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Abstract Three heat shock protein 70 (HSP70) genes, *HSPA1L*, *HSPA1A*, and *HSPA1B*, are located within the human leukocyte antigen (HLA) class III region. HSPs act as stress signals and regulate natural killer cell response to cancer. *HSP70* gene polymorphisms show disease associations partly due to their linkage disequilibrium with *HLA* alleles. To systematically evaluate their associations with childhood acute lymphoblastic leukemia (ALL), we examined the three functional single nucleotide polymorphisms (SNPs) rs2227956 (T493M) in *HSPA1L*, rs1043618 in *HSPA1A* 5'UTR, and rs1061581 (Q351Q) in *HSPA1B* by TaqMan assays or polymerase chain reaction–restriction fragment length polymorphism in 114 ALL cases and 414 controls from Wales (UK), in 100 Mexican Mestizo ALL cases and 253 controls belonging to the same ethnic group, and in a panel of 82 *HLA*-typed reference cell line samples. Homozygosity for *HSPA1B* rs1061581 minor allele G was associated with protection (odds ratio (OR)=0.37, 95% confidence interval (CI)=0.16–0.78; $P=0.007$) with gene-dosage effect (additive model) reaching significance ($P=0.0001$) in the Welsh case–control group. This association was replicated in the second case–control group from Mexico (OR (recessive model)=0.49, 95% CI=0.24–0.96; $P=0.03$), and the pooled analysis yielded a strong associa-

tion (Mantel–Haenszel OR=0.43, 95% CI=0.27–0.69, $P=0.0004$). The association was stronger in males in each group and in the pooled analysis. A three-SNP haplotype including the major allele A of rs1061581 showed a highly significant increase in Welsh cases compared with respective controls (6.7% vs 1.8%; $P=0.0003$) due to the difference between male cases and controls. The protective allele of rs1061581 occurred more frequently on the *HLA-DRB3* haplotypes (especially *DRB1*03*) in the cell line panel, but the *HSPA1B* association was independent from the *HLA-DRB4* association previously detected in the same case–control group from Wales (adjusted $P=0.001$). Given the cancer promoting roles played by HSPs intracellularly as well as roles in immune surveillance when expressed on the cell surface and the known correlations between expression levels and the *HSP* polymorphisms, these results are likely to indicate a primary association and warrant detailed assessment in childhood ALL development.

Keywords Genetic predisposition to disease · Heat shock protein gene polymorphism · HLA complex · Sex effect · Association study · Childhood leukemia susceptibility

Introduction

Three heat shock protein (HSP) genes, *HSPA1L* (HSP-HOM), *HSPA1A* (HSP70-1), and *HSPA1B* (HSP70-2), are located within the human leukocyte antigen (HLA) class III region. HSPs are chaperone proteins and also have a regulatory role on natural killer cell response to cancer by acting as stress signals. HSP gene polymorphisms have been associated with autoimmunity (Partanen et al. 1993; Pociot et al. 1993) mainly due to their linkage disequilibrium (LD) with *HLA* alleles, in particular *HLA-DRB1*03*.

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Associations in several cancers including breast, liver, and gastric cancers have also been reported (Chouchane et al. 1997; Mestiri et al. 2001; Jeng et al. 2008; Shibata et al. 2009). The most commonly examined polymorphism is the *Pst*I polymorphism in nucleotide 1267 of *HSPA1B* (rs1061581). This A to G substitution causes a synonymous change (Q351Q) in the HSP70-2 protein and shows correlations with mRNA levels (Temple et al. 2004; Kee et al. 2008). Likewise, the *HSPA1L* polymorphism (T493M) is also reported to influence inducible HSP70 levels (Singh et al. 2006a, b, 2007). The *HSPA1A* single nucleotide polymorphism (SNP) rs1043618 is reported to reduce luciferase expression in a promoter reporter assay (He et al. 2009).

Members of the HSP family (Hsp60, Hsp70, Hsp90, gp96) act as danger-signaling molecules to the innate immune system (Romanucci et al. 2008). Professional antigen-presenting cells can be activated by endogenous substances released by damaged or stressed tissue, and HSPs seem to be candidate molecules that signal cellular stress to the innate immune system (Multhoff et al. 1999; Wallin et al. 2002; Gehrmann et al. 2005). HSPs may also be operational in adaptive immune system (Javid et al. 2007). They act as carriers of antigenic peptides derived from tumor and virus-infected cells (Srivastava et al. 1998). An immunoregulatory cytokine role for HSP70 is also evident as it stimulates cytokine production (Asea et al. 2000, 2002) which may contribute to its role in cancer immune surveillance (Sherman and Multhoff 2007).

The most consistent association of HLA-linked *HSP70* genes has been reported in longevity (Singh et al. 2006a, b, 2007). These studies also confirmed the roles played by *HSP70* polymorphisms in gene transcription. Given the presumed correlation between cancer susceptibility and aging (Finkel et al. 2007), it is plausible that the *HSP70* gene variants may also play a role in cancer susceptibility. Due to the strong haplotypic relationships between *HSP70* gene polymorphisms and *HLA* haplotypes (Favati et al. 1997; Dorak et al. 2006), *HSP70* polymorphisms may even account for some of the *HLA* associations observed in cancers. An association between higher HSP70 serum levels and increased lung cancer risk has been reported but in males only (Suzuki et al. 2006).

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children affecting 30–45 per 1,000,000 children per year in developed countries (Stiller 2004). Like most childhood cancers, ALL is also more common in males for unknown reasons. ALL originates from B cell lineage in around 70% of cases (Stiller 2004). Cytogenetic and immunophenotypic markers are commonly used to distinguish ALL subtypes, and more recently, genome-wide studies identified somatic alterations in ALL (Mullighan and Downing 2009). The role of inherited alleles in ALL development is less well established. Two

genome-wide association studies have been performed in childhood ALL with similar results on the risk modification by previously unsuspected loci involved in transcriptional regulation and differentiation of B cell progenitors (Papaemmanuil et al. 2009; Treviño et al. 2009).

We have been exploring genetic risk markers for childhood ALL, and the *HLA* class II association we have found is male-specific (Dorak et al. 1999a). We have recently identified another male-specific association with a variant in the interferon regulatory factor 4 gene and its possible molecular mechanism (Do et al. 2009). Using the same case–control group in which the original *HLA* association was found, we examined the three common *HSP70* gene polymorphisms that show correlations with gene expression levels. The same analysis was also carried out in a second replication group to increase the confidence levels in the results.

Materials and methods

Cases and controls

All associations were examined in the original case–control group (Dorak et al. 1999a) with additional controls as described elsewhere (Dorak et al. 2002). In brief, the 114 cases were incident childhood (≤ 15 years) ALL cases diagnosed over a 10-year period (1990–1999) in South Wales (UK), and 414 controls were anonymously collected cord blood samples from newborns also in South Wales (UK). The collections were made from sequential births at two South Wales Hospitals during 1996–1998. Only births at full term and from vaginal delivery following noncomplicated pregnancies were included. The South Wales Research Ethics Committee (UK) provided favorable opinion for the use of these samples in genetic association studies.

All SNPs were also genotyped in a second case–control group from Mexico. These were 100 childhood (≤ 15 years) ALL cases and 253 adult controls (mean age=33 years). All Mexican cases and controls were from the Mexican Mestizo population. Mexico is mainly composed of Mestizos, constituting 95% of the actual population, who are formed by a triracial admixture of European genes of Spaniard origin, African, and Oriental genes (from a pool from the 68 different native groups of Amerindians; Gorodezky 1992). The cases were diagnosed between 1998 and 2004 at different hospitals: Instituto Mexicano del Seguro Social, Instituto de Seguridad Social al Servicio de los Trabajadores del Estado, Hospital de Petróleos Mexicanos, Hospital Infantil de México, Instituto Nacional de Pediatría, and Hospital Militar. Written informed consent was obtained from the participants or their parents, and the

study protocol was approved by the local ethic and research committees of the Instituto de Diagnostico y Referencia Epidemiologicos and the different hospitals where samples were drawn.

Reference cell line panel

International Histocompatibility Working Group *HLA*-typed reference cell line DNA samples ($n=82$) were purchased from the International Cell and Gene Bank (Seattle, WA, USA) and genotyped for all SNPs that were examined in cases and controls. This panel includes homozygous examples of all conserved extended haplotypes (CEH) except CEH37.1 which is represented as a heterozygous example (for a description of CEHs, see Dorak et al. 2006). This panel was genotyped to evaluate the relationships of *HSP* gene variants with *HLA* haplotypes.

Gene variants and genotypings

We examined *HSPA1L* rs2227956 (T493M), *HSPA1A* rs1043618 (5'UTR), and *HSPA1B* rs1061581 (Q351Q). Details of the SNPs are shown in Table 1 and the map position of *HSP* loci and locations of these SNPs in Fig. 1.

HSPA1L SNP rs2227956 and *HSPA1A* SNP rs1043618 genotypings were achieved by TaqMan allelic discrimination assays (purchased from Applied Biosystems (ABI), Foster City, CA, USA). *HSPA1B* SNP rs1061581 was genotyped by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) using the restric-

tion endonuclease *Pst*I (Dorak et al. 2006). This PCR-RFLP method is equivalent to the *Pst*I RFLP by Southern blotting analysis in earlier reports (Dorak et al. 1994; Favatier et al. 1997).

Statistical analysis

Statistical analysis was performed on Stata/IC v.10 (StataCorp, College Station, TX, USA). Statistical significance of associations with minor allele positivity (dominant model) or minor allele homozygosity (recessive model) was assessed by logistic regression and odds ratios (OR), and their 95% confidence intervals (CI) were obtained. In these models, the wild-type homozygous group is the reference group for comparisons. We also used gene-dosage effect analysis by a genotype-based trend test (additive model) which reveals associations that depend additively upon the minor allele. This test compares the heterozygote and then homozygote frequencies with wild-type homozygote frequency and yields an odds ratio for stepwise changes with the number of minor allele in the genotype, which is called OR per allele or combined OR. The additive model was examined by coding the three genotypes 0 (wild type), 1 (heterozygosity), and 2 (variant allele homozygosity) and running logistic regression or Cochran–Armitage trend test using the case–control status as the outcome variable. Independence of associations was assessed by multivariable logistic regression. The two case–control groups were analyzed separately and also pooled following demonstration of the lack of statistically significant heterogeneity. To

Table 1 HSP gene variants examined in the study

Gene	SNP type	dbSNP # (chr6 nucleotide position)	MAF ^d (W) ^e	MAF (MX)	MAF (EUR)	MAF (HISP)	MAF (YRI)	MAF (HCB)	MAF (JPT)
<i>HSPA1L</i>	Coding missense, A>G (T493M) ^a	rs2227956 (31 886 251)	0.198 (0.219) ^f	0.071 (0.066)	0.292	0.068	0.000	0.189	0.102
<i>HSPA1A</i>	Regulatory, G>C (5' UTR) ^b	rs1043618 (31 891 486)	0.403 (0.344)	0.296 (0.212)	0.342	0.318	0.908	0.311	0.198
<i>HSPA1B</i>	Coding silent, A>G (Q351Q) ^c	rs1061581 (31 904 759)	0.424 (0.286)	0.504 (0.429)	0.289	0.667	0.000	0.523 ^g	0.523 ^g

W Welsh (2n=828), MX Mexican (2n=494), EUR Europeans, HCB Han Chinese, JPT Japanese, YRI sub-Saharan African, Yoruba tribe

^a Human Genome Variation Society (HGVS) name: NP_005518.3:p.T493M

^b Also known as -27G>C or +190G>C (HGVS name: NM_005345.4:c.-27G>C; in Africa C>G)

^c Also known as +A1267G; in the African population sample, this site is monomorphic (G; see text). The rare allele is “G” in the Welsh group, but it is “A” in the Mexican group. The minor allele frequency given here is for allele G in Mexicans

^d MAF is minor allele frequency in controls except that in the Mexican controls, rs1061581 minor allele was reversed but for consistency, the European minor allele G was treated as minor allele

^e MAF denotes the frequency of the European minor allele in data from other populations (EUR, HCB, JPT, YRI) are from the HapMap project (except that all frequencies for rs1061581 and all Hispanic (HISP) frequencies are from the EGP SNP Study; <http://egp.gs.washington.edu>) which are available through NCBI ENTREZ SNP

^f Frequencies in brackets are from respective case groups

^g These frequencies are from EGP-ASIAN-PANEL

Fig. 1 **a** Classical HLA complex and the location of the three HSP genes in the class III region; **b** locations of the SNPs in the three HSP genes and LD plots in the control groups. The numbers in cells

correspond to the LD parameter $D' \times 100$ (the missing D' value for LD between SNPs 1 and 2 in Mexican controls is equal to 1.0)

test for associations after pooling, we calculated a Mantel–Haenszel P value (Mantel and Haenszel 1959) and accompanying weighted OR. All P values presented are two-sided.

We used the Stata command “genhwcci” which tests the Hardy–Weinberg equilibrium (HWE) for genotypic counts of cases, under the assumption that the genotypic counts of controls are under HWE (Cui 2000).

Haplotype construction was achieved on Haploview v4.0, which is freely available (<http://www.broad.mit.edu/haploview/haploview>). Haploview was also used for statistical assessment of allelic and haplotypic frequency differences between cases and controls.

Results

General characteristics of the case–control groups

In both case groups, the male-to-female ratios were higher than 1 (63:51 in Welsh and 58:42 in Mexican samples) as expected from generally higher incidence of childhood ALL in males. Because of the male predominance in cases,

sex-stratified analysis was performed. In the Welsh group, subtypes of ALL and age at diagnosis were known for each case, but subgroup analysis was not attempted due to small numbers.

Hardy–Weinberg equilibrium

All loci were in HWE ($P > 0.01$) in the control groups. The null hypothesis that “cases under HWE (given controls under HWE)” was rejected for all SNPs ($P < 0.01$) as an indication of an association.

Linkage disequilibrium

LD was high for any pair of the three SNPs in controls in both groups ($D' > 0.8$; Fig. 1b). This finding corroborates with a recent report that used the same three SNPs in an association study in two European populations (Konings et al. 2009) and another report which used three SNPs from the same genes with one SNP being different (Singh et al. 2006b). LD plots in the control groups are shown in Fig. 1b confirming comparable LD patterns in the two groups despite different allele frequencies in one of the SNPs (see below).

Allele frequencies

In Table 1, we present allele frequencies in each case–control group as well as publicly available data from other populations. The case groups had lower minor allele frequencies of *HSPA1B* SNP than in controls in both groups ($P<0.001$ and $P=0.09$ in the Welsh and Mexican groups, respectively), and the allele frequencies for *HSPA1L* rs2227956 in the two control groups were very different. The most interesting observation was that according to the data available on National Center for Biotechnology Information (NCBI) ENTREZ SNP, both *HSPA1B* and *HSPA1L* SNPs are monomorphic in the African population, and only the European minor alleles are present in sub-Saharan Africa (Table 1). The HapMap data on *HSPA1A* rs1043618 also shows a remarkable minor allele switch between Europeans and Africans. The European major allele of rs1043618 has a very low frequency (<0.10) in Africa.

Haplotype frequencies

Haplotype frequencies obtained from the two control groups are shown in Tables 2 and 3. One haplotype (1-2-1) showed a highly significant increase in Welsh cases (0.067 vs 0.018; $P=0.0003$). This difference was mainly due to the difference in male cases and controls (0.107 vs 0.027, $P=0.0006$; frequencies were 0.021 and 0.017 in female cases and controls). In the Welsh case–control group, two cases were homozygous for the *HSP* risk haplotype 1-2-1, but no controls showed homozygosity for this haplotype. These two cases were homozygous for *HLA-DRB1*15* and *HLA-DRB1*10*. In the Mexican group, the haplotype frequency for 1-2-1 was very low and did not differ between cases and controls (this male-specific risk haplotype did not occur in 151 male controls at all). There were two other haplotype frequency differences between

Mexican cases and controls that reached statistical significance. In the Welsh group, the differences were in the same direction but remained statistically nonsignificant.

Genotypic associations

Genotype frequencies in each group are shown in Table 4. Of the three SNPs, the *HSPA1B* SNP rs1061581 was associated with protection in the Welsh group by its minor allele G being less common in cases. Heterozygosity for allele G reduced the childhood ALL risk as measured by odds ratio (OR=0.58; $P=0.01$) and even more strongly when existed in two copies (OR=0.28, $P=0.001$). This stepwise linear change in the risk resulted in a strong additive model association ($P=0.0001$). When stratified by sex, this association remained statistically significant only in males, and homozygosity conferred very strong protection (with the wild-type homozygosity being the reference group, OR=0.16, 95% CI=0.05–0.55, $P=0.004$).

The rs1061581 association was replicated in the second case–control group from Mexico despite the switch in minor alleles. The European minor allele G was the major allele in Hispanics in the present study and in the Environmental Genome Project (EGP) SNP Study (Table 1). The association with allele G homozygosity was also statistically significant in the Mexican case–control group (OR=0.49, 95% CI=0.24–0.96, $P=0.03$). When the two groups were pooled using the raw data as in a meta-analysis, the association attained robust statistical significance (Mantel–Haenszel $P=0.0004$). In the stratified analysis of the pooled data, the male specificity of the *HSPA1B* association was confirmed ($P=0.0007$ in males and $P=0.13$ in females). When these analyses were repeated for the dominant model (i.e., variant allele positivity), both *HSPA1A* and *HSPA1A* associations were statistically significant in the pooled analysis (Table 5). The protective association of rs1061581 in males was in

Table 2 Three SNP haplotype frequencies obtained in Haploview in Welsh cases and controls

Haplotype ^{a,b}	Cases overall (<i>n</i> =114)	Controls overall (<i>n</i> =414)	Cases females (<i>n</i> =51)	Controls females (<i>n</i> =214)	Cases males (<i>n</i> =63)	Controls males (<i>n</i> =200)	Cell lines (<i>n</i>) ^c
111 (AGA)	0.427	0.375	0.435	0.379	0.422	0.372	31
122 (ACG)	0.269	0.380	0.306	0.372	0.237	0.389	28
211 (GGA)	0.206	0.185	0.228	0.193	0.186	0.175	7
112 (AGG)	0.018	0.030	0.011	0.026	0.023	0.033	3
121 (ACA)	0.067	0.018	0.021	0.017	0.107	0.027	0

^a The SNP order in the haplotypes is rs2227956 (A>G), rs1043618 (G>C), and rs1061581 (A>G). 1 and 2 denote major and minor alleles, respectively

^b Haplotypes 212 and 221 reached 0.01 frequency only in male cases (0.015 and 0.010, respectively) with haplotype 212 represented by the cell line PLH and no cell line homozygous for the 221 haplotype. Frequencies shown in bold were different between cases and controls with statistical significance

^c Number of cell line samples homozygous for the haplotype

Table 3 Three SNP haplotype frequencies obtained in Haploview in Mexican cases and controls

Haplotype ^{a,b}	Cases overall (n=99)	Controls overall (n=247)	Cases females (n=41)	Controls females (n=96)	Cases males (n=58)	Controls males (n=151)	Cell lines (n) ^c
111 (AGA)	0.506	0.414	0.495	0.435	0.514	0.401	31
122 (ACG)	0.197	0.288	0.247	0.304	0.160	0.277	28
211 (GGA)	0.065	0.066	0.069	0.062	0.060	0.069	7
112 (AGG)	0.219	0.215	0.173	0.177	0.254	0.239	3
121 (ACA)	0.013	0.011	0.000	0.014	0.012	0.000	0

^a The SNP order in the haplotypes is rs2227956 (A>G), rs1043618 (G>C), and rs1061581 (A>G). 1 and 2 denote major and minor alleles, respectively

^b Haplotypes 212 and 221 were not detected in more than 0.01 frequency. Frequencies shown in bold were different between cases and controls with statistical significance

^c Number of cell line samples homozygous for the haplotype

agreement with the decreased minor allele G frequency in cases and increased frequency of the haplotype 1-2-1 in cases (this haplotype bears the major allele of HSPA1L, minor allele of HSPA1A, and major allele A of rs1061581).

The minor allele G of rs1061581 is known to be in LD with *HLA-DRB1*03* and also with the *HLA-DRB3* lineage (Dorak et al. 2006), and the cell line panel data confirmed this observation (see below). In the original study that used the same Welsh case-control group, *HLA-DRB4* haplotypes showed a strong male-specific risk association (Dorak et al. 1999a). Although *HLA-DRB1*03* did not show any

association, the *DRB3* lineage was in general protective. We examined the *HSPA1B* association in multivariable models and obtained risk estimates adjusted for the presence of these *HLA* class II markers. The recessive *HSPA1B* association ($P=0.008$, OR=0.20) was independent from the male-specific *HLA-DRB4* risk association (adjusted $P=0.005$, OR=0.16) and *DRB3* protective association (adjusted $P=0.02$, OR=0.22; both in males) observed in the original study (Dorak et al. 1999a). This analysis ruled out the possibility that the protective *HSPA1B* association was not due to its being in negative LD with the risk marker *HLA-DRB4*.

Table 4 Genotype frequencies and accompanying association test results (additive model) in the Welsh and Mexican case-control groups

Genotypes	Welsh sample (114 cases and 414 controls)			Mexican sample (100 cases and 253 controls)		
	Frequencies (cases vs controls)	OR per allele (95% CI)	P^c	Frequencies (cases vs controls)	OR per allele (95% CI)	P^c
rs2227956		1.12 (0.79–1.59)	0.53		0.92 (0.50–1.71)	0.80
AA	67 vs 246			87 vs 214		
AG	30 vs 103			11 vs 27		
GG ^a	8 vs 22			1 vs 4		
rs1043618		0.79 (0.58–1.08)	0.14		0.65 (0.45–0.96)	0.03
GG	47 vs 137			62 vs 127		
CG	45 vs 162			32 vs 98		
CC ^b	14 vs 66			5 vs 25		
rs1061581		0.55 (0.40–0.75)	0.0002		0.74 (0.52–1.04)	0.09
AA ^c	58 vs 139			27 vs 61		
AG	47 vs 199			51 vs 111		
GG ^d	9 vs 76			14 vs 63		

^a Associated with lower inducible HSP70 production (Singh et al. 2006a)

^b Reduces luciferase expression in vitro (He et al. 2009)

^c Due to linkage disequilibrium with a promoter SNP (rs6457452), associated with lower HSP70 production in response to LPS (Temple et al. 2004)

^d By inference, allele G is associated with higher HSP70 production in response to LPS

^e These are P trend values for the additive model estimated by logistic regression

Table 5 Individual and pooled association test results (dominant model) in the Welsh and Mexican case-control studies

SNP	Welsh (OR, 95% CI)	Mexican (OR, 95% CI)	Homogeneity test (<i>P</i>)*	Pooled (OR, 95% CI)
<i>HSPA1L</i> (rs2227956)	1.12 (0.69–1.80)	0.95 (0.43–2.01)	0.71	1.07 (0.73–1.56) <i>P</i> =0.74
<i>HSPA1A</i> (rs1043618)	0.75 (0.48–1.20)	0.62 (0.37–1.02)	0.54	0.69 (0.50–0.95) <i>P</i> =0.02
<i>HSPA1B</i> (rs1061581)	0.50 (0.32–0.77)	0.84 (0.48–1.51)	0.13	0.61 (0.44–0.84) <i>P</i> =0.003

Dominant model corresponds to variant allele positivity (heterozygosity and homozygosity combined) with wild-type genotype being the reference group

**P*>0.05 indicates the lack of heterogeneity and allows pooling of the results

Cell line data

The protective allele G of *HSPA1B* rs1061581 occurred more frequently on the *HLA-DRB3* haplotypes (consisting of all *DRB1**03, *DRB1**11/12, *DRB1**13/14 haplotypes) in the cell line panel but especially on *HLA-DRB1**03 haplotypes with no exception as already known (Favatier et al. 1997; Dorak et al. 2006). Most *HLA-DRB4* haplotypes (consisting of all *DRB1**04, *DRB1**07, *DRB1**09 haplotypes) which showed a risk association in males in the same group of cases and controls (Dorak et al. 1999a) carried the risk allele A as reported before (Dorak et al. 2006). The other SNPs did not show any preferential association with *HLA* haplotypes or lineages. No cell line sample was homozygous for the risk haplotype 1-2-1. The frequencies of *HSP* haplotypes correlated well with the number of cell line samples homozygous for those haplotypes (Table 2). The most common *HSP* subregion haplotype consisting of major alleles of the three SNPs (1-1-1) also had the highest number of homozygote samples (*n*=31) in the cell line panel. This haplotype did not show any *HLA* class II lineage specificity: It was present in *HLA-DRB3/DR52* (conserved extended haplotype (CEH) 35.5 in the cell line J0528239), all major *DRB4/53* (CEH44.1/2/3/ and 62.1 haplotypes in cell lines AWELLS, PITOUT, MOU-MANN, and BOLETH) and *DRB5/51* (CEH18.1 in the cell line DO208915) lineages. The second most common haplotype 1-2-2 was present mainly on the *DRB3/52* haplotypes, in particular on the major *DRB1**03 haplotypes in Europeans (CEH8.1/COX; CEH18.2/QBL), Africans (CEH42.1/RSH), and Asians (CEH58.1/HAU). It was also present on relatively rare *DRB4/53* haplotypes (as in CEH13.1/BER). Altogether, 28 of the samples in the panel were homozygote for this haplotype. The haplotype 2-1-1 was present in homozygous form in seven cell lines including the major *DRB5/51* haplotype CEH7.1 (PGF) and the *DRB4/53* haplotypes CEH46.1 (T7526) and CEH57.1 (DBB). The only other *HSP* subregion haplotype appeared in homozygous form was 1-1-2 (in three cell lines) on the

cell line CB6B representing CEH62.1 and two other haplotypes that are not CEH.

Discussion

The present study revealed a protective association with the *HSPA1B* SNP rs1061581, which was replicated in a second case-control sample and shown to be male specific. There was also a haplotypic risk association involving all three *HSP* SNPs. Most importantly, we made sure that these associations were not due to LD between these *HSP* gene variants and previously found *HLA* complex associations with childhood ALL. These are the first *HSP* genetic associations in childhood ALL, and two separate case-control groups yielded comparable findings with combined analysis providing a highly significant result.

The *HLA* complex class III region where the *HSP* genes reside is the most gene-dense region of the genome (Xie et al. 2003), and it is important to exclude confounding by locus due to LD between variants of different loci in the interpretation of the results. We have confirmed the independence of the *HSP* associations by using *HLA*-typed reference cell line samples and also statistical tests. We have preliminary data on associations of other *HLA* complex polymorphisms with childhood ALL, and none showed a correlation with the *HSP* associations reported in the present study (Dorak et al. 2008). In other words, the *HSP* associations were independent and not due to linkage disequilibrium with variants of other candidate genes within the *HLA* complex (data not shown). This aspect of our study separates the present one from other reported *HSP* associations in cancer (Jeng et al. 2008; Shibata et al. 2009). In fact, the same polymorphism was found to be associated also with chronic myeloid leukemia (Dorak et al. 1994), but it was attributed to its LD with the *HLA* class II susceptibility marker. Our detailed analysis in childhood ALL by multivariable logistic regression showed that the *HSPA1B* association is independent from the *HLA* class II

region association reported in the same case–control group from Wales (Dorak et al. 1999a).

Another important finding in the present study was the male specificity of the *HSP* association. The sex effect in extended *HLA* complex associations with childhood ALL has already been reported by us and others (Taylor et al. 1998; Dorak et al. 1999a, b; Ng et al. 2006). Notably, the positive correlation between high HSP serum levels and lung cancer susceptibility is also observed in males only (Suzuki et al. 2006). We did not have serum samples from our cases and controls to examine associations with serum levels in childhood ALL. It is still unclear why there may be a sex effect in *HLA* complex associations, but in light of similar findings in other diseases (Meyer et al. 2001; Fisher et al. 2002; Hensiek et al. 2002; Tian et al. 2006), this aspect of the association we are reporting here warrants further study. In two rat models, HSP70 has a higher expression level in males in response to a viral infection (Klein et al. 2004) or following acute tail-shock stress (Nickerson et al. 2006) in lymphoid organs. Given the constitutive upregulation of NF- κ B in most cancers and its involvement in regulation of HSP gene transcription together with the suppressive effect of estrogen on NF- κ B activity (Stice and Knowlton 2008), a sex differential in HSP70 expression levels is plausible and may play a role in the general sex differential in cancer incidence (Cook et al. 2009) besides childhood ALL.

Having confirmed that the *HSP* association is likely to be independent, it is important to consider its biological plausibility. The selected SNPs are most likely to be markers for variability in production of HSP. These SNPs or others in LD with them correlate with differences in gene expression (Temple et al. 2004; Singh et al. 2006a, b; Kee et al. 2008; He et al. 2009) or serum levels (Afzal et al. 2008). Although the *HSPA1B* silent SNP rs1061581 itself does not directly influence *HSPA1B* mRNA levels (Pociot et al. 1993; Schroeder et al. 2000), the (A) allele of rs1061581 is in linkage with the major (C) allele of *HSPA1B* promoter SNP rs6457452 (–179C>T) which is associated with lower levels of *HSPA1A/B* gene expression (Temple et al. 2004). In the *HSPA1L* gene, the missense variant rs2227956 shows a correlation with inducible HSP70 levels (Singh et al. 2006a). Subjects with minor allele homozygosity (CC) respond poorly to *HSP70* induction. As shown in Tables 2 and 3, this *HSPA1L* minor allele occurs in one haplotype (211) which includes the major allele of *HSPA1B*. Thus, the alleles at *HSPA1L* and *HSPA1B* associated with low HSP70 levels are always on the same haplotype. The *HSPA1B* variant allele associated with high *HSPA1A/B* mRNA levels (Temple et al. 2004; Kee et al. 2008) and protection from childhood ALL (the present study) is always in the same haplotype as the variant of the *HSPA1L* SNP (that is associated with higher

induced HSP70 levels (Singh et al. 2006a)). If these two SNPs indeed have independent effects on HSP70 production, the two alleles may even have synergistic effects or, as the haplotype structure suggests, at least they do not have antagonistic effects.

There are numerous associations with *HSPA1A/B/L* SNPs in many diseases most of which with successful replications (Favatier et al. 1997; Temple et al. 2004; Singh et al. 2006a, b; Kee et al. 2008; Jeng et al. 2008; Shibata et al. 2009; Konings et al. 2009). This is in agreement with pleiotropic functions of HSPs. The most relevant functions of HSPs in the development of childhood ALL are their role as stress signal molecules, regulators of apoptosis, involvement in immune surveillance, and overall maintenance of cellular homeostasis (Romanucci et al. 2008). Their roles in cancer immune surveillance as the source of activating signals for antigen-presenting and natural killer cells and as potent stimulators of antitumor immune response are important in protection from cancers (Sherman and Multhoff 2007). It is therefore plausible that the protective association of the *HSPA1B* variant allele, which increases *HSPA1A/B* production (Temple et al. 2004; Kee et al. 2008), is the primary or causative association for protection from childhood ALL through its effect on immune surveillance of leukemic transformation.

The protective *HSPA1B* allele G corresponds to the 8.5-kb allele by *Pst*I RFLP by Southern blotting (Dorak et al. 1994; Favatier et al. 1997). Its strong LD with *DRB1*03* is well established. Examination of publicly available data presented in NCBI ENTREZ SNP (and in part, in Table 1) suggests that the allele G is the only allele at this locus in a small African population sample analyzed by the Environmental Genome Project. Likewise, the European *HSPA1A* minor allele also shows a switch between European and African samples. There is limited data on the HSP subregion SNPs in ENTREZ SNP, but one more SNP (rs2763979 in the 5' flanking region of *HSPA1B*) also shows an allelic frequency switch between continental groups (Table 1). Large allele frequency differences between populations are not uncommon and attributed to genetic drift (Hofer et al. 2009). However, it is an extreme case that a SNP that has a high allele frequency elsewhere is possibly monomorphic in Africa. The fixed nucleotide at this position in the African sample corresponds to the protective allele in the European and Mexican samples in the present study. This raises the question whether it may contribute to the lower frequency of childhood ALL in populations of African origin (Smith et al. 1999). In a complex disease like leukemia, a single SNP is not expected to influence the disease incidence but it may contribute to fluctuations. Preferential examination of SNPs that show such allele frequency differences between Africans and Europeans may be a worthwhile

effort in the study of genetic susceptibility to childhood ALL.

HSPA1A/B polymorphisms have been associated with several cancers (Chouchane et al. 1997; Mestiri et al. 2001; Jeng et al. 2008; Shibata et al. 2009). These findings suggest consistency in the *HSPA1A/B* associations with cancer, but there is need for a cautionary note. The previously reported *HSPA1B* rs1061581 associations in several cancer types by Chouchane et al. (1997) and Mestiri et al. (2001) are based on the same control group and similar to the one we report here. It has to be noted that the heterozygote frequency in the control groups of those studies is above 70%. Since heterozygosity rate for a biallelic polymorphism can never be more than 50%, those studies seem to have suffered from genotyping or data handling errors, which lower the confidence level in the results. In the present study, all genotype frequency distributions were in Hardy–Weinberg equilibrium in controls, and consistency of the results was confirmed by similarities in a second sample. Furthermore, the recent studies in liver cancer (Jeng et al. 2008) and gastric cancer (Shibata et al. 2009) report opposite associations with the same *HSPA1B* genotype. While the statistically more robust risk association in liver cancer of P2/P2 genotype (corresponding to the genotype AA) corresponds to our finding of a protective association of the genotype GG in childhood ALL, the marginally significant association in gastric cancer is the opposite. Assuming all these associations are real, different mechanisms may operate in different cancers due to relative importance of intracellular or extracellular functions of HSP70 in each cancer (Schmitt et al. 2007). Recently, another duality in the effect of cell surface expression on prognosis of cancer has been reported (Pfister et al. 2007). HSP70 membrane positivity correlated with better prognosis in cancers which metastasize to the liver where natural killer cells exist in abundance, but worse prognosis was noted in cancers that are not exposed to natural killer surveillance. It can thus be inferred that high expression of HSP70 in cells is beneficial for the cancer cell survival due to intracellular cancer promoting effects of HSP70 (Ciocca and Calderwood 2005; Schmitt et al. 2007; Yaglom et al. 2007) unless immune surveillance has a role in the control of the particular cancer where HSP70 membrane positivity acts as a danger signal for natural killer cells (Multhoff et al. 1999; Wallin et al. 2002; Gehrmann et al. 2005). The association we report here between high-expression-associated *HSPA1B* genotype and protection from childhood ALL therefore suggests the involvement of immune surveillance in the development of ALL.

Besides the usual limitations of case–control studies, the present study had a relatively small sample size in each group. Each group still provided significant results, and the

lack of heterogeneity also allowed us to pool them as in a meta-analysis to obtain more robust results. The replication group did not have similar allele frequencies in the SNPs examined. Although this is not ideal, the differences did not reach statistical significance in heterogeneity tests and also provided an opportunity to examine the consistency of association despite a switch in minor and major alleles between the populations. A limitation was not to be able to examine associations with clinical progress to see whether these genotypes could be used as prognostic markers since markers for primary susceptibility may also become markers for clinical progress or relapse risk. *HSPA1B* rs1061581 allele A is, for example, also a marker for survival in hepatocellular carcinoma (Jeng et al. 2008). Prospective studies may include the *HSP* genotypes to examine their role in clinical outcome.

The present study showed an association of *HLA*-linked *HSP70* gene polymorphisms independent of the *HLA* association shown in the same case–control group. The results were supported by findings in a second case–control study. Given the roles played by HSPs in immune surveillance as danger signals (Multhoff et al. 1999; Wallin et al. 2002; Gehrmann et al. 2005) and the known correlations between HSP gene variants and gene expression (Temple et al. 2004; Singh et al. 2006a, b; He et al. 2009) or serum levels (Afzal et al. 2008), these results are likely to indicate a primary association and warrant more detailed assessment of the *HSP70* genes in childhood ALL development.

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