$$\exp\left(\log\hat{\psi}\pm z_{\alpha/2}\cdot\sqrt{\frac{1}{2n_{11}+n_{21}}+\frac{1}{2n_{31}+n_{21}}+\frac{1}{2n_{12}+n_{22}}+\frac{1}{2n_{32}+n_{22}}}\right). \tag{1}$$

Hardy–Weinberg disequilibrium (HWD) is often encountered when experimental errors occur in the SNP typing. However, even after the careful quality control of the genotyping, the genotype distribution may depart from HWE for a variety of other reasons, such as stratification, selection, inbreeding, assortative or disassortative mating (Wright 1951, 1965; Nei 1987). Under such a Hardy–Weinberg disequilibrium (HWD), the standard error of the estimated allelic odds ratio given in the last term of Eq. (1) will either be overestimated or underestimated. In order to solve this problem, Schaid and Jacobsen (1999) provided a correction method based on determining the correct variance for the observed allele frequency difference $\left(\hat{P}_{11}-\hat{P}_{12}\right)$ between cases and controls, and quantified the effect on the type I error rate of Pearson's chi-square test induced by HWD. Additionally, the standard error of relative risk under HWD was shown by Zaykin et al. (2004). In this article, we present a generalized formula for calculating the CI of the allelic odds ratio based on the estimated standard error, which is valid under both HWE and HWD, and then examine the effect of this generalization in a genome-wide association study.

Materials and methods

Derivation of the generalized method of CI calculation

In case-control studies, allelic frequencies are compared between cases and controls. Assuming that two alleles X and x exist at a certain SNP locus, the genotype data are given in a 3×2 contingency table as shown in Table 1, the observed frequencies (n_{1j}, n_{2j}, n_{3j}) being distributed as a trinomial distribution Tn (n_j; π_{1j}, π_{2j}, π_{3j}) for $j=1$ (case) and $j=2$ (control), where (π_{1j}, π_{2j}, π_{3j}) are the population proportions of genotype (XX, Xx, xx), respectively, and n_j ($j=1, 2$) is the sample

size for each population. Of course, $\pi_{1j}+\pi_{2j}+\pi_{3j}=1$ and $n_{1j}+n_{2j}+n_{3j}=n_j$ ($j=1, 2$).

Let the population proportions of allele X in cases and controls be P_{11} and P_{12}. Then $P_{11}=\pi_{11}+\pi_{21}/2$ and $P_{12}=\pi_{12}+\pi_{22}/2$, and they are estimated as $\hat{P}_{1j}=(2n_{1j}+n_{2j})/(2n_{.j})(j=1,2)$ (Li and Horvitz 1953; Sasieni 1997) in Table 2. The estimator of allelic odds ratio $\psi=\frac{P_{11}(1-P_{12})}{(1-P_{11})P_{12}}$ is given by Eq. 2. (See Appendix.)

$$\hat{\psi}=\frac{\hat{P}_{11}\left(1-\hat{P}_{12}\right)}{\left(1-\hat{P}_{11}\right)\hat{P}_{12}}. \tag{2}$$

When $n_{.1}$ and $n_{.2}$ are large, $\log\hat{\psi}$ is asymptotically distributed as normal with mean and variance given by Eqs. 3 and 4, respectively. (See Appendix.)

$$E\left\{\log\hat{\psi}\right\}\approx\log(\psi). \tag{3}$$

$$V\left\{\log\hat{\psi}\right\}\approx\left(\frac{1}{2n_{.1}P_{11}}+\frac{1}{2n_{.1}(1-P_{11})}\right)(1+F_1)$$
$$+\left(\frac{1}{2n_{.2}P_{12}}+\frac{1}{2n_{.2}(1-P_{12})}\right)(1+F_2), \tag{4}$$

where F_1 and F_2 are fixation indices of case and control populations, respectively.

Based on the estimated standard error $SE\left(\log\hat{\psi}\right)$ that is given by Eqs. 5 and 6, an approximate $100(1-\alpha)\%$ CI for ψ is given by Eq. 7. (See Appendix.)

$$\left(SE\left(\log\hat{\psi}\right)\right)^2=\left(\frac{1}{2n_{11}+n_{21}}+\frac{1}{2n_{31}+n_{21}}\right)\left(1+\hat{F}_1\right)$$
$$+\left(\frac{1}{2n_{12}+n_{22}}+\frac{1}{2n_{32}+n_{22}}\right)\left(1+\hat{F}_2\right), \tag{5}$$

$$\hat{F}_j=1-\frac{2n_{.j}n_{2j}}{(2n_{1j}+n_{2j})(2n_{3j}+n_{2j})}\quad j=1,2. \tag{6}$$

$$\exp\left(\log\hat{\psi}\pm z_{\alpha/2}\cdot SE\left(\log\hat{\psi}\right)\right). \tag{7}$$

Table 1 A 3×2 contingency table

Genotype	Case	Control
XX	n_{11}	n_{12}
Xx	n_{21}	n_{22}
xx	n_{31}	n_{32}
Total	$n_{.1}$	$n_{.2}$

Table 2 A 2×2 allele frequency table

Allele	Case	Control
X	$2n_{11}+n_{21}$	$2n_{12}+n_{22}$
x	$2n_{31}+n_{21}$	$2n_{32}+n_{22}$
Total	$2n_{.1}$	$2n_{.2}$

2×2 Contingency table for alleles constructed from Table 1

When HWE is true without doubt, Eq. 5 should be changed to $\hat{F}_1 = \hat{F}_2 = 0$ and then Eq. 7 reduces to Eq. 1, which implies that calculating CI by Eq. 7 is a generalization of the usual method. The essential derivation idea of the generalized method is to introduce the fixation index (F_j) into the population probabilities of genotypes $(\pi_{1j}, \pi_{2j}$ and $\pi_{3j})$. In actuality, as F_j approaches 0, one automatically arrives at the usual Eq. 1.

Numerical evaluation of the difference of the two formulas

It is obvious from Eq. 5 that the calculated CI is wider in the generalized method than the one in the usual method if $\hat{F}_1 > 0$ and $\hat{F}_2 > 0$, while it is narrower if they are less than 0. However, the difference of the two methods should be evaluated numerically, because it is influenced by sampling errors of F_1 and F_2. We evaluated the difference by a numerical calculation of expected upper and lower confidence limits for various values of the fixation indices and sample sizes in the case of P_{11}=0.10 and P_{12}=0.15. In the calculation, we used a normal approximation to the trinomial distribution and the software SAS for computing.

Simulation experiment to examine the influence of generalization

In SNP data analysis, we simultaneously investigate the association between thousands of SNPs and a disease. Some SNPs among them may be under HWD with a distribution of fixation index, while others may be under HWE $(F=0)$. We have to examine the performance of the generalized method for CI calculation, assuming that the fixation indices have a distribution among thousands of SNPs. Consequently, we conducted a Monte Carlo simulation experiment to statistically identify disease-associated SNPs using the decision rule that an association was judged as positive if the calculated CI did not include 1.0.

As the framework of simulation, we set the following conditions referring to the genome-wide association study (Sato et al. 2004):

Condition 1 The total number of SNPs to be examined was set as N=10,000 and the number of disease-associated SNPs (positive SNPs) was set as N_p=50, referring to the literature (Sing et al. 1996; Wright et al. 1999; Pharoah et al. 2002; Ponder 2001).

Condition 2 Allelic odds ratio for positive N_p SNPs was ψ=1.5 or 2.0, but ψ=1.0 for the remaining $N-N_p$ SNPs.

Condition 3 The sample size was varied as $n=n_{.1}=n_{.2}$=188, 376 or 752.

Condition 4 The proportion P_{12} of allele X in the control population was a random variable uniformly distributed in unit interval (0.05, 0.95), and P_{11} in the case population was automatically determined by P_{12} through Eq. 20 in Appendix. This condition was set with reference to Fig. 1, to which a uniform distribution is plausible, for the distribution of alleles in the database of Japanese Single Nucleotide Polymorphisms (Haga et al. 2002; Hirakawa et al. 2002). In our genome-scan, we did not include these SNPs with low allele frequency (P_{11}>0.95 or P_{11}<0.05). Note that (π_{1j}, π_{2j}, π_{3j}, j=1, 2) were fixed through Eq. 12 in Appendix when (P_{11}, P_{12}, F) or, equivalently, (P_{11}, ψ, F) was determined.

Condition 5 In a case-group, the fixation index F was specified by a mixed distribution of a constant 0 with probability $1-w$ and a normal distribution $N(\mu, 0.10^2)$ with probability w, where w=0.02, 0.06 or 0.10, and μ was set as 0.0 (in the null case), 0.2, or 0.4. On the other hand, F was set to 0 for a control group. Note that this condition was set referring to Figs. 2 and 3 taken from a database, Genome Medicine Database of Japan. In order to determine whether normally distributed or not, we showed a quantile–quantile plot in Fig. 2. It showed that the core data reasonably fit a normal distribution, but the tail data do not. Therefore, the distribution of observed F does not have a normal distribution with mean 0. Moreover, around 2% of the larger tail area in Fig. 3 was laid outside the distribution of observed F under the null hypothesis that the
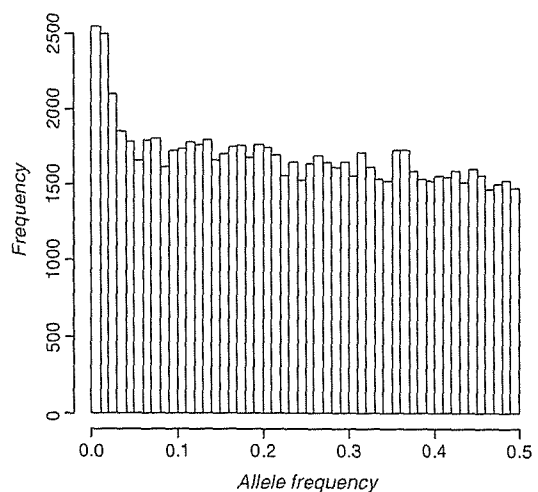

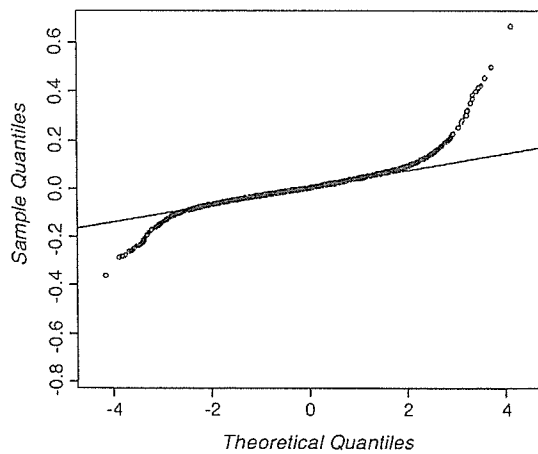**Fig. 1** An example of the minor allele frequency distribution of SNP. The data are from the JSNP database (http://www.snp.ims.u-tokyo.ac.jp/)

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Fig. 2 Quantile–quantile plot for fixation index F in a case-group obtained from Genome Medicine Database of Japan, http://www.gemdbj.nibio.go.jp/dgdb/)

fixation index was equal to 0 and the mean of the outlying values was around 0.2 or more.

Condition 6 The criteria to evaluate the performance of the decision rule were two indicators, positive predictive value R_p and sensitivity R_s, defined by Eqs. 8 and 9 with notations in Table 3.

$$R_p = \frac{N_{TP}}{N_{P.}}, \tag{8}$$

$$R_s = \frac{N_{TP}}{N_{.P}}. \tag{9}$$

Condition 7 The Monte-Carlo simulation to observe R_p and R_s was repeated 1,000 times, and the mean values, together with the mean number of N_{TP} and N_{FP}, were used for comparison of the two methods.
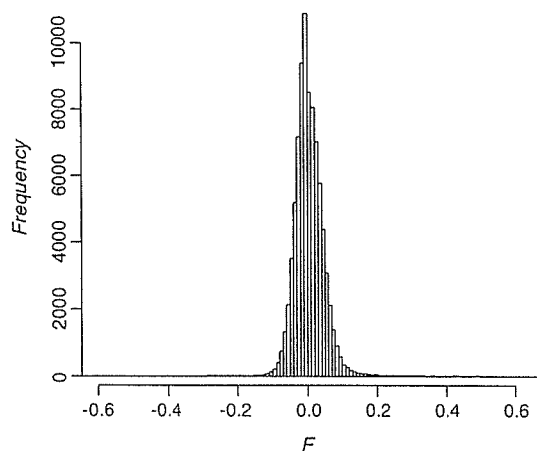

Fig. 3 An example of the frequency distribution of fixation index F in a case-group obtained from Genome Medicine Database of Japan

Table 3 The contingency table for schematic outcomes of a judgment

	True association		Total
	Positive	Negative	
Judgment			
Positive	N_{TP}	N_{FP}	$N_{P.}$
Negative	N_{FN}	N_{TN}	$N - N_{P.}$
Total	$N_{.P}$	$N - N_{.P}$	N

Notation for defining R_p and R_s. Positive predictive value: $R_p = N_{TP}/N_{P.}$, sensitivity: $R_s = N_{TP}/N_{.P}$

Note that $N_{.P}$ was a constant fixed by Condition 1, whereas $N_{P.}$ was a random variable realized as the sum of N_{TP} and N_{FP} in the simulation experiment. Note further that these N_{TP} and N_{FP} have a trade-off relationship depending on the nominal confidence level, but that we fix the nominal confidence level as $1 - \alpha = 0.999$, taking the multiplicity of SNPs into consideration.

The procedure to conduct the simulation experiment was as follows:

Step 1. Assign a set of values to N, $N_{.P}$, ψ, and n according to the above-described conditions.

Step 2. Assign the value $\psi = 1.5$ or 2.0 to the first $N_{.P}$ SNPs and $\psi = 1.0$ to the remaining $N - N_{.P}$ SNPs.

Step 3. Generate 10,000 random numbers of F according to Condition 5 and assign them to 10,000 SNPs.

Step 4. Generate random numbers (n_{11}, n_{21}, n_{31}) and (n_{12}, n_{22}, n_{32}) distributed as $Tn(n, \pi_{11}, \pi_{21}, \pi_{31})$ and $Tn(n, \pi_{12}, \pi_{22}, \pi_{32})$, respectively, for each 10,000 SNPs.

Step 5. Calculate CIs using Eq. 1 (usual method) and Eq. 8 (Generalized method) with $\alpha = 0.001$ and calculate N_{TP}, N_{FP}, R_p, and R_s for each 10,000 SNPs.

Step 6. Repeat Steps 1–5 1,000 times and calculate the mean of the realized values.

Step 7. Repeat Steps 1–6, changing parameters ψ in Condition 2, n in Condition 3, and w and μ in Condition 4.

Results

A summarized result of numerical evaluation of the expected confidence limits in a typical case is shown in Table 4 for various values of the fixation index $F = F_1 = F_2$ when the sample size was set at $n_{.1} = n_{.2} = 188$ or

752. Table 4 suggests that the difference of the two methods is not ignorable, on average, judging by statistical significance when $F \geq 0.4$, because the CI by the generalized method included 1.0, whereas CI by the usual method did not.

The essential feature of the influence of the generalized method on the judgment of association can be seen in Table 5, which is the mean of R_P, R_S, N_{TP}, and N_{FP} obtained from the 1,000 simulation repetitions. When $\psi=1.5$ or 2.0, $w=0.02$, 0.06 or 0.10 and $\mu=0.2$ or 0.4, the false positive number of SNPs in the generalized method was, on average, slightly less than that in the usual method.

Discussion

The essential improvement achieved by the generalized method is summarized in Table 5. In this table, for example, the average number of falsely detected SNPs by the usual method was 22.0 ($n=188$) or 22.0 ($n=752$), whereas it was 20.4 ($n=188$) or 19.7 ($n=752$) by the generalized method when $\psi=2.0$, $w=0.10$ and $\mu=0.4$. The amount of the improvement was not great, but it may be appreciated in certain research circumstances, because a difference of even a few SNPs would be highly significant in the advanced stages of gene hunting following an association study, such as large-scale, multiethnic replication studies or lengthy functional analyses on model animals. It should be noted that a substantial investment in the post-association study is often necessary, especially in a hypothesis-free genome scan, in which a prior probability of the gene is minimal.

Deviation from HWE is not a rare, exceptional case in association studies. Figure 2 shows an example of the distribution of the fixation index in a large-scale SNP typing project, in which 84,542 SNP typing data on autosomal chromosomes were obtained for 940

individuals in the Millennium Genome Project of Japan (Haga et al. 2002; Yoshida and Yoshimura 2003). In this dataset, the operating protocol of our SNP typing laboratory includes routine quality check steps to filter simple experimental errors. However, even after the careful check for the genotyping errors, a sizable fraction of about 2% of the 84,542 SNPs showed a fixation index outside the normal range of variation under the hypothesis that the population fixation index was 0.

As for other data, Wittke-Thompson et al. (2005) did a survey of HWD in several recent reviews of association studies (Xu et al. 2002; Gyorffy et al. 2004; Kocsis et al. 2004a, b; Osawa et al. 2004) and identified 41 studies with 60 polymorphisms showing a departure from HWE: 35 polymorphisms that depart from HWE in cases only, 21 that departed in controls only, 2 that departed in the same direction in cases and controls, and 1 that departed in the opposite direction in cases and controls. Wittke-Thompson et al. (2005) emphasized the importance not only of correctly assessing HWE for genotype data but also of understanding whether an observed HWD was consistent with a genetic model of disease susceptibility.

In a previous study, Schaid and Jacobsen (1999), Zaykin et al. (2004) and Salanti et al. (2005) each recommended the correction of the variance of the observed statistics which is allele frequency difference, relative risk or odds ratio under HWD, respectively, because the type I error for gene-disease associations tested on the level of alleles was inflated when the estimated inbreeding coefficient was positive, while the error deflates for the negative coefficient. However, under circumstances where the assumptions of HWE in controls and codominance between the alleles do not hold well, Sasieni (1997) recommended simply to abandon the allelic odds ratio for an association study, because the allelic odds ratio and chi-square statistics are not robust under such circumstances. These previ-

Table 4 Difference of confidence interval between two methods for various fixation indices $F=F_1=F_2$ at $P_{11}=0.15$ and $P_{12}=0.10$

Fixation index F	$n=n_1=n_2=188$				$n=n_1=n_2=752$			
	Usual method		Generalized method		Usual method		Generalized method	
	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
−0.10	1.047	2.551	1.071	2.494	1.282	1.994	1.298	1.972
0.00	1.048	2.556	1.049	2.554	1.283	1.995	1.283	1.995
0.20	1.054	2.577	1.012	2.684	1.284	1.997	1.258	2.039
0.40	1.059	2.594	0.978	2.809	1.286	2.001	1.236	2.083
0.60	1.066	2.620	0.949	2.946	1.289	2.006	1.216	2.126

The range of fixation index (F_j) depends on the population probabilities allele X and is shown by the following equation:
$-\frac{P_{1j}}{1-P_{1j}} \leq F_j \leq 1 (j = 1, 2)$

Table 5 Observed means of positive predicative value (R_p), sensitivity (R_s), true positive SNPs (N_{TP}), and false positive SNPs (N_{FP}) obtained in the simulation experiment ($F_1 > 0$ and $F_2 = 0$)

ψ	w	μ	Method	n=188		n=376		n=752	
				Usual	General	Usual	General	Usual	General
1.5	0.02	0.2	R_p	0.502	0.503	0.641	0.645	0.695	0.701
			R_s	0.411	0.412	0.727	0.724	0.924	0.921
			N_{TP}	20.6	20.6	36.3	36.2	46.2	46.0
			N_{FP}	20.4	20.3	20.4	19.9	20.3	19.6
		0.4	R_p	0.496	0.500	0.640	0.646	0.646	0.701
			R_s	0.410	0.411	0.728	0.725	0.924	0.922
			N_{TP}	20.5	20.6	36.4	36.3	36.2	46.1
			N_{FP}	20.8	20.6	20.5	19.8	19.8	19.7
	0.06	0.2	R_p	0.499	0.504	0.636	0.645	0.689	0.700
			R_s	0.414	0.415	0.730	0.725	0.923	0.919
			N_{TP}	20.7	20.7	36.5	36.3	46.1	46.0
			N_{FP}	20.8	20.4	20.9	20.0	20.8	19.7
		0.4	R_p	0.492	0.501	0.629	0.640	0.687	0.703
			R_s	0.414	0.412	0.725	0.720	0.922	0.919
			N_{TP}	20.7	20.6	36.2	36.0	46.1	45.9
			N_{FP}	21.3	20.5	21.4	20.2	21.0	19.4
	0.10	0.2	R_p	0.490	0.498	0.636	0.647	0.688	0.702
			R_s	0.410	0.410	0.731	0.727	0.924	0.920
			N_{TP}	20.5	20.5	36.6	36.4	46.2	46.0
			N_{FP}	21.3	20.6	20.9	19.8	20.9	19.5
		0.4	R_p	0.483	0.499	0.626	0.646	0.679	0.700
			R_s	0.410	0.408	0.725	0.720	0.923	0.919
			N_{TP}	20.5	20.4	36.3	36.0	46.2	45.9
			N_{FP}	21.9	20.5	21.7	19.7	21.8	19.7
2.0	0.02	0.2	R_p	0.677	0.675	0.702	0.706	0.707	0.715
			R_s	0.849	0.845	0.961	0.958	0.992	0.991
			N_{TP}	42.4	42.2	48.1	47.9	49.6	49.6
			N_{FP}	20.2	20.3	20.4	19.9	20.5	19.8
		0.4	R_p	0.672	0.673	0.697	0.704	0.706	0.715
			R_s	0.850	0.846	0.959	0.956	0.992	0.991
			N_{TP}	42.5	42.3	48.0	47.8	49.6	49.6
			N_{FP}	20.8	20.5	20.8	20.1	20.7	19.7
	0.06	0.2	R_p	0.672	0.675	0.700	0.708	0.704	0.716
			R_s	0.849	0.845	0.962	0.959	0.992	0.991
			N_{TP}	42.4	42.2	48.1	47.9	49.6	49.6
			N_{FP}	20.8	20.3	20.7	19.8	20.9	19.7
		0.4	R_p	0.666	0.674	0.694	0.707	0.704	0.719
			R_s	0.849	0.844	0.961	0.958	0.992	0.991
			N_{TP}	42.5	42.2	48.0	47.9	49.6	49.6
			N_{FP}	21.3	20.4	21.2	19.8	20.9	19.3
	0.10	0.2	R_p	0.670	0.676	0.697	0.708	0.702	0.716
			R_s	0.851	0.847	0.960	0.957	0.992	0.992
			N_{TP}	42.6	42.3	48.0	47.9	49.6	49.6
			N_{FP}	20.9	20.2	20.9	19.8	21.1	19.7
		0.4	R_p	0.657	0.673	0.687	0.706	0.693	0.716
			R_s	0.846	0.839	0.963	0.960	0.992	0.991
			N_{TP}	42.3	42.0	48.1	48.0	49.6	49.5
			N_{FP}	22.0	20.4	22.0	20.0	22.0	19.7

The fixation index (F) in a case group was specified by a mixed distribution of a constant 0 with probability $1-w$ and a normal distribution $N(\mu, 0.10^2)$ with probability w, where $w = 0.02$, 0.06 or 0.10, and μ was set as 0.2, or 0.4. In a control group F was set to 0

ous studies were targeted at a candidate–gene association study or meta-analysis and did not examine a genome-wide association study. Here, we scrutinized the situation in a genome-wide association study and showed that around 2% of the large tail area was laid outside the distribution of F, suggesting the importance of the correction under HWD. Because the cardinal feature of the genome-wide association study is a screening, we believe that Sasieni's recommendation may be too conservative to be accepted, and the

generalized method should be applied as a sensitivity analysis in a genome-wide association study to improve both false positive rate (for $F>0$) and false negative rate (for $F<0$).

Appendix: Mathematical details

Let the population probabilities of genotypes "XX", "Xx", and "xx" be π_1, π_2 and π_3 ($\pi_1 + \pi_2 + \pi_3 = 1$), respectively, then those of alleles "X" and "x" for a SNP are given by Eq. 10.

$$P_1 = \pi_1 + \pi_2/2, \quad P_2 = \pi_3 + \pi_2/2 (= 1 - P_1). \tag{10}$$

When we use the fixation index (Li et al. 1953) defined by

$$F = 1 - \frac{\pi_2}{2(\pi_1 + \pi_2/2)(\pi_3 + \pi_2/2)}, \tag{11}$$

(π_i; $i=1, 2, 3$) are expressed as Eq. 12.

$$\left.\begin{array}{l} \pi_1 = P_1^2 + FP_1P_2 \\ \pi_2 = 2P_1P_2 - 2FP_1P_2 \\ \pi_3 = P_2^2 + FP_1P_2 \end{array}\right\} \tag{12}$$

Therefore, (π_1, π_2, π_3) is equivalent to (P_1, P_2, F).

When the Hardy–Weinberg equilibrium (HWE) holds, $F=0$ and Eq. 12 reduces to Eq. 13; that is, the second term in the right side of Eq. 12 represents the degree of disequilibrium.

$$\pi_1 = P_1^2, \quad \pi_2 = 2P_1P_2, \quad \pi_3 = P_2^2. \tag{13}$$

For a random sample of size n, the observed frequency (n_1, n_2, n_3); ($n_1 + n_2 + n_3 = n$) of genotypes (XX, Xx, xx) is distributed as trinomial distribution Tn(n; π_1, π_2, π_3) and, therefore, the maximum likelihood estimator of π_i ($i=1, 2, 3$) is $p_i = n_i/n$ ($i=1, 2, 3$). Likewise, the maximum likelihood estimators of P_1 and allele odds $P_1/(1 - P_1)$ are $\hat{P}_1 = p_1 + p_2/2 = (2n_1 + n_2)/(2n)$, and $\hat{P}_1 / \left(1 - \hat{P}_1\right)$, respectively.

Since the means, variances, and covariance of p_1 and p_2 are given (Bishop et al. 1975; Agresti 2001) by

$$\left.\begin{array}{l} E\{p_1\} = \pi_1 = P_1^2 + FP_1P_2 \\ E\{p_2\} = \pi_2 = 2P_1P_2 - 2FP_1P_2 \\ V\{p_1\} = \frac{1}{n}\pi_1(1 - \pi_1) = \frac{1}{n}\left(P_1^2 + FP_1P_2\right)\left(1 - P_1^2 - FP_1P_2\right) \\ V\{p_2\} = \frac{1}{n}\pi_2(1 - \pi_2) = \frac{1}{n}(2P_1P_2 - 2FP_1P_2) \\ \qquad\qquad \times (1 - 2P_1P_2 + 2FP_1P_2) \\ \mathrm{Cov}\{p_1, p_2\} = -\frac{1}{n}\pi_1\pi_2 = -\frac{1}{n}\left(P_1^2 + FP_1P_2\right) \\ \qquad\qquad \times (2P_1P_2 - 2FP_1P_2) \end{array}\right\}, \tag{14}$$

the mean and variance of $\hat{P}_1$ is, after a simple but tedious algebra, derived as Eqs. 15 and 16.

$$\begin{aligned} E\left\{\hat{P}_1\right\} &= E\{p_1\} + \frac{1}{2}E\{p_2\} \\ &= P_1^2 + FP_1P_2 + \frac{1}{2}2(P_1P_2 - FP_1P) \\ &= P_1(P_1 + P_2) = P_1, \end{aligned} \tag{15}$$

$$\begin{aligned} V\left\{\hat{P}_1\right\} &= V\{p_1\} + 2\frac{1}{2}\mathrm{Cov}\{p_1, p_2\} + \left(\frac{1}{2}\right)^2 V\{p_2\} \\ &= \frac{1}{n}\left(P_1^2 + FP_1P_2\right)\left(1 - P_1^2 - FP_1P_2\right) \\ &\quad - \frac{1}{n}\left(P_1^2 + FP_1P_2\right)(2P_1P_2 - 2FP_1P_2) \\ &\quad + \frac{1}{4n}(2P_1P_2 - 2FP_1P_2)(1 - 2P_1P_2 + 2FP_1P_2) \\ &= \frac{P_1P_2}{2n}\left(2F(P_1 + P_2)^2 + 1 - F\right) \\ &= \frac{P_1(1 - P_1)}{2n}(1 + F). \end{aligned} \tag{16}$$

When $F=0$, the last term is the well-known formula for binomial proportion for the size $2n$ and probability P_1. It reflects that the distribution of the frequency of allele X under HWE is the same as that of allele X randomly chosen from $2n$ alleles with P_1 as the proportion of X.

Since $\hat{P}_1$ tends to P_1 in probability when n tends to infinity, the logarithm of estimated allelic odds, $\log\left(\hat{P}_1 / \left(1 - \hat{P}_1\right)\right)$, can be approximated by the first order Taylor expansion as Eq. 17.

$$\log\left(\frac{\hat{P}_1}{1 - \hat{P}_1}\right) \approx \log\left(\frac{P_1}{1 - P_1}\right) + \frac{1}{P_1(1 - P_1)}\left(\hat{P}_1 - P_1\right). \tag{17}$$

Consequently, the mean and variance of $\log\left(\hat{P}_1 / \left(1 - \hat{P}_1\right)\right)$ are asymptotically approximated by Eqs. 18 and 19:

$$E\left\{\log\left(\frac{\hat{P}_1}{1-\hat{P}_1}\right)\right\} \approx \log\left(\frac{P_1}{1-P_1}\right), \tag{18}$$

$$\begin{aligned} V\left\{\log\left(\frac{\hat{P}_1}{1-\hat{P}_1}\right)\right\} &\approx \frac{1}{P_1^2(1-P_1)^2}V\left\{\hat{P}_1\right\} \\ &= \frac{1}{2nP_1(1-P_1)}(1+F) \\ &= \left(\frac{1}{2nP_1}+\frac{1}{2n(1-P_1)}\right)(1+F). \end{aligned} \tag{19}$$

When we consider the populations of cases and controls of a disease, the association between allele and disease is conventionally represented by the allele odds ratio ψ defined by Eq. 20, where the case and the control are differentiated with the second subscript 1 (case) and 2 (control).

$$\psi = \frac{P_{11}}{1-P_{11}}\Big/\frac{P_{12}}{1-P_{12}}. \tag{20}$$

Consider we have random samples of size $n_{.1}$ and $n_{.2}$ from cases and controls, respectively. Then the maximum likelihood estimator $\hat{\psi}$ of ψ is given by Eq. 21, where $\hat{P}_{11}$ and $\hat{P}_{12}$ are the maximum likelihood estimators based on samples of case and control, respectively.

$$\hat{\psi} = \frac{\hat{P}_{11}}{1-\hat{P}_{11}}\Big/\frac{\hat{P}_{12}}{1-\hat{P}_{12}}. \tag{21}$$

Since the sample of case and that of control can be assumed independent, we obtain Eqs. 22 and 23.

$$\begin{aligned} E\left\{\log\hat{\psi}\right\} &= E\left\{\log\left(\frac{\hat{P}_{11}}{1-\hat{P}_{11}}\right)\right\} - E\left\{\log\left(\frac{\hat{P}_{12}}{1-\hat{P}_{12}}\right)\right\} \\ &\approx \log\left(\frac{P_{11}}{1-P_{11}}\right) - \log\left(\frac{P_{12}}{1-P_{12}}\right) = \log(\psi), \end{aligned} \tag{22}$$

$$\begin{aligned} V\left\{\log\hat{\psi}\right\} &= V\left\{\log\left(\frac{\hat{P}_{11}}{1-\hat{P}_{11}}\right)\right\} + V\left\{\log\left(\frac{\hat{P}_{12}}{1-\hat{P}_{12}}\right)\right\} \\ &\approx \left(\frac{1}{2n_{.1}P_{11}}+\frac{1}{2n_{.1}(1-P_{11})}\right)(1+F_1) \\ &+ \left(\frac{1}{2n_{.2}P_{12}}+\frac{1}{2n_{.2}(1-P_{12})}\right)(1+F_2), \end{aligned} \tag{23}$$

where F_1 and F_2 are fixation indices of case and control, respectively.

When we construct an asymptotic confidence interval of $\log(\psi)$ with confidence level $1-\alpha$, we should replace $V\left\{\log\left(\hat{\psi}\right)\right\}$ with its estimator given by Eq. 24.

$$\begin{aligned} \hat{V}\left\{\log\hat{\psi}\right\} &= \left(\frac{1}{2n_{11}+n_{21}}+\frac{1}{2n_{31}+n_{21}}\right)\left(1+\hat{F}_1\right) \\ &+ \left(\frac{1}{2n_{12}+n_{22}}+\frac{1}{2n_{32}+n_{22}}\right)\left(1+\hat{F}_2\right), \end{aligned} \tag{24}$$

where $\hat{F}_1, \hat{F}_2$ are as follows:

$$\hat{F}_j = 1 - \frac{2n_{2j}n_{.j}}{(2n_{1j}+n_{2j})(2n_{3j}+n_{2j})} \quad j = 1,2. \tag{25}$$

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Meeting Report

Mouse Lymphoma Thymidine Kinase Gene Mutation Assay: Follow-up Meeting of the International Workshop on Genotoxicity Testing—Aberdeen, Scotland, 2003—Assay Acceptance Criteria, Positive Controls, and Data Evaluation

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The Mouse Lymphoma Assay (MLA) Workgroup of the International Workshop on Genotoxicity Testing (IWGT), comprised of experts from Japan, Europe, and the United States, met on August 29, 2003, in Aberdeen, Scotland, United Kingdom. This meeting of the MLA Workgroup was devoted to reaching a consensus on the appropriate approach to data evaluation and on acceptance criteria for both the positive and negative/vehicle controls. The Workgroup reached consensus on the acceptance criteria for both the agar and microwell versions of the MLA. Recommendations include acceptable ranges for mutant frequency, cloning efficiency, and suspension growth of the negative/vehicle controls and on criteria to define

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an acceptable positive control response. The recommendation for the determination of a positive/negative test chemical response includes both the requirement that the response exceeds a defined value [the global evaluation factor (GEF)] and that there also be a positive dose–response (evaluated by an appropriate statistical method). Environ. Mol. Mutagen. 47:1–5, 2006.

INTRODUCTION

The Mouse Lymphoma Assay (MLA) Workgroup of the International Workshop on Genotoxicity Testing (IWGT), comprised of experts from Japan, Europe, and the United States, met on August 29, 2003, in Aberdeen, Scotland, United Kingdom. This meeting of the MLA Workgroup was devoted to reaching a consensus on the appropriate approach to data evaluation and on acceptance criteria for the positive controls. In addition, on the basis of new data, the group slightly revised the acceptance criteria for the negative controls that had been agreed upon at the last group meeting, held on June 28 and 29, 2002 in Plymouth, England, United Kingdom [Moore et al., 2003].

Since its first meeting, held as a part of the IWGT Procedures in Washington, DC, in the spring of 1999, the MLA Workgroup has been endeavoring to address three main issues of importance to the assay. These include (1) the conduct of a data-based analysis upon which to base a final recommendation for measuring cytotoxicity; (2) the criteria for data acceptance and appropriate approaches for data evaluation; and (3) the issues related to the International Committee for Harmonization (ICH) recommended use of a 24-hr treatment time (including the ability of the assay to detect aneugens). This is the 4th meeting of the Workgroup in which consensus has been reached and reported. The previous three meetings are reported in Moore et al. [2000, 2002, 2003].

ACCEPTANCE CRITERIA FOR NEGATIVE/VEHICLE CONTROLS

During the June 2002 meeting of the Workgroup in Plymouth, acceptance criteria were agreed upon for the MLA [Moore et al., 2003]. Following the meeting, the various laboratories revisited their methods for scoring mutant colonies. One laboratory conducting the microwell version of the assay and counting stained colonies determined that many of the colonies previously scored were below the size normally considered by others to be scoreable mutants. Additional experiments using unstained wells resulted in an overall lowering of the background mutant frequencies (MFs) from that laboratory and in a modification of the overall multiple laboratory distribution. The new global distribution for the negative/vehicle control MFs for the microwell version of the assay is shown in Figure 1. Based on this new information, the

majority of the Workgroup members agreed that the acceptable ranges for the negative/vehicle control MF for the microwell version of the assay should be revised to $(50–170) \times 10^{-6}$.

For both methods, the cloning efficiency (CE) referred to in the criteria is the absolute CE obtained at the time of mutant selection. The suspension growth (SG) of the negative/vehicle control refers to the growth during the 2-day expression period following 3- or 4-hr treatments. It is defined as the fold-increase of the cell number during this 2-day period. The SG is calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number.

The acceptance criteria for all of the negative/vehicle control parameters for the soft agar and microwell methods of performing the MLA are now as follows:

	Agar method	Microwell method
MF	$(35–140) \times 10^{-6}$	$(50–170) \times 10^{-6}$
CE	$(65–120)\%$	$(65–120)\%$
SG	8–32	8–32

ACCEPTANCE CRITERIA FOR POSITIVE CONTROLS

Positive control cultures should be included in every MLA experiment. This is done to assure that the assay is working within historical experience and that both the large and small-colony thymidine kinase (Tk) mutants are being adequately enumerated in each experiment. Because of the importance of adequate small colony mutant recovery, the Workgroup has chosen to develop criteria for the positive control that includes an appropriate increase in the small-colony Tk MF.

During the meeting in Plymouth, the Workgroup agreed to investigate a single reference chemical treatment that would be used for the MLA without S9 activation, and a single reference chemical treatment that would be used with S9 activation [Moore et al., 2003]. Following the meeting, it became apparent that achieving this goal required more effort than anticipated. Therefore, the Workgroup developed a different strategy for this requirement to provide assurance that adequate small and large colony mutant recovery was attained in an individual experiment.

The majority of the Workgroup members agreed upon two different approaches to assuring an adequate positive control response. Either approach is acceptable. In the
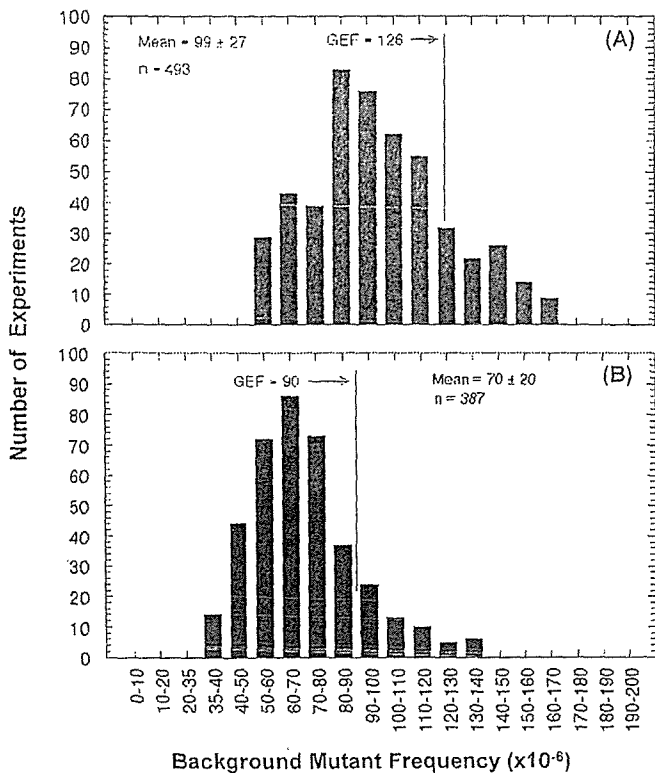
Fig. 1. Distribution plots of the negative/vehicle control MFs were as follows: (A) six laboratories conducting the microwell version and (B) four laboratories conducting the agar version of the MLA assay. The number of experimental observations is plotted in histogram form for incremental increases in mutant frequency ($\times 10^{-6}$). Units of 10 were used for the increments with the exception of the agar data where increments of 15 or 5 are used for the 20–35 and 35–40 increases, respectively. The GEF, calculated as the mean of the distribution plus one standard deviation, is indicated for each version of the MLA.

first approach, the laboratory should use a dose of a chemical that yields an absolute increase in total MF, that is an increase above the spontaneous background MF [an induced MF (IMF)] of at least 300×10^{-6}. At least 40% of the IMF should be reflected in the small colony MF. For instance, in a culture showing an IMF of 300×10^{-6}, the small colony IMF should be at least 120×10^{-6}. The second approach requires the use of a dose of a chemical that increases the small colony MF at least 150×10^{-6} above that seen in the concurrent negative/vehicle control (a small colony IMF of 150×10^{-6}).

Furthermore, the upper limit of cytotoxicity observed in the positive control culture should be the same as for the experimental cultures. That is, the relative total growth (RTG) should be greater than 10% [Moore et al., 2002]. While some laboratories prefer to use more than one dose of their positive control, it is sufficient to use a single dose (or one of the doses of the positive control cultures if more than one dose is used) to demonstrate that the acceptance criteria for the positive control have been satisfied.

DATA EVALUATION

Proper assay evaluation includes assuring that the negative/vehicle controls are acceptable and that the appropriate test chemical dose range has been obtained. For more detail on the Workgroup recommendations on the steps for proper assay evaluation, the reader is referred to the summaries of the New Orleans and Plymouth meetings [Moore et al., 2002, 2003]. Once the criteria for experimental acceptance have been satisfied, the data from each individual experiment can be evaluated to determine whether the response is positive, negative, or equivocal.

The Workgroup agreed that none of the previously used methods to evaluate MLA data is entirely satisfactory. With the goal of recommending one or more statistical methods, the group utilized actual experimental data collected from 10 laboratories (six microwell labs and four agar labs) and applied 29 statistical methods to these 398 data sets. A brief summary of this analysis and a complete list of the statistical methods are included in the Plymouth Meeting Report [Moore et al., 2003]. A complete description of this analysis will be written. The statistical methods used can be defined by the dependent variable (MF, log MF, or rank MF) and the independent variable(s) (dose, dose categories, dose + dose × dose, log dose, or rank dose) and the weight [none, variance (dependent variable)].

There were several conclusions from the analyses of the statistical methods. When $P < 0.05$ was used to define a data set as positive, all of the statistical methods agreed on the positive or negative call in approximately 40% of the data sets. However, for approximately 60% of the data sets, the various statistical methods gave divergent positive/negative results. Based on this analysis, it was impossible to select one or more statistical methods that the Workgroup could confidently recommend to determine whether a chemical was positive or negative in the MLA. That is, for a large portion of the evaluated data sets, statistical methods could be selected that would result in either a positive or negative call.

It should be noted that a large percentage of the database was composed of chemicals that gave only small increases in MF. That is, the data were representative of that generally seen in laboratories testing chemicals of unknown mutagenicity. It is easy to classify chemicals that induce large increases in MF as positive. The difficulty arises in those situations where the increase in MF is very small. Statistical methods are designed to estimate the probability that a particular response occurred by chance. Therefore, those experiments having little variability between duplicate cultures or data points fitting tightly to a linear (or quadratic) curve are more likely to be judged positive (by statistical analysis) than experiments showing large variability. It is clear that the actual variability for any dose of a test chemical is not

adequately represented in any single experiment. In fact, from our analysis, the response distribution is not well defined until approximately 50 experiments are included [Moore et al., 2003]. Therefore, a positive or negative call should not be based solely upon the variability observed within an individual experiment.

It should also be noted that the data evaluation issues mentioned earlier are not unique to the MLA. Other genetic toxicology assays have similar issues in regard to data evaluation and data interpretation.

The background MF for the MLA shows a broad range of values (Fig. 1). It is clear that all laboratories generate some experiments where a "statistically" positive dose–response curve can fit within the normal range of the background MF.

Based on these considerations and taking into account previous guidance documents (i.e., FDA Redbook [http://www.cfsan.fda.gov/~redbook/red-toca.html] and OECD [1997]), which state that biological relevance should be a major factor in data evaluation, the majority of the members of the Workgroup decided to take a different approach to data analysis.

The majority of the Workgroup members agreed that a biologically relevant approach to MLA data evaluation might be achieved by requiring that the IMF exceed some value based on the global background MF for each method (agar or microwell). The basic concept for this approach was discussed at the meeting in Plymouth and a complete discussion of the rationale for this approach can be found in Moore et al. [2003]. This value would be the global evaluation factor (GEF). In addition, statistical analysis should be applied to determine whether there is a dose-related increase in MF. Such a procedure would disregard small increases in IMF, judged by experts in the assay to be of little or no toxicological significance, but would include statistical analysis to evaluate the presence of a positive dose-related trend.

The majority of the Workgroup members agreed that it would be desirable to use the multilaboratory distributions shown in Figure 1 to calculate the GEF for the agar and the microwell versions of the assay and that factor would be used for evaluating MLA experiments in all laboratories. After considerable discussion, the majority of the Workgroup members agreed that the GEF should be defined as the mean of the negative/solvent MF distribution plus one standard deviation. For the agar version of the assay the GEF would therefore be 90 and for the microwell version it would be 126 (see Fig. 1). The GEF would be applied as in the following example: If the negative/vehicle control MF in a microwell experiment is 50×10^{-6}, then one of the test cultures must have a MF of at least $50 + 126$ (the microwell GEF) $= 176 \times 10^{-6}$ to meet the GEF criterion for a positive call. An appropriate statistical trend test should be applied to determine whether there was a positive dose-related increase. This

approach should be applied both to experiments that utilize single cultures and those using duplicate, triplicate, etc. cultures for each data point. Of course, some statistical methods can only be used for experiments in which at least duplicate cultures are used.

A test agent response in an experiment is positive if *both* the IMF for any treatment meets or exceeds the GEF and a positive trend test is obtained. A test agent response is clearly negative if *both* the trend analysis and the GEF are negative. It is recognized that there will be experiments in which either (but not both) the GEF or the statistical analysis indicates a positive response. These situations should be evaluated on a case-by-case basis. It is generally advisable to conduct one or more additional experiments to better define the assay response (particularly in the (30–10)% RTG cytotoxicity range).

The majority of the Workgroup members feel that only those responses positive for both the GEF and the statistical trend analyses are biologically relevant. However, particular attention should be given to test agents, after rigorous testing, that are negative for the GEF, yet consistently show a positive trend test. It is possible that the test agent is very weakly mutagenic under the test conditions or contains a small amount of a mutagenic substance. These compounds may require further chemical characterization and, perhaps additional evaluation for mutagenic potential. The metabolism of these chemicals under the in vitro test conditions should be evaluated and the possibility considered that the in vitro metabolic conditions might be inadequate and that their in vivo metabolites might be mutagenic.

CURRENT WORKGROUP ACTIVITIES

To date, the Workgroup has completed its recommendations for two of the three issues identified during its meeting in Washington, DC. Currently, the Workgroup is beginning to address the issues related to the ICH recommendation for a 24-hr treatment in the absence of S9 metabolic activation. This recommendation has been in place long enough for laboratories to have substantial databases for the 24-hr treatment. Presently, the Workgroup is collecting data from which to base additional recommendations for this treatment condition.

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Influence of SNPs in cytokine-related genes on the severity of food allergy and atopic eczema in children

Negoro T, Orihara K, Irahara T, Nishiyama H, Hagiwara K, Nishida R, Takagi H, Satoh K, Yamamoto Y, Shimizu S, Hagiwara T, Ishii M, Tanioka T, Nakano Y, Takeda K, Yoshimura I, Iikura Y, Tobe T. Influence of SNPs in cytokine-related genes on the severity of food allergy and atopic eczema in children.
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Although many single nucleotide polymorphism (SNP) studies have reported an association of atopy, allergic diseases and total serum immunoglobulin E (IgE) levels, almost all of these studies sought risk factors for the onset of these allergic diseases. Furthermore, many studies have analyzed a single gene and hardly any have analyzed environmental factors. In these analyses, the results could be masked and the effects of other genes and environmental factors may be decreased. Here, we described the correlation between four genes [interleukin (IL)-4 (C-590T), IL-4 receptor (A1652G), FCER1B (G6842A) and STAT6 (G2964A)] in connection with IgE production; the role of IL-10 (C-627A) as a regulatory cytokine of allergy; and the severity of food allergy (FA) and atopic eczema (AE) in 220 Japanese allergic children. In addition to these SNPs, environmental factors, i.e., patient's attitude, indoor envirmonment, and so on, were also investigated in this study.
Our study was retrospective, and the correlation was analyzed by our defined clinical scores divided into three terms: worst symptoms, recent symptoms and general amelioration at the most recent examination during the disease course. Our results indicated that IL-10 AA, the genotype with lower IL-10 production, is associated with higher IgE levels in the serum (p < 0.0001, estimate; 0.912). Marginal liver abnormalities were observed in the subject group with both FA and AE (p < 0.1191, estimate; 0.1490).
Our defined clinical scores enabled evaluation of various aspects of disease severity. Based on the scores, while no single SNP selected in this study determined severity, the combination of the SNP with laboratory data and environmental factors appeared to determine severity.

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Atopy is a common familial trait characterized by increased specific or total serum immunoglobulin E (IgE) and underlies food allergy (FA) and atopic eczema (AE) among other childhood allergic diseases. As much as 95% of childhood allergy is associated with atopy and the cause of allergy involves both environmental and genetic risk factors. Genetic studies of atopic diseases using genome screens and candidate gene approaches suggest that several genes have been implicated in the pathogenesis of the allergic diseases (1–6).

Although many genetic studies have analyzed single nucleotide polymorphisms (SNPs) as risk factors for allergic diseases (7–13), the aim of our research was to study the correlation between

SNPs of seven genes and the severity of FA and AE. Recently, the hygiene hypothesis focused on the prevention of atopy acquisition. In this theory, the surrounding environments during infancy play an important role in atopy predisposition for life (14, 15). Therefore, we used a questionnaire and clinical recordings to analyze the effect of environmental factors. Allergic pediatric subjects are mostly complicated by FA, AE, bronchial asthma (BA) and allergic rhinitis (AR). Therefore, we used the complicated subjects as the proper group for this study of genetic and environmental factors.

We focused on four candidate genes, interleukin-4 (IL-4), IL-4 receptor α-chain (IL-4Rα)), the β-subunit of the high-affinity IgE receptor (FCER1B) and signal transducer and activator of transcription 6 (STAT-6), for enhancement of IgE production and action (7–9, 16–18). Correlation with severity of allergic diseases was speculated to be much stronger with a combined analysis of a series of proteins for IgE production than single gene analysis. Furthermore, we also analyzed IL-10, which was reported as a regulatory cytokine that controls the balance between Th1/Th2 (19–21). Thus, low production of IL-10 appeared to lead to chronic inflammation at local sites (20). We selected these five SNPs (single SNP per one gene) to be functionally important on each gene and to be associated with atopy or allergy (18, 19, 21–26).

In the present study, we evaluated the disease severity of three aspects categorized by pharmacological medications and clinical manifestation and then correlated the categorized disease severity and the aforementioned seven genes. Thus, we revealed the masked effect of the other genes in the single gene analysis. We demonstrated that genetic and environmental factors played a part in exacerbations of atopic diseases.

Materials and methods
Subjects

Two hundred and twenty Japanese allergic subjects, who were regularly treated by only one doctor, were recruited for this study from our outpatient department. Thirty-three subjects were excluded from the study for various reasons and 19 nuclear families were included. The mean age of this group was 7.3 yr (range: 0–19 yr), with a median of 6.5 yr (s.d. = 4.5). The proportion of males to females in this group was about three to two. These subjects had complications of other allergic disorders, such as asthma, allergic rhinitis, and allergic conjuncti-

vitis other than AE and FA. Ethical approval was granted by the Showa University Medical and Pharmaceutical Ethics Committee Board, and written consent was obtained from all subjects and their parents. The clinical data extracted from clinical recordings were as follows: age, sex, family history, total serum IgE levels, eosinophil (%), IgE CAP RAST score, oral allergy syndrome symptoms (pruritus and angioedema of the lips, tongue and palate), blood biochemical parameters of liver function (GOT, GPT, LDH, and ALP), patient surrounding environmental conditions, and medications.

Classification of clinical data

The individual clinical data was gradually scored into five classes. We divided the family history, a risk factor of atopy, into five classes based on the munber of family members with allergies as follows: 1: none; 2: one grandparent: 3: one parent; 4: a parent and a grandparent; and 5: both parents. In general, most children with AE have an allergic response to certain foods. Thus, the specific antibody or allergic response to food was detected in the allergic subjects with FA or AE. Positive responses to these foods (egg, milk, wheat, tree nuts, shellfish, peanuts, buckwheat, latex, and others) were expressed as the number of CAP RAST or oral allergy syndrome (OAS) symptoms. These responses were separated into five classes based on the presence of CAP RAST and OAS symptoms as follows: 1: none; 2: one or two; 3: three or four; 4: five or six; and 5: greater than seven. The GOT, GPT, LDH, and ALP data were used as an index of liver function. Evaluation of each value was based on Showa University Hospital's criteria. Liver function was divided into five classes based on the number of abnormal liver function tests as follows: 1: none; 2: one; 3: two; 4: three; 5: all. Total serum IgE (IU/mL) level was separated into five classes as follows: 1: below 100; 2: 100–500; 3: 500–1000; 4: 1000–5000; and 5: over 5000. The percentage of eosinophils (%) was divided into five classes as follows: 1: below 4; 2: 4–7; 3: 7–10; 4: 10–13; and 5: over 13. Mite RAST score was divided into five classes as follows: 1: zero ; 2: one; 3: two or three; 4: four or five; and 5: six. Environmental conditions, including patient's attitude, indoor environment, cleaning, bathing, and discipline, of allergic subjects were extracted from clinical recordings and the questionnaire. As shown in Table 1, patient's attitude and indoor environment were assessed in five items, respectively. Conditions were separated into five classes based on the number of applicable criteria

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Table 1. Primers and PCR conditions for PCR–RFLP.

Positions	Primer sequences	Primer	Restriction enzymes	Annealing temperature (°C)	PCR cycles	Mg^{2+} (mM)	Size (bp) Amplified fragment	Size (bp) Restricted fragment
IL-4 C-590T	5′-CACTAAACTTGGGAGAACATGGT-3′	F	Ava II	58	43	3.5	256	233, 23
	5′-TGCTTTGCATAGAAGGGA-3′	R						
IL-4Rα 1652A/G Gln592Arg	5′-CCCCACCAGTGGCTACC-3′	F	Msp I	58	43	1.5	105	89,16
	5′-GCCTTGTAACCAGCCTCTCCT-3′	R						
FCER1B 6842G/A Glu237Gly	5′-CAGGTTCCAGAGCATCGTG-3′	F	Xmn I	58	50	3.5	103	80,23
	5′-CTTATAAATCAATGGGAGGAAACA-3′	R						
IL-10 -627C/A	5′-GAAACATGTGCCTGAGAATCC-3′	F	Rsa I	58	43	3.5	198	126,72
	5′-TTAGGCAGTCACCTTAGGTCTC-3′	R						
STAT6 G/A	5′-GAAGTTCAGGCTCTGAGAGAC-′3	F	Hga I	58	50	2.5	215	194,21
	5′-GCCTCTAGTGTAAATGTGTCTG-′3	R						

F; Forward, R; Reverse, PCR; polymerase chain reaction, RFLP; restriction enzyme fragment length polymorphism.

as follows: 1: zero; 2: one of five; 3: two of five; 4: three of five; and 5: four of five. Cleaning and bathing were described as the number of times rooms were cleaned or the patient bathed a day. They were divided into three classes as follows: 1: over twice daily; 2: once daily; and 3: none daily. Discipline was expressed as exercises such as swimming that can reduce the amount of sweat on the skin, breathing relaxation techniques, or drinking cold water for management of autonomic nerve balance. This was separated into three classes as follows: 1: daily; 2; every few days; and 3: rarely.

Classification of medication

Disease severity was evaluated in three frames. The worst symptoms were designated a score based on the drugs or diets used at the worst stage of disease. Recent symptoms were designated a score based on the drugs or diets in current use. The worst or recent symptoms of AE were evaluated by the prescribed drugs based on Japanese AE guidelines. FA symptoms were evaluated by the extent of allergen elimination in diets based on Japanese FA guidelines. Symptoms were separated into five classes as follows: 1: none; 2: mild; 3: moderate; 4: severe; and 5: most severe. General amelioration of FA and AE was represented by a score based on the change in symptoms of allergic subjects from the previous to the latest examination. This was evaluated by clinical records and the questionnaire. While this characteristic is based on subjective subjects, this was felt to be appropriate for the study as only one doctor performed the evaluations. Amelioration was divided into five classes as follows: 1: no treatment required; 2: very good improvement; 3: good improvement; 4: slight improvement; 5: poor control.

Genotyping of SNPs

DNA samples were extracted from whole peripheral blood with the Wizard Genomic DNA Purification Kit (Promega, WI, USA) and were genotyped for SNPs by polymerase chain reaction–restriction enzyme fragment length polymorphism (PCR–RFLP).

The region surrounding the polymorphisms was amplified by PCR using the conditions listed in Table 1. PCR was carried out in a volume of 50 μL containing 100 ng of DNA and 0.5 μmol/L of each primer. The PCR of FCER1B and STAT6 were performed using the hot-start method with 1 U of KOD-DNA polymerase (TOYOBO, Osaka, Japan). The parameters for thermocycling were as follows: denaturation at 95°C for 5 min, followed by 50 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. This was followed by a final extension at 72°C for 6 min. The amplifications of IL-4, IL-4R, and IL-10 genes were performed with 1 U of EX Taq-DNA polymerase (TAKARA, Osaka, Japan). The conditions for amplification of these genes were as follows: denaturation at 95°C for 5 min, followed by 43 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. This was followed by a final extension at 72°C for 6 min.

Identification of the SNPs in PCR products was performed by incubation with a restriction enzyme chosen to cut one of the two alleles (Table 1), followed by electrophoresis on 2% agarose gels or 8% polyacrylamide gels (Fig. 1).

Statistical analysis

This study was necessary to determine of the extent to which the severity of atopic diseases are

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IL-4 -590   IL-4R   1652   FCER1B   6842

M   TT   CT   CC   AA   AG   M   M   AA   AG   GG

IL-10   -627   STAT6   2964

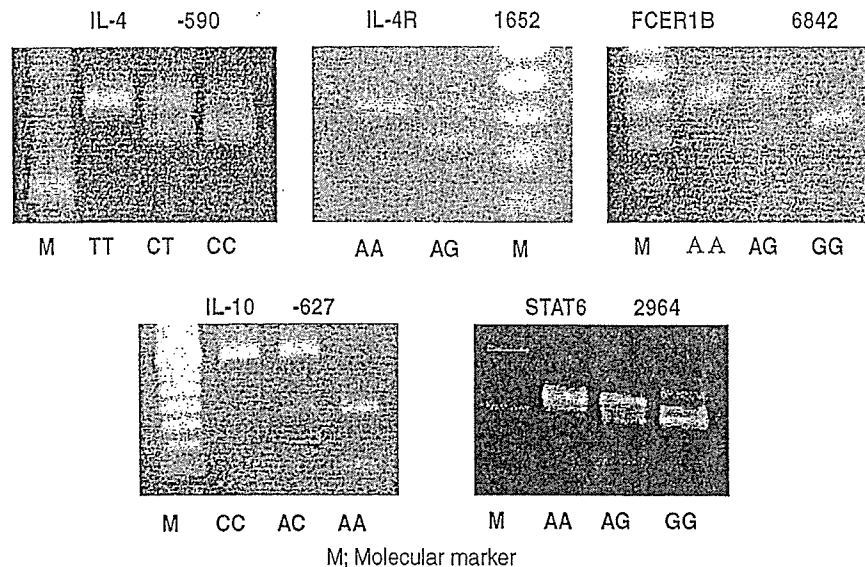M   CC   AC   AA   M   AA   AG   GG

M; Molecular marker

Fig. 1. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP)-based genotyping of cytokine gene polymorphisms. Seven allergy-specific genes were analyzed with the PCR–RFLP method. Restricted or non-restricted PCR products with specific enzymes were loaded on 2% agarose gel or 8% polyacrylamide gel and stained with ethidium bromide (see Table 1).

based on the host and environmental factors. For comparisons of factors divided into two groups based on a yes/no response, chi-square analysis was used. First, all interactions were considered under a value of $p < 0.2$. Second, we listed all interactions to be biologically considered. The evaluations of the worst symptoms, recent symptoms, and general amelioration of each allergic disease were used as response variables, and all laboratory data, SNPs, and interactions in the study were used as explanatory variables. The p value was estimated using simple regression analysis.

All testing was done using a JMP program (SAS Institute, Cary, NC, USA) for Windows computers, developed from the SAS-based system.

Results

Genotype frequencies

A total of 220 DNA samples from the Japanese children were amplified by PCR in order to genotype the polymorphisms of the four cytokines, two receptors, and one transcription factor genes. The bi-allelic polymorphisms were coded into three classes and the genotype distribution was assessed. The allele frequencies are shown in Table 2. The IL-10*-627A allele was major and the C allele was minor. Similarly, the IL-4*-590T and IL-4Rα*1652A alleles were major.

Influence of genetic and environmental factors on disease severity

Table 3 showed that the AA genotype, with low production of IL-10, was inadequate to regulate the IgE level ($p = 0.0001$). The subpopulation with both an IL-4 CT and STAT6 AG genotype

easily elevated IgE ($p = 0.003$). Similarly, the subpopulation with both the IL-4R AA and STAT6 AG genotype was strongly correlated with the eosinophil percentage. This suggested that these subpopulations easily increased the aforementioned laboratory data. Naturally, the serum IgE level, the eosinophil percentage, and the mite-specific IgE score were strongly correlated with each other. As environmental factors, discipline and bathing were good actions for ameliorating IgE levels or mite-specific IgE scores, respectively.

Influence of genetic and environmental factors on disease severity of FA (Table 4)

FA refers to an adverse immunologic response to proteins in food. In the food allergic reaction, there are both IgE-mediated and non-IgE-mediated gastrointestinal reactions. Infant FA generally associates with atopic diseases (i.e.,

Table 2. Genotype frequencies

Genes	No. of cases	Genotypes					
		TT		CT		CC	
IL-4	186	99	53% GG	79	42% AG	8	4% AA
IL-4Rα	177	3	2% GG	53	30% AG	121	68% AA
FCER1B	185	10	5% AA	77	42% AC	98	53% CC
IL-10	185	73	39% GG	93	50% AG	19	10% AA
STAT6	173	16	9%	72	42%	85	49%

Table 3. Simple linear regression analysis of Laboratory Data

Factor	IgE			Mite			Eosinophile			Positive Food		
	P value	Estimate	Genotype	P value	Estimate	Genotype	P value	Estimate	Genotype	P value	Estimate	Genotype
IL-4R										0.0200*	0.5200	AG
IL-10	0.0001***	0.9120	AA									
STAT6	0.0102*	0.6350	G allele									
IL-4R*STAT6							0.0179*	0.8030	AA-AG			
IL-4*STAT6	0.0030*	0.9160	CT-AG									
IgE				0.0001***	0.7910		0.0001***	0.5970				
Mite	0.0001***	0.6300										
Eosinophile	0.0001***	0.4780										
Bathing				0.0685	−0.5490							
Discipline	0.0448*	−0.4790										

*p < 0.05, **p < 0.01, ***p < 0.001.

Table 4. Simple linear regression analysis of Food Allergy

Factors	Genotypes or groups	Worst symptoms		Recent symptoms		General amelioration	
		P value	Estimate	P value	Estimate	P value	Estimate
Age	Matured					0.0354*	0.0127
FCER1B	AA			0.0135*	0.3750		
Food-specific IgE	Many	0.0709	0.2172	0.0001***	0.4760	0.0873	0.1856
FCER1B* Liver Function	AA* Bad	0.2438	0.1673	0.0137*	0.3750		
Eosinophile* Discipline	High* Well					0.0765	−0.7705
IgE* Discipline	High* Well					0.2193	−0.2759

*p < 0.05, **p < 0.01, ***p < 0.001.

atopic eczema and asthma). In this study, 93% of total FA subjects also had atopic eczema (Fig. 2). Therefore, the finding that the number of food-specific IgE antibodies in pediatric FA subjects was correlated with the severity of disease is consistent with this general opinion (Worst symptoms: p = 0.0709; recent symptoms: p = 0.0001; general amelioration: p = 0.0873). FA symptoms are usually ameliorated with maturation but are
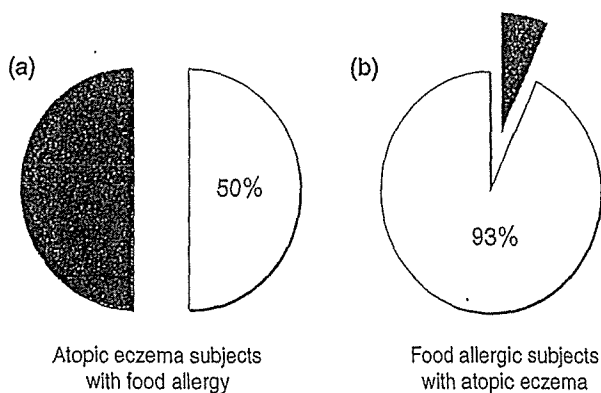

Fig. 2. Ratio of complications between food allergy (FA) and atopic eczema (AE). AE was a complication in 93% of food allergic subjects. FA complicated 49% of AE subjects. The frequency of food allergic subjects with AE was very high.

difficult to ameliorate when teenagers experience FA (general amelioration: p = 0.0354).

Interestingly, the FCER1B 6842AA genotype was correlated with significantly more severe FA than the other genotypes (recent symptoms: p = 0.0135). Furthermore, the combination of FCER1B 6842AA and abnormalities of liver function were more related to disease severity than any single factor (recent symptoms: p = 0.0137). However, previous reports indicated that the G genotype transmitted much stronger cellular signaling than the A genotype and was associated with childhood atopic asthma. It is speculated that the weak signal was appropriate for FA and chronic marginal inflammation of the liver gradually disturbed oral tolerance.

In specific subpopulations with high levels of eosinophils or IgE, discipline had a positive effect on general amelioration (p = 0.0765 and p = 0.2193, respectively).

Influence of genetic and environmental factors on disease severity of AE (Table 5)

AE is a chronic inflammatory skin disease that is considered familial with allergic features. It often

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Table 5. Simple linear regression analysis of Atopic Eczema

Factors	Genotypes or groups	Worst symptoms		Recent symptoms		EGeneral amelioration	
		P value	Estimate	P value	Estimate	P value	Estimate
IL-10	AA	0.0153*	0.3485	0.0148*	0.5549	0.0439*	0.3765
Food-specific IgE	Many	0.0607	0.2911			0.1506	0.2924
IgE	High	0.0004***	0.5018	0.0001***	1.1458	0.0004***	0.6501
STAT6* Eosinophile	A allele* High	0.0615	0.3020	0.0011**	0.8344	0.0001***	0.8876
IL-10* Liver Finction	AA* Bad	0.2376	0.1308	0.0092**	0.8328	0.0048**	0.7563
Mite* Cleaning	High* Bad			0.1296	0.5619	0.0905	0.5103

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

occurs in patients with other atopic disorders, such as asthma and allergic rhinitis. Figure 2 showed that almost 50% of patients with AE also had allergic rhinitis. Consistent with general opinions, a high level of serum IgE was significantly related to the severity of AE (worst symptoms: $p = 0.0004$; recent symptoms: $p = 0.0001$; general amelioration: $p = 0.0004$). A combination of the IL-10 AA genotype and abnormalities of liver function was much more correlated with disease severity than the IL-10 AA genotype alone. Thus, low producers of IL-10 could not regulate IgE production but did have exacerbations of AE symptoms. In this specific population, marginal inflammation in the liver affected atopic skin disease. The children with hyperreactivity to food allergens also had severe AE. In the specific subpopulation with a high level of mite-specific IgE, AE symptoms were influenced by indoor surroundings. Interestingly, the STAT6 A genotype seemed to be linked with eosinophilia in local inflammatory sites, especially in the specific subpopulation with high levels of eosinophils in the blood.

Discussion

Atopy is a heterogeneous disease determined by genetic and environmental interactions. The genetic contribution to atopic allergy was calculated to vary between 36 and 79% in twin studies (27–29). We investigated the correlation of disease severity of FA or AE with SNPs in seven genes previously identified to be putative atopy genes by linkage and association studies. In addition to single SNP analysis, the combination of the SNP analysis, laboratory data, and environmental factors were analyzed in this study.

In our study, a couple of SNPs were observed to be correlated with increased severity of FA and AE in Japanese children. IL-10, which is produced by Th_2 and T regulatory 1 (Tr_1) cells, was initially identified in mice as the inhibitory

factor of antigen-specific Th_1 cell proliferation and production of IFN-γ (30). IL-10 is a very important molecule as it inhibits many functions of immunocytes as a negative feedback regulator. Rosenwasser et al. (20) reported that the A/A genotype of the IL-10 -627C/A promoter polymorphism diminished promoter strength more than the C/C genotype. They speculated that persistent inflammation leads to prolonged and severe asthma episodes owing to lower IL-10 levels. Consistent with this report, our data indicated that lower production of IL-10, as in the AA genotype, was significantly correlated with severity of AE. It was observed that these genotypic subjects tended to produce high levels of IgE, which is typically associated with severity of AE.

Moreover, Iikura et al. (31) reported that the serum transaminase level was often slightly increased in infants who suffered from food-induced AE. The liver plays an important role in induction of oral tolerance. Liver $B220^+$ DC have the ability to induce apoptosis of activated T cells and generate immune regulatory activity by promoting Tr_1 cell differentiation (32). In addition, marginal liver damage caused impairment of DC activity following Tr_1 cell differentiation. The Tr_1 cell exhibits regulatory function following IL-10 production. Thus, it was speculated that the IL-10 AA genotype with marginal liver inflammation easily exacerbated FA and AE symptoms as a result of disruption of oral tolerance. Surprisingly, the IL-10 A/A genotype was more correlated with disease severity in subjects with both FA and AE than the other genotypes (Fig. 3). However, as serum total IgE level was not correlated with severity of FA, the regulatory mechanisms of FA and AE might differ. Therefore, in the specific subgroup with both illnesses, the IL-10 SNP was related to severity of illness. These data indicated that IL-10-sensitive and insensitive groups may exist among the FA subjects.

(a)

(b)   Classification of recent symptoms

Fig. 3. The correlation between total serum IgE levels and the promoter polymorphism of the IL-10 gene. Total serum IgE levels were higher in allergic subjects with the IL-10 promoter -627 AA genotype than the other genotype. The IL-10 AA genotype was 3.35 ± 0.18 (mean ± s.e.). The other genotype was 2.43 ± 0.15.
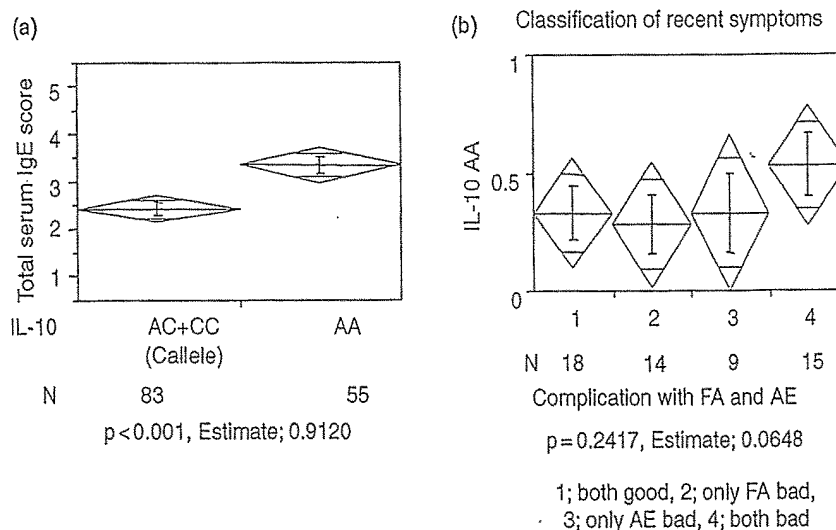
The number of food-specific IgE was significantly related to the severity of FA and AE. Thus, several food antigens may be responsible for the slight impairment of the system of oral tolerance. The FCER1B 6842A/G (E237G) polymorphism is adjacent to an immunoreceptor tyrosine-based activator motif (ITAM) site, which interacts with γ chains and influences the efficiency of IgE cell signaling involving *lyn* and *syc* (33). The 6842 A/G SNP was tightly linked with the -426 T/C and -654 C/T SNPs, which were responsible for the association with atopy (34). These promoter SNPs enhanced the transcriptional activity and increased the expression of FCER1B in blood basophils. However, in the present data, the genotype with lower transcriptional activity and abnormal liver function had more severe FA. This may be because of the effect of other promoter SNPs of FCER1B on FA. Second, while marginal liver damage existed in patients, lower expression levels of FCER1B in basophils and mast cells might play an important role in FA.

Shirakawa et al. (8) reported that the G allele was present in 10% of Japanese atopic asthma children. They also noted that genetic effects of FCER1B were much more prominent in childhood asthma. The proportion of the 6842A/G polymorphism in the study was quite different from our data, in which the G allele was present in 23.5% of the Japanese pediatric population. The difference may be explained by the possibility that genetics facilitated exacerbations in our population, e.g., comprised of various allergic diseases such as FA, AE, and asthma.

Furthermore, environmental factors may significantly affect the severity of FA and AE in specific subpopulations. Well-performed bathing and discipline improved high levels of serum total IgE and mite-specific IgE. Naturally, this

suggested that the effect of SNPs was masked by these factors in these specific subpopulations, i.e., the high level of serum IgE group. However, strict adherence to disciplines improved only the general amelioration of FA in groups with high levels of eosinophils or total serum IgE. These results indicated that more subjective evaluations might be represented in the diagnosis. For this reason, we also adopted medication and laboratory data for objective evaluation.

As the number of subjects was small and most subjects had complications, we did not have large statistical power. Therefore, the interactions of only two factors could be analyzed with a sufficient p value and estimate. In addition, we could not study non-allergic control children for ethical reasons. As we did not research all SNPs per one gene, we could not suggest whether other single SNP not analyzed in this study was associated with severity of FA and AE in Japanese children. However, the present study provides useful preliminary data.

Our data presented the laboratory data of liver function and SNPs, especially IL-10 and FCER1B, which might serve in the evaluation of disease severity. Moreover, our results indicated that these genetic polymorphisms may be correlated with risk factors not only for the onset but also for the severity of allergy. The combination of environmental factors and SNP analysis easily detected specific subpopulations. We believe that this kind of analysis is useful for research on the correlation of disease severity and SNPs.

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