Pediatr Allergy Immunol 2006: 17: 578–582
DOI: 10.1111/j.1399-3038.2006.00468.x

PEDIATRIC ALLERGY AND IMMUNOLOGY

Polymorphisms of the Bcl-2 family member *bfl-1* in children with atopic dermatitis

Gray A, Stewart H, Pravica V, Fryer AA, Lenney W, Hutchinson IV, Arkwright PD. Polymorphisms of the Bcl-2 family member *bfl-1* in children with atopic dermatitis.
Pediatr Allergy Immunol 2006 17: 578–582.

T lymphocytes accumulating in the skin of patients with atopic dermatitis (AD) have a prolonged survival and are key mediators of this inflammatory disease. The anti-apoptotic *bfl-1* gene is unique in that it is the only member of the Bcl-2 family that is transcriptionally regulated by inflammatory cytokines and might therefore be important in promoting the survival of effector T cells in patients with AD. The aim of this study was to determine whether polymorphisms in the *bfl-1* gene are associated with a predisposition to childhood AD. Four *bfl-1* gene, single nucleotide polymorphisms (SNPs) were studied by ARMS-PCR in 105 Caucasian children with moderately severe AD and 110 non-atopic adult controls. In addition to the known polymorphisms of exon 1 (+141*A/G, +202*G/T, +303*A/G), we described a novel polymorphism in the promoter region of the gene (−1182*G/C). We found a significant difference in *bfl-1* +141 genotype [OR (95% CI) 5.1 (1.0–25.2)], as well as *bfl-1* −1182:+141:+202:+303 G:A:G:A/G:A:G:A diplotype frequencies [3.5 (1.0–12.2)] in AD ($p < 0.05$). The study thus provides evidence for an association between *bfl-1* polymorphisms and the genetic predisposition to AD.

Andrew Gray[1], Helen Stewart[1], Vera Pravica[1], Anthony A. Fryer[2], Warren Lenney[2], Ian V. Hutchinson[1] and Peter D. Arkwright[3]

[1]School of Biological Sciences, University of Manchester, [2]Keele University, Stoke on Trent, Child Health and [3]University of Manchester, United Kingdom

Key words: atopic dermatitis; apoptosis; *bcl-2*; polymorphisms; children

P. D. Arkwright, Booth Hall Children's Hospital, Charlestown Rd., Manchester, M9 7AA, United Kingdom
Tel.: + 44 161 918 5535
Fax: + 44 161 918 5143
E-mail: peter_arkwright@lineone.net

Accepted 10 July 2006

Atopic dermatitis (AD) is characterized by an infiltrate of cutaneous-lymphocyte-associated antigen$^+$ CD45RO$^+$ T lymphocytes into the skin, and their longevity once there. These T lymphocytes are key mediators of the chronic inflammatory response characteristic of this condition (1). Inhibition of apoptosis and thus prolonged survival of these immune effector cells in the skin, but not blood, is important in the pathogenesis of AD (2). Topical corticosteroid and calcineurin inhibitors, effective in the treatment of AD have been shown to induce apoptosis of cutaneous T lymphocytes (3).

Lymphocyte apoptosis is regulated by intracellular proteins, which include members of the large Bcl-2 family (4). Bfl-1 is a 175 amino acid Bcl-2 member with anti-apoptotic activity (5, 6). It is unique in that rather than being constitutionally expressed, it is transcriptionally regulated by growth factors, such as granulocyte macrophage-colony stimulating factor (GM-CSF) and inflammatory cytokines including tumour necrosis factor (TNF)-α and interleukin (IL)-1β, all of which are present in increased amounts in the skin of patients with AD (7–10). We have previously shown that polymorphisms in GM-CSF and transforming growth factor (TGF-β) are associated with a genetic predisposition to AD (11, 12). High levels of these factors in the skin might influence Bfl-1 expression promoting survival of activated T lymphocytes and thus a chronic inflammatory response.

The NC/Nga-inbred mouse strain is an animal model of human AD in that it spontaneously develops dermatitis with many of the clinical, histological and immunological features of human AD (13, 14). Experiments crossing NC/Nga mice and the MSM/MS strain that does not develop dermatitis have shown that the major genetic locus for the dermatitis phenotype maps to a region of chromosome 9 (15). Although the exact gene or genes responsible are still

unknown, this region of chromosome 9 includes the *A*1 murine homologue of the human *bfl-1* gene, which has similar anti-apoptotic activity (16).

Based on these data, we hypothesized that variations in the *bfl-1* gene may predispose certain individuals to AD. The human *bfl-1* gene has four known polymorphisms (+141*A/G, +202*G/T, +303*A/G and +629*C/T) (Fig. 1) (10). The first three of these polymorphisms occur in the first of the three exons of the *bfl-1* coding region and all result in amino acid changes, leaving open the possibility of changes in Bfl-1 protein structure and function. The fourth polymorphism is located in the sequence encoding the 3′ untranslated region of the mRNA. This study tests this hypothesis by comparing the *bfl-1* genotype and allele frequencies of children with AD and a non-atopic control group.

Fig. 1. Sequence of *bfl-1* promoter and coding regions. The coding sequence was derived from DNA complementary to *bfl-1* mRNA (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=1079557). The locations of polymorphisms are indicated. The grey box indicates the *bfl-1* 5′ regulatory region. Open boxes indicate the sequences encoding exons 1 and 2.

Methods

Subjects

One hundred and five Caucasian children aged 1–16 yr with AD and 110 healthy Caucasian adult controls having no atopic diseases (no eczema, asthma or hay fever), a total IgE concentration < 100 IU/l, negative skin prick tests to a panel of seven aeroallergens and similar sex distribution were genotyped for *bfl-1* polymorphisms. The diagnosis of eczema was based on the UK working party's diagnostic criteria for AD (17). All children recruited from the regional eczema clinic had moderate to severe disease [median surface area affected 27% (range 8–46%), which was incompletely controlled with mild topical corticosteroid preparations. The demographic features of the two groups are shown in Table 1. The study was cleared with the local research ethics committee and written consent was obtained from all participants.

Sequencing *bfl-1* promoter region

DNA of the *bfl-1* promoter region that had been amplified by standard PCR was sequenced commercially (Lark Technologies, Saffron Walden, UK). Genomic DNA from five individuals was used as a template in separate PCR reactions. A total of 10 human chromosomes were sequenced.

bfl-1 polymorphism genotyping

DNA was extracted from buccal scrapings from children and blood from adults as described previously (11). Amplification refractory mutation system (ARMS)–PCR assays were used to screen the three known polymorphisms in exon 1. The general ARMS–PCR method has been described previously (11, 12). The primer sequences are shown in Table 2 and their position in the *bfl-1* gene and promoter region are illustrated in Fig. 1.

Table 1. Demographic profiles of the patients with atopic dermatitis (AD) and controls

	Control	AD
Number	110	105
Ethnic origin	Caucasian (North-West England)	Caucasian (North-West England)
Age (yr)	40.8 ± 6.6	6.0 ± 4.6
Sex ratio (male/female)	0.44/0.56	0.56/0.44
Total serum immunoglobulin E (IU/l)	all < 100	6233 ± 5794*

*Mean and standard deviation.

Table 2. Primers used for genotyping *bfl-1* polymorphisms

bf1-1 −1182	Generic primer (antisense) 5′-tgcactccagtctgggcaa-3′
	Primer G (sense) 5′-ccaggctgttttgtagtctg-3′
	Primer C (sense) 5′-ccaggctgttttgtagtctc-3′
bfl-1 +141	Generic primer (antisense) 5′-aatatggttacaattcttcccc-3′
	Primer A (sense) 5′-gctcaggactatctgcagta-3′
	Primer G (sense) 5′-gctcaggactatctgcagtg-3′
bfl-1 +202	Generic primer (antisense) 5′-ccacatccggggcaatttg-3′
	Primer G (sense) 5′-cgtccagagtgctacaaaag-3′
	Primer T (sense) 5′-acgtccagagtgctacaaaat-3′
bfl-1 +303	Generic primer (sense) 5′-tttggatatatttacaggctgg-3′
	Primer A (antisense) 5′-attcttccccagttaatgatgt-3′
	Primer G (antisense) 5′-ttcttccccagttaatgatgc-3′
Internal control primer 1 (sense) 5′-gccttcccaacccttccctta-3′	
Internal control primer 2 (antisense) 5′-tcacggatttctgttgtgtttc-3′	

Binding of the transcription factor NF-κB to the −783 to −932 regulatory region of the *bfl-1* promoter is essential for gene expression (9). We therefore also looked for additional polymorphisms in this region of the gene by examining the DNA of 10 chromosomes from five individuals commercially sequenced as detailed previously. One novel polymorphism in the promoter region (−1182*C/G) was found (GenBank accession number DQ081729). The cohort was therefore also screened for the novel −1182*C/G polymorphism using the primer sequences listed in Table 2. Details of the PCR protocols used are shown in Table 3.

Statistical analysis

Analyses were performed using the SPSS software package (version 12.0, SPSS Inc., Chicago, USA). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using binary logistic regression analysis. Hardy-Weinberg equilibrium was calculated using a standard formula (18).

Table 3. PCR annealing temperature protocol

Standard protocol for the PTC-100 PCR machine (MJ Research):		
95°C	1 min	
95°C	15 s	10 cycles
61°C	50 s	
72°C	40 s	
95°C	20 s	20 cycles
55°C	50 s	
72°C	50 s	
Modified protocol with lower annealing temperatures for the *bfl-1* +141 primers:		
95°C	1 min	
95°C	15 s	10 cycles
59°C	50 s	
72°C	40 s	
95°C	20 s	20 cycles
53°C	50 s	
72°C	50 s	

Results

The demographic characteristics of the AD and control group are shown in Table 1, illustrating the non-atopic nature of the control group in comparison with the children with moderately severe AD.

All four polymorphisms, including the newly described *bfl-1* −1182 polymorphism were in Hardy-Weinberg equilibrium and the genotype and allele frequency results are shown in Table 4. Genotyping was successful in over 95% of samples analysed as detailed in this table. Binary logistic regression analysis revealed a significant association between the *bfl-1* +141 AA genotype, but none of the other *bfl-1* genotypes, and AD.

The 196 of 220 patients (89%) where genotyping of all four polymorphisms was possible had 14 different *bfl-1* −1182:+141:+202:+303 diplotypes. Three common diplotype combinations, determined by direct sequencing of the *bfl-1* gene accounted for 80% of the total (Table 5). The remaining 20% (40 patients) had nine further diplotype combinations with low individual frequencies (1–7 patients/diplotype). Binary logistic regression showed that the G:A:G:A/G:A:G:A homozygous diplotype was significantly more common in patients with AD than in controls [OR (95% CI): 3.5 (1.0–12.2), $p < 0.05$).

Table 4. Allele and genotype frequencies of *bfl-1* polymorphisms in children with atopic dermatitis (AD) and controls

	Control	AD	OR (95% CI)
bfl-1 −1182*C/G			
Number	110	104	
Alleles			
C	163 (74%)	154 (74%)	
G	57 (26%)	54 (26%)	0.9 (0.6–1.5)
Genotype			
CC	56 (51%)	57 (55%)	
GC	51 (46%)	40 (38%)	0.7 (0.4–1.3)
GG	3 (3%)	7 (7%)	2.3 (0.6–9.3)
bfl-1 +141*A/G			
Number	109	101	
Alleles			
G	159 (73%)	134 (66%)	
A	59 (27%)	68 (34%)	1.3 (0.9–2.2)
Genotype			
GG	52 (48%)	41 (41%)	
GA	55 (50%)	52 (51%)	1.2 (0.7–2.1)
AA	2 (2%)	8 (8%)	5.1 (1.0–25.2)*
bfl-1 +202*G/T			
Number	109	105	
Alleles			
T	161 (74%)	146 (70%)	
G	57 (26%)	64 (30%)	0.8 (0.5–1.3)
Genotype			
TT	57 (52%)	52 (50%)	
GT	47 (43%)	42 (40%)	1.0 (0.6–1.7)
GG	5 (5%)	11 (10%)	2.4 (0.8–7.4)
bfl-1 +303*A/G			
Number	110	100	
Alleles			
G	156 (71%)	134 (67%)	
A	64 (29%)	66 (33%)	0.7 (0.5–1.1)
Genotype			
GG	50 (45%)	42 (42%)	
GA	56 (51%)	50 (50%)	1.1 (0.6–1.9)
AA	4 (4%)	8 (8%)	2.4 (0.7–8.5)

Number (percentage).
Odds ratio (OR) (95% confidence interval, CI) compared with first allele or genotype; *$p < 0.05$.

Table 5. *bfl-1* −1182:+141:+202:+303 diplotype frequencies in children with atopic dermatitis (AD) and controls

	Control	AD	OR (95% CI)
Number	106	90	
C:G:T:G/C:G:T:G	45 (42%)	32 (36%)	
C:G:T:G/G:A:G:A	38 (36%)	27 (30%)	1.0 (0.5–2.0)
G:A:G:A/G:A:G:A	4 (4%)	10 (11%)	3.5 (1.0–12.2)*
Nine other diplotype combinations	19 (18%)	21 (23%)	1.6 (0.7–3.4)

Number (percentage).
Odds ratio (OR) (95% confidence interval, CI) compared with C:G:T:G/C:G:T:G diplotype; *$p < 0.05$.

Discussion

This is the first study to examine the possible association between the *bfl-1* anti-apoptotic gene and atopic disease, specifically AD. The gene was suspected as possibly contributing to the susceptibility to AD for two main reasons. First, a major qualitative trait locus responsible for AD-like lesions in the NC/Nga mouse model maps to a chromosomal region which includes the mouse homologue of *bfl-1* (15). Second, the survival of T lymphocytes homing to the skin of patients with AD is known to be prolonged and the local milieu known to express increased levels of inflammatory cytokines and grown factors which induce the expression of this specific anti-apoptotic gene (2, 5, 7). The current study describes a novel polymorphism in the promoter region of this transcriptionally regulated anti-apoptotic *bfl-1* gene, which would seem functionally most relevant if pro-inflammatory cytokines prevented apoptosis of effector T lymphocytes by inducing its expression. In keeping with our *a priori* hypothesis, we found a significant association between the *bfl-1* +141 genotype and the homozygous *bfl-1* −1182:+141:+202:+303 G:A:G:A/G:A:G:A diplotype and a predisposition to childhood AD. The described association is a weak one based on the 95% CI of the data and a large cohort of subjects are required to confirm these findings.

Hardy-Weinberg equilibrium was maintaining ruling out population biasing and technical problems. The older control group was chosen deliberately to make sure they were of a completely atopic disease free-phenotype. If children of the same age would have been selected then there would be a real possibility that some may go on to develop atopic diseases (particularly asthma and hay fever) in later childhood or early adult life, which would have reduced the discriminatory power of the study. Furthermore all the controls were non-atopic with normal serum immunoglobulin E (IgE) concentrations and negative skin prick tests. All subjects in the study were Caucasian and came from the same part of England in order to control as far as possible for other genetic or environmental factors that might affect the clinical phenotype of the two groups.

The *bfl-1* gene is unique amongst the Bcl-2 family of apoptotic regulators in that it is the only one whose activity is controlled by inflammatory cytokines. Individuals with atopic diseases do not have a generalized increase in T-lymphocyte survival, but an increased survival of T lymphocytes at the site of inflammation is well documented (2). Our results suggest that polymorphisms in the anti-apoptotic gene *bfl-1* may help to explain the increased survival of T lymphocytes in the skin or an individual's genetic predisposition to AD. It may be that this genetic predisposition works in conjunction with other genetic and environmental factors to further increase the survival and homing of T lymphocytes to skin (19). In support of this hypothesis, changes in expression of a number of genes involved in T-cell homing, proliferation and apoptosis have recently been found to be altered in lymphocytes isolated from patients with AD (20).

Acknowledgments

We are grateful to Andy Vail, Medical Statistician, Hope Hospital, Manchester, UK for his helpful comments and suggestions concerning statistical analysis.

References

1. Leung DYM, Bieber T. Atopic dermatitis. Lancet 2003: 361: 151–60.
2. Trautmann A, Akdis M, Blaser K, Akadis CA. Role of dysregulated apoptosis in atopic dermatitis. Apoptosis 2000: 5: 425–9.
3. Hoetzenecker W, Ecker R, Kopp T, Stuetz A, Stingl G, Elbe-Burger A. Pimecrolimus leads to an apoptosis-induced depletion of T cells but not Langerhans cells in patients with atopic dermatitis. J Allergy Clin Immunol 2005: 115: 1276–83.
4. Green D, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004: 305: 626–9.
5. Karsan A, Yee E, Kaushansky K, Harlan JM. Cloning of a human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells. Blood 1996: 87: 3089–96.
6. Ko JK, Lee MJ, Cho SH, et al. Bfl-1S, a novel alternative splice variant of Bfl-1, localises in the nucleus via its C-terminus and prevents cell death. Oncogene 2003: 22: 2457–65.
7. Mandal M, Borowski C, Palomero T, et al. The BCL2A1 gene as a pre-T cell receptor-induced regulator of thymocyte survival. J Exp Med 2005: 201: 603–14.
8. Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes Dev 1999: 13: 400–11.
9. Edelstein LC, Lagos L, Simmons M, Tirumala H, Gelinas C. NF-κB-dependent assembly of an enhanceosome-like complex on the promoter region of apoptosis inhibitor Bfl-1/A1. Mol Cell Biol 2003: 23: 2749–61.
10. Choi SS, Park IC, Yun JW, Sung YC, Hong SI, Shin HS. A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. Oncogene 1995: 11: 1693–8.
11. Arkwright PD, Chase JM, Babbage S, Pravica V, David TJ, Hutchinson IV. Atopic dermatitis is associated with a low-producer transforming growth factor β1 cytokine genotype. J Allergy Clin Immunol 2001: 108: 281–4.
12. Rafatpanah H, Bennett E, Pravica V, et al. Inheritance of a specific GM-CSF genotype is associated with complete protection against severe atopic dermatitis. J Allergy Clin Immunol 2003: 112: 593–8.
13. Vestergaard C, Yoneyama H, Matsushima K. The NC/Nga mouse: a model for atopic dermatitis. Mol Med Today 2000: 6: 209–10.
14. Shiohara T, Hayakawa J, Mizukawa Y. Animal models for atopic dermatitis: are they relevant to human disease? J Dermatol Sci 2004: 36: 1–9.
15. Kohara Y, Tanabe K, Matsuoka K, et al. A major determinant quantitative-trait locus responsible for atopic dermatitis-like skin lesions in NC/Nga mice is located on chromosome 9. Immunogenetics 2001: 53: 15–21.
16. Lin EY, Orlofsky A, Berger MS, Prystowsky MB. Characterization of A1, a novel hemopoietic-specific early response gene with sequence similarity to bcl-2. J Immunol 1993: 151: 1979–88.
17. Williams HC, Burney PG, Hay RJ, et al. The U.K. working party diagnostic criteria for atopic dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. Br J Dermatol 1994: 131: 383–96.
18. Emery AEH. Hardy-Weinberg equilibrium and the estimation of gene frequencies. In: Methodology in Medical Genetics. An Introduction to Statistical Methods, 2nd edn, chap. 2. London, U.K., Churchill Livingstone, 1986: 3.
19. Sengler C, Lau S, Wahn U, Nickel R. Interaction between genes and environmental factors in asthma and atopy: new developments. Respir Res 2002: 3: 7.
20. Hijnen D, Nijhuis E, Bruin-Weller M, et al. Differential expression of genes involved in skin homing, proliferation, and apoptosis in CD4 + T cells of patients with atopic dermatitis. J Invest Dermatol 2005: 125: 1149–55.