nature genetics

Two new susceptibility loci for Kawasaki disease identified through genome-wide association analysis

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To find new candidate loci predisposing individuals to Kawasaki disease, an acute vasculitis that affects children, we conducted a genome-wide association study in 622 individuals with Kawasaki disease (cases) and 1,107 controls in a Han Chinese population residing in Taiwan, with replication in an independent Han Chinese sample of 261 cases and 550 controls. We report two new loci, one at *BLK* (encoding B-lymphoid tyrosine kinase) and one at *CD40*, that are associated with Kawasaki disease at genome-wide significance ($P < 5 \times 10^{-8}$). Our findings may lead to a better understanding of the role of immune activation and inflammation in Kawasaki disease pathogenesis.

Kawasaki disease (MIM 611775) is an acute, self-limiting vasculitis that affects infants and young children. Symptoms include prolonged fever, polymorphous skin rash, swollen glands, red eyes, mouth inflammation and swollen hands and feet[1]. Coronary aneurysms develop in 15–25% of untreated children with Kawasaki disease[1,2], and this disease is the leading cause of acquired heart disease among children in industrialized countries.

Genetic determinants have been suggested to contribute to disease susceptibility. Populations in Asian countries have higher incidence rates of Kawasaki disease than those in Western countries: Japan has the highest annual incidence rate[3,4], followed by Korea[5] and Taiwan[6,7]. Although the cause of Kawasaki disease is unknown, clinical and epidemiological findings suggest that an infectious agent triggers an inflammatory response, leading to host immune dysregulation in genetically predisposed individuals[8,9]. Thus, in addition to loci related to cardiovascular function, genes with a role in immune activity have been a focus of candidate gene studies of Kawasaki disease susceptibility and disease outcome[10]. A genome-wide linkage analysis conducted in samples from Japanese sibling pairs with Kawasaki disease[11,12] and four genome-wide association studies (GWAS) in individuals of European ancestry and in Korean and Taiwanese populations identified biologically plausible candidate loci for Kawasaki disease[13–16]. However, these loci do not fully explain the genetic risk for Kawasaki disease, suggesting that additional genetic factors remain to be discovered.

We performed a case-control GWAS to search for loci associated with increased risk of Kawasaki disease using the Affymetrix 6.0 SNP chip. We initially analyzed 905,358 SNPs in 627 Kawasaki disease cases and 1,118 controls in a Han Chinese population residing in Taiwan. After strict quality control filtering (**Supplementary Table 1**), we analyzed 716,935 SNPs (79.19%) in 622 Kawasaki disease cases and 1,107 controls. Analysis of population structure by principal-component analysis (PCA) did not give any significant evidence of population stratification between Kawasaki disease cases and controls (**Supplementary Fig. 1**). The genomic inflation factor was 1.000.

The association results for Kawasaki disease susceptibility in the 622 Kawasaki disease cases and 1,107 controls are shown (**Fig. 1**). We found 101 SNPs associated with Kawasaki disease at $P < 1 \times 10^{-4}$ (**Fig. 1** and **Supplementary Table 2**). We validated these SNPs by Sequenom MassARRAY and further genotyped the validated SNPs in an independent cohort of 261 Kawasaki disease cases and 564 controls (**Supplementary Table 2**). After kinship analysis, 261 Kawasaki disease cases and 550 controls remained in the replication cohort.

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Received 30 August 2011; accepted 29 February 2012; published online 25 March 2012; doi:10.1038/ng.2227

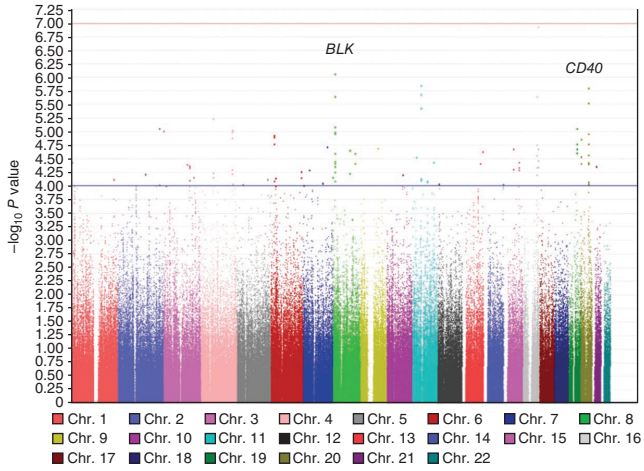

Figure 1 Results of genome-wide association analysis ($-\log_{10} P$) shown in chromosomal order for 716,935 SNPs tested for association in initial sample of 622 Kawasaki disease cases and 1,107 controls. The *x* axis represents each of the SNPs used in the primary scan. The *y* axis represents the $-\log_{10} P$ value of the trend test. Horizontal lines indicate $-\log_{10} P = 4$ and 8. Signals in the *BLK* and *CD40* regions are indicated.

A total of 23 SNPs showed nominal evidence of replication ($P < 0.05$) (**Supplementary Table 3**). All these SNPs showed similar evidence for association with Kawasaki disease after PCA adjustment (for components 1 to 10) in the GWAS collection samples (**Supplementary Table 4**). We observed no strong evidence of heterogeneity between samples from the GWAS and the replication study for these SNPs ($I^2 = 0$, $P_{het} > 0.43$) (**Supplementary Table 5**). A joint analysis of both GWAS and replication samples for these 23 SNPs resulted in three SNPs located at two loci exceeding the threshold for genome-wide significance ($P < 1 \times 10^{-8}$; **Table 1**).

The rs2736340 ($P = 9.01 \times 10^{-10}$; odds ratio (OR) = 1.54) and rs2618476 ($P = 1.96 \times 10^{-9}$; OR = 1.52) SNPs were found to be in strong linkage disequilibrium (LD) ($D' = 0.988$ and $r^2 = 0.971$; **Fig. 2a** and **Supplementary Fig. 2a**) and mapped to a 12.2-kb LD block (position 11,378,539–11,390,744) at 8p23.1; the block comprises the promoter and the first intron of *BLK* (encoding B-lymphoid tyrosine kinase). Ten nearby SNPs clustered in the first intron of *BLK* did not reach genome-wide significance; however, their *P* values were significantly associated with Kawasaki disease ($P = 2.68 \times 10^{-6}$ to 2.44×10^{-8}) in the joint analysis (**Fig. 2a** and **Supplementary Table 3**). Subsequent logistic regression analyses conditioned on rs2736340 indicated that most of the observed associations resulted from strong LD with rs2736340 (**Supplementary Fig. 2b**). We performed haplotype analysis to investigate the effect of combinations of these Kawasaki disease–associated SNPs; however, no haplotype showed stronger association than the single-marker association of rs2736340 (strongest $P = 9.35 \times 10^{-7}$).

The third SNP to reach genome-wide significance was rs1569723 ($P = 5.67 \times 10^{-9}$; OR = 1.42; **Table 1**), which mapped to a 17.2-kb LD block (position 44,164,170–44,181,354) at 20q13.12 located upstream of the *CD40* gene (**Fig. 2b**). Five nearby SNPs encompassing the region upstream of *CD40* and *CD40* itself showed suggestive associations ($P = 1.46 \times 10^{-6}$ to 1.93×10^{-7}) in the joint analysis (**Fig. 2b** and **Supplementary Table 3**). These SNPs are in LD with rs1569723 ($0.75 < D' < 0.89$ and $0.51 < r^2 < 0.67$) (**Fig. 2b** and **Supplementary Fig. 3a**). In two-point logistic regression analyses conditioned on rs1569723, the significant associations at the other SNPs disappeared (**Supplementary Fig. 3b**), indicating that the associations at the six SNPs were not independent of each other.

BLK is a Src family tyrosine kinase that transduces signals downstream of the B-cell receptor. Expression of BLK is highly restricted to the B-cell lineage and is dependent on developmental stage[17,18]. B-cell receptor signaling is important for establishing the B-cell repertoire during development of these cells[19] and has a critical role in B-cell activation and antibody secretion. Genetic variants in the region upstream of the transcription initiation site of *BLK* have been associated with expression levels of *BLK* and increased risk of systemic lupus erythematosus (SLE)[20] (rs13277113: OR = 1.39; $P = 1 \times 10^{-10}$). The SLE-associated SNP rs13277113 was in strong LD with the Kawasaki disease–associated SNP rs2736340 ($D' = 1$ and $r^2 = 0.957$ in the HapMap Japanese in Tokyo (JPT) and Han Chinese in Beijing (CHB) populations). Very recently, the rs2736340 SNP was shown to be associated with rheumatoid arthritis[21] ($P = 5.69 \times 10^{-9}$; OR = 1.19). Altered BLK protein levels could influence tolerance mechanisms during B-cell development and B-cell activation, predisposing individuals to systemic autoimmunity. Our finding that *BLK* is associated with increased risk for Kawasaki disease suggests that autoimmunity and antibody-mediated immune responses may be involved in Kawasaki disease pathogenesis. We also observed that the distribution of rs2736340 in *BLK* alleles differs according to ancestry, in agreement with the prevalence of Kawasaki disease in Europeans and Asians. The frequency of the T allele is higher in Asians (0.68–0.77) and lower in Western Europeans (0.239) (based on 1000 Genomes Project data). Further elucidation of the role of *BLK* in Kawasaki disease susceptibility and its association with differences in Kawasaki disease prevalence among Europeans and Asians is required.

CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily. It is expressed on the surface of B cells and is inducibly

Table 1 Association analyses for the three SNPs reaching genome-wide significance in the joint analysis

Chr.	SNP	Position	Gene	Allele format	Risk allele	Stage	RAF controls	RAF cases	Trend *P*	Risk allele OR (95% CI)
8	rs2736340	11381382	*BLK*	CT	T	GWAS	0.722	0.797	8.74×10^{-7}	1.51 (1.28–1.79)
						Replication	0.718	0.802	2.56×10^{-4}	1.59 (1.24–2.05)
						Combined			9.01×10^{-10}	1.54 (1.34–1.77)
8	rs2618476	11389950	*BLK*	AG	G	GWAS	0.725	0.797	2.23×10^{-6}	1.49 (1.26–1.76)
						Replication	0.719	0.804	2.08×10^{-4}	1.60 (1.24–2.07)
						Combined			1.96×10^{-9}	1.52 (1.33–1.75)
20	rs1569723	44175471	*CD40*	AC	A	GWAS	0.548	0.632	1.55×10^{-6}	1.41 (1.23–1.63)
						Replication	0.570	0.655	6.69×10^{-4}	1.43 (1.15–1.78)
						Combined			5.67×10^{-9}	1.42 (1.26–1.59)

Chr., chromosome; gene, genes containing the SNP or the closest gene up to 50 kb upstream or downstream of the SNP; RAF controls, risk allele frequency in controls; RAF cases, risk allele frequency in Kawasaki disease cases; OR, odds ratio; 95% CI, 95% confidence interval. **Supplementary Table 3** reports all SNPs with $P < 1 \times 10^{-4}$ in the Kawasaki disease GWAS collection and with $P < 0.05$ in the Kawasaki disease replication collection and the results of the joint analysis.

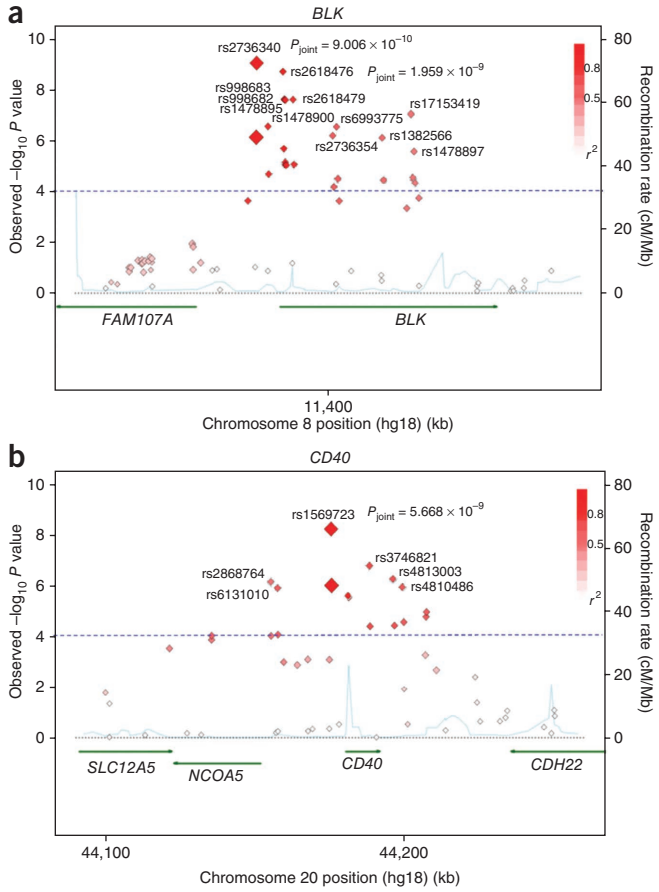

Figure 2 Association plots for the *BLK* and *CD40* regions. (**a**,**b**) Regional association plot, recombination rate and LD for the *BLK* region on chromosome 8 (**a**) and the *CD40* region on chromosome 20 (**b**), with gene annotations superimposed. Each SNP is plotted with respect to its chromosomal location (*x* axis) and its $-\log_{10}$ *P* values (left *y* axis) for the trend test from the primary GWAS scan and joint analysis at that region of the chromosome. The results from the joint analysis for key SNPs are indicated with their rs numbers. The estimated recombination rates (right *y* axis) based on the combined JPT, CHB and Chinese in Denver (CHD) samples from the HapMap Project are plotted in light blue. The color of each SNP symbol reflects its LD (using the *D′* algorithm) with the top SNP (large red diamond) within the association locus. *D′* values were calculated using data from the GWAS study.

expressed on a variety of immune and nonimmune cell types[22]. CD40 potentially contributes to inflammation and autoimmune disease processes through the selection of autoreactive T cells in the thymus[23] and the activation of B and T cells[24]. In addition, increased CD40 signaling leads to the production of proinflammatory cytokines and chemokines within targeted tissues, which contributes to tissue destruction and inflammatory cell influx. It has been proposed that aberrant expression of CD40 is a contributing factor for the initiation of autoimmunity in Graves' disease[25], type 1 diabetes[26], multiple sclerosis[27], psoriasis[28], Crohn's disease[29], rheumatoid arthritis[30] and SLE[31]. A functional polymorphism located −1 to the start codon of *CD40*, rs1883832, was previously reported to alter the translation efficiency of *CD40*. This polymorphism was associated with increased risk of Graves' disease[32–34] and may have an effect on susceptibility to rheumatoid arthritis[35] and multiple sclerosis[36]. The significant SNP (rs1569723) identified in the current study in the *CD40* region was in strong LD with this functional polymorphism (*D′* = 0.96 and $r^2 = 0.93$; in 1000 Genomes Project JPT and CHB data). Strategies for alleviating these autoimmune diseases by inhibiting CD40 signaling have been explored[37]. There is increasing evidence that interactions between CD40 and CD40 ligand on T lymphocytes and platelets have an important role in acute coronary syndrome[38]. Furthermore, Kawasaki disease is characterized by overactivation of the immune system that specifically targets vascular endothelium, resulting in systemic vasculitis or even coronary artery aneurysm. Indeed, the expression of CD40 ligand on $CD4^+$ T cells and platelets is associated with coronary artery lesions and disease progression in Kawasaki disease[39]. These findings support our observation of an association between *CD40* and Kawasaki disease susceptibility and shed light on a possible mechanism for Kawasaki disease pathogenesis. We suggest that inhibiting CD40 signaling may be an effective strategy for treating Kawasaki disease.

The strongest Kawasaki disease susceptibility loci identified to date are on chromosome 19q13 and at *FCGR2A*. The 19q13 region was initially identified in Japanese sibling pairs[11]. Subsequent finer-scale mapping and further *in vitro* functional analysis identified a functional SNP (rs28493229, $P = 1.2 \times 10^{-8}$) in *ITPKC* that affects *ITPKC* expression and T-cell activation and thus may be involved in Kawasaki disease susceptibility[12]. More recently, a large-scale international study identified two SNPs that were associated with Kawasaki disease susceptibility[16]. One SNP was located in *FCGR2A* (rs1801274, $P = 7.35 \times 10^{-11}$), and the other SNP was located upstream of the *MIA* and *RAB4B* genes at 19q13 (rs2233152, $P = 2.51 \times 10^{-9}$). The previously identified functional SNP in *ITPKC* was also verified in that study and showed the strongest association with Kawasaki disease susceptibility (rs28493229, $P = 1.68 \times 10^{-12}$). These three SNPs were genotyped in a portion of our GWAS cohort comprising 438 cases and 446 controls and showed nominal association with Kawasaki disease (rs2233152, $P = 0.0036$; rs1801274, $P = 6.30 \times 10^{-4}$; rs28493229, $P = 1.50 \times 10^{-4}$)[16]. In addition, we observed that rs10401344 (*NUMBL*), rs17713068 (*SNRPA*), rs2233152 (*MIA*) and rs10403040 (*RAB4B*) at 19q13 were suggestively associated with Kawasaki disease (all with $P < 1 \times 10^{-4}$) in the current GWAS (**Supplementary Fig. 4** and **Supplementary Table 2**). To establish independent evidence for association, the results for these four SNPs in our previously reported GWAS samples[15] and the additional individuals genotyped in the current study are listed (**Supplementary Table 6**). Although three SNPs (rs10401344, rs17713068 and rs2233152) were genotyped in our replication samples, they failed to replicate; however, the low minor allele frequency (MAF; 0.06 in controls) in the small replication sample size may have affected our ability to observe an association (**Supplementary Table 7**). Because the *ITPKC*, *MIA* and *RAB4B* genes in this region are plausible biological candidates for Kawasaki disease susceptibility, detailed resequencing of this region and functional studies are required to provide further information and to identify disease-modifying variants. Although we did not observe any SNPs in *FCGR2A* that reached the threshold ($P < 1 \times 10^{-4}$) for evaluation in the replication collection in our GWAS, six SNPs located in *FCGR2A* did show an association with Kawasaki disease at $P < 0.005$. The association of these six SNPs in the previous genotyped samples and the additional individuals genotyped in the current study are given (**Supplementary Table 8**). Our new results provide further support for an association between Kawasaki disease and the 19q13 region but do not add support for the *FCGR2A* association.

Early genetic studies of Kawasaki disease were focused on major histocompatibility complex (MHC) antigens[10]. We examined the MHC region in our GWAS, but we did not identify any SNP associated with $P < 1 \times 10^{-4}$ in this region. However, some SNPs

located in the MHC region did show nominal association ($P < 0.01$) (**Supplementary Fig. 5**). Whether the MHC has a role in Kawasaki disease susceptibility needs to be further investigated by traditional human leukocyte antigen (HLA) genotyping methods.

In summary, we have identified and replicated the *BLK* and *CD40* regions as two new loci associated with increased Kawasaki disease susceptibility and confirmed the previously identified *ITPKC* locus. All of these candidate loci are involved in immune and inflammatory responses and therefore broadly fit the current consensus regarding Kawasaki disease pathogenesis. Both BLK and CD40 signaling pathways are potential targets for the treatment of Kawasaki disease.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank all affected individuals and their families who devoted their time and effort to participate in this study. We gratefully acknowledge the members of the Translational Resource Center (TRC) (NSC100-2325-B-001-023) for Genomic Medicine and the National Center for Genome Medicine (NCGM) (NSC100-2319-B-001-001) at Academia Sinica for their support in subject recruitment, genotyping and statistical analysis. We especially thank H. Lue for his inspirational discussion. This study was supported by the Academia Sinica Genomic Medicine Multicenter Study, Taiwan (40-05-GMM). The funders had no role in study design, data collection or analysis, the decision to publish or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

Y.-T.C., F.-J.T. and J.-Y.W. are the principal investigators who conceived and obtained funding for this project. Y.-C.L., C.-H.C. and J.-Y.W. organized and supervised the GWAS and replication genotyping pipeline and devised the overall analysis plan. Y.-C.L. wrote the first draft of the manuscript with input from C.-H.C. and J.-Y.W. Y.-C.L., L.-C.C. and C.-H.C. analyzed the data. C.-D.L., J.-S.C., L.-Y.C., L.-M.H., M.-R.C., H.-C.K., H.C., F.-Y.H., M.-L.L., Y.-C.H., B.H., N.-C.C., K.-P.H., P.-C.L., Y.-M.L., Y.-J.C. and the Taiwan Pediatric ID Alliance coordinated and contributed subject and database phenotype collections.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Ethical statement. The study was approved by the Institutional Review Board and the Ethics Committee of the Institutional Review Board of China Medical University Hospital, National Taiwan University Hospital, Changhua Christian Hospital, Taipei Veterans General Hospital, Kaohsiung and Linkou Chang Gung Memorial Hospital, Mackay Memorial Hospital and Academia Sinica, Taiwan. Written informed consents were obtained from the subjects' parents in accordance with institutional requirements and Declaration of Helsinki principles.

Study subjects and phenotype definition. Individuals with Kawasaki disease (n = 627) (including the 250 Kawasaki disease cases in the GWAS and the 208 Kawasaki disease cases in the replication study in our previous study[15], which were also used in the international replication study[16]) were consecutively recruited in Taiwan from the China Medical University Hospital in Taichung, the National Taiwan University Hospital in Taipei, Changhua Christian Hospital in Changhua, Taipei Veterans General Hospital in Taipei and Chang Gung Memorial Hospital in Kaohsiung and Linkou in collaboration with the Translational Resource Center (TRC) for Genomic Medicine of Taiwan. The 261 Kawasaki disease cases in the replication study were also recruited from these hospitals. All of the cases were diagnosed according to criteria for Kawasaki disease[40,41] and were recruited as in our previous report. The 1,118 control subjects in the GWAS and the 564 control subjects in the replication study were randomly selected from the Taiwan Han Chinese Cell and Genome Bank in Taiwan, as reported previously[42]. The prevalence of Kawasaki disease in the Taiwanese population is less than 0.01%; hence, the controls were presumably disease free. The demographic and clinical characteristics of participants in the GWAS and replication study after kinship filtering are listed (**Supplementary Table 9**).

Genotyping and quality control. Genomic DNA was extracted from blood using the Puregene DNA Isolation Kit (Gentra Systems). Each individual was genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 (with a total of 906,600 SNPs) according to the manufacturer's protocols by the National Center for Genome Medicine (NCGM) at Academia Sinica. All of the sample call rates were >98%, and the mean individual sample call rate was 98.4 ± 0.7%. First-degree relatives (parent-offspring and full sibling pairs) in Kawasaki disease cases and in the control samples were identified by kinship analysis and were excluded from further analysis. Genotyping quality control for each SNP was further evaluated by determining the total call rate (successful call rate) and MAF in cases and controls. SNPs were excluded from further analysis if only one allele appeared in cases and controls, the total call rate was <0.95 or the total MAF was <0.05 and the total call rate was <0.99. In addition, SNPs that departed significantly from Hardy-Weinberg equilibrium were excluded ($P < 1 \times 10^{-4}$).

Statistical analysis. Detection of possible population stratification that could influence association analysis was carried out using EIGENSTRAT 2.0 to conduct PCA[43]. We also estimated the variance inflation factor for genomic control. Genome-wide association analysis was carried out to compare allele and genotype frequencies between cases and controls using the Cochran-Armitage trend test. A quantile-quantile plot was used to examine the P value distribution (**Supplementary Fig. 1f**). Two-point analyses were performed using a logistic regression model, regressing the affected status of two SNPs and their interaction. SNPs were coded as 0, 1 and 2 for the number of minor alleles and were treated as continuous variables. Heterogeneity tests (I^2 and P values of the Q statistics) between GWAS and replication groups were performed using described methods[44].

Validation and replication. The top SNPs ($P < 1 \times 10^{-4}$) from the genome-wide association analysis of the 622 Kawasaki disease cases and 1,107 controls were further validated in 94 controls and 188 Kawasaki disease cases using MALDI-TOF mass spectrometry (MassARRAY, Sequenom), and the SNP genotypes with over 99% successful rate and over 99% concordance between two platforms were then genotyped in an additional 261 Kawasaki disease cases and 564 controls for replication.

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doi:10.1038/ng.2227