Arch Gynecol Obstet
DOI 10.1007/s00404-010-1812-7

MATERNO-FETAL MEDICINE

The effect of prepregnancy body mass index on singleton cesarean delivery among term nulliparous women in Japanese population

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Received: 29 July 2010 / Accepted: 8 December 2010

Abstract
Purpose Overweight or obesity is a known risk factor for cesarean delivery although there is minimal data among Japanese women. The aim of the study was to examine the effect of prepregnancy body mass index (BMI) on singleton cesarean delivery among term nulliparous women using a national sample from the Human Milk Survey.
Methods Data from the Human Milk Survey between 1998 and 2008 were used for the secondary analysis. Women were categorized as underweight (BMI < 18.5 kg/m^2), normal weight ($18.5 \leq$ BMI < 25.0), or overweight (BMI ≥ 25.0) based on their prepregnancy BMI. The association between maternal prepregnancy BMI and cesarean delivery was assessed using logistic regression models.
Results A total of 915 women were included in the analysis. The proportion of cesarean section was 10.1%. Overall, 17.1% of the women were underweight while 6.0% were overweight. After adjusting for maternal age, smoking status, pregnancy complications, and infant birthweight, overweight women were 2.7 times more likely to have a cesarean delivery compared to normal weight women (adjusted odds ratio [adjusted OR] = 2.7, 95% confidence interval [CI] = 1.4–5.4), and underweight women were half as likely to have a cesarean delivery compared to normal weight women (adjusted OR = 0.5, 95% CI = 0.2–1.1).
Conclusions Being overweight before pregnancy more than doubled the risk of cesarean delivery independent of age, smoking, pregnancy complications, and infant birthweight among term nulliparous women. Overweight Japanese women should be advised to achieve normal prepregnancy BMI in their preconception period to prevent cesarean delivery.

Keywords Epidemiology · Cesarean delivery · Prepregnancy BMI · Maternal overweight · Pregnancy · Japanese

Introduction

In Japan, cesarean rate has doubled over the last two decades, from 8.5% in 1987 to 18.4% in 2008, while the total number of deliveries has decreased [1]. Although cesarean section is regarded to be a low-risk procedure both by health professionals and patients in developed countries, maternal intraoperative and postoperative complication rates have been reported to be high [2, 3]. Some may claim that the increase in cesarean section rates was necessary to improve perinatal outcomes. However, recent WHO surveys conducted in Latin America and Asia suggested that increasing rates of cesarean section do not necessarily lead to improved perinatal outcomes but may be associated with maternal mortality and morbidity [4, 5]. In addition, cesarean delivery costs more than a vaginal birth, contributing to rising health costs [6].

Overweight or obesity is a known risk factor for cesarean delivery [7–11]. While increasing numbers of women of childbearing age in western countries are overweight or obese [12, 13], a similar trend has not been observed in Japan. According to a national survey of non-pregnant women in Japan, in 2007, the prevalence of overweight (defined as body mass index [BMI] of ≥ 25.0 kg/m^2) women in their twenties and thirties were 5.9 and 11.1%,

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Published online: 30 December 2010

respectively, and the prevalence has not changed over the last 20 years [14]. In Japan, the government does not monitor the prevalence of obesity (defined as BMI of $\geq$30.0 kg/m^2) for non-pregnant women. In pregnant women in Japan, there is no national prevalence data of overweight or obesity. National data indicate that Japanese women are having their babies at older age compared to a few decades ago. According to vital statistics, the average maternal age at delivery rose from 27.4 years in 1975 to 31.0 years in 2009 [15]. Because the prevalence of overweight increases as women age [14], it is likely that the overall prevalence of overweight among pregnant women have increased over the years. This may have contributed to the rise in cesarean section rate in Japan, to some degree.

The association between overweight and cesarean delivery has not been well documented among Japanese women. To our knowledge, in Japan, there is only one published study investigating the association between overweight women and cesarean delivery in the English language and it is a single institution study [16]. Therefore, our aim of the study was to examine the effect of prepregnancy body mass index on singleton cesarean delivery among term nulliparous women using a national sample from the Human Milk Survey.

Methods

Data from the Human Milk Survey between 1998 and 2008 were extracted for secondary analysis. The primary purpose of the survey was to investigate the dioxin levels in human breast milk and their effects on child development. The details of the survey methodology are described elsewhere [17]. In brief, healthy nulliparous women carrying singleton babies in their twenties and thirties were recruited and interviewed by public health nurses in 19 prefectures and one city. Women were first interviewed during the third trimester of pregnancy, and they provided maternal information including prepregnancy BMI and smoking status. Women were again interviewed approximately 1 month post-delivery, at which time, delivery and infant information were obtained. The information was self-reported and confirmed by the public health nurses from each mothers' maternal and child health handbook, a document issued by municipal governments in Japan. By law, health providers in charge of prenatal visits and/or delivery are responsible for documenting information such as maternal weight, pregnancy complications, and mode of delivery in the handbooks.

Between 1998 and 2008, there were 1,021 participants in the Human Milk Survey. Women with non-cephalic fetuses and post-term delivery were excluded from the analyses since malpresentation and post-term delivery are both associated with cesarean delivery, and we wanted to separate those effects. After excluding those missing mode of delivery ($n = 5$), those missing gestational age at delivery ($n = 2$), preterm delivery defined as less than 37 weeks gestation ($n = 26$), post-term delivery defined as 42 weeks gestation or more ($n = 16$), those missing information on prepregnancy BMI ($n = 2$), and those with non-cephalic fetuses ($n = 48$) or missing information on fetal presentation ($n = 7$), a total of 915 women and their infants were included in the analysis.

The exposure variable of interest was prepregnancy BMI, calculated using prepregnancy weight and height as self-reported by the mother during the 3rd trimester of pregnancy. Prepregnancy BMI was categorized into underweight (BMI $<$ 18.5 kg/m^2), normal weight (18.5 kg/m^2 $\leq$ BMI $<$ 25.0 kg/m^2), and overweight (BMI $\geq$ 25.0 kg/m^2) according to the definition by the Japan Society for the Study of Obesity [18]. The outcome variable was a dichotomous variable of whether or not the mother had a cesarean delivery, as self-reported by mother 1 month post-delivery. Bivariate analyses were conducted using a contingency table approach to assess the association between prepregnancy BMI and demographic and clinical characteristics of the mothers and infants. The association between three-category prepregnancy BMI variable and cesarean delivery variable was assessed using logistic regression models. Covariates and potential effect modifiers considered were maternal age, smoking status, region of residence (Japan is officially divided into 8 regions, including Hokkaido, Tohoku, Kanto, Chubu, Kinki, Chugoku, Kyushu, and Okinawa), pregnancy complications (presence of either one of the following: preeclampsia/hypertension, uterine anatomic abnormality, gestational diabetes, and threatened labor), gestational age, and infant birthweight (in 100 g). A p value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using the Statistical Analysis System® (SAS, version 9.1; SAS Institute, Cary, NC).

Results

Table 1 demonstrates the demographic and clinical characteristics of women and infants in the study. Proportion of cesarean delivery was 10.1%. Women were aged between 24 and 36 years (mean 29.3 years, median 29 years). The study participates were from six regions (19 prefectures and 1 city) out of eight regions (47 prefectures) of Japan. Prepregnancy BMI ranged from 16.0 to 36.2 kg/m^2, and the mean (SD) and median were 20.7 (2.7) kg/m^2 and 20.2 kg/m^2, respectively. Overall, 17.1% of the women were underweight while 6.0% were overweight. Pregnancy complications (preeclampsia or hypertensive disorder

Table 1 Characteristics of term nulliparous women and infants in cephalic position, the human milk survey, 1998–2008 ($n = 915$)

Characteristic	n (%)[a]
Maternal age (years)	
24–27	276 (30.2)
28–31	440 (48.1)
32–36	199 (21.8)
Region of residence	
Tohoku	133 (14.5)
Kanto	225 (24.6)
Chubu	261 (28.5)
Kinki	145 (15.9)
Chugoku	101 (11.0)
Kyushu	50 (5.5)
Smoking status	
Current smoker (3rd trimester)	33 (3.6)
Quit smoking during pregnancy	162 (17.8)
Non-smoker	716 (78.6)
Missing $n = 4$	
Prepregnancy BMI status	
Underweight (BMI < 18.5 kg/m^2)	156 (17.1)
Normal (18.5 kg/m^2 ≤ BMI < 25.0 kg/m^2)	704 (76.9)
Overweight (BMI ≥ 25.0 kg/m^2)	55 (6.0)
Pregnancy complications	
Present	36 (4.0)
Absent	873 (96.0)
Missing $n = 6$	
Sex of the infant	
Male	468 (51.4)
Female	443 (48.6)
Missing $n = 4$	
Birth weight	
Low birthweight (<2,500 g)	54 (5.9)
Normal birthweight (2,500 g ≤ BW < 4,000 g)	855 (93.4)
High birthweight (≥4,000 g)	6 (0.7)
Mode of delivery	
Cesarean	92 (10.1)
Vaginal	823 (90.0)

[a] Pecentages may not add up to 100% because of rounding

during pregnancy, uterine anatomic abnormality, gestational diabetes, and threatened labor) were reported in 4.0% of women.

Bivariate analyses that looked at the association between prepregnancy BMI categories and characteristics of women and infants using contingency table approach showed that, in general, increasing maternal age and greater birthweight were associated with greater maternal prepregnancy BMI categories (Table 2). Maternal smoking status and pregnancy complications were not significantly associated with prepregnancy BMI status. In addition, there was no regional difference in prepregnancy BMI status (data not shown).

The results of logistic regression analyses to test the association between maternal and infant characteristics and mode of delivery (cesarean vs. vaginal) are presented in Table 3. The crude logistic regression models showed that prepregnancy BMI, maternal age, the presence of pregnancy complications, and infant birthweight were associated with cesarean delivery. After adjusting for maternal age, smoking status, pregnancy complications, and infant birthweight, overweight women were 2.7 times more likely to have a cesarean delivery compared to normal weight women (adjusted odds ratio [OR] = 2.7, 95% confidence interval [CI] = 1.4–5.4). On the other hand, underweight women were half as likely to have a cesarean delivery compared to normal weight women (adjusted OR = 0.5, 95% CI = 0.2–1.1) although the association was marginally significant ($p = 0.076$). Adding gestational age to the final model did not alter the results.

Discussion

Overweight women were 2.7 times more likely to have a cesarean delivery compared to normal weight women even after controlling for pregnancy complications, maternal age, smoking status, and birthweight. This result indicates that overweight women are likely to deliver cesarean not only because of pregnancy complications such as preeclampsia and diabetes but also because they may have difficulty delivering vaginally for anatomical reasons: dystocia due to increases in pelvic soft tissues [9, 19]. Although overweight women are likely to have high birthweight infants [20], our result also suggests that infant birthweight was not the only reason for the overweight women to have a cesarean delivery. It is likely that abnormal labor (i.e., protraction and arrest disorders) resulted in cesarean delivery. In addition, obstetricians may tend to recommend a cesarean delivery to overweight women once the labor is prolonged because they may fear that vaginal delivery may not be possible for those women. Further research is needed to reveal whether the perception and practice patterns of obstetricians in Japan differ by prepregnancy BMI status.

To our knowledge, this is the first national multicenter study in Japan that investigated the association between prepregnancy BMI categories and cesarean delivery. A previous study of 633 women by Murakami et al. involving one hospital showed that the odds of a cesarean delivery were 2.42 times (95% CI = 1.05–5.58) higher among overweight women (prepregnancy BMI ≥ 25 kg/m^2) compared with normal weight women after adjusting for

Table 2 Characteristics of the study population and mode of delivery by prepregnancy BMI categories, the human milk survey, 1998–2008 ($n = 915$)

Characteristic	Underweight ($n = 156$)	Normal weight ($n = 704$)	Overweight ($n = 55$)	p Value[a]
Mean maternal age (in years) (SD)	28.8 (2.5)	29.3 (2.7)	30.0 (2.5)	0.008
Smoking status				0.278
Current smoker (%)	5.8	3.3	1.8	
Quit smoking during pregnancy (%)	19.9	16.9	23.6	
Non-smoker (%)	74.4	79.9	74.6	
Sex of the infant				0.578
Male (%)	51.3	50.9	58.2	
Birth weight (g) (SD)	2974.7 (358.3)	3073.1 (358.5)	3198.7 (429.6)	<0.001
Birth weight (3 category)				<0.001
Low birthweight (<2,500 g) (%)	9.6	5.4	1.8	
Normal birthweight (2,500 g ≤ BW < 4,000 g) (%)	89.7	94.3	92.7	
High birth weight (≥4,000 g) (%)	0.6	0.3	5.5	
Pregnancy complications				0.861
Present (%)	3.2	4.2	3.6	
Mode of delivery				<0.001
Cesarean delivery (%)	4.5	10.1	25.5	

SD standard deviation

[a] p Values were calculated using the Chi-square test, Fisher's exact test, and one-way analysis of variance test as appropriate

Table 3 Results from logistic regression models to test the association between maternal and infant characteristics and mode of delivery (cesarean vs. vaginal), the human milk survey, 1998–2008 ($n = 915$)

	Crude		Adjusted[a]	
	OR (95% CI)	p Value	OR (95% CI)	p Value
Prepregnancy BMI				
Underweight (BMI < 18.5 kg/m^2)	0.4 (0.2–0.9)	0.032	0.5 (0.2–1.1)	0.076
Normal (18.5 kg/m^2 ≤ BMI < 25.0 kg/m^2)	Reference		Reference	
Overweight (BMI ≥ 25.0 kg/m^2)	3.0 (1.6–5.9)	0.001	2.7 (1.4–5.4)	0.003
Maternal age (years)	1.1 (1.1–1.2)	0.002	1.1 (1.0–1.2)	0.011
Smoking status				
Current smoker	0.6 (0.1–2.5)	0.470	0.7 (0.2–3.2)	0.672
Non-smoker	Reference		Reference	
Quit during pregnancy	1.1 (0.7–2.0)	0.649	1.1 (0.6–2.0)	0.664
Pregnancy complications				
Present	3.2 (1.4–7.0)	0.004	3.0 (1.3–6.7)	0.009
Absent	Reference		Reference	
Birth weight (in 100 g)	1.1 (1.0–1.1)	0.036	1.0 (1.0–1.1)	0.121

OR odds ratio, *CI* confidence interval

[a] The multivariable model is adjusted for all the other variables listed in the table

maternal age, parity, smoking, weight gain during pregnancy, and gestational age [16]. The study was conducted at a tertiary hospital, and included women of multiparity, teenagers, those over 40 years of age, as well as deliveries between 24 and 42 weeks gestation. The women in our study were low-risk because the survey targeted healthy pregnant women in their twenties and thirties who were planning to breastfeed. Nonetheless, our study involving low-risk women from the general population showed that being overweight is a significant risk factor for cesarean delivery, consistent with the previous hospital-based study.

Japan has lower rates of cesarean delivery compared to western countries: The overall cesarean rate in Japan was 18.4% in 2008 while the rate was 32% in the United States

in 2007 [21]. Even though our study involved low-risk, term nulliparous women with cephalic fetuses, overweight women in our sample had a high cesarean delivery rate of 25.5%. This is comparable to a US cesarean delivery rate of 26.5% among nulliparous overweight women with term live births from the Pregnancy Risk Assessment Monitoring System between 1998 and 2000 [22] although overweight was defined slightly different (26.1 kg/m^2 $\leq$ BMI $\leq$ 29.0 kg/m^2) in the US.

The results of the current study and the previous study by Murakami et al. suggest that overweight Japanese women may have higher risk of cesarean delivery compared with women from western countries, in terms of the magnitude of risk of overweight women compared to normal weight women. A meta-analysis of studies conducted in western countries by Chu et al. showed that the OR of a cesarean delivery were 1.46 (95% CI = 1.34–1.60), 2.05 (95% CI = 1.86–2.27), and 2.89 (95% CI = 2.28–3.79) among overweight, obese, and severely obese women, respectively, compared with normal weight pregnant women [23]. Although our study and the study by Murakami et al. combined the overweight and obese categories into one category of overweight (prepregnancy BMI $\geq$ 25.0 kg/m^2) as suggested by the Japan Society for the Study of Obesity [18], the majority of these women are between the prepregnancy BMI of 25.0 and 29.9 kg/m^2 (78% in our sample). Therefore, the OR of 2.7 in our study and OR of 2.42 in the previous study most likely represent the odds ratios of women with prepregnancy BMI between 25.0 and 29.9 kg/m^2. These magnitudes of risk are comparable to the risk among obese women in western countries.

There are some limitations in our study. The study used prepregnancy weight and height to calculate prepregnancy BMI, which were self-reported during the third trimester of pregnancy and may have been inaccurate. Although self-reported weight and height are reported to be generally reliable among middle-aged Japanese women [24], no validation study has been conducted among Japanese pregnant women. Another limitation is that other important variables associated with cesarean delivery such as weight gain during pregnancy [25, 26] and socioeconomic status [27, 28] were not available. Future studies should investigate the association between prepregnancy BMI and cesarean delivery in relation to these variables in Japan.

Furthermore, the generalizability of the study is limited. The study population was low-risk since the survey targeted healthy pregnant women in their twenties and thirties who were planning to breastfeed. The cesarean rate among term nulliparous women in our study was 10.1% while the overall cesarean rate for Japan was between 14.7 and 18.4% during the same period [1]. Nonetheless, the study was a multicenter study, had a large sample size of 915, and provided useful insight on this subject. Further research is needed among adolescents, older women, and high-risk women in Japan.

In conclusion, our study showed that being overweight more than double the risk of cesarean delivery even among low-risk nulliparous women in Japan, where women are leaner compared to women in the western countries. Overweight Japanese women should be advised to achieve normal prepregnancy BMI through dietary and life-style modifications in their preconception period to prevent primary cesarean delivery.

Acknowledgments This study was conducted as a part of the study on dioxins in human milk and child development supported by Health and Labour Science Research Grants (Principal investigator: Professor Hiroshi Tada).

Conflict of interest We declare that we have no conflict of interest.

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SGA児の長期予後

神経学的障害と発達予後

河野 由美

はじめに

Small for gestational age(SGA)は体重，身長，頭囲が在胎期間相当かどうかにより定義され，その多くは，子宮内発育不全(intrauterine fetal growth restriction：IUGR)によるものである。すなわち，児自身のもつ先天的異常や子宮内での不良な環境のため，予定されている発育より劣っている状態にあるといえる。一般にSGA児の精神運動発達予後は，AGA(appropriate for gestational age)児に比べ劣るとされることが多いが，胎児発育に影響した因子が何かにより，また出生後の発育状態によって児の予後や発達は異なる。本稿では，主に胎盤機能不全など子宮内環境に起因したSGA児の神経発達予後について，在胎期間そのものが予後に大きな影響を与えることを考慮し，可能な範囲で早産児と正期産児に分けて文献を基に論じる。

脳性麻痺(cerebral palsy：CP)

1．正期産SGA児，late preterm SGA児

在胎期間に相応する出生体重が小さいこととCPリスクの増加との関連がいくつかの研究で示唆されている。米国のNCPP研究では，痙性両麻痺の頻度は，正期産では出生体重が2,501 gより小さいSGA児は，2,501 g以上のAGA児よりCPのリスクが有意に高率であった[1]。最近のヨーロッパの多施設研究によると，出生時発育基準値，あるいは胎児発育基準値をもとに出生体重10パーセンタイル未満のSGA児(Z-scoreでは－1.28未満に相当)と25～75パーセンタイルのAGA児(Z-scoreは－0.67～0.67に相当)を比較すると，在胎期間32週以降からSGA児でCP合併率の上昇が認められ(**図1**)，在胎32～42週のSGA児はAGA児の4～6倍のCPリスクであった[2]。これらの結果は，正期産のSGA児と，早産の中でもいわゆるlate pretermのSGA児でCPリスクが高いことを示している。鈴木ら[3]は，滋賀県のCPに関する疫学的検討で，正期産CP例では19%に，早産CP例では8%に出生体重－1.5 SD未満のIUGRを認め，正期産のほうが高率であった。IUGRのあったCP例の障害原因として，脳形成異常が最も多く，ほかには胎内感染，脳室周囲白質軟化症(periventricular leukomalacia：PVL)，低酸素性虚

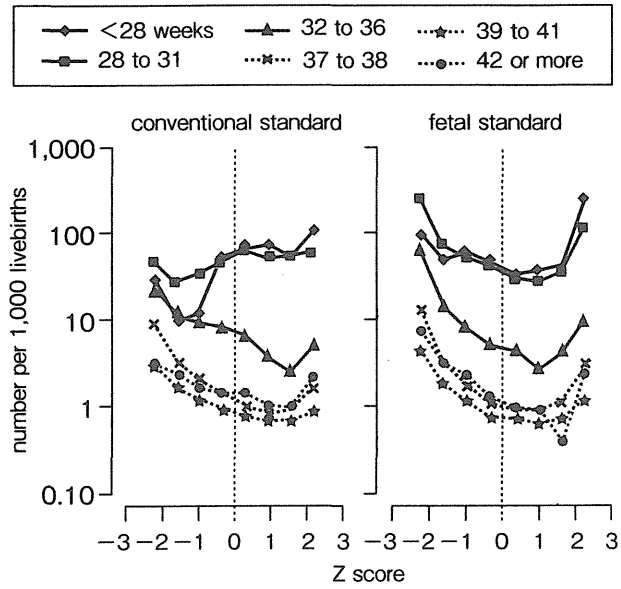

図1　2種類の発育基準値による出生体重のZスコアと在胎期間別CP有病率(出生1,000当たり)(Jarvisら，2003より引用一部改変)[2]
在胎28週未満，28～31週のグループはCPの有病率が高いがZスコアと有病率には明らかな関連は認めない

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血性脳症(hypoxic ischemic encephalopathy：HIE)，頭蓋内出血(intraventricular hemorrage：IVH)であった。正期産，late preterm のSGA児の絶対数は早産より多く，児に先天異常を伴っている割合も高いことがCPのリスク要因となることに関連している。

2. 早産SGA児

図1に示されているように，在胎32週未満ではSGAとCPの関連はそれほどクリアではない。著しい早産で未熟な状態で出生したことによる脳への損傷のリスクが，SGAであることのリスクより上回るためと考えられる。早産児のCPの原因として，第1にPVLがあげられるが，PVLの合併頻度をSGAとAGAで厳密に比較した研究は少ない。在胎22～32週の早産児1,902名を対象としたcystic PVLのリスク要因の検討によると，cystic PVLは全体で5.4%に認め，SGAは性別，在胎期間，出生前ステロイド投与を調整した回帰分析で，妊娠高血圧症候群合併の非SGAと比較して，オッズ比0.9(95%C.I. 0.3～2.3)で，有意なリスク因子ではなかった[4]。

早産児のCPのその他の原因となるIVHについて，妊娠高血圧症候群合併のSGA児では内因性のコルチコステロイドの分泌を促進することにより児の脳神経系の成熟を促しIVHのリスクを減少させ得ることが報告されている[5]。しかし，極低出生体重(very low birth weight：VLBW)児，約2万人を対象としたVermont Oxford networkの報告では出生体重が10パーセンタイル未満のSGA児において，在胎期間，出生前ステロイド投与の有無，性別，人種等の要因を調整した解析でIVHのリスクはオッズ比1.13(0.99～1.29)，IVH 3度以上のリスクのオッズ比は1.25(0.98～1.59)であり，有意な関係は認められていない[6]。

知　能

これまでにSGAと知能，学業成績の関連について多くの報告があり，SGAは低知能や低成績のリスク要因とされる。同時に起こり得る早産や新生児合併症などの周産期要因，養育環境や経済状況などの社会的要因とSGAの影響を厳密に分離して評価することは，実際には困難なことが多い。**表**は西オーストラリアの約22万を対象としたコホート研究による理想の出生体重からの隔たりと10歳時の知的障害(intellectual disability：ID)の関係をオッズ比で示している[7]。SGAの程度は在胎期間に相応する理想体重の何%に相当するかにより示され，85%未満はおよそ10パーセンタイル未満に相当する。白人の85%未満の出生体重では理想出生体重(95～104%)に比べ，男児，女児，早産児(37週未満)，正期産児いずれにおいても軽度～中等度IDの有意なリスク要因であった。75%未満の出生体重では，男児，女児，正期産児で重度IDのリスク要因であったが，早産児では有意でなかった。

注目すべきは，知能は出生時SGAであるかどうかに加え，その後の成長によりキャッチアップするかどうかで差が認められている点である。1973～1978年出生男性25万人の18歳時の知的能力を調査したスウェーデンのコホート調査で，出生体重－2 SD未満の児は出生体重が±2 SD内の児に比し有意に知的パフォーマンススコアが低いこと，さらに，18歳時の身長がキャッチアップ(正常成人の－2 SDを超える)するかどうかでスコアが異なり，キャッチアップしないほうがよりスコアが低いことが示された[8]。頭囲のキャッチアップの有無による差についても同様の報告がみられる[9]。

1. 正期産SGA児，late preterm SGA児

Sommerfeltら[10]は正期産SGA児(出生体重15パーセンタイル未満)338名の5歳児のIQ(WPPSI-R)をAGA児と比較し，ほかの生物学的要因や母の喫煙や疾病，養育力，経済状況などを考慮しても，言語性IQで3点，動作性IQで4点，SGA児のほうが有意に低値であった。正期産のSGA児とその兄弟のAGA児で7歳での知能指数(IQ)を比較した研究では，出生時の頭囲が兄弟の頭囲より3 cm以上小さい児(小頭を伴う児)ではIQおよび視覚–運動機能スコアが低かったが，頭囲の差が3 cm以内のものはIQに差がなかったとされ[11]，いわゆるbrain-sparingが起こり，頭囲発

表　理想出生体重からの隔たり(パーセンテージ)による知的障害のリスク(Leonard ら，2008 より引用，一部改変)[7]

Ethnicity and subgroup	Percentage of optimal birth weight											
	<75		75～84		85～94		95～104	105～114		115～124		>124	
	AOR †	95% C.I.	AOR	95% C.I.	AOR	95% C.I.	(referent)	AOR	95% C.I.	AOR	95% C.I.	AOR	95% C.I.
Mild-moderate intellectual disability													
Caucasian													
Males	2.28	1.69, 3.09	1.83	1.50, 2.24	1.13	0.95, 1.34	1	0.99	0.80, 1.21	0.94	0.69, 1.29	1.37	0.86, 2.17
Females	2.67	1.88, 3.79	1.60	1.24, 2.07	1.04	0.83, 1.30	1	0.96	0.74, 1.24	1.00	0.68, 1.48	0.83	0.41, 1.69
Preterm	1.71	1.06, 2.77	1.57	1.03, 2.41	0.88	0.57, 1.36	1	1.04	0.65, 1.65	1.09	0.61, 1.92	0.77	0.41, 1.44
Term	2.42	1.88, 3.12	1.69	1.43, 2.00	1.12	0.97, 1.29	1	0.96	0.81, 1.14	0.93	0.71, 1.22	1.29	0.86, 1.95
Severe intellectual disability													
Caucasian													
Males	2.79	1.22, 6.39	0.61	0.25, 1.46	1.08	0.64, 1.81	1	0.46	0.21, 1.01	1.07	0.45, 2.58	1.83	0.56, 6.02
Females	5.42	2.22, 13.23	1.49	0.64, 3.48	1.09	0.54, 2.20	1	0.75	0.31, 1.83	0.67	0.15, 2.93		
Preterm	0.90	0.24, 3.32	0.54	0.15, 2.01	0.55	0.18, 1.64	1			0.62	0.13, 2.89		
Term	4.79	2.59, 8.83	0.91	0.48, 1.74	1.13	0.73, 1.76	1	0.65	0.36, 1.18	0.83	0.35, 1.96	1.50	0.46, 4.84

AOR †：出生年，母の結婚，母の出生国，保険の種類，父の職業，経済状況を調整したオッズ比
C.I.：95%信頼区間

育が正常の SGA 児では，必ずしも知能との関連は明らかではない。

学童期の知能と学業について，オーストラリアの正期産児 7,388 名のコホート研究の結果，Ravens IQ score の平均値は，出生体重 3 パーセンタイル未満では 97.2，3～10 パーセンタイルでは 99.9，10 パーセンタイル以上では 100.2 であり SGA と非 SGA で有意差を認めなかった[12]。しかし，WRAT(Wide Range Achievement test)の Reading スコアは非 SGA に比較し SGA で－1 SD 未満の低値の割合が有意に高率であった。

SGA に多くみられる母の喫煙や疾病，低い養育力や経済力は知能や学習能にも影響する要因であり，SGA そのものの影響に環境要因が加算された結果が，SGA 児での低い知能，学業成績につながると予想される。

2. 早産 SGA 児

早産は認知能低下のリスク要因であることはメタアナリシスでも示されているが，早産児のみで SGA と AGA の知能を比較した研究は限られる。Gutbrod ら[13]は VLBW の SGA と，体重がマッチした AGA(AGA-BW，在胎期間が最も短い)，在胎週がマッチした AGA(AGA-GA)の 3 グループの 5 カ月，20 カ月，56 カ月での発達検査と知能検査の結果を比較したところ，AGA-GA グループがすべての時期の検査で有意に高いスコアであった。しかし新生児期の合併症，多胎などの要因を加えるとこれらの差は有意でなく，児の未熟性や多胎に起因する合併症を考慮すると VLBW 児において SGA か AGA かは知能に長期的な影響は認めなかったと結論している。一方，25～35 週の早産児で同様なコントロールグループを用いた Feldman

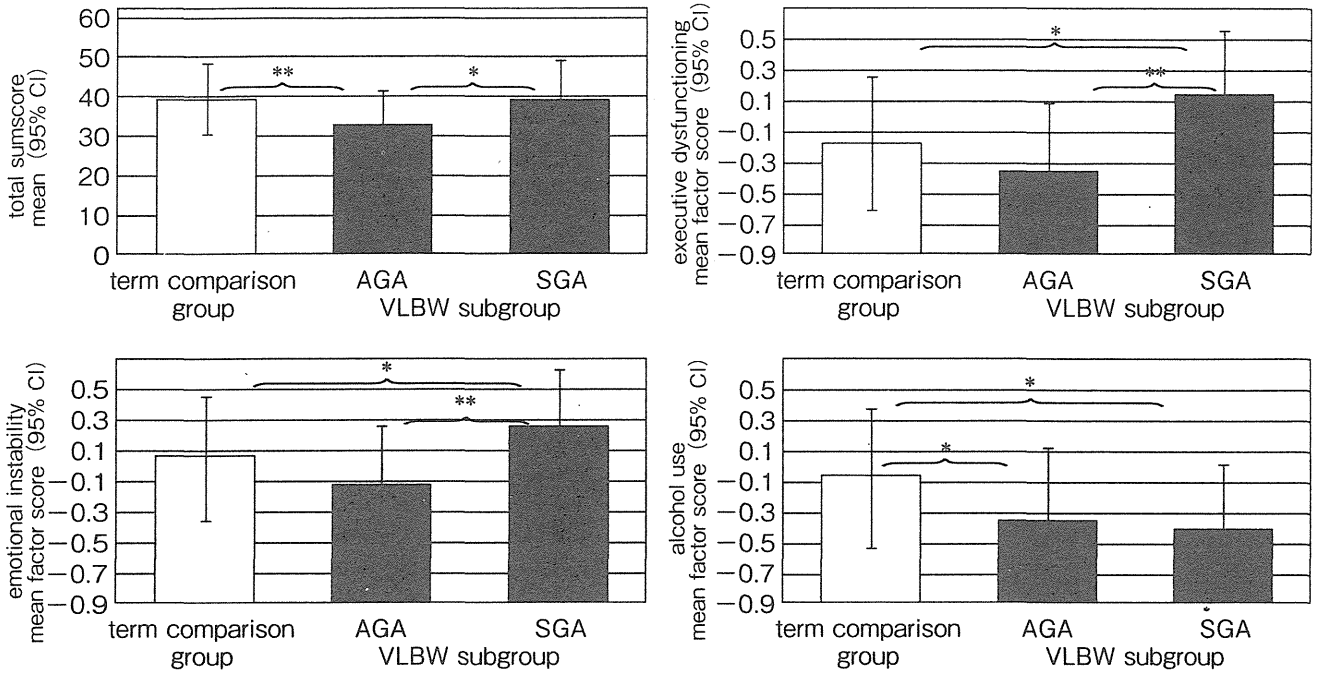

図2　VLBW-AGA，VLBW-SGA と正期産コントロール群の Adult Problem Questionnaire による情緒・行動スコアの平均値(Strang-Karlsson ら，2008)[18]

性別，年齢，BMI，両親の教育歴，妊娠中の喫煙を調整した平均値

AGA：appropriate for gestational age(n=110)，SGA：small for gestational age(n=52)

$^{*}p \leq 0.05$，$^{**}p < 0.01$

ら[14]によると，24カ月での Balyey 検査の MDI スコアは，SGA グループが最も低く，SGA であることは MDI スコアの有意な予測要因であった。早産児における SGA の知能への影響は早産の程度と早産に至った理由も関係し一定の結論は得られていない[15]。

行　動

IUGR をきたすような不適切な胎児環境が，小児期，成人期の疾病に影響すること，いわゆる DOHaD(developmental origins of health and disease)仮説が知られ，成人では生活習慣病のみでなくうつ病，統合失調症などの精神疾患や，小児期では行動パターンや行動障害との関連が示されている。

1. 正期産 SGA 児，late preterm SGA 児

5～6歳時の ADHD との関連を，正期産児267名を対象とした調査で，出生時のやせの程度を表す ponderal index(kg/m^3)が小さい(＝やせている)こと，頭囲が小さいこと，頭囲/身長比が小さいことが ADHD と有意に関連し，出生体重そのものではなく IUGR の程度と頭囲の発育が ADHD と関連することが示された[16]。前述のオーストラリアのコホート研究では，SGA 児と AGA 児の思春期における高次脳機能を比較し，SGA 児は学習障害，注意欠陥障害の頻度が高率であった[12]。

SGA は学習障害のリスク因子としてもあげられ，その病態として短期記憶の障害とそれに関連する言語プロセスの障害が推察されている。この短期記憶の障害は未熟性，新生児期の合併症，発育のキャッチアップの有無との関連はないことから，子宮内での発育不全が脳神経系の変化，特に海馬の低形成と関連していることが示唆されている[17]。

2. 早産 SGA 児

早産児あるいは VLBW 児が ADHD のハイリスク群であることはこれまでに多く報告され，幼少期の ADHD の行動徴候が高率であり，年齢とともにその率は低下するといわれている。成人となっ

たVLBW児を対象としたヘルシンキスタディで，SGA-VLBW，AGA-VLBW，AGA-termの3群でADHDの行動徴候に関する調査を行ったところ，SGA-VLBW群はほかの2群に比べ，ADHDと関連のある感情の不安定さ，高次脳機能障害のスコアが有意に高く，AGA-VLBW群はコントロールと有意差を認めなかった(**図2**)[18]。SGA児ではADHDの徴候が成人まで継続する率が高く，長期の経過観察が必要であるといえる。

以上の研究報告から，SGA児は正期産，早産にかかわらず，軽度の知的障害，学業成績低下，ADHDのリスク要因と考えられる。早産児では未熟性に起因する合併症や障害の影響がSGAであることの影響に上回るため有意差として認められないことがある。また，SGAとなる胎児環境は，児の知能や学業に影響する養育環境と共通する部分が多く，リスクの加算が起こっている可能性が高い。胎内の発育制限が発達に影響するメカニズムとしてプログラミングによるエピジェネティックな変化が示唆されるが未だ十分な解明に至っていない。

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282 低出生体重児の精神運動発達
Psychomotor development in low birth weight infants

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Key words：極低出生体重児，発達検査，知能検査，発達障害

概　念

早産低出生体重児の救命率の向上とともに，児の予後が調査され明らかになってきている。長期予後には，脳性麻痺や視覚・聴覚障害といった神経・感覚器の障害以外に，言語を含めた知的発達，行動面の発達，学習能力などの評価も含まれ，低出生体重児はこれらの発達の遅れや障害のリスクが高いといわれる。「精神運動発達」には，運動発達と精神発達(知能)の両面があるが，その障害(遅滞)は乳幼児期の運動発達の遅れや言葉の遅れなどから気づかれ，徐々に全般的な精神機能の遅れが明らかになってくることが多いことから，特に乳幼児期には区別しないで，「精神運動発達」として評価されることが多い。また発達障害として，自閉性障害，アスペルガー障害，そのほかの広汎性発達障害や注意欠陥多動性障害(attention deficit hyperactivity disorder：ADHD)についての評価も重要である。

疫　学

主な精神運動発達の障害の頻度を，日本全国の施設を対象とした極低出生体重児あるいは超低出生体重児の予後調査の結果から表に示した[1,2]。障害の定義がほかの報告と同一でないこと，低出生体重児の中でも限られた対象であり，フォローアップから脱落している児がいることなどのバイアスを考慮しなければならないが，低出生体重児，特に極・超低出生体重児において障害の頻度が高いことは明らかである。

運動発達の評価と対応

1. 運動発達の指標と評価

早産児の運動機能の評価では，脳性麻痺の早期発見が重要な課題となる。頸定，四つん這い，独歩などの発達の指標も重要であり，これらの指標は出産予定日から修正した修正月年齢で評価する。修正で評価した場合でも，より小さい出生体重や在胎期間では，より獲得時期が遅くなる傾向にある[3]。また，乳児期から幼児期早期では精神遅滞などのほかの発達障害との鑑別も必要であり，原始反射や姿勢反応など神経生理学的評価が不可欠である。発達における正常範囲の variation があること，運動機能は精神発達と相まって進むことを考慮し，時間をかけて経過を観察しながら評価を行う。

表　精神運動発達の障害の頻度

	超低出生体重児 6 歳[1]	極低出生体重児 3 歳[2]
脳性麻痺	15.5%	8.5%
視力障害(両眼，片眼失明)	2.0%	1.4%
聴力障害	0.5%	0.7%
知能・発達評価：遅滞	20.3%	15.4%
ADHD	1.4%	No data

[1]6 歳で予後評価を行った 1995 年出生の超低出生体重児 394 名に対する割合
[2]3 歳で予後評価を行った 2003，2004 年出生の極低出生体重児 1,826 名での各項目の評価数に対する割合

2. 運動発達の支援

運動発達の評価の結果，下肢の痙性などの異常が認められる場合は，両親への十分な説明の上でリハビリテーションを開始する。神経学的異常は明らかでないが発達の遅れがある場合，運動発達の支援のためのポジショニングやハンドリングを外来で指導し，次回の受診を確認する。

精神発達の評価と対応

1. 低出生体重児の精神発達の特徴

早産・低出生体重児では，胎内での栄養を含めた環境，早く，小さく産まれたことに伴うさまざまな疾病の合併や栄養状態などの影響を受け，一般児に比べ精神発達の遅れのリスクが高い。評価の際には，身体発育の評価と同様に乳幼児早期は修正年月齢を用いて評価をすることが望ましい。いつまで修正年齢を用いるかは定まっていないが，ハイリスク児フォローアップ研究会では 3 歳以降は暦年齢で評価を行っているが，より早い在胎期間は出生体重の児ほど追いつく時期は遅くなる傾向にある。乳幼児期の発達は変化が大きく，1〜2 歳は発達の遅れがみられた子どもが，その後就学までに同年代の子どもに追いついてくることも少なくない。また，極低出生体重児の発達予後に関連する要因を分析した報告

では，両親の社会経済的な状態に影響を受けることが知られている。この観点から養育環境を調査し，整えることは極めて重要と考えられる。

2．発達・知能検査

新生児フォローアップ外来で行われる主な個別発達・知能検査には，新版K式発達検査，WISC知能検査，WPPSI知能検査，田中・ビネー式知能検査，K-ABC心理教育アセスメントバッテリーなどがあり，適応年齢に応じて検査を選択する。個別検査では，発達指数(DQ)＝(発達年齢/生活年齢)×100，知能指数(IQ)＝(精神年齢/生活年齢)×100が求められる。IQが同年齢の標準集団の平均から標準偏差を単位としてどの程度隔たっているかで，正常，境界，遅滞に判定される。WISC知能検査は，2010年末に日本語版WISC-IVが発行された。WISC-IIIでは全検査IQのほかに言語性IQ，動作性IQを算出したが，WISC-IVでは，全検査IQは言語理解指標(VCI)，知覚推理指標(PRI)，ワーキングメモリー指標(WMI)，処理速度指標(PSI)の四つの指標から成り立っている。

3．評価の注意点と家族への支援

発達・知能検査は，全体的な発達やその子のもっている能力を測ることが可能であるが，結果は検査の実施場面での結果であること，乳幼児の発達は変化することがあり経過を追って評価することが重要であること，いくつかの検査法を組み合わせて判断することなどに注意が必要である。検査結果のみを安易に伝えて家族の不安を増長させないように配慮する必要がある。子どもの発達の特徴，苦手な部分，かかわりのコツなどを，実際に検査を行った心理士とともに家族に伝えサポートする。また，適切な療育は子どもの発達を促すために必要なことを伝え，理解を得た上で専門施設を紹介することが望ましい。

ADHD，広汎性発達障害，学習障害

年長となった早産・低出生体重児の調査では，学習障害やADHDの出現率が高いことが報告されている。ADHDについては，用いる定義の差などにより日本の低出生体重児での頻度は未だ明確なものは見当たらない。海外のメタアナリシスでは学童期を迎えた早産児のADHDの相対リスクは2.64(95% CI 1.85～3.87)と，正常出生体重の児に比べ有意に高い[4]。自閉症スペクトラムの日本の低出生体重児での正確な頻度も不明である。自閉性障害の頻度は対照と差がないようであるが，そのほかの広汎性発達障害について，低出生体重児での発生率が高いとする報告は少なからずあり，診断基準を統一した全国調査が必要である。学習障害(LD)については，原[5]は極低出生体重児の小学校中学年での典型的LD 14%，LD疑い30%と報告しており，欧米の研究での頻度20～30%(対照の3～5倍)と一致している。この頻度は出生体重，在胎期間が小さいほど多い傾向も報告されている。いずれにしても，これらの発達障害は，学校や社会での適応に大きく関係するものであり，新生児にかかわる医療者も知っておく必要がある。

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Genetic variations in MyD88 adaptor-like are associated with atopic dermatitis

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Received December 9, 2010; Accepted January 20, 2011

DOI: 10.3892/ijmm.2011.645

Abstract. Toll-like receptors (TLRs) are important pathogen-associated molecular pattern recognition receptors involved in initiating immune responses. The adaptor protein MyD88 adaptor-like (Mal), involved in signaling downstream of TLRs, plays a crucial role in mediating NF-κB activation. The association of Mal polymorphisms with allergic diseases has not previously been defined. The objective of this study was to detect polymorphisms in the Mal gene and to investigate their association with allergic diseases. Mal gene polymorphisms were genotyped in 310 subjects. The functional effects of Mal variants were analyzed *in vitro*. One Mal polymorphism, c.303 G>A (Q101Q), was found at a significantly lower frequency in atopic dermatitis patients (p=0.016). Q101Q is in linkage disequilibrium with -103 A>G (rs1893352) and c.539 C>T (S180L) (rs8177374) in the HapMap database. The A allele of -103 A>G showed significantly reduced transcription of Mal compared with the G allele. In addition, three rare variants were identified in this study, c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D), and were shown to lead to loss-of-function of Mal. It is possible that gene polymorphisms in Mal could affect atopic dermatitis by influencing the innate immune system. We show that Q101Q, which is in linkage disequilibrium with -103 A>G and S180L, may play a protective role against atopic dermatitis. Furthermore, we propose that loss-of-function variants of Mal could predispose individuals to atopic dermatitis or other immunological disorders.

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Abbreviations: Mal, MyD88 adaptor-like; TIR, Toll/interleukin-1 receptor; TIRAP, Toll/interleukin-1 receptor domain-containing adaptor protein; TLR, Toll-like receptor; PAMPs, pathogen associated molecular patterns; MyD88, myeloid differentiation primary response protein 88; TRIF, TIR domain-containing adaptor protein including IFNβ; TRAM, TRIF-related adaptor molecule; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; NF-κB, nuclear factor-κB; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; SNPs, single nucleotide polymorphisms; NCBI, National Center for Biotechnology Information; LD, linkage disequilibrium; HEK, human embryonic kidney; SD, standard deviation; AD, atopic dermatitis

Key words: atopic dermatitis, innate immunity, Mal/TIRAP, polymorphism, Toll-like receptor

Introduction

The toll-like receptor (TLR) protein family plays a central role in the activation of the innate immune system. In humans, CD14 and TLRs are involved in extracellular recognition of pathogen associated molecular patterns (PAMPs), which activate the initial host defense system. Downstream of the TLRs, five Toll/interleukin-1 receptor (TIR) domain containing adaptor proteins have been identified: myeloid differentiation primary response protein 88 (MyD88), MyD88 adaptor-like (Mal) [also known as Toll/interleukin-1 domain-containing adaptor protein (TIRAP)], TIR domain-containing adaptor protein including IFNβ (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and heat-armadillo motifs. These adaptor proteins function in mediating the intracellular signaling pathways that lead to inflammatory gene expression (1). One of these adaptor proteins, Mal/TIRAP (hereafter referred to as Mal), is involved in the MyD88-dependent signaling pathway downstream of TLR2 and TLR4 (2,3). The Mal/MyD88-dependent signaling pathway induces the sequential activation of interleukin-1 receptor-associated kinase (IRAK)4, IRAK1 and tumor necrosis factor receptor-associated factor-6 (TRAF6) (1). Mal plays specific roles in interactions with various TIR domain-containing adaptors; Mal mediates the interaction between TLR4 and MyD88 (4,5), and directly interacts with the downstream component TRAF6 (6).

Allergic diseases are caused by inappropriate immunological responses to harmless antigens, driven by T helper type 2 (Th2)-mediated immune responses. Insufficient stimulation of TLRs by PAMPs results in decreased production of T helper type 1 (Th1) cytokines such as, IL-12 and IFNs, which can attenuate the activity of down-regulators of Th2 responses, in turn leading to allergic disease (7). It has been

Table I. Clinical information on patients with allergic diseases and control subjects.

Characteristics	Patients with allergic diseases (n=207)		Control subjects (n=103)
	AD (n=99)	Other allergic diseases (n=108)	
Gender			
Male	62	72	56
Female	37	36	47
Serum total IgE (IU/ml)			
Mean	2491.63	950.17	38.11
Median	461	390	21
SD	4835.59	1882.37	39.36
Min	2.36	1.91	4.34
Max	24838	16000	152

AD, atopic dermatitis; SD, standard deviation.

reported that endotoxin can induce MyD88-deficient dendritic cells to support Th2 cell differentiation (8). Therefore, the Mal/MyD88-dependent signaling pathway may be implicated in allergic diseases caused by overactive Th2 responses.

Genetic variations leading to alterations in proteins involved in PAMP recognition or downstream signaling may alter the balance between Th1 and Th2 immune responses and may therefore influence the individual's susceptibility to developing allergic diseases. Previous studies have found single nucleotide polymorphisms (SNPs) in TLRs and TLR-related proteins to be associated with allergic diseases. For example, the polymorphism R753Q in the TLR2 gene increases the frequency of atopic dermatitis (9,10); the A-16934T promoter polymorphism of the TLR2 gene is associated with severe atopic dermatitis (11); polymorphism D299G of the TLR4 gene is associated with a high prevalence of asthma and reduced IL-12 responses (12); and polymorphisms in IRAK4 and promoter polymorphisms of CD14 are associated with total serum IgE levels (13,14).

In contrast to these associations with allergic disease, the Mal polymorphism S180L (rs8177374) has been associated with protection against some diseases (15). However, there is conflicting evidence for the role of S180L in some ethnic populations (16,17). Recently, the rare mutation D96N (rs8177400) was described as a loss-of-function variant of Mal that leads to reduced TLR2/TLR4 signaling activity (18,19). These recent results suggest that the effects of SNPs in Mal are important in some immunological diseases.

In this study, we detected SNPs in the Mal gene in Japanese population samples with or without allergic diseases and explored whether we could detect associations of SNPs with allergic diseases. Further functional analyses were performed to identify the functional effects of the variants of Mal.

Subjects and methods

Subjects. The association between Mal gene polymorphisms and the presence of atopic dermatitis (AD) (n=99), other allergic diseases (n=108) and non-allergic control subjects (n=103) was analyzed in Japanese subjects. Non-allergic unrelated control subjects had no history of atopic dermatitis or other allergic diseases including bronchial asthma, food allergies, allergic rhinitis or allergic conjunctivitis. Detailed clinical information on the subjects is presented in Table I. The diagnosis of atopic dermatitis was made according to the criteria of Hanifin (20). All subjects provided informed consent to participate in the study.

Polymorphism detection and linkage disequilibrium (LD) analysis. Genomic DNA was extracted from neutrophils using a SepaGene kit (Sanko Junyaku, Tokyo, Japan). Three primer sets (Table II) were designed based on the coding region of the Mal gene sequence available from the National Center for Biotechnology Information (NCBI Reference Sequence: NC_000011.9) to amplify the two coding exons. Genotyping was performed using the ABI 3100 DNA auto-sequencer program (Applied Biosystems). Japanese HapMap data were obtained from http://www.hapmap.org/ (21). Pairwise LD was calculated as the r^2-values by using the Haploview 4.1 program.

Vector preparation and in vitro mutagenesis. The wild-type coding region of the Mal gene (235-aa isoform; Accession number: NM_148910) was cloned into pFLAG-CMV6a (Sigma-Aldrich). This plasmid was used for generating substituted types of Mal using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI). Primers containing the following substituted sites in Mal were designed for generating the six different variations of Mal (S55N, Q101Q, E132K, R143Q, S180L and E190D). Fragments (7500-7808 bp of NC_000011.9) contained in the promoter region of Mal and containing the Mal intron 3 replicated from the genome of individuals with or without the -103G variation, were cloned into pGL4.11 (luc2P) (Promega). These were used to analyze the transcriptional activities of Mal/-103A and Mal/-103G.

Cell culture. Human embryonic kidney (HEK) 293T cells and 293-hTLR4/MD2-CD14 cells (InvivoGen, CA, USA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen,

Table II. Primer details for amplifying exon 4 and 5 in the Mal gene by PCR.

Exon	Primer	Primer sequence	Length of amplified DNA	Annealing temp (°C)
Exon 4	S4 (F)	5'GTCTGGCCCTAATCTCATGA3'		
Exon 4	A4 (R)	5'CACTCTCCACAAAGCATCCAG3'	479 bp	60
Exon 5	S5 (F)	5'GAGAATAAGATGTTTCCCAGTGC3'		
Exon 5	A5 (R)	5'GCAGCATCTGGTACTTGCACCA3'	557 bp	62
Exon 5	S6 (F)	5'GGTCTCCTACTTGGAAGGCA3'		
Exon 5	A6 (R)	5'CAATGGAAACCTGTTGGTCAG3'	569 bp	60

F, forward sense; R, reverse antisense; temp, temperature.

Carlsbad, CA) supplemented with 10% FCS, 100 U/ml penicillin and 100 pg/ml streptomycin. THP-1 cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS, 0.01 M HEPES, 100 U/ml penicillin and 100 pg/ml streptomycin. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Luciferase assay. HEK 293T cells ($2x10^4$ cells/well in 96-well plates) were transfected with 100 ng pFLAG-CMV6a empty vector or pFLAG-CMV6a-Mal wild-type or variants (S55N, Q101Q, E132K, R143Q, S180L and E190D) using Lipofectamine 2000 (Invitrogen). NF-κB luciferase reporter vector (50 ng) [pGL4.32 (luc2P/NF-κB-RE/Hygro)] and 50 ng *renilla* luciferase reporter control vector (pGL4-hRluc-TK) were co-transfected into the appropriate wells. At 48 h after transfection, NF-κB luciferase reporter gene activity was analyzed using the Dual-Luciferase Reporter assay system (Promega). For dose-dependent assays, 293-hTLR4/MD2-CD14 cells were transfected with 10, 40 and 80 ng plasmids DNA of Mal variants (E132K, R143Q and E190D), respectively. The DNA content was adjusted to a total volume of 80 ng with mock plasmid DNA. Cells were stimulated with lipopolysaccharide (LPS) (100 ng/ml) for 6 h and NF-κB luciferase reporter gene activity was then analyzed. THP-1 cells are derived from the human monocytic cell line, which expresses TLRs and TLR adaptor proteins. Therefore, we used THP-1 cells to perform transcription activity assays and cytokine production experiments. THP-1 cells ($1x10^6$ cells/ml) were transfected using Lipofectamine LTX (Invitrogen) with 800 ng pGL4.11 (luc2P) control vector, pGL4.11 (luc2P)-Mal promoter (-103A) or pGL4.11 (luc2P)-Mal promoter (-103G). pGL4-hRluc-TK (50 ng) was co-transfected into all wells as an internal control. Transcriptional enhancer activities were measured at 24 h after transfection. Each of the luciferase assays was performed at least three times.

Measurement of TNF-α and IL-12. THP-1 cells ($1x10^6$ cells/ml) were transfected with Mal variant plasmids as described above or with the pSV-β-galactosidase control vector as an internal control using Nucleofector (Amaxa, Program V-001). After 24 h of transfection, cells were cultured with or without 100 ng/ml LPS for a further 24 h. Supernatants were collected at 48 h after transfection and the concentrations of TNF-α and IL-12 in the supernatants were determined using human TNF-α or human IL-12 ELISA kits (BioSource International, Carlsbad, CA).

Western blotting. HEK 293T cells ($8x10^5$ cells/well for 6-well plates) were transfected as described above. After 48 h, cells were lysed in 200 μl lysis buffer (10 mM Tris HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 10% glycerol and 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The concentration of each expressed protein was measured by the Lowry method and the quantity of each expressed protein was adjusted to load equal protein quantities for Western blots analysis. Samples were analyzed by Western blotting using an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO).

Statistical analysis. Genotype frequencies were analyzed using a chi-square (χ^2) test. A p-value of <0.05 was considered statistically significant. The differences in luciferase activities and cytokine values between wild-type Mal and the Mal variants were analyzed using the Dunnett's test. The difference between the transcriptional activity of -103A and -103G was analyzed by the Bonferroni test. Each assay was performed with at least three samples.

Protein structure modeling. Structure modeling of the TIR domain of Mal was performed using the MyD88-TIR structure (PDB code: 2z5v) as a template on MOE software according to our previously described method (5). The figure of this structure was made using PyMOL (22).

Results

Characterization of Mal polymorphisms in Japanese subjects. Eight polymorphisms were discovered in the 235-aa coding region of Mal (Fig. 1A). Four of these polymorphisms were already described in the NCBI, while the other four polymorphisms were novel [c.297 C>T (A99A), c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D)]. The three non-synonymous polymorphisms in the Mal gene [c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D)] are relatively rare missense variants. Two of three were only discovered in AD subjects (Table III). These three rare missense variants were found as heterozygotes. On the other hand, one of the synonymous SNPs, c.303 G>A (Q101Q)

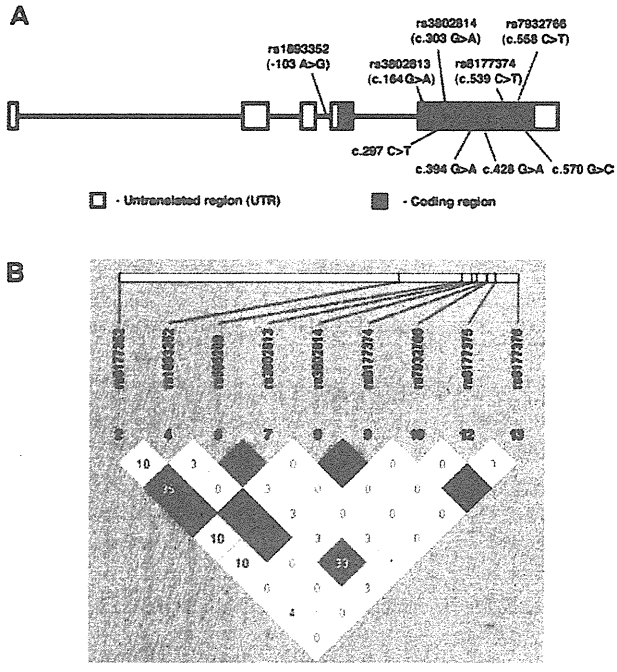

Figure 1. (A) Exon-intron structure of the Mal gene and locations of genotyped SNPs. c.297 C>T (A99A), c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D) are novel variants. The fragment of the genome containing the novel variants was sequenced for at least three times under different conditions to confirm that these novel variants are not PCR errors. (B) Linkage disequilibrium (LD) plot surrounding the Mal SNPs. The LD plot was generated by Haploview version 4.1 using the HapMap JPT database. The colors of the squares illustrate the magnitude of the pairwise r^2 values on a black and white scale, where black indicates a perfect LD ($r^2 = 1.00$).

(rs3802814), was found at a significantly different frequency in the three categorized groups (p=0.038, Table III). When we statistically tested the difference between AD and control groups, we found this SNP at a significantly lower frequency in AD subjects than in control subjects (p=0.016, data not shown). We did not find a significant difference between the other allergic disease groups and the control group.

Functional analyses of synonymous and non-synonymous SNPs of Mal. Overexpression of wild-type Mal led to a strong induction of NF-κB reporter gene activity, and three of the studied polymorphisms (S55N, Q101Q and S180L) were comparable to wild-type Mal in their ability to activate NF-κB. Interestingly, the variants E132K, R143Q and E190D were severely incompetent in NF-κB activation (Fig. 2A). The expression of E132K, R143Q and E190D variants in HEK 293T cells was confirmed by Western blotting (Fig. 2B).

The variants S55N, Q101Q and S180L produced comparable levels of TNF-α and IL-12 to the levels produced by wild-type Mal. However, E132K, R143Q and E190D did not produce TNF-α or IL-12 in the presence or absence of LPS (Fig. 2C). These analyses suggest that these three non-synonymous SNPs of Mal are loss-of-function variants.

Dose-dependent assays were performed to classify the mechanism of behavior of the variants E132K, R143Q and E190D. As shown in Fig. 4A, E132K showed a dominant negative inhibitory effect in LPS/TLR4 dependent activation of NF-κB, while R143Q and E190D did not show a significant dominant negative effect.

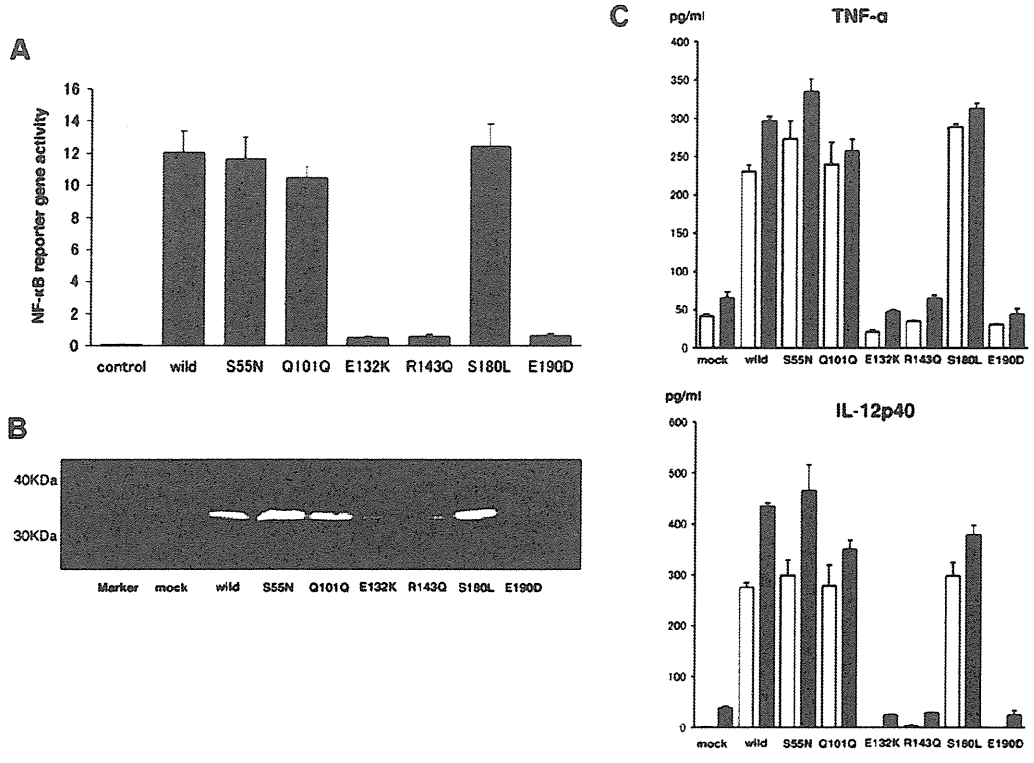

Figure 2. (A) Mal signaling induces NF-κB-driven reporter gene activity. HEK 293T cells were equally transfected with 100 ng vector DNA (pFLAG Mal wild-type or variants of Mal). (B) Protein expression levels of Mal variants. (C) TNF-α and IL-12 production assay. White bars show the cytokine production levels from THP-1 cells without LPS stimulation, black bars show cytokine production levels from LPS stimulated THP-1 cells. These results are shown as the mean of triplicate determinations ± SD.

Table III. Mal single nucleotide polymorphism (SNP) frequencies in the AD (n=99), other allergic diseases (n=108) and control (n=103) groups.

SNP (amino acid change)	dbSNP ID	Genotype	AD (%)	Other allergic diseases (%)	Controls (%)	P-value
c.164 G>A	rs3802813	GG	66 (66.7)	77 (71.3)	64 (62.1)	
(S55N)		GA	32 (32.3)	29 (26.85)	39 (37.9)	0.34
		AA	1 (1.0)	2 (1.85)	0	
c.297 C>T		CC	99 (100)	107 (99.1)	103 (100)	
(A99A)		CT	0	1 (0.9)	0	0.39
		TT	0	0	0	
c.303 G>A	rs3802814	GG	91 (91.9)	94 (87.0)	82 (79.6)	
(Q101Q)		GA	8 (8.1)	14 (13.0)	21 (20.4)	0.038[a]
		AA	0	0	0	
c.394 G>A		GG	97 (98.0)	108 (100)	103 (100)	
(E132K)		GA	2 (2.0)	0	0	0.11
		AA	0	0	0	
c.428 G>A		GG	98 (99.0)	108 (100)	100 (97.1)	
(R143Q)		GA	1 (1.0)	0	3 (2.9)	0.17
		AA	0	0	0	
c.539 C>T	rs8177374	CC	94 (94.9)	104 (96.3)	97 (94.2)	
(S180L)		CT	5 (5.1)	4 (3.7)	6 (5.8)	0.77
		TT	0	0	0	
c.558 C>T	rs7932766	CC	89 (89.9)	102 (94.4)	96 (93.2)	
(A186A)		CT	10 (10.1)	5 (4.6)	7 (6.8)	0.38
		TT	0	1 (1.0)	0	
c.570 G>C		GG	98 (99.0)	108 (100)	103 (100)	
(E190D)		GC	1 (1.0)	0	0	0.34
		CC	0	0	0	

[a]Statistically significant; AD, atopic dermatitis.

-103 A>G and Q101Q are in LD. As displayed in Table III, the polymorphism Q101Q was found at a significantly lower frequency in the AD group. However, this polymorphism did not show a significantly higher ability than wild-type Mal in stimulating NF-κB luciferase activity and producing inflammatory cytokines (Fig. 2). We searched the LD surrounding the Mal gene using the HapMap JPT database to detect any SNPs acting in LD with Q101Q. We found that the SNP -103 A>G (rs1893352) shows complete LD (r^2=1.00) with Q101Q (rs3802814) (Fig. 1B). A luciferase reporter gene assay was performed to assess the functional effect of this SNP on the transcriptional ability of the Mal promoter. As shown in Fig. 3, the -103A allele significantly reduced the transcriptional activity of Mal (p=0.009), showing a 2.6-fold lower activity than the -103G allele. Additionally, we found another SNP, S180L (rs8177374), to be in LD with Q101Q.

Discussion

Many SNPs in the Mal gene have been described, including A9P, R13W, S55N, D96N, Q101Q, D102H, S180L, A186A and V197I (23). Other polymorphisms have been found specifically in some ethnic groups, such as A100T, S131S and R143W in the coding region of Mal (24). With the SNPs discovered in our study (E132K, R143Q and E190D) added to this list, Mal can be considered a relatively polymorphic gene among TIR domain-containing adaptors (25).

In this study, we found Q101Q to be present at a significantly lower frequency in the AD group compared with control subjects. Though we could not explain the exact protective function of Q101Q, -103 A>G was found to be in LD with Q101Q and S180L, and the latter has been previously described as protective factor against bacterial infection (15). Thus, we speculate that Q101Q plays a protective role against the onset of atopic dermatitis via increasing transcriptional activity of Mal.

We searched for transcription factor binding sites in the promoter region of Mal by using Genomatix (http://www.genomatix.de/) to detect whether the polymorphism -103 A>G acts to influence transcription factor. We found that one transcription factor associated with the Mal promoter, named CTCF, precisely binds to the nucleotide sequence 5'tctctct ACCCtctgtaggatggctgc3', in which the capital letters denote the core sequence used by MatInspector (Genomatix: Matrix Library information). The capital A in the core sequence is the A allele of -103 A>G. It has been reported that CTCF

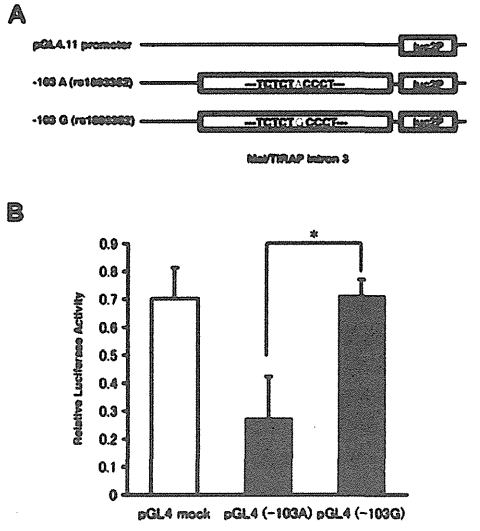

Figure 3. (A) pGL4 plasmid constructs used for transfection. (B) Transcriptional activities of the promoter of Mal with -103 A>G (rs1893352). THP-1 cells were transfected with the pGL4 vector as a control and the pGL4-Mal/-103A and pGL4-Mal/-103G variants. Transcriptional activities were measured by the luciferase reporter assay. Values of relative luciferase activity are shown as the means ± SD. *P=0.009, Bonferroni test.

is a vertebrate insulator protein that blocks the interaction between enhancers and upstream promoters, thereby regulating imprinted expression (26). Therefore, we hypothesize that the variant G allele alters the CTCF binding site sequence and thus inhibits the binding of the insulator protein CTCF to the Mal promoter, thereby enhancing Mal transcriptional activity. This is consistent with the result of our transcriptional activity assay showing that the A allele of -103 has reduced activity compared with the G allele of -103 (Fig. 3).

S180L was the first identified functional SNP in Mal, as described by Khor *et al* (15). L180 led to a failure of Mal to interact with TLR2 but not MyD88. In contrast, Ferwerda *et al* showed increased production of proinflammatory cytokines (TNF, IL-6 and IFN-γ) in response to endotoxin in the S180L heterozygous individuals but not in S180 homozygous individuals (27). Although the overexpression assays of the S180L variants in this study and previously reported studies did not show differences in the activity between the wild-type Mal and S180L (18,19), the linkage of S180L with Q101Q may be another modification factor for Q101Q.

The novel variants identified in this study (E132K, R143Q and E190D) all showed a significantly lower ability to activate NF-κB and induce TNF-α and IL-12 (Fig. 2). IL-12 is an essential signal for the differentiation of naive Th cells into Th1 cells, which produce IFN-γ and thereby inhibit Th2 differentiation and IgE production (28,29). A failure to produce IL-12 can lead to allergic diseases. Because MyD88-dependent pathways are essential for the development of protective IL-12-mediated Th1 responses (30), a loss-of-function or a dominant-negative inhibitory effect of Mal may also lead to polarized Th2 responses, which could increase susceptibility to AD.

E132K is predicted to be located in the BB loop in the TIR domain of Mal (Fig. 4B). One of the important mutations of Mal, P125H, which is also located in the BB loop, has been found to fail to co-immunoprecipitate with TLR4 and to strongly inhibit activation of NF-κB (2,3). Similarly, E132K is considered a critical residue located in the interaction region of the TIR domain. Interestingly, the E132K variant also showed a dominant-negative inhibitory effect in TLR4 signaling (2) (Fig. 4A). Moreover, this variant was found in two AD patients in this study, but was not found in the control subjects (Table III). These results suggest that the E132K variant may increase the risk of developing AD.

Our study found that the Mal variants R143Q and E190D also lead to loss-of-function (Figs. 2 and 4A). R143Q was found in both AD and control subjects, and we did not find a clear role for R143, but we hypothesize that R143Q affects the stability of the Mal protein, since R143 lies in a core position of the Mal TIR domain (Fig. 4B). The loss of the positively charged arginine in this polymorphism may affect the stability of Mal. On the other hand, in a novel feature that distinguishes Mal from MyD88, Mal has been found to possess a TRAF6 interaction motif, P-P-E-L-R-F, at amino acid position 188-193. This motif is essential for the signaling function of Mal. Mansell *et al* experimentally mutated the glutamic acid at position 190 to alanine and found that the mutant failed to induce TLR2- and TLR4-mediated activation of the NF-κB, JNK and MAP kinase pathways (6). These data suggest that E190D can inhibit NF-κB activation and thereby may be associated with susceptibility to developing immunological diseases.

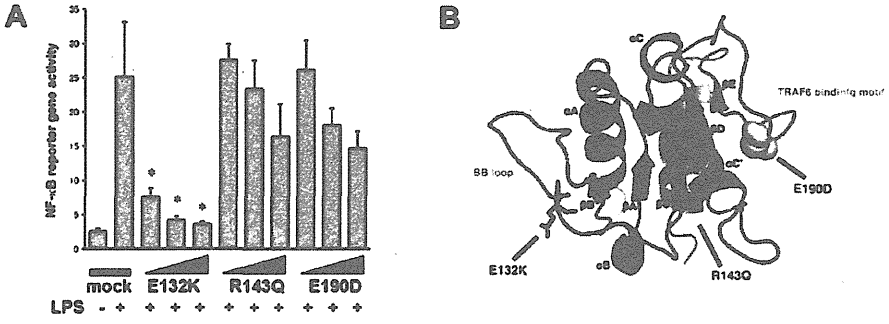

Figure 4. (A) The dose-dependent NF-κB reporter gene assay of Mal variants. Mal variants were cotransfected (10 ng, 40 ng, or 80 ng) in 293-hTLR4/MD2-CD14 cells. E132K showed significant inhibition of LPS/TLR4-mediated NF-κB activation in a dose-dependent manner. *P<0.01. (B) Model structure of the Mal TIR domain. The illustrated structure was created from the protein structure of MyD88-TIR as a template. This structure comprised a central five-stranded parallel β-sheet (βA-βE) surrounded by four α-helices (αA-αC and αE). The orientations of the side chain of E132 and R143 are shown with red or magenta color, respectively. The TRAF6 binding motif on Mal is highlighted with green and E190 is shown with yellow spheres.

The TLR signaling pathways are crucial for the innate immune system to act in efficient host defense. Deletion mutations, compound heterozygous or homozygous missense mutations in MyD88 or IRAK4 have all been associated with immunodeficiency (31,32). Recently, two MyD88 non-synonymous SNPs were reported as loss-of-function variants (33). In addition, Mal knock-out mice have been shown to be susceptible to *Klebsiella pneumonia* (34). Therefore, it is likely that homozygosity or compound heterozygosity of the loss-of-function variants described in this study, which are located in TIR domain of Mal, could lead to immunodeficiency disorder due to an impaired innate immune response.

Efficient expression and appropriate function of Mal is necessary for the induction of sufficient proinflammatory cytokines and in initiating a timely Th1 immune response, thereby helping to balance Th2 and Th1 immune responses and defend against allergic diseases. The polymorphism -103 A>G, linked with Q101Q and/or S180L, may play a key protective role against atopic dermatitis. In contrast, dominant negative or loss-of-function variants in TIR domain of Mal could impair Mal-mediated signaling and lead to deficient production of proinflammatory cytokines, which may lead to AD or the other immunological disorders. The new identified polymorphisms and the functional characteristics described in our study may facilitate genetic diagnosis and lead to new therapies for atopic dermatitis.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports Science, Culture and Technology of Japan, by Health and Labour Science Research Grants for Research on Intractable Disease from the Ministry of Health, Labour and Welfare, Japan, and by Environmental Restoration and Conservation Agency of Japan.

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