

A genome-wide association scan for asthma in a general Australian population

J. Hui · A. Oka · A. James · L. J. Palmer ·
A. W. Musk · J. Beilby · H. Inoko

Received: 3 December 2007 / Accepted: 25 January 2008
© Springer-Verlag 2008

Abstract To date, almost every chromosome has been implicated in genetic susceptibility to asthma to some degree. When compared with single nucleotide polymorphism, microsatellite markers exhibit high levels of heterozygosity and therefore provide higher statistical power in association. The objective of this study was to perform a genome-wide association study using 23,465 in-house microsatellite markers to detect asthma susceptibility regions in the Busselton population. In this study, three separate pooled DNA screenings yielded 18 markers with significantly different estimated frequencies in the three

separate “case and control” pools: each pool consisting of 60 males and 60 females. These markers were evaluated by individual typing in 360 cases and 360 controls. Two markers showed significant differences between cases and controls ($P = 0.001$ and $P = 0.003$). Regions surrounding the two markers were subjected to high-density association mapping with a total of 14 additional markers. We were able to confirm and fine map the association in these two regions by typing 14 additional microsatellite markers (1805A09 (D18S0325i), $P = 0.002$; 1806D05 (D18S0181i), $P = 0.001$). Each region contains a predicted gene that showed strong associations with asthma. Further studies are underway to characterize the novel candidate asthma susceptibility genes identified in this genome-wide study.

J. Hui (✉) · L. J. Palmer
Western Australian Institute for Medical Research
and UWA Centre for Medical Research, B Block,
QEII Medical Centre, The University of Western Australia,
Nedlands, WA 6009, Australia
e-mail: jhui@cyllene.uwa.edu.au

J. Hui · A. Oka · H. Inoko
Department of Molecular Life Science, Tokai University School
of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan

A. James
Western Australian Sleep Disorders Research Institute,
Sir Charles Gairdner Hospital, Nedlands, WA 6009, Australia

A. W. Musk
Department of Respiratory Medicine,
Sir Charles Gairdner Hospital, Nedlands, WA 6009, Australia

J. Beilby
Clinical Biochemistry, PathWest Laboratory Medicine Western
Australia, QEII Medical site Nedlands, Australia School
of Surgery and Pathology, University of Western Australia,
Nedlands, WA 6009, Australia

J. Beilby
School of Surgery and Pathology,
University of Western Australia, Nedlands, WA 6009, Australia

Introduction

A large number of studies have been carried out and numerous candidate genes have been investigated for asthma (Holloway and Koppelman 2007). Like many other diseases, it has been shown that the genetic predisposition of asthma varies with ethnicity. To date, almost every chromosome has been implicated in genetic susceptibility to asthma to some degree. However, genes that are associated with asthma in one population may not be associated with asthma in another population (Chanock et al. 2007). Environmental triggers have been suggested to be one of the factors resulting in the failure to replicate associations between different studies. The involvement of multiple genes and gene–gene interactions are also likely to play a significant role in asthma.

Family and twin studies have confirmed that genetics plays an important role in the development of the disease. However, attempts to pinpoint the associated mutations or

polymorphisms of asthma have proved largely fruitless and produced inconsistent results. For example, ADAM33 (Van Eerdewegh et al. 2002) was the first putative asthma gene identified by positional cloning. However, the strong initial association was not consistently replicated in subsequent studies (Raby and Weiss 2004). Other positional candidate genes for asthma, such as PHF11 (Anderson et al. 2002; Zhang et al. 2003), DPP10 (Allen et al. 2003), CYFIP2 (Noguchi et al. 2005) and HLA-G (Nicolae et al. 2005), have been identified with similar inconsistent results from replication studies (Hersh et al. 2007). It is therefore important to identify other genes that may play a role in the development of asthma in specific ethnic groups.

Our laboratory has successfully mapped and localized susceptibility loci for a number of diseases such as Behcet's (Ota et al. 1999), psoriasis vulgaris (Oka et al. 1999), atopic dermatitis (Iizuka et al. 2002), non-obstructive azoospermia (Matsuzaka et al. 2002) and diffuse panbronchiolitis (Keicho et al. 2000) using a microsatellite genome-wide scan DNA pooling approach. From these studies, sequence data on a total of 23,465 in-house microsatellite markers have been developed. In this study, we performed a genome-wide association (GWA) study using these microsatellite markers to detect asthma susceptibility regions. Microsatellite markers are abundant and interspersed throughout the human genome with a high degree of polymorphism. When compared with single nucleotide polymorphism (SNPs), microsatellite markers exhibit high levels of heterozygosity and therefore provide higher statistical power in association analyses (Tamiya et al. 2005; Ohashi and Tokunaga 2003). Furthermore, unlike a candidate gene approach, GWA studies investigate the whole genome at the same time in a hypothesis-independent, unbiased fashion.

Methods

Patients and unaffected individual

The Busselton Health Study (BHS) includes a series of six cross-sectional population health surveys of adults (all people listed on the electoral roll) residing in the Shire of Busselton, Western Australia, since 1966. Busselton has a population that is predominantly of European origin. In 1994/1995 all traceable subjects were contacted for a follow-up study. These data, stored DNA and long-term follow-ups provide a unique opportunity to assess in an Australian population, the potential for genetic polymorphisms in asthma. From this population, 360 unrelated cases and 360 unrelated controls were randomly selected from those attending the 1994/1995 follow-up survey. Prediction of multi-locus identity-by-state had previously

been carried out in this population to ensure no cryptic relationships existed amongst unrelated cases and controls selected. Subjects were considered asthmatic if they answered yes (in 1994 or at any preceding survey) to "Has your doctor ever told you that you have asthma?" Other phenotypes, such as atopic status, airway hyperresponsiveness and lung function data, are also available for these individuals but were not analysed in this study. Sex-matched individuals who answered no (in 1994 and at all previous surveys attended) to "Has your doctor ever told you that you have asthma?" were selected as controls. The studies were approved by the Human Rights Committee of the University of Western Australia and were performed in accordance with the Declaration of Helsinki, 1964.

Preparation of pooled DNA samples for GWA

A DNA pooling method was used to reduce the cost and technical burden of genotyping across the whole genome in each individual. Firstly, all DNA concentration was normalized with a median of $\pm 5\%$ between each sample. DNAs were then quantitated in triplicate as described in Collins et al. (2000) using a PicoGreen (Molecular Probes) detection kit. In addition, to minimize pipetting errors, all DNAs were measured side by side by the same technician using one set of calibrated pipette. Genomic DNA samples were adjusted to 8 ng/ μ l, and the samples were equally divided into three case pools and three control pools with equal numbers of males and females.

Microsatellite typings for GWA

A GWA study was performed using 23,465 well-characterized microsatellite markers in a nested case/control sample to identify asthma susceptibility loci. A multi-stage pooling approach was used followed by individual typing and fine mapping. Microsatellite markers were used as described in Tamiya et al. (2005). In brief, PCR primers were designed for amplifying fragments with annealing temperature of 57°C. Forward primers were labelled at the 5' end with 6FAM or HEX (Applied Biosystems). PCR on pooled DNA was performed in 20 μ l reactions containing a total of 48 ng of pooled DNA, 0.5 U of DNA polymerase (AmpliTaq Gold), 1 $\times$ reaction buffer with 1.5 mM MgCl₂, 5 μ M of each primer and 0.25 mM of dNTP with same amplification conditions as described in Kawashima et al. (2006).

Additional microsatellite markers for high-density association mapping were obtained from the University of California-Santa Cruz (UCSC) Genome Browser database (<http://www.genome.ucsc.edu/>) (March 2006). PCR primers were designed and labelled as described earlier.

Microsatellite analyses

The frequency of the allele at each locus was analysed. Comparison between patients and controls was carried out to investigate significant differences between asthma patients and controls.

Statistical analyses

The primary binary outcome analysed was presence of physician-diagnosed asthma (ever) or treatment of asthma/bronchial asthma (ever). The principal explanatory variables were the microsatellite markers, which were analysed categorically by allele frequency. The result from each individual microsatellite typing was tested for a departure from Hardy–Weinberg equilibrium (Van Eerdeghe et al. 2002) using an expectation–maximisation approach as implemented in the GENEPOP (Raymond and Rousset 1995).

Genotype

Disease associations were assessed by Fisher's exact test, with the use of 2×2 contingency tables for the marker allele frequencies in the cases versus controls. The smallest P -value for each marker was selected. Allele frequencies in pooled-DNA typing were estimated from the height of peaks: each allele divided by the summed height of all alleles. In individual typing, case–control associations with microsatellite markers were also evaluated by Fisher's exact tests, with the use of $2 \times m$ (where m = number of alleles for that marker) and 2×2 contingency tables. Statistical significance was defined at the 5% level.

Results

Table 1 summarizes the number of markers used on each chromosome and their physical mean interval. On average, these markers were designed to chart the human genome with a mean interval of around 120 kb. Three stepwise screenings were performed to sequentially replicate the results in each of the pooled samples, thereby reducing the number of pseudo-positives resulting from type I errors (Barcellos et al. 1997). An experimental flow diagram of this GWA study using 23,465 microsatellite markers in 360 doctor-diagnosed asthma cases and 360 controls is shown in Fig. 1. Table 2 shows the characteristics of the study population, including lung function data. For completeness, information on dyspnoea, wheeze and current wheeze are included.

Table 1 Number of markers used on each chromosome and their estimated physical mean interval

Chromosome	No. of markers	Mean interval (kb)
1	1,895	118.7
2	2,053	115.8
3	1,650	118.0
4	1,554	120.5
5	1,500	118.5
6	1,369	122.2
7	1,370	113.1
8	1,103	129.3
9	956	125.7
10	1097	120.0
11	1076	121.9
12	1128	115.5
13	806	118.6
14	709	124.5
15	610	133.3
16	631	125.0
17	626	124.3
18	649	115.0
19	454	122.9
20	501	118.8
21	296	115.4
22	251	138.8
X	1,105	136.7
Y	76	337.5
Total	23,465	121.8

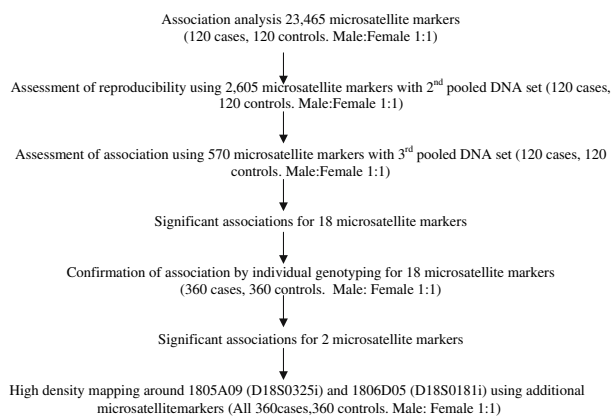


Fig. 1 Experimental flow diagram of genome-wide association analyses using 23,465 microsatellite markers in 360 doctor-diagnosed cases and 360 controls

Pooled and individual typings

Table 3 shows the results for pooled and individual typings. Allele frequencies were estimated from the height of

Table 2 Characteristics of study population

	Asthmatics		Non-asthmatics	
	Male (<i>n</i> = 169)	Female (<i>n</i> = 180)	Male (<i>n</i> = 175)	Female (<i>n</i> = 179)
Age (SD)	54.78 (16.95)	54.03 (15.76)	58.35 (14.12)	57.97 (14.91)
BMI (SD)	26.58 (3.60)	25.71 (4.53)	26.41 (3.18)	25.39 (4.22)
Height (SD)	1.75 (0.07)	1.62 (0.06)	1.75 (0.07)	1.61 (0.07)
FEV1(L) (SD)	3.05 (1.18)	2.28 (0.77)	3.32 (0.83)	2.43 (0.63)
FVC (L) (SD)	4.21 (1.28)	3.07 (0.88)	4.40 (0.96)	3.13 (0.75)
FEV1/FVC (SD)	0.71 (0.12)	0.73 (0.09)	0.75 (0.07)	0.77 (0.06)
*DRS (SD)	27.44 (69.31)	48.93 (100.50)	3.63 (11.41)	4.01 (9.34)
Dyspnoea	82.10%	80.20%	95.50%	92.00%
Wheeze	82.90%	80.70%	18.80%	19.30%
Current wheeze	60.30%	53.20%	11.40%	9.10%

Table 3 Results of *P* values for pooled and individual typings

Marker		Pooled DNA typing						Individual DNA typing	
		1st		2nd		3rd		2×2	$2 \times m$
		2×2	$2 \times m$	2×2	$2 \times m$	2×2	$2 \times m$		
0113C11	1p36.23	1.84E-07	6.63E-06	0.037	0.412	3.97E-05	3.97E-05	0.197	0.411
0216A11	2q37.1	0.025	0.159	0.018	0.049	0.007	0.042	0.065	0.123
0401F12	4p14	0.020	0.053	0.028	0.167	0.007	0.014	0.008	0.022
0610D06	6p22.3	0.024	0.059	0.022	0.022	0.003	0.009	–	–
0709A02	7p21.1	0.044	0.044	0.003	0.035	0.003	0.003	0.109	0.538
1005B02	10q21.3	3.12E-04	1.38E-04	0.038	0.404	5.47E-06	3.94E-06	0.174	0.778
1011D04	10q23.31	1.95E-07	6.39E-06	0.038	0.287	9.11E-09	3.40E-08	0.004	0.083
1102C03	11q14.2	0.012	0.127	0.017	0.376	2.47E-06	3.88E-05	–	–
1109E12	11q22.3	0.004	0.030	0.006	0.016	3.58E-04	0.056	0.073	0.764
1111F07	11q13.4	0.005	0.163	4.46E-04	0.026	1.57E-13	0.000	0.034	0.563
1307C09	13q21.31	4.27E-05	4.62E-05	0.006	0.039	8.38E-04	0.004	0.026	0.442
1307H09	13q21.31	0.003	0.006	0.006	0.013	0.001	0.004	–	–
1401A11	14q22.1	0.035	0.324	0.021	0.016	0.003	0.001	0.396	0.277
1805A09	18q23	1.28E-07	1.28E-07	3.13E-05	3.13E-05	0.022	0.022	0.001	0.002
1806D05	18q21.3	0.043	0.118	0.027	0.298	0.002	0.012	0.004	0.001
1901G11	19p12	3.77E-05	1.27E-04	5.25E-04	9.02E-04	7.04E-07	0.000	–	–
9911C08	Yp11.2	0.016	0.121	2.29E-04	0.002	7.41E-08	0.000	–	–
9911E05	Yp11.2	5.43E-04	0.002	0.005	0.009	7.78E-06	1.94E-05		

After three pooled DNA screenings, 18 markers with significantly different estimated frequencies in the three separated “case and control” pools were genotyped individually in 360 cases and 360 controls. Two markers (1805A09 and 1806D05) showed significant differences between cases and controls ($P = 0.002$ and $P = 0.001$)

individual peaks. No correction for multiple comparisons was performed to minimize false-negative inferences.

Significant differences between allele frequencies were found in 2,605 markers after the first round of pooled DNA typing (Fig. 2). To reduce Type I error, two further screening steps were performed using different case/control pools. Firstly, the 2,605 markers which showed significant differences between case and control pools in the initial genome-wide scan were genotyped in different case and control pools. After the second round of genotyping, allele

frequencies for 570 of the 2,605 markers were found to be significantly different between cases and controls. Finally, the 570 markers from the second stage were genotyped in a third set of case and control pools where 18 markers were consistently shown to be significant in all three pooled DNA typing. These 18 microsatellite markers were then typed on individuals in a full sample of 360 cases and 360 controls. The exact 2×2 and $2 \times m$ values for each of the pooled DNA typing round and the subsequent individual typing for each of the 18 markers were shown in Table 3.



Fig. 2 Results from the GWS showing 23,465 markers and their P -values

Two markers (1805A09 (D18S0181i) and 1806D05 (D18S0325i)) showed significant differences between cases and controls ($P = 0.002$ and $P = 0.003$, respectively) (Table 3). To further define the candidate gene regions, additional polymorphic markers were designed in the surroundings of these two markers. To cover approximately 248 and 234 kb regions around markers 1806D05 (D18S0181i) and 1805A09 (D18S0325i), respectively, an additional 14 microsatellite markers were genotyped to fine-map the genomic regions around these two markers (Table 4). We were able to confirm and narrow the susceptibility regions using high-density association mapping analyses (Fig. 3).

Discussion

In the current study, we have performed a genome-wide scan for asthma status using a dense set of 23,465 well-characterized microsatellite markers evenly spread across the entire genome. After four rounds of screenings (3 pooled DNA and one individual screen) on 360 asthma patients and 360 controls, we have identified 2 candidate

asthma loci on chromosome 18. These two asthma loci contain a predicted gene on the reverse strand on chromosome 18q registered in the UCSC Genome Browser (<http://genome.ucsc.edu/>). The characteristics and functions of these predicted proteins are currently unknown. A previous genetic study conducted in the National Heart, Lung and Blood Institute Family Heart Study (Wilk et al. 2003) has also identified two loci on chromosome 18 which may harbour genes related to COPD by influencing lung growth and development. One of these loci (around 79 cM, near D18S858) is located near marker 1806D05 (D18S0181i) (approximately 513 kb away). Using the ProtFun protein prediction database (<http://www.cbs.dtu.dk/services/ProtFun/>), the predicted protein within marker 1806D05 (D18S0181i) is predicted to be a signal peptide with extra-cellular domain. This structural protein is predicted to be important in translational processes.

Asthma possibly comprises multiple overlapping syndromes with varying origins and heterogeneous pathobiology (Kiley et al. 2007). We appreciate that improved phenotypic characterisation and knowledge of underlying pathobiology is necessary to ascertain the relative contribu-

Table 4 Results for fine mapping around markers 1805A09 and 1806D05

Marker ID	Public ID	Human March 2006 Assembly	Primer sequence		Results of individual typings							
			Position 1	Position 2	Forward	Reverse	Mean number of allele	Significant allele	Exact 2 × 2 P-value	Allele freq (controls)	Allele freq (cases)	Exact 2 × m P-value
1806N09	–	53612736	53613086		TCTCTGGAAAGTGGCTGTGG	TGCTCTCACTGAGCTGGCAG	6	339	0.118	0.000	0.004	0.462
1806N08	–	53643160	53643312		CTATGAACAGAACAAATTGCTGGAGG	CAGAGTGAGACTCCATCAAAAAGAAAG	9.7	143	0.090	0.041	0.061	0.751
9909A05	D18S0426i	53652278	53652508		TATGTGGTGTCACCTGAGAGCC	TTAGCAGTTGGATCAAAACAAG	8.3	228	0.042	0.404	0.351	0.487
1806N06	–	53675486	53675639		CTGAATACGTTTTTCCCAAAATTC	GGGATATGATGGCTTAGCTTG	8.7	140	0.022	0.025	0.009	0.151
1806D05*	D18S0181i	53715498	53715882		GTTGGAGCTAAGGAAAGAATC	AAACTCCTGACTTCAAGTGATC	19	246	0.004	0.068	0.033	0.001
1806N01	–	53719611	53719874		TGTAGTGGACTGAATAATGGC	CCTCCATCTTCATATCACC	15.7	284	0.085	0.004	0.013	0.591
1806N02	–	53779842	53780209		GTGGTGAAGTCAACTGTGAAAGG	TTGTGGTGATGGAAACATTCACAG	6.7	365	0.052	0.015	0.032	0.258
1802F04	D18S0161i	53791666	53791963		ATGTCCACCTAGACTTAGCATC	GCTGGTACAATCTGAGCAC	6.3	282	0.106	0.000	0.005	0.371
1806N04	–	53860580	53860736		GCTAGTGAGGGTAGGTAGAGAAGCAC	TCCTTTGATAAACAAAAGGTACACGTG	5	152	0.028	0.000	0.007	0.170
9901H03	HUMUT576	71307090	71307247		CACTGCATTCGGGGCAAC	AGGCTCTTGCTCTCGGAAT	5.7	156	0.453	0.007	0.003	0.914
1805N07	–	71370305	71370464		CGACATGCTACAGAGAGTGCA	GAATGTGGGCAATACAAAGC	4	157	0.001	0.060	0.025	0.003
1805A09*	D18S0325i	71461581	71461788		CAGAAATGATTAGGAATAAGAAGGTAG	AATCTCCATCCACATGGATAG	3.7	117	0.001	0.133	0.198	0.002
1805N01	–	71472571	71472726		TTTTGTGCCCATGTATTG	CCTGCTATTAAAGGACACAGAT	9.7	160	0.001	0.102	0.160	0.006
1805N02	–	71489702	71489854		AACCAAGTACGCTTGTGTTTC	TTTACAGGGAATATCAGGATG	8.7	156	0.018	0.081	0.049	0.313
1805N04	–	71515155	71515492		ACTCAATACTCAAACTGTGTTCC	GAGAGTGTGCTGAACAGAATC	7.3	335	0.016	0.288	0.349	0.226

 $P = 0.002$, $P = 0.001$

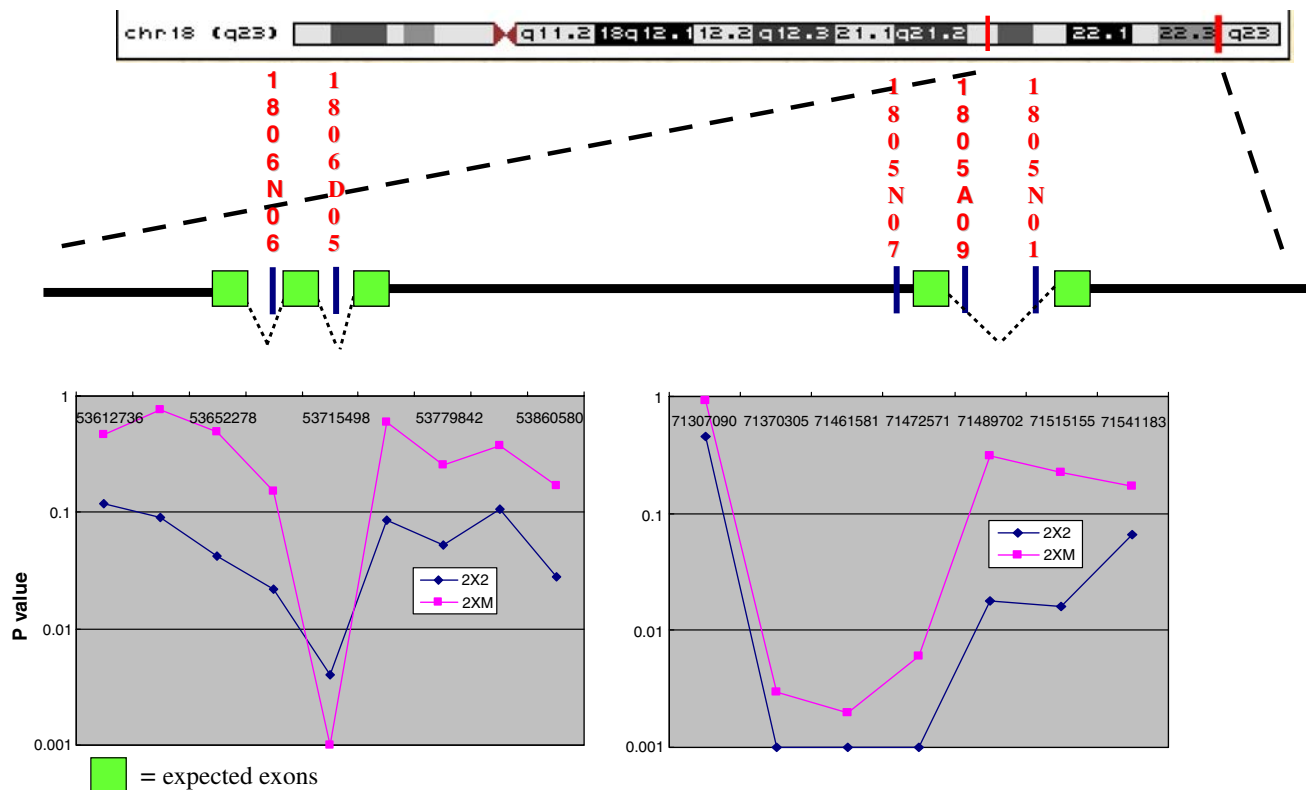


Fig. 3 Fine mapping results showing location and predicted susceptibility gene location on chromosome 18. The predicted exons are shown. Markers with significant *P* values are located within the introns of the predicted genes

tion of genetic factors to disease manifestations. In the present study, we have chosen to use positive response to questions on doctor-diagnosed asthma or ever been treated for asthma/bronchial asthma as the definition of asthma in this study. Since there is no universally accepted ‘gold standard’ definition of ‘asthma’, misclassification of case status is possible in our study. However, this would remain a limitation even if other operant definitions of asthma, such as current wheeze plus hyperresponsiveness or current use of asthma medications (Peat et al. 1992), are used. Potential misclassifications will be non-differential and therefore favour the null hypothesis. The associations that we have identified using this conservative approach will therefore remain valid. Other operant definitions of asthma (such as current wheeze plus airway hyperresponsiveness, current use of asthma medications) may alter the prevalence of asthma but will suffer from the same degree of misclassification as using doctor-diagnosed asthma.

Linkage to a non-specific IgE locus was previously reported on chromosome 18 near D18S844 (Mathias et al. 2001). D18S844 is within 1 cM of nuclear factor of activated T cells 1 (NFATC1) and approximately 900 kb from 1805A09 (D18S0325i). Suppression of NFATC1 was associated with highly increased serum IgE levels in patients with severe atopic dermatitis (Wierenga et al. 1999). These results suggest that a gene in this region of chromosome 18

could control IgE levels which may play a role in the pathogenesis of asthma. Further studies are required to determine the expression pattern of the two novel genes within chromosome 18 identified in this GWA study.

The prevalence of asthma has increased over the past 30 years, and its social and economic costs are a major problem in many developed countries (Rachelefsky 2007).

The human genome is now charted by approximately 30,000 highly polymorphic microsatellites (Kawashima et al. 2006; Tamiya et al. 2005). In this study, we have performed a high-density GWA study using microsatellite markers instead of SNPs for several reasons. Firstly, the average length of linkage disequilibrium for 30,000 microsatellites is approximately 100 kb (Tamiya et al. 2005), which is considerably higher than that of SNPs and therefore allows the capture of a larger region of linkage disequilibrium per marker when compared to SNP. Polymorphic microsatellite markers also provide a higher information content when compared with biallelic SNPs. Furthermore, interpolation variability is known to be smaller for microsatellite markers (Jorde et al. 2000; Sawyer et al. 2005). In addition, SNP haplotypes are virtual products, based on statistical hypothesis that depend on other conditions (for example, $r^2 > 0.8$, minor allele frequency > 0.1). Often, recombination events cannot be included in the deduced haplotypes due to the lack of a heterozygous SNP at the

relevant position. This can result in the generation of incorrect haplotype blocks which can, in turn, lead to false negative in GWA studies based on SNPs only. Microsatellites, on the other hand, do not generally suffer from these problems and can therefore be used as a stand-alone source of markers in GWA studies (Bahram and Inoko 2007). Microsatellites may also have important intrinsic functional relevance (Kashi and King 2006). Allelic differences in microsatellite markers are known to cause a wide range of hereditary disorder including Fragile X (Tassone et al. 2007), Huntington's disease (Vassos et al. 2007), spinocerebellar ataxia (Stevanin and Brice 2007) and cleidocranial dysplasia (Vaughan et al. 2002).

In this study, three separate pooled DNA screenings yielded 18 markers with significantly different estimated frequencies in the three separate "case and control" pools: each pool consisting of 120 individuals. In a study with 100 controls at an association strength of 0.1 with $\alpha = 5\%$, the maximum power is 0.81 when frequency of control is 0.1, and the minimum power is 0.52 when the frequency of control is 0.5 (Barcellos et al. 1997).

In this study, in the confirmation stage, 360 cases and 360 controls were individually typed. The maximum power when number of controls is 300 at an association strength of 0.1 with $\alpha = 5\%$ is 1 when frequency of control is 0.1 and power = 0.94 when the control frequency is 0.5. With a three-stage screenings and a final confirmation using a total of 720 individuals (360 controls and 360 cases), we therefore have enough power to detect association even when the control frequency is 0.5.

In a multi-stage GWA study, it is important to ensure that sufficiently large proportion of markers with effect are selected for the subsequent stage. False discoveries can always be eliminated in future stages but markers with effects that have been eliminated cannot be recovered (van den Oord et al. 2007).

Asthma, as it is defined clinically and epidemiologically, is based on symptoms which may result from a number of patho-physiological mechanisms related to airway inflammation and remodelling. Airway pathology may also be present in the absence of symptoms. Therefore, asthma remains phenotypically heterogeneous. This may partly explain why no consistent positive associations for asthma have been identified to date (Bosse and Hudson 2007). Conflicting findings may also be due to genetic heterogeneity and poor study design. Result from a recent GWA study of childhood onset asthma identified markers on chromosome 17q21 to be associated with childhood onset asthma (Moffatt et al. 2007). Further analyses concluded that genetic variants regulating ORMDL3 expression are responsible for childhood onset asthma. We did not detect any consistent significant differences in our adult asthma cases and controls on chromosome 17. One explanation is

that no stratification was performed in relation to the age of onset of asthma in the current study. Genetic susceptibility for early onset asthma may also represent a different type of disease. Further analyses of additional phenotypes with consideration of interaction effects as well as characterisation of the two predicted proteins are underway.

In conclusion, this is the first asthma GWA study using in excess of 23,000 microsatellite markers, evenly spaced within the genome to identify asthma susceptibility gene in an unbiased fashion. Our results suggest that chromosome 18 harbours at least two novel asthma susceptibility loci which warrant further investigation. We also demonstrated the feasibility of utilising pooled DNA genome-wide microsatellite approach for identifying possible candidate genes in other diseases and as an efficient and economical alternative approach to the individual level genome-wide chip-based genotyping technique.

Acknowledgments This work was supported by a Grant-in-Aid for Science Research from the Japanese Ministry of Education and by Tokai University of Medicine, Japan. J. Hui is supported by a post-doctoral fellowship from Japan Society for the Promotion of Science (JSPS), Tokai University of Medicine and the Western Australia Institute for Medical Research. The author would like to thank the Busselton Population Medical Research Foundation and the Busselton community for their participation in the study. We also thank Euzebiusz Jamrozik and Mark Divitini for statistical advices and Kaori Yamaguchi, Hiromi Kamura, Erika Matsushita and Hiromi Takase at the Department of Molecular Life Science, Tokai University School of Medicine, for technical assistance in the genome-wide screens.

References

- Allen M, Heinzmann A, Noguchi E, Abecasis G, Broxholme J, Ponting CP, Bhattacharyya S, Tinsley J, Zhang Y, Holt R, Jones EY, Lench N, Carey A, Jones H, Dickens NJ, Dimon C, Nicholls R, Baker C, Xue L, Townsend E, Kabesch M, Weiland SK, Carr D, von Mutius E, Adcock IM, Barnes PJ, Lathrop GM, Edwards M, Moffatt MF, Cookson WO (2003) Positional cloning of a novel gene influencing asthma from chromosome 2q14. *Nat Genet* 35:258–263
- Anderson GG, Leaves NI, Bhattacharyya S, Zhang Y, Walshe V, Broxholme J, Abecasis G, Levy E, Zimmer M, Cox R, Cookson WO (2002) Positive association to IgE levels and a physical map of the 13q14 atopy locus. *Eur J Hum Genet* 10:266–270
- Bahram S, Inoko H (2007) Microsatellite markers for genome-wide association studies. *Nat Rev Genet* 8:164
- Barcellos LF, Klitz W, Field LL, Tobias R, Bowcock AM, Wilson R, Nelson MP, Nagatomi J, Thomson G (1997) Association mapping of disease loci, by use of a pooled DNA genomic screen. *Am J Hum Genet* 61:734–747
- Bosse Y, Hudson TJ (2007) Toward a comprehensive set of asthma susceptibility genes. *Annu Rev Med* 58:171–184
- Chanock SJ, Manolio T, Boehnke M, Boerwinkle E, Hunter DJ, Thomas G, Hirschhorn JN, Abecasis G, Altshuler D, Bailey-Wilson JE, Brooks LD, Cardon LR, Daly M, Donnelly P, Fraumeni JF Jr, Freimer NB, Gerhard DS, Gunter C, Guttmacher AE, Guyer MS, Harris EL, Hoh J, Hoover R, Kong CA, Merikangas KR, Morton CC, Palmer LJ, Phimister EG, Rice JP, Roberts J, Rotimi C, Tucker MA, Vogan KJ, Wacholder S, Wijsman EM, Winn DM, Col-

- lins FS (2007) Replicating genotype–phenotype associations. *Nature* 447:655–660
- Collins HE, Li H, Inda SE, Anderson J, Laiho K, Tuomilehto J, Seldin MF (2000) A simple and accurate method for determination of microsatellite total allele content differences between DNA pools. *Hum Genet* 106:218–226
- Hersh CP, Raby BA, Soto-Quiros ME, Murphy AJ, Avila L, Lasky-Su J, Sylvia JS, Klanderman BJ, Lange C, Weiss ST, Celedon JC (2007) Comprehensive testing of positionally cloned asthma genes in two populations. *Am J Respir Crit Care Med* 176(9):849–857
- Holloway JW, Koppelman GH (2007) Identifying novel genes contributing to asthma pathogenesis. *Curr Opin Allergy Clin Immunol* 7:69–74
- Iizuka M, Katsuyama Y, Mabuchi T, Umezawa Y, Matsuyama T, Ozawa A, Kawada H, Inoko H, Morita E, Ota M (2002) Genetic association analysis using microsatellite markers in atopic dermatitis. *Tokai J Exp Clin Med* 27:51–56
- Jorde LB, Watkins WS, Bamshad MJ, Dixon ME, Ricker CE, Seielstad MT, Batzer MA (2000) The distribution of human genetic diversity: a comparison of mitochondrial, autosomal, and Y-chromosome data. *Am J Hum Genet* 66:979–988
- Kashi Y, King DG (2006) Simple sequence repeats as advantageous mutators in evolution. *Trends Genet* 22:253–259
- Kawashima M, Tamiya G, Oka A, Hohjoh H, Juji T, Ebisawa T, Honda Y, Inoko H, Tokunaga K (2006) Genomewide association analysis of human narcolepsy and a new resistance gene. *Am J Hum Genet* 79:252–263
- Keicho N, Ohashi J, Tamiya G, Nakata K, Taguchi Y, Azuma A, Ohishi N, Emi M, Park MH, Inoko H, Tokunaga K, Kudoh S (2000) Fine localization of a major disease-susceptibility locus for diffuse panbronchiolitis. *Am J Hum Genet* 66:501–507
- Kiley J, Smith R, Noel P (2007) Asthma phenotypes. *Curr Opin Pulm Med* 13:19–23
- Mathias RA, Freidhoff LR, Blumenthal MN, Meyers DA, Lester L, King R, Xu JF, Solway J, Barnes KC, Pierce J, Stine OC, Togias A, Oetting W, Marshik PL, Hetmanski JB, Huang SK, Ehrlich E, Dunston GM, Malveaux F, Banks-Schlegel S, Cox NJ, Bleeker E, Ober C, Beaty TH, Rich SS (2001) Genome-wide linkage analyses of total serum IgE using variance components analysis in asthmatic families. *Genet Epidemiol* 20:340–355
- Matsuzaka Y, Makino S, Okamoto K, Oka A, Tsujimura A, Matsumiya K, Takahara S, Okuyama A, Sada M, Gotoh R, Nakatani T, Ota M, Katsuyama Y, Tamiya G, Inoko H (2002) Susceptibility locus for non-obstructive azoospermia is localized within the HLA-DR/DQ subregion: primary role of DQB1*0604. *Tissue Antigens* 60:53–63
- Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, Depner M, von Berg A, Bufe A, Rietschel E, Heinzmann A, Simma B, Frischer T, Willis-Owen SA, Wong KC, Illig T, Vogelberg C, Weiland SK, von Mutius E, Abecasis GR, Farrall M, Gut IG, Lathrop GM, Cookson WO (2007) Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* 448:470–473
- Nicolae D, Cox NJ, Lester LA, Schneider D, Tan Z, Billstrand C, Kuldanek S, Donfack J, Kogut P, Patel NM, Goodenbour J, Howard T, Wolf R, Koppelman GH, White SR, Parry R, Postma DS, Meyers D, Bleeker ER, Hunt JS, Solway J, Ober C (2005) Fine mapping and positional candidate studies identify HLA-G as an asthma susceptibility gene on chromosome 6p21. *Am J Hum Genet* 76:349–357
- Noguchi E, Yokouchi Y, Zhang J, Shibuya K, Shibuya A, Bannai M, Tokunaga K, Doi H, Tamari M, Shimizu M, Shirakawa T, Shibasaki M, Ichikawa K, Arinami T (2005) Positional identification of an asthma susceptibility gene on human chromosome 5q33. *Am J Respir Crit Care Med* 172:183–188
- Ohashi J, Tokunaga K (2003) Power of genome-wide linkage disequilibrium testing by using microsatellite markers. *J Hum Genet* 48:487–491
- Oka A, Tamiya G, Tomizawa M, Ota M, Katsuyama Y, Makino S, Shiina T, Yoshitome M, Iizuka M, Sasao Y, Iwashita K, Kawakubo Y, Sugai J, Ozawa A, Ohkido M, Kimura M, Bahram S, Inoko H (1999) Association analysis using refined microsatellite markers localizes a susceptibility locus for psoriasis vulgaris within a 111 kb segment telomeric to the HLA-C gene. *Hum Mol Genet* 8:2165–2170
- Ota M, Mizuki N, Katsuyama Y, Tamiya G, Shiina T, Oka A, Ando H, Kimura M, Goto K, Ohno S, Inoko H (1999) The critical region for Behcet disease in the human major histocompatibility complex is reduced to a 46-kb segment centromeric of HLA-B, by association analysis using refined microsatellite mapping. *Am J Hum Genet* 64:1406–1410
- Peat JK, Haby M, Spijker J, Berry G, Woolcock AJ (1992) Prevalence of asthma in adults in Busselton, Western Australia. *BMJ* 305:1326–1329
- Raby BA, Weiss ST (2004) ADAM33: where are we now? *Am J Respir Cell Mol Biol* 31:1–2
- Rachelefsky GE (2007) Difficult-to-control asthma: underlying factors, clinical implications, and treatment strategies. *Curr Med Res Opin*
- Raymond M, Rousset F (1995) GENESPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J Hered* 86:248–249
- Sawyer SL, Mukherjee N, Pakstis AJ, Feuk L, Kidd JR, Brookes AJ, Kidd KK (2005) Linkage disequilibrium patterns vary substantially among populations. *Eur J Hum Genet* 13:677–86
- Stevanin G, Brice A (2007) Spinocerebellar ataxia 17 (SCA17) and Huntington's disease-like 4 (HDL4). *Cerebellum* 1–9
- Tamiya G, Shinya M, Imanishi T, Ikuta T, Makino S, Okamoto K, Furugaki K, Matsumoto T, Mano S, Ando S, Nozaki Y, Yukawa W, Nakashige R, Yamaguchi D, Ishibashi H, Yonekura M, Nakami Y, Takayama S, Endo T, Saruwatari T, Yagura M, Yoshikawa Y, Fujimoto K, Oka A, Chiku S, Linsen SE, Giphart MJ, Kulski JK, Fukazawa T, Hashimoto H, Kimura M, Hoshina Y, Suzuki Y, Hotta T, Mochida J, Minezaki T, Komai K, Shiozawa S, Taniguchi A, Yamanaka H, Kamatani N, Gojobori T, Bahram S, Inoko H (2005) Whole genome association study of rheumatoid arthritis using 27 039 microsatellites. *Hum Mol Genet* 14:2305–2321
- Tassone F, Adams J, Berry-Kravis EM, Cohen SS, Brusco A, Leehey MA, Li L, Hagerman RJ, Hagerman PJ (2007) CGG repeat length correlates with age of onset of motor signs of the fragile X-associated tremor/ataxia syndrome (FXTAS). *Am J Med Genet B Neuropsychiatr Genet* 144:566–569
- van den Oord E, McClay J, York T, Murrelle L, Robles J (2007) Genetics and diagnostic refinement. *Behav Genet* 37:535–545
- Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, Walsh A, Liu Z, Hayward B, Folz C, Manning SP, Bawa A, Saracino L, Thackston M, Bencheekroun Y, Capparelli N, Wang M, Adair R, Feng Y, Dubois J, FitzGerald MG, Huang H, Gibson R, Allen KM, Pedan A, Danzig MR, Umland SP, Egan RW, Cuss FM, Rorke S, Clough JB, Holloway JW, Holgate ST, Keith TP (2002) Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 418:426–430
- Vassos E, Panas M, Kladi A, Vassilopoulos D (2007) Effect of CAG repeat length on psychiatric disorders in Huntington's disease. *J Psychiatr Res*
- Vaughan T, Pasco JA, Kotowicz MA, Nicholson GC, Morrison NA (2002) Alleles of RUNX2/CBFA1 gene are associated with differences in bone mineral density and risk of fracture. *J Bone Miner Res* 17:1527–1534
- Wierenga EA, Walchner M, Kick G, Kapsenberg ML, Weiss EH, Messer G (1999) Evidence for suppressed activity of the transcription

- factor NFAT1 at its proximal binding element P0 in the IL-4 promoter associated with enhanced IL-4 gene transcription in T cells of atopic patients. *Int Immunol* 11:297–306
- Wilk JB, DeStefano AL, Arnett DK, Rich SS, Djousse L, Crapo RO, Leppert MF, Province MA, Cupples LA, Gottlieb DJ, Myers RH (2003) A genome-wide scan of pulmonary function measures in the National Heart, Lung, and Blood Institute Family Heart Study. *Am J Respir Crit Care Med* 167:1528–1533
- Zhang Y, Leaves NI, Anderson GG, Ponting CP, Broxholme J, Holt R, Edser P, Bhattacharyya S, Dunham A, Adcock IM, Pulleyn L, Barnes PJ, Harper JJ, Abecasis G, Cardon L, White M, Burton J, Matthews L, Mott R, Ross M, Cox R, Moffatt MF, Cookson WO (2003) Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma. *Nat Genet* 34:181–186