BMC Cancer

Research article

Association of HLA Class I and Class II genes with *bcr-abl* transcripts in leukemia patients with t(9;22) (q34;q11)

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Abstract

Background: Based on the site of breakpoint in t(9;22) (q34;q11), *bcr-abl* fusion in leukemia patients is associated with different types of transcript proteins. In this study we have seen the association of HLA genes with different types of *bcr-abl* transcripts. The association could predict the *bcr-abl* peptide presentation by particular HLA molecules.

Methods: The study included a total of 189 patients of mixed ethnicity with chronic myelogenous leukemia and acute lymphocytic leukemia who were being considered for bone marrow transplantation. Typing of *bcr-abl* transcripts was done by reverse transcriptase PCR method. HLA typing was performed by molecular methods. The *bcr-abl* and HLA association was studied by calculating the relative risks and chi-square test.

Results: Significant negative associations ($p < 0.05$) were observed with HLA-A*02 (b2a2, e1a2), -A*68 (b2a2, b3a2, e1a2), -B*14 (b2a2, b3a2, e1a2), -B*15 (b2a2, b3a2), -B*40 (b2a2), -DQB1*0303 (b2a2, b3a2), -DQB1*0603 (b2a2), -DRB1*0401 (e1a2), -DRB1*0701 (b3a2), and -DRB1*1101 (b2a2).

Conclusions: The negative associations of a particular *bcr-abl* transcript with specific HLA alleles suggests that these alleles play a critical role in presenting peptides derived from the chimeric proteins and eliciting a successful T-cell cytotoxic response. Knowledge of differential associations between HLA phenotypes and *bcr-abl* fusion transcript types would help in developing better strategies for immunization with the *bcr-abl* peptides against t(9;22) (q34;q11)-positive leukemia.

Background

The t(9;22) (q34;q11) translocation seen in 90% of patients with chronic myelogenous leukemia (CML) and about 10% patients with acute lymphocytic leukemia (ALL) results in juxtaposition of the 3' segment of the *c-abl* proto-oncogene on chromosome 9 with the 5' segment on the *bcr* gene on chromosome 22 [1-6]. Breaks in *c-abl* gene generally involve exon 2, also known as a2. Breaks in the *bcr* occur in one of the three following regions: the major

breakpoint cluster region (M-*bcr*), the minor breakpoint cluster region (m-*bcr*) or the micro breakpoint cluster region (μ-*bcr*) [7]. Breakpoints occurring in m-*bcr* involve introns 13 and 14 and join exon 13 or 14 with *abl*, resulting in the fusion transcripts e13a2 (also known as b2a2) and e14a2 (also known as b3a2), respectively. These transcripts lead to production of a 8.5-kb transcript coding for a 210-kD chimeric protein (p210) [8,9]. Breakpoints in m-*bcr* involve the first intron of *bcr*, exon 1 joins with *abl*,

resulting in a smaller fusion transcript, e1a2, that codes for a 190-kD protein (p190) [10]. Breakpoints in the μ-*bcr* involve intron 19, the joining of exon 19 with *abl* results in a fusion transcript e19a2, that codes for a 230-kD protein (p230) [11]. Although tyrosine kinase is activated in all *bcr-abl* fusion transcripts, the p190 form has been shown to have more transforming potential than p210 in vitro and in vivo [12-14]. Fusion transcripts b3a2 and b2a2, which are translated into p210, account for the majority of CML cases, while the fusion transcript e1a2, which is translated into p190, is seen primarily in t(9;22)-positive ALL and the blastic phase of CML [10,15]. The rare fusion transcript e19a2, resulting in p230, is associated primarily with the entity known as chronic neutrophilic leukemia [16].

Several reports indicate an association between t(9;22) (q34;q11) and different human leukocyte antigen (HLA) alleles. This association suggests a possible role for T-cell cytotoxicity in the pathogenesis of diseases linked to *bcr-abl* fusion proteins resulting from t(9;22) (q34;q11). Different HLA alleles have different predilections for the sequence of peptides they can present to T cells. In order to elicit a T-cell response, a peptide must bind to the HLA molecule prior to its presentation to the T cell. Since a given HLA allele can bind and present only peptides with certain sequence constraints, the ability of an individual to elicit a successful T-cell cytotoxic response to cells carrying foreign or newly mutated proteins depends on the set of HLA alleles that individual has inherited. Thus, individuals carrying certain HLA alleles capable of binding peptides derived from *bcr-abl* fusion transcripts can in principle be considered to have a biological advantage in fighting the disease over individuals lacking these particular HLA alleles.

In studying the association between the distribution of HLA alleles in patients with different types of *bcr-abl* transcripts, a positive association suggests that a particular HLA allele does not effectively bind and present peptides derived from a given *bcr-abl* transcript. Likewise, a negative association suggests that the binding and presentation of a peptide is effective and elicits a successful T-cell cytotoxic response with destruction of leukemia cells.

The aim of this study was to analyze the disease association between HLA alleles and *bcr-abl* fusion transcripts in greater detail than has so far been studied. First, we assessed the HLA association with a particular *bcr-abl* transcript, i.e., b2a2 or b3a2 or e1a2, and not with CML in general. Second, unlike earlier studies that were limited to low-resolution allele typing, this study used high-resolution typing for HLA class II genes.

Methods

A total of 189 patients comprising of 163 with CML and 26 with ALL, all with t(9;22) (q34;q11) and known HLA types, were included in this study. Of these patients, 28 expressed b2a2 and b3a2 transcripts, while 68 expressed the b2a2 transcript, 76 the b3a2 transcript, and 17 the e1a2 transcript. The comparisons were made with healthy, potential bone marrow donors that were HLA typed at our laboratory. 376 donors were typed for HLA class I and 267 donors for HLA class II. The donor population is a multiracial group of both related and unrelated prospective bone-marrow donors with no significant clinical history. The gene frequencies are not at odds with reported gene frequencies in general populations for prevailing races. We decided to use our internal gene frequencies from this donor population for three reason: First, it is a multiracial group in which no racial bias was made, more representative of the population the reported patients came from than any specific racial group typically used to determine HLA gene frequencies. Second, it is a group for which high-resolution high-quality typing is readily available making it possible to evaluate allelic gene frequency differences, rather than differences at the serologic low-resolution level. Third, it is a group for which not only the HLA phenotype is available, but also the genotype, that is, the haplotypes were determined in each case, particularly in regard to class II genes.

Typing of bcr-abl *fusion transcripts by reverse-transcriptase PCR*

Leukocytes prepared by erythrocyte lysis of bone marrow aspirate (1–2 ml) and peripheral blood (10 ml) samples collected in ethylene diamine tetraacetic acid were used for RNA isolation. Total RNA was isolated by using Trizol reagent (*Invitrogen Lifetechnologies*, USA) according to the manufacturer's instruction. The integrity of RNA was determined by gel electrophoresis prior to reverse transcription (RT). Total RNA (1–5 μg) from samples with intact 28 s and 18 s RNA was converted to cDNA by using random hexamers and Superscript II reverse transcriptase (*Invitrogen Lifetechnologies*) according to the recommendations of the manufacturer.

Each sample was amplified in duplicate for *bcr-abl* in a multiplex RT-PCR using an *abl* primer in combination with *bcr* b2- and e1-specific primers [17]. The t(9;22)-positive cell lines KBM7, K562, and B15, which carry b2a2, b3a2, and e1a2 fusion genes, respectively, served as positive controls. [18,19] The HL60 cell line was used as negative control.

HLA typing

HLA typing was performed by molecular methods. For class I, HLA typing was done at the intermediate-resolution level by using enzyme linked probe hybridization

assay with sequence-specific oligonucleotide probe (ELPHA-SSOP) (*Biotest*, Germany). The sequence-specific oligonucleotide probes were used to identify polymorphic sequence motifs. The hybridization between probe and target DNA from the series of amplified PCR products was detected by a method adapted from the protein enzyme linked immunosorbent assay (ELISA) technique. For class II, HLA typing was done at the high-resolution level by using the sequence specific primers (SSP) (*Genovision*, USA or *Pel-freez*, USA). The technique uses a battery of known sequence specific primers to amplify specific alleles or group of alleles. This typing method is based on the fact that a completely matched primer will be used more efficiently in the PCR reaction than a partially mismatched primer. The electrophoresis bands generated were compared with the kit standards.

Statistical analysis

HLA gene frequencies (not phenotype frequency) were calculated in two different populations: (1) Healthy individuals typed as potential candidates for donating bone marrow; and (2) Patients diagnosed as having CML or ALL in whom *bcr-abl* transcripts were identified.

The sizes of the gene pools of these populations for HLA typing are shown in Table 1. In order to obtain more accurate gene frequencies, the largest population of typed donors was used. This resulted in a larger population for class I genes (376 individuals) than for class II genes (268 individuals). The frequencies of the HLA-A, -B, -DRB1 and -DQB1 alleles for the three groups of CML patients with different *bcr-abl* transcripts were compared with their gene frequencies in the healthy donor group. The frequencies of HLA alleles in patients were evaluated in 192 chromosomes of patients with b2a2 transcirpts (96 individuals, 68 with only b2a2 and 28 with both b2a2 and b3a2); 208 chromosomes of patients with b3a2 (104 individuals, 76 with only b3a2 and 28 with both b2a2 and b3a2); and 34 chromosomes of patients with e1a2 (17 individuals). The comparisons were made by calculating the relative risks. Chi-squares were also calculated to assess the statistical significance of the differences. With $(\nu) = (r-1)(c-1) = 1$ degree of freedom and a significance level of $\alpha = 0.05$ needed to reject the null hypothesis, the χ^2 statistic must be > 3.84. Since ours is an international referral center with a patient population of diverse racial backgrounds, it was not possible to compare the patient data with the standard HLA frequencies based on ethnicity. We therefore used the healthy bone marrow donor population for comparison.

The binding scores were calculated on the basis of the amino acid sequence of the peptide and the HLA molecule as described elsewhere [20-22]. The sequences of e1a2, b3a2, and b2a2 junctional peptides are EGAFHG-

Table 1:

HEALTHY DONORS*		CML + ALL*	
HLA Class I:	752	b2a2:	192
HLA Class II:	538	b3a2:	208
		e1a2:	34

*Numbers of HLA genes available in the different populations to calculate HLA allele frequencies (not the number of individuals in these populations taking into account that an individual carries two genes for each loci).

DAEALQRPVAS [10,15], ATGFKQSSKALQRPVAS [23], and IPLTINKEEALQRPVAS [24].

Results and discussion

Our findings are summarized in Tables 2,3,4, which include only statistically significant observations with $\chi^2 >$ 3.84, corresponding to a significance level of $p < 0.05$. Relative risk > 1 indicates that a particular HLA allele is more frequent in a patient population with a particular transcript than within the corresponding healthy population. Relative risk < 1 indicates a negative association, with a gene frequency significantly smaller than in the corresponding general population. Significant negative associations with b2a2 were seen with HLA-A*02, -A*68, -B*14, -B*15, -B*40, -DQB1*0303, -DQB1*0603, and -DRB1*1101. Significant positive association was seen with HLA-A*01, -A*23, -A*66, -B*37, -B*38, -B*42, -B*45, -B*49, -B*53, -B*56, -B*62, -DQB1*0201, -DQB1*0402, -DQB1*0609, -DRB1*0301, -DRB1*0302, -DRB1*0901, -DRB1*1001, -DRB1*1201, -DRB1*1202, and -DRB1*1503 (Table 2).

Significant negative associations with b3a2 were seen with HLA-A*68, -B*14, -B*15, -DQB1*0303, and DRB1*0701. Significant positive association were seen with HLA-A*25, -B*37, -B*49, -B*51, -B*53, -B*56, -B*60, -B*62, -DQB1*0609, -DRB1*0405, -DRB1*0802, -DRB1*0901, -DRB1*1001, and -DRB1*1503 (Table 3).

Significant negative associations with e1a2 were seen with HLA-A*02, -A*68, -B*14, and -DRB1*0401. Significant positive association were seen with HLA-A*01, -A*11, -A*26, -B*08, -B*49, -B*53, -B*62, -DQB1*0201, -DQB1*0502, -DRB1*0301, -DRB1*0802, and DRB1*1601 (Table 4).

The relative maximum binding scores of various peptides with the HLA types are also shown in the corresponding tables. The scores are particularly high for HLA-A*02, -A*68, -B*14, and -B*53 (all with b2a2), HLA-A*68, -B*14, and -B*62 (all with b3a2) and HLA-A*01, -A*02, -B*08, and -B*14 (all with e1a2).

Table 2: b2a2 association with HLA Class I and II in leukemia patients and healthy donors with statistical analysis

HLA	ALLELE	LEUKEMIA PATIENTS*	HEALTHY DONORS*	RELATIVE RISK	CHI-SQUARE	BINDING SCORE
A	01	32	78	1.76	6.35	3
A	02	50	275	0.63	6.66	6
A	23	9	13	2.85	6.12	
A	66	5	4	5.09	7.15	
A	68	5	107	0.16	19.20	6
B	14	9	99	0.32	10.88	7
B	15	6	60	0.37	5.56	
B	37	3	2	5.94	4.87	
B	38	7	11	2.55	3.89	
B	40	3	64	0.17	11.22	
B	42	3	1	11.90	7.40	
B	45	5	5	3.99	5.48	
B	49	3	2	5.94	4.87	
B	53	9	3	12.26	22.38	9
B	56	4	2	7.97	7.99	
B	62	6	0	45060000.00	23.62	0
DQB1	0201	23	36	1.89	5.29	
DQB1	0303	4	30	0.36	3.90	
DQB1	0402	10	9	3.22	6.95	
DQB1	0603	8	50	0.42	5.11	
DQB1	0609	4	0	21480000.00	11.25	
DRB1	0301	23	36	1.89	5.29	
DRB1	0302	3	0	16110000.00	8.43	
DRB1	0901	4	1	11.40	7.47	
DRB1	1001	5	2	7.15	7.41	
DRB1	1101	10	53	0.50	3.89	
DRB1	1201	3	0	16110000.00	8.43	
DRB1	1202	2	0	10740000.00	5.61	
DRB1	1503	5	1	14.33	10.13	

*Figures shown are the gene frequencies. For a significance level of = 0.05 needed to reject the null hypothesis, the chi square statistic must be > 3.84

Interestingly, 1.6% of patients with t(9;22) (q34;q11) lacked HLA-DRB3, -DRB4, and -DRB5 alleles. This contrasts with the healthy group of which only 0.13% lacked these alleles. The 2 × 2 contingency table analysis yielded a likelihood ratio of 25 and a χ^2 of 42.8, indicating a statistical significance of $p < 0.001$.

The presence of *bcr-abl* transcripts in patients with leukemia appears to be associated with both HLA class I and II alleles. In general, exogenous antigens are processed to peptides in the endocytic compartment and are presented by HLA class II to CD4+ T cells. On the other hand, endogenous peptides resulting from cleavage of proteins are transferred by the transport-associated proteins to the endocytoplasmic reticulum. In the endocytoplasmic reticulum, these peptides bind to newly synthesized HLA class I molecules, and the resulting complexes are transported to the cell surface to be recognized by CD8+ T cells. It has been shown however, that endogenous antigens can be processed by both class I and class II HLA molecules [25-27]. The *bcr-abl* transcripts in leukemia cells would be

endogenous peptides and would normally be presented by HLA class I molecules. In our study and other studies cited in Table 5, negative associations with both HLA class I and II alleles have been seen. A possible explanation is that the *bcr-abl* transcripts are presented by both HLA class I and class II.

There are published reports of both negative and positive associations of *bcr-abl* transcripts with HLA class I and class II alleles (Table 5). By a positive association it is meant that individuals having a particular *bcr-abl* transcript are more likely to be endowed with a particular HLA phenotype. By a negative association it is meant that individuals showing a particular *bcr-abl* transcript are less likely to have a given HLA phenotype. Most of these studies were done with low-resolution HLA typing data. Posthuma *et al* claimed that HLA-A3 and -B8 are associated with a diminished risk of development of CML [28]. On the basis of a large multi-center data from the European bone marrow transplant program (n = 1462), they also claimed that HLA-DR4 is associated with diminished risk

Table 3: b3a2 association with HLA Class I and II in leukemia patients and healthy donors with statistical analysis

HLA	ALLELE	LEUKEMIA PATIENTS*	HEALTHY DONORS*	RELATIVE RISK	CHI-SQUARE	BINDING SCORE
A	25	4	4	3.74	3.94	
A	68	7	107	0.21	17.80	8
B	14	5	99	0.16	19.57	5
B	15	5	60	0.28	8.04	
B	37	5	2	9.22	10.27	
B	49	6	2	11.12	13.50	
B	51	13	19	2.57	6.99	4
B	53	5	3	6.14	7.91	3
B	56	3	2	5.48	4.34	
B	60	8	0	60080000.00	29.13	
B	62	6	0	45060000.00	21.80	8
DQB1	0303	4	30	0.33	4.62	
DQB1	0609	3	0	16110000.00	7.78	
DRB1	0405	4	2	5.25	4.51	
DRB1	0701	21	99	0.50	7.72	
DRB1	0802	3	1	7.84	4.43	
DRB1	0901	4	1	10.51	6.78	
DRB1	1001	4	2	5.25	4.51	
DRB1	1503	3	1	7.84	4.43	

*Figures shown are the gene frequencies. For a significance level of = 0.05 needed to reject the null hypothesis, the chi square statistic must be > 3.84

of CML. They compared patient data with data from unaffected individuals from the registry of bone marrow donors worldwide (n = 500596). The patients and controls were matched by country. The odds ratio for HLA-DR3 was 0.86 (95% confidence interval CI 0.75–0.98) and for -DR4 combined with -B8, was 0.84 (95% CI 0.72–0.98) [29]. A limitation of this study was that no high-resolution class II typing was done. It was also assumed that all the CML patients expressed the p210 protein, which is present in a majority but not all cases of CML. Pawelec and Wagner have questioned their claim on the basis of its reliance on low-resolution typing and have stressed the need for high-resolution HLA typing [30]. Our present study not only used high-resolution HLA class II typing but also included all three types of *bcr-abl* transcripts (b2a2, b3a2, and e1a2). Significant negative associations with $p < 0.05$ was seen with HLA-A*02 (b2a2, e1a2), -A*68 (b2a2, b3a2, e1a2), -B*14 (b2a2, b3a2, e1a2), -B*15 (b2a2, b3a2), -B*40 (b2a2), -DQB1*0303 (b2a2, b3a2), -DQB1*0603 (b2a2), -DRB1*0401 (e1a2), -DRB1*0701 (b3a2), and -DRB1*1101 (b2a2). In a similar study using high-resolution HLA-DRB1 typing, Yasukawa *et al* reported positive association with HLA-DRB1*1201 (b2a2), and -DRB1*0403, -DRB1*0802, -DRB1*1403, DRB1*1405 (all with b3a2), and a negative association with -DRB1*0405, -DRB1*08032, -DRB1*1502 (all with b2a2) and -DRB1*08032, and -DRB1*1501 (both with b3a2) in a Japanese population (Table 5) [36]. However, the study included only 50 cases and included no patients with the e1a2 transcript. Fur-

thermore, association with HLA-DQB1 typing was not assessed. Our study had a larger population (n = 189) though of mixed ethnicity. The common findings of the two studies include positive association with HLA-DRB1*1201 (b2a2) and -DRB1*0802 (b3a2). There were no common HLA alleles with a negative association.

Tabe 6 shows HLA class II haplotypes with increased frequency in patients with the various types of bcr-abl transcripts.

The patients in our study had HLA class II typing that included HLA-DRB1*, -DRB3*, -DRB4*, -DRB5*, -DQB1* and -DPB1*. We included in our results the statistical data on HLA-DRB1* and -DQB1*. Our study is unique among published reports by incorporating HLA-DQB1 data. Unlike HLA-DRB1 and -DQB1, HLA-DRB3, -DRB4, and -DRB5 are not expressed by all individuals; expression depends on the haplotype. An interesting observation was the fact that the frequencies of absence of HLA-DRB3, -DRB4 and -DRB5 phenotype were higher in the patient pool then in the healthy donors. This could imply that HLA-DRB3, -DRB4, and -DRB5 alleles are associated with *bcr-abl* transcript presentation.

The *bcr-abl* transcripts can generate a T-cell response [31-34]. Successful allogeneic, autologous, and HLA-identical sibling dendritic cell immunization strategies against CML with b3a2-p210 peptide have also been tried, with generation of T-cell response [35-39]. There are reports of

Table 4: e1a2 association with HLA Class I and II in leukemia patients and healthy donors with statistical analysis

HLA	ALLELE	LEUKEMIA PATIENTS*	HEALTHY DONORS*	RELATIVE RISK	CHI-SQUARE	BINDING SCORE
A	01	9	78	3.18	8.91	5
A	02	6	275	0.38	4.76	8
A	11	4	27	3.65	5.93	3
A	26	3	16	4.54	6.37	
A	68	0	107	0.00	5.48	3
B	08	6	45	3.36	7.27	7
B	14	0	99	0.00	5.13	7
B	49	1	2	11.35	6.11	
B	53	1	3	7.56	4.15	3
B	62	3	0	22530000.00	66.52	2
DQB1	0201	7	36	3.61	8.85	
DQB1	0502	2	4	8.33	8.12	
DRB1	0301	7	36	3.61	8.85	
DRB1	0401	0	56	0.00	3.93	
DRB1	0802	1	1	16.24	6.95	
DRB1	1601	2	2	16.72	13.96	

*Figures shown are the gene frequencies. For a significance level of = 0.05 needed to reject the null hypothesis, the chi square statistic must be > 3.84.

Table 5: Published data on HLA association with *bcr-abl* transcripts

	b2a2	b3a2	e1a2
POSITIVE ASSOCIATION	DRB1*1201 [42]	DRB1*0423 [42] DRB1*0802 [42] DRB1*1423 [42] DRB1*1425 [42]	
NEGATIVE ASSOCIATION	DRB1*0425 [42] DRB1*08032 [42] DRB1*1502 [42]	DRB1*08032 [42] DRB1*1501 [42]	
PEPTIDE PRESENTATION	A2.1 [43] A3 [28, 44, 45] A11 [28, 44] B8 [28, 44] DR1 [23, 30, 46] DR2 [30, 46] DR3 [30, 46] DR4 [30, 46]	A2.1 [43] A3 [28, 44, 45, 47] A11 [28, 44, 47] B8 [28, 44, 47] DR1 [23, 30, 46] DR2 [30, 46] DR3 [30, 46] DR4 [30, 46] DR11 [44] DRB1*0101 [23] DRB1*0301 [48] DRB1*0423 [46] DRB1*0901 [42]	DR4 [29] DRB1*1501 [40, 41]

successful in vitro generation of HLA-DRB1*1501-restricted p190 minor *bcr-abl* (e1a2)-specific CD4+ T lymphocytes in ALL [40,41]. The restriction was confirmed by loss of the cytotoxic effect in the presence of anti-HLA-DR and preservation of the effect when anti-HLA class I was used.

Conclusions

It seems that *bcr-abl* peptides are presented by many different HLA types. The negative association could occur because the early leukemogenic events are contained by a T-cell cytotoxic response and this prevents disease proliferation. A positive association could mean that the HLA molecules do not present the *bcr-abl* transcripts. We hold

Table 6: HLA class II haplotypes positively associated with *bcr-abl* transcripts. (Each line represents a haplotype. An "X" indicates positive association with the respective transcript.)

DRB1	DRB3	DRB4	DRB45	DQB1	b2a2	b3a2	e1a2
090102		01030101		030302	X	X	
1503			010101	0602	X	X	
1001				050101	X	X	
080201				0402		X	
040501		01030101		0302		X	
160101			0202	050201			X
030101	010101			0201			X

the theoretical assumption that HLA-restricted T-cell cytotoxicity performs an immunosurveillance role in the pathogenesis of *bcr-abl*-transcript leukemias. Should this assumption hold, knowledge of the HLA association with different *bcr-abl* transcripts would have diagnostic and prognostic implications. It would also help in improving strategies of immunization with the *bcr-abl* peptides against t(9;22) (q34;q11) leukemia.

Competing interests
None declared.

Authors' contributions
SM carried out the data collection and analysis and writing the manuscript. The *bcr-abl* typing was done in the lab of RL. HLA typing was done in the lab supervised by PC. RL and PC did the study design and analysis of the data. All authors read and approved the final manuscript.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/4/25/prepub