also thank the pediatricians (Dr. Makimto, Dr. Kitamura, Dr. Inami, Dr. Chinen and residents) and nurses of the NICU. Finally, we thank the parents who consented to inclusion of their babies in this study.

Competing interests.

We certify that we had no sponsors in this study and no potential conflicts of interest.

Funding: None

Figure legends

Fig.1

Changes in systolic and diastolic blood pressure during the first 120 hours after birth.

Fig.2

Changes in the daily amount of urine volume during the first 120 hours after birth.

What is already known about this topics and What this study adds sections.

What is already known about this topic.

- An important intervention that reduces the need for subsequent blood transfusion is the maximization of circulatory blood volume at birth.
- In premature infants, administration of circulatory blood volume by placental transfusion at birth is sufficient to prevent hypotension.

What dose this study adds.

- Umbilical cord milking may facilitate early stabilization of both the blood pressure and urine output in very low birth weight infants.

References

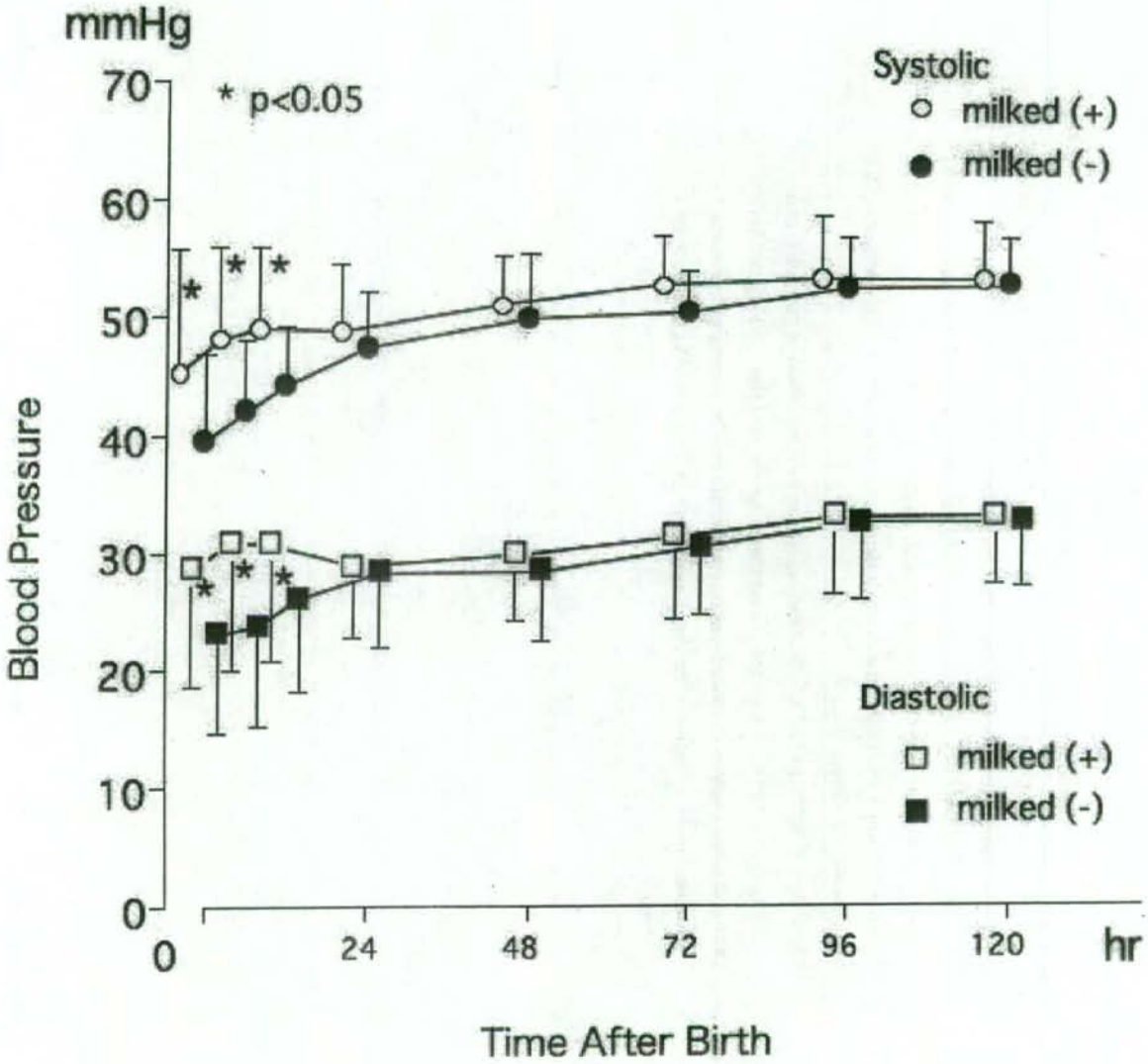
1. Osborn DA, Evans N, Kluckow M, Bowen JR, Rieger I. Low superior vena cava flow

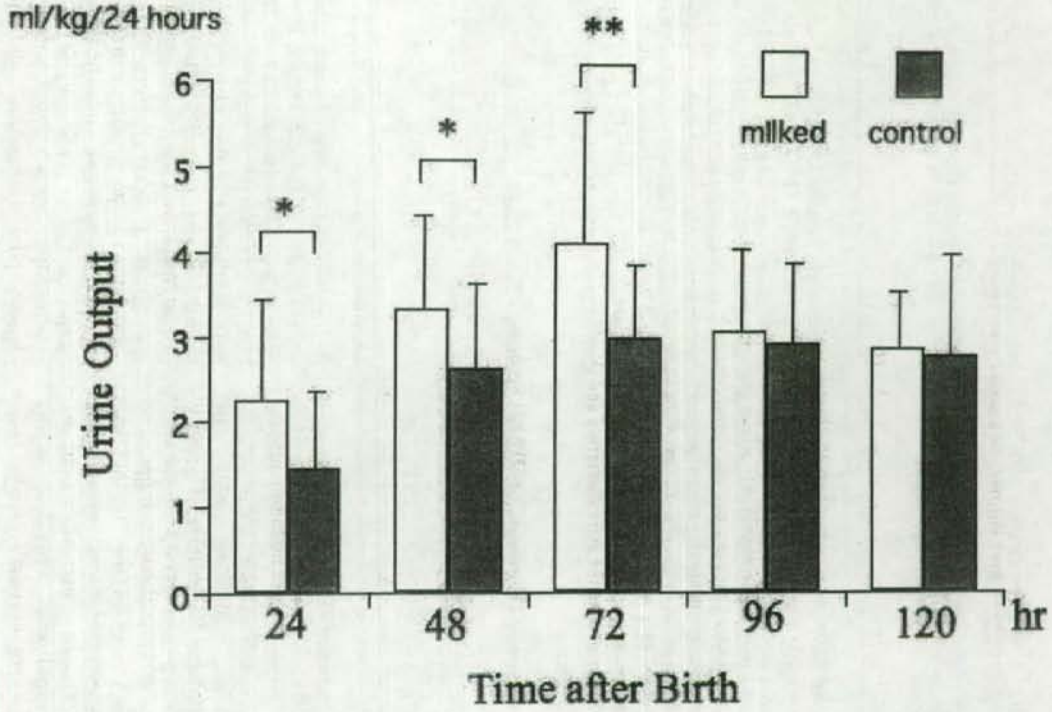
and effect of inotropes on neurodevelopment to 3 years in preterm infants. Pediatrics. 2007; 120: 372-80

2. Fanaroff AA, Fanaroff JM: Short- and long-term consequences of hypotension in ELBW infants. Semin Perinatol. 2006 ;30:151-5.
3. Noori S, Seri I. Pathophysiology of newborn hypotension outside the transitional period. Early Hum Dev. 2005;81:399-404
4. Osborn DA, Paradisis M, Evans N. Early volume expansion for prevention of morbidity and mortality in very preterm infants. Cochrane Database Syst Rev. 2004; CD002055.
5. Rabe H, Reynolds G, Diaz-Rossello J. Early versus delayed umbilical cord clamping in preterm infants. Cochrane Database Syst Rev. 2004: CD003248.
6. Hosono S, Mugishima H, Kitamura T, Inami I, Fujita Y, Hosono A, Minato M, Okada T, Takahashi S, Harada K. Effect of hemoglobin on transfusion and neonatal adaptation in extremely low birth weight infants. Pediatr Int. 2008;50:306-11
7. Hosono S, Mugishima H, Fujita H, Hosono A, Minato M, Okada T, Harada K. Umbilical cord milking reduces the need for red cell transfusions and improves neonatal adaptation in infants born less than 29 weeks' gestation: a randomized controlled trial. Arch Dis Child Neonatal Fetal ed. 2008;93:F14-9
8. Jones JG, Wardrop CA. Measurement of blood volume in surgical and intensive care practice. Br J Anaesth. 2000;84:226-35
9. Report of the Second Working Group of the British Association of Perinatal Medicine, Guidelines for good practice in the management of neonatal respiratory distress syndrome. British Association of Perinatal Medicine. Arch Dis Child 1992; 67:1221-1227
10. Seri I. Management of hypotension and low systemic blood flow in the very low birth weight neonate during the first postnatal week. J Periantol. 2006;26:S8-S13
11. Rabe H, Reynolds G, Diaz-Rossello J. A systematic review and meta-analysis of a brief delay in clamping the umbilical cord of preterm infants. Neonatology. 2008;93:138-44.
12. Mercer JS, Vohr BR, McGrath MM, Padbury JF, Wallach M, Oh W. Delayed cord clamping in very preterm infants reduces the incidence of intraventricular hemorrhage and late-onset sepsis: a randomized, controlled trial. Pediatrics. 2006;117:1235-42.
13. Grönlund JU, Korvenranta H, Kero P, Jalonen J, Välimäki IA. Elevated arterial blood

pressure is associated with peri-intraventricular haemorrhage. Eur J Pediatr. 1994;153:836-41.

14. Seri I, Noori S. Diagnosis and treatment of neonatal hypotension outside the transitional period. Early Hum Dev. 2005;81;405-11
15. Oh W, Lind J, Gessner IH. The circulatory and respiratory adaptation to early and late cord clamping in newborn infants. Acta Paediatr Scand. 1966; 55: 17-25.
16. Oh W, Oh MA, Lind J. Renal function and blood volume in newborn infant related to placental transfusion. Acta Paediatr Scand. 1966;56:197-210
17. Linderkamp O. Placental transfusion: determinants and effects. Clin Perinatol.1982; 9: 559-92.
18. Osborn DA, Evans N. Early volume expansion for prevention of morbidity and mortality in very preterm infants. Cochrane Database Syst Rev. 2004;(2):CD002055.
19. American Academy of Pediatrics and American Heart Association. Lesson 6 Medications Text Book of Neonatal Resuscitation 5th Ed John M.D. Kattwinkel ed 2006


mmHg
* p<0.05
Systolic
milked (+)
milked (-)
Diastolic
milked (+)
milked (-)
Blood Pressure
70
60
50
40
30
20
10
0
0
24
48
72
96
120
hr
Time After Birth


ml/kg/24 hours
6
5
4
3
2
1
0
Urine Output
*
*
**
mllked
control
24
48
72
96
120
hr
Time after Birth

CASE REPORTS

Coronary Artery Dilatation in LEOPARD Syndrome. A Child Case and Literature Review

Yoko Iwasaki, MD,* Hitoshi Horigome, MD, PhD,* Miho Takahashi-Igari, MD,* Yoshiaki Kato, MD,* M. Abdur Razzaque, PhD,† and Rumiko Matsuoka, MD, PhD†

*Department of Child Health, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba; †Division of Pediatric Cardiology, International Research and Educational Institute for Integrated Medical Sciences, Tokyo Women's Medical University, Tokyo, Japan

ABSTRACT

LEOPARD syndrome (LS) is a rare inherited disease with multiple somatic abnormalities. LS and Noonan syndrome (NS) share many features, including cardiovascular disorders, and PTPN11 gene mutation is commonly reported in both syndromes. We report a 10-year-old male patient who was diagnosed as LS based on typical phenotypes including multiple lentigines, electrocardiographic abnormalities, ocular hypertelorism and deafness. Although the most prevalent cardiovascular abnormalities in LS are pulmonary stenosis and hypertrophic cardiomyopathy, diffuse bilateral dilatation of the coronary arteries was found on angiography in addition to apical hypertrophic cardiomyopathy in the present case. The vessels showed slight increases in diameter on angiography conducted at an interval of 6 years. A literature review identified several case reports describing coronary ectasia in patients with NS as well as LS. Considering both syndromes share the mutation of PTPN11 gene, coronary arterial involvement could be related to the gene aberration and should be screened even if the patient shows no symptoms of ischemic heart disease.

Key Words. Coronary Artery Dilatation; LEOPARD Syndrome; *PTPN11* Gene

Introduction

LEOPARD syndrome (LS) is an autosomal dominant inherited disorder characterized by multisystemic abnormalities including multiple Lentigines, Electrocardiographic abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormalities of genitalia, Retardation of growth, and Deafness. LS shares many phenotypes with Noonan syndrome (NS), although lentigines and deafness are usually not present in the latter, though protein tyrosine phosphatase, nonreceptor-type 11 (*PTPN11*) gene mutation is commonly observed in both syndromes.[1] Pulmonary stenosis and hypertrophic cardiomyopathy are the most common cardiac abnormalities in patients with NS as well as those with LS.[1] We report a patient with LS and apical hypertrophic cardiomyopathy who also had diffuse bilateral dilatation of coronary arteries.

Case Report

A 10-year-old boy was referred to our hospital for evaluation of the cardiovascular system. The patient had been diagnosed with LS at age 5 based on characteristic features, including multiple lentigines, ocular hypertelorism, growth retardation, and sensorineural hearing loss. Electrocardiography showed left axis deviation and low-voltage T wave in the inferiolateral leads without ST segment deviation. Family history showed no significant inherited phenotype suggestive of LS or cardiovascular disease. Echocardiography on admission showed myocardial hypertrophy of the septum and apical portions. Septal hypertrophy was not prominent with maximum thickness of the interventricular septum of 10 mm. Cardiac catheterization was then conducted. There was no pressure gradient in both the right and left ventricular outflow

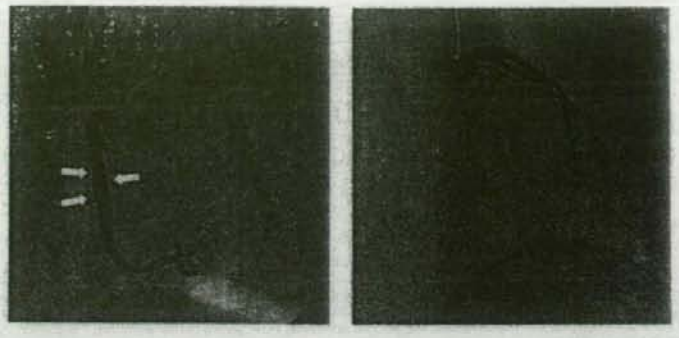


Figure 1. Coronary arteriograms at age 16, showing diffuse bilateral dilatation of coronary arteries without thrombus formation in the lumen. Arrows indicate slightly irregular vessel wall of the right coronary artery.

tracts. Selective coronary angiography revealed diffuse bilateral dilatation of coronary arteries with a maximum diameter of 6.8 mm for the right and 5.7 mm for the left anterior descending branch. There was no coronary arterial fistula or aneurysm formation indicative of Kawasaki disease.

Follow-up coronary arteriography performed at age 16 revealed slight increase in the vessel diameter, with a maximum diameter of 7.9 mm for the right and 7.3 mm for the left anterior descending branch. The vessel wall of the right coronary artery was slightly irregular (Figure 1). Squeezing of the peripheral portion of the right coronary artery was also noted. Left ventriculography showed the characteristic shape of apical hypertrophic cardiomyopathy (Figure 2). However, electrocardiography still showed no ST deviation. *PTPN11* gene sequence analysis using peripheral blood identified a missense mutation in exon 7 (Tyr279Cys).

The patient did not complain of chest pain, arrhythmias or other cardiac symptoms during the follow-up period of 12 years without any medication.

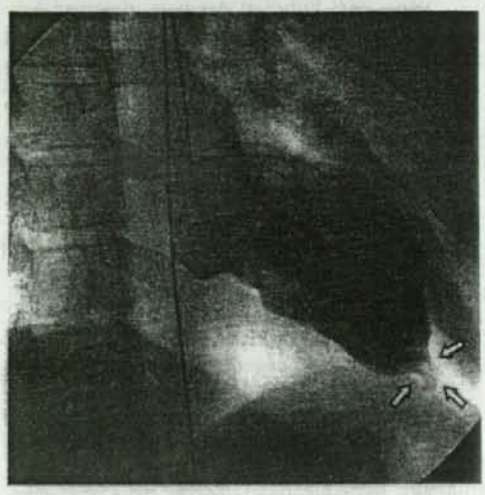


Figure 2. Left ventriculogram at age 16, showing a typical configuration of left ventricular cavity to apical hypertrophic cardiomyopathy (arrows).

Discussion

The present case demonstrated that LS can be complicated by coronary artery dilatation as well as hypertrophic cardiomyopathy and pulmonary stenosis. Aneurysm formation or ectasia of coronary arteries is usually the secondary manifestation of a congenital coronary fistula or, more prevalently, Kawasaki disease; both disorders could be excluded in the present case based on history taking and angiography. A careful search of the 1990–2008 PubMed database identified five reported cases of LS complicated with coronary artery dilatation[2–4] (Table 1). Four of them were recently reported by Limongelli et al.[4] among 26 patients with LS (one of them overlapped with the case of reference[3]). Thus, the estimated incidence of coronary dilatation in LS is 15%. Four out of the five reported cases had *PTPN11* mutation as in the present case. Although the type of *PTPN11* mutation varied among cases, one had the same mutation (Tyr279Cys) and showed LV morphological abnormality similar to our case.[3]

It is intriguing that coronary artery dilatation has also been demonstrated in patients with NS,

Table 1. Noonan Syndrome and LEOPARD Syndrome with Coronary Dilatation

Case	Authors (year)	Reference no.	Age/Gender	LS or NS	Cardiac Manifestation	*PTPN11* Mutation
1	Nomura et al. (2000)	5	10/M	NS	HOCM	ND
2	Ucar et al. (2005)	6	12/F	NS	CAN, PS, ASD, VSD	ND
3	Wong et al. (1990)	7	23/M	NS	CoA, AVA, PDA	ND
4	Loukas et al. (2004)	8	26/F	NS	PS	ND
5	Limongelli et al. (2007)	4	2	LS	AVA, MVA	Thr468Met
6	Limongelli et al. (2007)	4	7	LS	PS, MVA, LVS	Tyr279Cys
7	Pacileo et al. (2008)	3	13/M	LS	HOCM	Exon 13, Arg498Leu
8	Hagspiel et al. (2005)	2	17/M	LS	PS, HOCM	ND
9	Limongelli et al. (2007)	4	39	LS	HOCM	Negative
10	Present case	—	10/M	LS	APH	Exon7 Tyr279 Cys

APH, apical hypertrophic cardiomyopathy; Arg, arginine; ASD, atrial septal defect; AVA, aortic valve abnormalities; CAN, coronary aneurysm; CoA, coarctation of aorta; Cys, cysteine; HOCM, hypertrophic obstructive cardiomyopathy; Leu, leucine; LS, LEOPARD syndrome; LVS, left ventricular shape abnormality; Met, methionine; MVA, mitral valve abnormalities; ND, not described; NS, Noonan syndrome; PDA, patent ductus arterioles; PS, pulmonary stenosis; Thr, threonine; Tyr, tyrosine; VSD, ventricular septal defect.

because *PTPN11* gene mutation has been identified in both syndromes. A careful search of the 1990–2008 PubMed database identified four reported cases of NS complicated with coronary dilatation,[5–8] including a case with giant aneurysm reported by Wong et al.[7] (Table 1). It is known that LS and NS have common cardiovascular manifestations, pulmonary stenosis, and hypertrophic cardiomyopathy, although the most common heart defect is different between the two syndromes. In NS, pulmonary stenosis is the most common and is associated with a codon 308 mutation hot spot.[1] On the other hand, hypertrophic cardiomyopathy is the most common in LS in association with mutation hot spots in exons 7 and 12.[1] Screening for coronary artery abnormalities is important in the management of patients with LS and NS because thrombus formation can develop in dilated coronary arteries. Also, prophylactic administration of anticoagulant and antiplatelet drugs was recommended in previous reports,[5] although ischemic heart disease associated with thrombus formation has not been reported in LS and NS with dilated coronary arteries. Surgical resection or reconstruction should also be considered for large coronary aneurysms to avoid complications such as rupture, thrombosis, and coronary embolization. However, the size criteria of surgical intervention in an asymptomatic patient have not been established, and most of the patients who underwent surgery in the literature had a giant aneurysm, e.g., >50 mm in diameter.[9] Therefore, simple observation or conservative therapy with antiplatelet drugs is justifiable for our patient. Further studies in a large number of patients are needed to determine whether prophylactic anticoagulation therapy and surgery should be considered in LS and NS.

Coronary artery ectasia because of atherosclerosis is rather common in adulthood, and two mechanisms have been proposed.[10] The favorable one is severe and chronic inflammation and the less favorable is vascular overstimulation by exogenous interstitial nitric oxide. The involvement of metalloproteinases and plasma soluble adhesion molecules was also reported as a possible mechanism. However, the abovementioned pathologies have not been reported in LS or NS, and are also unlikely in the present case. As a congenital mechanism of coronary ectasia, jet blood flow produced by bicuspid aortic valve was reported in three neonates.[11] Although the presence of bicuspid aortic valve is common in NS, the present case did not have such a lesion. Further, the dilated site produced by jet flow tends to be confined to the proximal portion of the coronary artery,[11] compared with the present case which showed diffuse dilatation at longer segments.

Although *PTPN11* gene mutation is a possible background of many kinds of congenital heart defects and is possibly involved in coronary dilatation, its role in the pathogenesis remains to be clarified. *PTPN11* gene maps to chromosome 12q22-pter and encodes the human Shp2, the nonreceptor-type protein tyrosine phosphatase, which is a signal-enhancing component of growth factor, cytokine, and extracellular matrix receptor signaling.[12] Shp2 is involved in the signaling cascade of the vascular endothelial growth factor through interaction with the angiopoietin-1 receptor (Tie-2), and exerts mitogenic effects on vascular smooth muscle cells under the nitric oxide-induced pathway.[13] These mechanisms may explain the vascular abnormalities, including coronary dilatation in LS patients. However, *PTPN11* mutations were evident in approximately 60–80% of LS and 30–50% of NS reported in the literature,[1,4,14] and mutations are located in the different domain of the protein. Further, a number of genes of the Ras mitogen-activated protein kinase

pathway are involved in the pathogenesis of LS and NS.[14] It is unclear how the specific mutation Tyr279Cys is causing the different phenotype. It is possible that there might be other genes or single nucleotide polymorphisms associated with coronary dilatation in LS. Further studies are needed to elucidate how the genotype influences the clinical phenotype in LS patients.

Conclusion

LS and NS share multisystemic phenotypes including abnormalities in cardiovascular system as well as *PTPN11* gene mutation. Coronary artery dilatation is an additional important cardiovascular complication in both syndromes, and we recommend screening such patients even if they show no symptoms of ischemic heart disease.

Corresponding Author: Hitoshi Horigome, MD, PhD, Department of Child Health, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan. Tel: (+81) 29-853-3210; Fax: (+81) 29-853-5635; E-mail: hhorigom@md.tsukuba.ac.jp

Conflict of interest: None.

Accepted in final form: October 15, 2008.

References

1 Sarkozy A, Conti E, Seripa D, et al. Correlation between PTPN11 gene mutation and congenital heart defects in Noonan and LEOPARD syndromes. *J Med Genet.* 2003;40:704–708.

2 Hagspiel KD, Candinas RC, Hagspiel HJ, Amann FW. LEOPARD syndrome: cardiac imaging findings. *AJR.* 2005;184:S21–S24.

3 Pacileo G, Calabro P, Limongelli G, et al. Diffuse coronary dilatation in a young patient with LEOPARD syndrome. *Int J Cardiol.* 2006;112:e35–e37.

4 Limongelli G, Pacileo G, Marino B, et al. Prevalence and clinical significance of cardiovascular abnormalities in patients with the LEOPARD syndrome. *Am J Cardiol.* 2007;100:736–741.

5 Nomura Y, Yanagi S, Kono Y, et al. Bilateral coronary artery dilatation in a child with Noonan syndrome. *Jpn Circ J.* 2000;64:481–483.

6 Ucar T, Atalay S, Tekin M, Tutar E. Bilateral coronary artery dilatation and supravalvular pulmonary stenosis in a child with Noonan syndrome. *Pediatr Cardiol.* 2005;26:848–850.

7 Wong CK, Cheng CH, Lau CP, Leung WH. Congenital coronary artery anomalies in Noonan's syndrome. *Am Heart J.* 1990;119:396–400.

8 Loukas M, Dabrowski M, Kantoch M, Ruzytto W, Waltenberger J, Giannikopoulas P. A case report of Noonan's syndrome with pulmonary valve stenosis and coronary aneurysms. *Med Sci Monit.* 2004; 10:CS80–CS83.

9 Mawatari T, Koshino T, Morishita K, Komatsu K, Abe T. Successful surgical treatment of giant coronary artery aneurysm with fistula. *Ann Thorac Surg.* 2000;70:1394–1397.

10 Manginas A, Cokkinos DV. Coronary artery ectasias: imaging, functional assessment and clinical implications. *Eur Heart J.* 2006;27:1026–1031.

11 Chakrabarti S, Thomas E, Wright JGC, Vettukattil JJ. Congenital coronary artery dilatation. *Heart.* 2003;89:595–596.

12 Keilhack H, David FS, McGregor M, Cantley LC, Neel BG. Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes. *J Biol Chem.* 2005;280:30984–30993.

13 Chang Y, Ceacareanu B, Dixit M, Sreejayan N, Hassid A. Nitric oxide-induced motility in aortic smooth muscle cells: role of protein tyrosine phosphatase SHP-2 and GTP-binding protein Rho. *Circ Res.* 2002;91:390–397.

14 Razzaque MA, Nishizawa T, Komoike Y, et al. Germline gain-of-function mutations in RAF1 cause Noonan syndrome. *Nat Genet.* 2007;39:1013–1017.

Eur J Pediatr (2009) 168:329–331
DOI 10.1007/s00431-008-0779-x

ORIGINAL PAPER

Late-onset adrenal hypoplasia congenita caused by a novel mutation of the *DAX-1* gene

Fan Yang · Keiichi Hanaki · Tomoe Kinoshita · Yuki Kawashima · Jun-ichi Nagaishi · Susumu Kanzaki

Received: 28 April 2008 / Accepted: 2 June 2008 / Published online: 8 July 2008

Abstract Mutation in the orphan nuclear receptor DAX-1 gene causes X-linked adrenal hypoplasia congenita (AHC). Affected male children classically suffer a salt-losing crisis and adrenal insufficiency in their early infancy or, in some rare exceptions, with late-onset subtype. We report here a patient manifesting late-onset adrenal hypoplasia congenita caused by the premature truncation of the C-terminus of the DAX-1 molecule, which is essential for its function as a transcriptional repressor. A 12-year-old boy was referred to us after being afflicted with generalized skin pigmentation for about 3 years, fatigue and headache. Primary adrenal insufficiency was determined on the basis of a low plasma cortisol level (3.9 μg/dl) despite an extremely high ACTH level (1200 pg/ml). Replacement therapy with hydrocortisone and fludorocortisone acetate was initiated soon thereafter. Hypogonadotropic hypogonadism was confirmed at the age of 18 years, at which time sexual infantilism had become apparent. Direct sequencing of the peripheral lymphocyte-derived DNA revealed a novel 1033del13 mutation on the ligand-binding domain of the *NR0B1* (DAX-1) gene, which generated a premature stop codon truncating the C-terminus. This mutation was considered de novo since we could not find it in his mother. This case demonstrates that even a truncated protein lacking the major functional domain of DAX-1 can present late-onset and latent adrenal failure.

Keywords Adrenal hypoplasia congenita · DAX-1 · Hypogonadotropic hypogonadism

F. Yang · T. Kinoshita · Y. Kawashima · J.-i. Nagaishi · S. Kanzaki
Division of Pediatrics and Perinatology, Faculty of Medicine,
Tottori University,
Yonago 683–8504, Japan

F. Yang
Department of Pediatrics, West China Second University Hospital,
Sichuan University,
Chengdu 610041, China

K. Hanaki (✉)
School of Health Sciences, Faculty of Medicine,
Tottori University,
Nishi-cho 86,
Yonago 683–8503, Japan
e-mail: hanaki@grape.med.tottori-u.ac.jp

Introduction

X-linked congenital adrenal hypoplasia (AHC, OMIM #300200) is a relatively rare disease that is caused by an abnormality in the DAX-1 gene (dosage-sensitive sex reversal–adrenal hypoplasia congenita critical region on the X chromosome gene 1, *NR0B1*) [9]. The affected adrenal cortex resembles that of the fetal zone and is made up of disorganized vacuolated cytomegalic cells. Boys with this disorder usually present with severe adrenal failure in their infancy because of the absence of the mature adrenal cortex. Early diagnosis and effectual replacement therapy enable an increasing number of patients with AHC to survive into adolescence. By that time hypogonadotropic hypogonadism (HH) characterized as gonadotropin deficiency and impaired spermatogenesis [1, 7] generally becomes apparent.

The DAX-1 gene consists of two exons and a single 3.4-kb intron, and it encodes a 470-amino acid protein that belongs to the orphan nuclear receptor superfamily (NRSF). The protein coded by this gene has a carboxyl-terminal region that shows a sequence similarity with the ligand-binding domain (LBD) of other nuclear receptors [9]. Functional studies suggest that DAX-1 acts as a

repressor of gene transcription in part by inhibiting the activity of steroidogenic factor-1 (SF-1), another nuclear receptor involved in sex differentiation [2]. To date, over 80 different mutations in the DAX-1 gene have been identified [6], and most have been reported to disrupt the LBD, regardless of whether being a missense or truncating mutation. At the carboxyl terminal, at least 11 amino acids have been found essential for normal adrenal cortical embryogenesis [5].

We describe here a novel DAX-1 gene 1033del33 mutation that generates a stop codon, truncating the C-terminus. Our observations provide an example of mild and late-onset adrenal phenotypes which can be caused by a DAX-1 molecule lacking the major functional domain.

Case report

A 12-year-old boy presented with a history of systemic skin pigmentation and fatigue. He was 151.0 cm tall (+0.2 SD) and weighed 47.0 kg (+0.3 SD). His sexual development was at the Tanner 1 stage for pubic hair growth. Blood pressure was 118/62 mmHg at the upper arm. Bone maturation measured at the left hand was 11.5 years of age.

Laboratory examination revealed hypocortisolemia (3.9 μg/dl; normal range 2.7 to approx.15.5 μg/dl) and an elevated adrenocorticotropic hormone (ACTH) level (1200 pg/ml; normal range <60 pg/ml), which is consistent with primary adrenal insufficiency. The serum level of dehydroepiandrosterone sulfate (<200 ng/ml; normal range 500 to approx. 3000 ng/ml) was also decreased. The 17-hydroxyprogesterone level was not high (0.7 ng/ml; normal range 0.1 to approx.1.7 ng/ml). Replacement therapy with hydrocortisone (25 mg/day) and fludorocortisone acetate (0.075 mg/day) was initiated soon thereafter, resulting in a rapid improvement in his clinical condition. At the age of 18 years, hypogonadism was confirmed by a low testosterone level (126 ng/dl; normal range 250 to approx.1100 ng/dl) and apparent sexual infantility. A lutenizing hormone–releasing hormone test and a human chorionic gonadotropin test were used to identify the HH. Azoospermia was diagnosed by semen analysis. No other males in his family were affected with this condition in any way.

Subjects and methods

DNA extraction, sequencing, PCR, and mutation analysis

After obtaining written informed consent, genomic DNA was extracted from 300 μl whole blood using a Genomic DNA Extraction kit (Wako Pure Chemical Industries, Osaka, Japan). The primer pairs used for the PCR were: DAX1.1 (forward, 5′-catgggcgaacacaccggagcgcagcac-3′; reverse, 5′-acccccctggcctctgcgcgaagtaggag-3′), DAX1.2 (forward, 5′-tcctgtaccgctgctgcttttgtggtga-3′; reverse, 5′-tcgttactgcccgcgcccctagat-3′) and DAX2 (forward, 5′-ctagcaaaggactctgtggtga-3′; reverse; 5′-atttgatttgagcaggttccatga-3′). The PCR conditions consisted of a 1-min predenaturation at 95°C; 35 cycles of 1 min at 95°C; annealing for 1 min at 60°C; extension for 1 min at 72°C and a final extension at 72°C for 5 min. The PCR product was purified using the DNA Gel Extraction kit (Millipore Corp, Bedford, MA). Forward and reverse direct sequencing was performed using a Big Dye Terminator Sequencing kit and an ABI 3100 automated sequencer (PE Applied Biosystems, Foster City, CA). Restriction fragment analysis was done to ensure mutation by digestion for 16 h with an *Msp*I restriction enzyme (MBI fermentas, Vilnius, Lithuania) using the buffer and temperature recommended by the manufacturer. Cleaved PCR products were fractioned by electrophoresis on a 3% agarose gel containing ethidium bromide and run in 1× TBE buffer.

Results

Direct DNA sequencing of the amplified PCR products showed a 13-bp deletion in the first exon of the DAX-1 gene (Fig. 1). This altered the *Msp*I restriction site, allowing confirmation of the mutational and normal statuses by restriction fragment analysis. This deletion caused a change in the reading frame, leading to the introduction of a stop codon and putatively truncating the carboxyl-terminus of the DAX-1 molecule by 104 amino acids. This mutation was considered to be de novo since we could not find it in the mother.

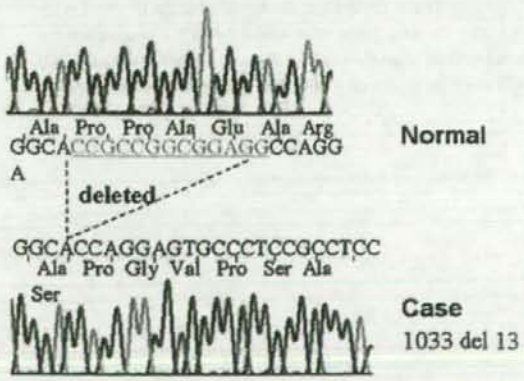


Fig. 1 Partial sequence of the DAX-1 gene from the patient and a normal control

Discussion

We report here a boy with AHC and HH who had a novel deletion in the DAX-1 gene. His main clinical feature was skin pigmentation starting at 9 years of age. In his infancy, there were no symptoms of adrenal insufficiency, such as a salt-losing crisis, which is a cardinal feature of AHC. Late-onset X-linked AHC has been described in four patients who developed such symptoms at 6 months of age or later. One patient, who had no previous history of hypoadrenalism, was referred to an endocrinologist for hypogonadism at 28 years of age. He was found to have a hemizygous Y380D missense mutation in the DAX-1 gene [4]. Another patient who presented with mild adrenal failure at 28 years of age had an I439S missense mutation [8]. The other two affected boys were brothers who presented with salt-losing adrenal crisis at the ages of 6 and 3 years, respectively. They were hemizygous for the W171X nonsense mutation [3]. Our patient was also confirmed as late-onset X-linked AHC. It is quite remarkable that a wide phenotypic variability in adrenal insufficiency has been reported among patients with different mutations, even in those with the same mutation in the DAX-1 gene.

The DAX-1 molecule has the property of transcriptional regulator similar to other members of the NRSF. The C-terminal end of the molecule, comprising approximately 207–470 amino acids, has a structural similarity to the LBD of NRSF, although no available ligand has yet been identified [6]. The fact that most of the missense mutations found so far are located in the LBD suggests its essential role for the repressor function. The LBD can be further divided into structural (amino acids 261–304 and 362–442) and ligand binding (amino acids 207–260, 305–361 and 443–470) subdomains [10]. In our patient, the 1033del13 mutation putatively disrupted at least one structural and two ligand binding subdomains in the LBD. It is noteworthy that, despite these definite mutational defects in the LBD, the patient showed only mild and late-onset symptoms of adrenal insufficiency as well as rather obvious HH symptoms. In light of the fact that the aforementioned patients with Y380D or I439S mutations still had obvious HH symptoms with latent adrenal symptoms, there may be some kind of site specificity in the LBD where adrenal insufficiency or hypogonadism is developed.

References

1. Habity RL, Boepple P, Nachtigall L, Sluss PM, Crowley WF Jr, Jameson JL (1996) Adrenal hypoplasia congenita with hypogonadotropic hypogonadism: evidence that DAX-1 mutations lead to combined hypothalamic and pituitary defects in gonadotropin production. J Clin Invest 98:1055–1062
2. Ito M, Yu R, Jameson JL (1997) DAX-1 inhibits SF-1-mediated transactivation via a carboxyl-terminal domain that is deleted in adrenal hypoplasia congenita. Mol Cell Biol 17(3):1476–14 83, Mar
3. Loke KY, Larry KS, Lee YS, Peter M, Drop SL (2000) Prepubertal diagnosis of X-linked congenital adrenal hypoplasia presenting after infancy. Eur J Pediatr 159(9):671–675
4. Mantovani G, Ozisik G, Achermann JC, Romoli R, Borretta G, Persani L et al (2002) Hypogonadotropic hypogonadism as a presenting feature of late-onset X-linked adrenal hypoplasia congenita. J Clin Endocrinol Metab 87(1):41–43
5. Nakae J, Tajima T, Kusuda S, Kohda H, Okabe T, Shinohara N, Kato M, Murashita M, Mukai T, Imanaka K, Fujieda K (1996) Truncation at the C-terminus of the DAX-1 protein impairs its biological actions in patients with X-linked adrenal hypoplasia congenita. J Clin Endocrinol Metab 81:3680–3685
6. Phelan JK, McCabe ER (2001) Mutations in NR0B1 (DAX-1) and NR5A1 (SF1) responsible for adrenal hypoplasia congenita. Hum Mutat 18:472–487
7. Seminara SB, Achermann JC, Genel M, Jameson JL, Crowley WF Jr (1999) X-Linked adrenal hypoplasia congenita: A mutation in *DAX1* expands the phenotypic spectrum in males and females. J Clin Endocrinol Metab 84:4501–4509
8. Tabarin A, Achermann JC, Recan D, Bex V, Bertagna X, Christin-Maitre S, Ito M, Jameson JL, Bouchard P (2000) A novel mutation in DAX1 causes delayed-onset adrenal insufficiency and incomplete hypogonadotropic hypogonadism. J Clin Invest 105(3):321–328
9. Zanaria E, Muscatelli F, Bardoni B, Strom TM et al (1994) An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature 372:635–641
10. Zhang YH, Guo W, Wagner RL, Huang BL, McCabe L, Vilain E et al (1998) DAX-1 mutations map to putative structural domains in a deduced three-dimensional model. Am J Hum Genet 62 (4):855–864

Original Article

Low adiponectin state is associated with metabolic abnormalities in obese children, particularly depending on apolipoprotein E phenotype

Elfi Wardaningsih[1], Takashi Miida[2], Utako Seino[1], Yuriko Fueki[1], Masayuki Ito[1], Keisuke Nagasaki[3], Toru Kikuchi[3], Makoto Uchiyama[3], Satoshi Hirayama[4], Osamu Hanyu[4], Kazunori Miyake[2] and Masahiko Okada[1]

[1]Division of Clinical Preventive Medicine, Department of Community Preventive Medicine, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi 1-757, Chuo-ku, Niigata 951-8510; [2]Department of Clinical Laboratory Medicine, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421; [3]Division of Pediatrics; [4]Division of Endocrinology and Metabolism, Department of Homeostatic Regulation and Development, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi 1-757, Chuo-ku, Niigata 951-8510, Japan
Corresponding author: Dr Takashi Miida. Email: tmiida@juntendo.ac.jp

Abstract

Background: Adiponectin links obesity with insulin resistance, which causes various metabolic abnormalities including dyslipidaemia. Apolipoprotein E (apoE) phenotypes also affect lipoprotein profiles. We aimed to determine whether low adiponectin concentrations are associated with insulin resistance and downstream metabolic abnormalities in obese children.
Methods: We measured fasting concentrations of lipids, apoE, glucose, insulin and adiponectin, as well as anthropometric parameters, in 191 obese children aged 6–15 years. ApoE phenotypes were determined by isoelectric focusing. Boys ($n = 79$) and girls ($n = 39$) with apoE3/3 were classified into tertiles according to their adiponectin concentrations. Metabolic parameters, were compared among these three groups in boys and girls separately.
Results: The low adiponectin groups had higher median homeostasis model assessment of insulin resistance (HOMA-IR) than the middle and high adiponectin groups in both boys [5.3 (low) versus 3.1 (middle; $P < 0.05$) and 3.5 (high; $P < 0.05$)] and girls [5.0 (low) versus 4.4 (middle) and 3.0 (high; $P < 0.05$)]. However, only boys who were in the low adiponectin group exhibited significantly higher concentrations of blood pressure, triglycerides, LDL-cholesterol, and remnant-like particle-cholesterol, and lower concentrations of HDL-cholesterol compared with the middle or high adiponectin groups.
Conclusion: Low adiponectin concentration is associated with insulin resistance in obese children. Furthermore, decreased adiponectin with E3/3 exhibited more prominent downstream metabolic abnormalities in obese boys than in obese girls.

Ann Clin Biochem 2008; 45: 496–503. DOI: 10.1258/acb.2008.007237

Introduction

The prevalence of childhood obesity in Japan has been steadily increasing because of the introduction of Westernized life styles, including a high-calorie, high-fat diet and low physical activity.[1] Similar to obese adults, obese children also suffer from multiple health problems closely related to insulin resistance.[2–4] In typical cases, they have hypertension,[3,4] dyslipidaemia (hypertriglyceridaemia and/or hypo-α-lipoproteinaemia),[2,3] and impaired glucose tolerance.[3] Studies have shown that these metabolic abnormalities inevitably accumulate in obese subjects because of imbalances in adipocytokines (bioactive substances secreted from adipocytes).[5]

Adiponectin is an anti-atherogenic adipocytokine, and serum concentrations have been found to be reduced in obese subjects.[6,7] In human serum, adiponectin exists as three types of multimers with differing molecular weights. Adiponectin multimers with high molecular weights bind to adiponectin receptors more efficiently than those with middle or low molecular weights.[8] Although subjects with low adiponectin concentrations have more metabolic abnormalities than those with high adiponectin concentrations, clinical manifestations vary considerably among subjects with similar adiponectin concentrations.[9–12] Such individual variations may result from the effects of other confounding factors such as apolipoprotein E (apoE).

ApoE phenotypes significantly affect serum concentrations of LDL-cholesterol (LDL-C), triglycerides (TG) and remnant-like particle cholesterol (RLP-C).[13-17] Furthermore, apoE phenotypes are thought to be associated with insulin resistance in obese women.[18]

The aim of this study was to determine whether low adiponectin concentrations are associated with insulin resistance and downstream metabolic abnormalities in obese children. To eliminate the confounding effects of differing apoE phenotypes, we selected only E3/3 children to be included in the study and compared metabolic abnormalities among subgroups classified by adiponectin concentrations.

Methods

Subjects

For this study, we enrolled 191 obese (132 boys and 59 girls) and 91 non-obese children (50 boys and 41 girls) in the age range of 6–15 years. The obese children were selected from public schools in Niigata Prefecture, Japan. A percentage of the standard weight (POW) was calculated according to sex, age and body height, as reported by the Ministry of Education, Sciences and Culture of Japan in 1990.[19] We defined obesity as POW equal to or more than 20%.

We measured several anthropometric parameters including height, weight and systolic and diastolic blood pressures (SBP and DBP) in all children. Fasting (at least 12 hours) and non-fasting venous blood was collected from obese and normal children, respectively, for measurement of lipoprotein and adiponectin later. Serum was separated with low-speed centrifugation, and aliquots were frozen at −80°C until used for later analysis. Informed consent for all children was given from the parents or guardians before blood sampling. This study was approved by the ethics committee of Niigata University Graduate School of Medical and Dental Sciences.

Determination of Apolipoprotein E phenotypes

ApoE phenotypes were determined by isoelectric focusing (IEF) followed by immunoblotting.[20] Briefly, a serum sample (100 μL) was mixed with the pretreatment buffer (0.005 mol/L dithiothreitol, 0.5% Tween 20) at 4°C for 15 minutes. IEF was carried out at 8°C and 13 W for 2000 Vh using a 5% polyacrylamide gel containing 5% ampholyte (pH 4.5–5.4 and pH 5–8, 1:2) and 3 mol/L urea. The separated proteins were transferred overnight onto the nitrocellulose membrane by simple diffusion at 4°C.

Nitrocellulose membranes were reacted with polyclonal rabbit antibodies against human apoE, and then horseradish-conjugated goat antibodies against rabbit immunoglobulin. After visualizing apoE bands by phosphate-buffered saline containing diaminobenzidine (0.072 g/L) and 0.1% hydrogen peroxide (v/v), we determined apoE phenotypes from the multiple banding patterns.[21]

In some cases with unclear results, we desialylated apoE bands prior to IEF.[22] Serum samples (10 μL) were incubated with 0.1 mol/L acetate buffer (pH 5.0; 20 μL) containing 2 unit/mL neuraminidase (10 μL) at 37°C for 4 hours. The mixture was delipidated with acetone/ethanol (1:1, v/v) at −80°C for 4 hours, acetone/ethanol solution at −80°C for 2 hours, and then diethyl ether at −80°C for 1 hour. The delipidated protein was dried under a stream of nitrogen. The desialylated samples were incubated with 100 μL sample buffer (0.01 mol/L Tris–HCl [pH 8.6] containing 0.01 mol/L dithiothreitol and 5.2 mol/L urea) for 1 hour at room temperature. IEF was carried out in the same manner as described above except that we used a polyacrylamide gel that contained 5.2 mol/L urea.

Measurements of lipoproteins and adiponectin

Total cholesterol (TC) and TG concentrations were measured enzymatically using an automatic analyser (Hitachi-7450, Tokyo, Japan). LDL-C and HDL-C concentrations were determined by homogeneous assays, and apoE concentration was measured by a turbidometric immunoassay. RLP-C concentrations were determined by detergent-based homogenous assay (RemL-C; Kyowa Medex, Tokyo, Japan).[23] The values obtained using these kits were highly correlated with those measured by the original methods of Nakajima *et al.*[24] using an immunoaffinity gel. Serum total adiponectin concentrations were measured by immunoassay using a commercial kit (Human Adiponectin ELISA Kit; Otsuka, Osaka, Japan). As we could not obtain fasting samples from non-obese children, TG, RLP-C and adiponectin were measured only in the obese children.

Insulin resistance

Fasting plasma glucose (FPG) concentration was measured using an enzymatic method. Serum insulin concentrations were measured with a commercial enzyme-linked immunoassay kit (LS 'Eiken' Insulin; Eiken Chemical Co., Ltd., Tokyo, Japan). Insulin resistance was evaluated with the homeostasis model assessment of insulin resistance (HOMA-IR) calculated by the following equation:

$$\frac{\text{FPG (mmol/L)} \times (\text{fasting insulin concentration (mU/L)})}{22.5}$$

Statistical analysis

In this study, we compared variables according to sex. Data were reported as the mean (±SD) or median (interquartile range). Data distributions were checked for normality using a one-sample Kolmogorov-Smirnov test. Markedly skewed data were logarithmically transformed prior to statistical analysis. Continuous variables between groups were compared by either unpaired Student's *t*-test or non-parametric Mann-Whitney *U* test. Multiple stepwise regression analysis was carried out to define the independent risk factors for insulin resistance using Statcel (OMS, Tokorozawa, Japan), an add-in software for Microsoft

Excel (Microsoft Japan, Tokyo, Japan). Differences were considered significant at P values <0.05 (two-sided tests).

Results

Apolipoprotein E phenotype and ε allele frequencies

Obese and non-obese children had similar relative frequencies of apoE phenotypes and ε alleles. In both groups, about two-thirds of the children were E3/3. The incidences of other major phenotypes (E4/3 and E3/2) were similar between the obese and non-obese children. As reflected by these data, the frequencies of ε2, ε3 and ε4 were similar between the two groups. One apoE5 carrier was found in the non-obese group, while four apoE7 carriers were detected in the obese group. Because of the small number of subjects for these two phenotypes, their prevalence did not reach statistical significance (Table 1).

In the following comparison, we focused our analyses on subjects with the three most common phenotypes (i.e. E2/3, E3/3 and E3/4) as the other phenotypes accounted for <1–3% of the study subjects. Two apoE3/3 children were excluded from the analysis because they were not fasting at the time of blood sampling.

Effects of apolipoprotein E phenotypes on lipoprotein profiles and insulin resistance

The effects of apoE phenotypes on lipoprotein profiles were more evident in obese children than in non-obese children. However, apoE phenotypes did not affect SBP or DBP concentrations in either group. In non-obese children, apoE concentration was the only metabolic parameter that differed significantly among the three most common apoE phenotypes. In boys, apoE concentration was highest in the E3/2 carriers, lowest in the E4/3 carriers and intermediate in the E3/3 carriers (Table 2). In girls, apoE concentration

Table 1 Comparisons of apoE phenotypes and ε allele frequencies between obese and non-obese children

	Obese children (n = 191)	Non-obese children (n = 91)	Total (n = 282)
ApoE phenotype			
E3/2	23 (12.0%)	7 (7.7%)	30 (10.6%)
E3/3	120 (62.8%)	59 (64.8%)	179 (63.5%)
E4/2	4 (2.1%)	–	4 (1.4%)
E4/3	39 (20.4%)	24 (26.4%)	63 (22.3%)
E4/4	1 (0.5%)	–	1 (0.4%)
E5/3	–	1 (1.1%)	1 (0.4%)
E7/3	3 (1.6%)	–	3 (1.1%)
E7/4	1 (0.5%)	–	1 (0.4%)
ε allele			
ε2	0.071	0.038	0.060
ε3	0.798	0.824	0.807
ε4	0.120	0.132	0.124
ε5	–	0.005	0.002
ε7	0.011	–	0.007

ApoE phenotypes were determined by isoelectric focusing followed by immunoblotting. The apoE5 and apoE7 bands were confirmed using serum pretreated with neuraminidase as described in the Subjects and Methods section

Table 2 Effects of apoE phenotypes on anthropometric and lipoprotein profiles in non-obese children

	Boys (n = 49)			Girls (n = 41)		
Parameters	E4/3 (n = 13)	E3/3 (n = 33)	E3/2 (n = 3)	4/3 (n = 11)	E3/3 (n = 26)	E3/2 (n = 4)
Age (years)	10.0 (0.3)	10.0 (0.4)	9.9 (0.4)	10.0 (0.6)	10.0 (0.4)	9.8 (0.5)
Body height (m)	1.35 (0.06)	1.36 (0.06)	1.35 (0.09)	1.41 (0.08)	1.37 (0.07)	1.37 (0.03)
Body weight (kg)	30.6 (5.5)	30.8 (5.5)	26.1 (4.7)	34.2 (6.0)	31.6 (6.4)	33.3 (3.0)
POW (%)	−1.1 (9.5)*	−3.1 (9.2)	−15.0 (6.9)	−1.2 (4.8)	−1.7 (10.2)	5.0 (6.9)
SBP (mmHg)	100 (8)	101 (9)	95 (9)	98 (10)	104 (9)	99 (6)
DBP (mmHg)	49 (7)	52 (6)	52 (5)	55 (6)	53 (6)	56 (8)
TC (mmol/L)	4.45 (0.68)	4.70 (0.73)	4.46 (0.28)	4.22 (0.46)	4.47 (0.63)	4.39 (0.45)
LDL-C (mmol/L)	2.35 (0.56)	2.47 (0.65)	2.11 (0.45)	2.42 (0.45)	2.49 (0.52)	2.33 (0.49)
HDL-C (mmol/L)	1.77 (0.39)	1.87 (0.38)	1.97 (0.09)	1.37 (0.21)	1.61 (0.32)	1.61 (0.21)
ApoE (mg/L)	31 (30, 34)*,†	41 (36, 44)*	52 (50, 53)	37 (33, 42)*	38 (33, 45)*	55 (42, 64)

POW, a percentage of the standard weight for age, height and sex; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; apoE, apolipoprotein E. Data are presented as the mean (SD) except for apoE (median (25th, 75th percentiles))

*$P < 0.05$ vs. E3/2 children

†$P < 0.05$ vs. E3/3 children

was significantly higher in E3/2 carriers than in E4/3 and E3/3 carriers.

In contrast, in obese children, TC and LDL-C concentrations were highest in the E4/3 carriers, lowest in the E3/2 carriers, and intermediate in the E3/3 carriers (Table 3). TG and RLP-C concentrations did not differ among the three phenotypes. As in the non-obese children, apoE concentration was highest in the E3/2 carriers independent of sex.

Note that E4/3 obese girls, but not boys, displayed abnormalities in parameters related to insulin resistance. Fasting insulin was greater in E4/3 carriers than in E3/3 ($P < 0.05$) and E3/2 carriers ($P = 0.07$). Furthermore, HOMA-IR values in obese girls tended to be higher in E4/3 carriers than in E3/3 ($P = 0.05$) and E3/2 carriers ($P = 0.07$).

Effects of adiponectin concentration on metabolic parameters in obese children

First, we examined the effects of adiponectin on metabolic parameters in obese children with various apoE phenotypes. Obese boys and girls were divided into tertiles according to their adiponectin concentrations. The median HOMA-IR value was higher in the low adiponectin group than either the middle or high adiponectin group in the obese boys (5.59 [4.07, 7.35] (low) versus 3.68 [2.64, 4.85] (middle), $P < 0.001$; versus 3.12 [2.09, 4.93] (high), $P < 0.001$) and girls [5.01 (3.73, 7.90) (low) versus 4.72 (3.28, 8.67) (middle), not significant; versus 2.98 (2.29, 4.03) (high), $P < 0.01$]. However, we failed to find any significant differences among the subgroups in both obese boys and girls for any other variables (data not shown).

Second, we performed the same analysis in obese children who had the same apoE phenotype. In both obese boys and girls, children with the same apoE phenotype were divided into tertiles according to their adiponectin concentrations. However, we did not examine obese girls with E4/3 and E3/2 because of the small number of individual subgroups. In the obese children with E3/3, fasting insulin concentrations were highest in the low adiponectin groups in both obese boys and girls, although FPG concentrations were not different among groups (Table 4). The low adiponectin groups had almost a two-fold higher HOMA-IR than either the middle or high adiponectin groups (Figure 1, a). The median values of both adiponectin and HOMA-IR were very similar between the corresponding groups of obese boys and girls (Figure 1, a).

In the obese boys with E4/3 and E3/2, fasting insulin concentrations were higher in the low adiponectin groups (28.9 ± 18.0 mU/L; 30.2 ± 17.8 mU/L) than in the middle (16.9 ± 4.8 mU/L; $P = 0.07$; 26.7 ± 8.8 mU/L) and high adiponectin groups (18.0 ± 15.1 mU/L; 14.2 ± 11.4 mU/L, $P = 0.1$). The mean FPG concentrations were very similar among groups. As a result, the mean values of HOMA-IR tended to be higher in the low adiponectin groups (6.7 ± 4.1; 6.8 ± 3.9) than in the middle (4.0 ± 1.0, $P = 0.08$; 6.2 ± 2.0) and high adiponectin groups (4.0 ± 3.2; 3.2 ± 2.6, $P = 0.1$).

Unexpectedly, the effects of adiponectin on anthropometric and lipoprotein parameters were more prominent

Table 3 Effects of apoE phenotypes on anthropometric and lipoprotein profiles, and HOMA-IR values in obese children

	Boys ($n = 123$)			Girls ($n = 57$)		
Parameters	E4/3 ($n = 28$)	E3/3 ($n = 79$)	E3/2 ($n = 16$)	4/3 ($n = 11$)	E3/3 ($n = 39$)	E 3/2 ($n = 7$)
Age (years)	9.9 (2.4)	10.3 (2.2)	10.6 (2.0)	11.1 (2.3)	10.0 (2.1)	10.4 (1.9)
Body height (m)	1.42 (0.17)	1.44 (0.14)	1.46 (0.18)	1.45 (0.09)	1.40 (0.12)	1.46 (0.13)
Body weight (kg)	56.6 (22.3)	57.9 (16.8)	61.5 (23.3)	59.0 (16.2)	53.8 (16.5)	56.5 (13.7)
POW (%)	53.1 (17.5)	52.6 (13.7)	55.0 (22.3)	50.8 (12.0)	53.8 (16.5)	47.9 (6.1)
SBP (mmHg)	114 (13)	117 (13)	119 (10)	114 (11)	115 (11)	113 (6)
DBP (mmHg)	57 (10)	56 (7)	58 (7)	58 (9)	55 (8)	57 (7)
TC (mmol/L)	5.09 (0.89)*†	4.56 (0.66)	4.34 (0.79)	5.12 (0.98)†	4.69 (0.62)†	4.03 (0.75)
LDL-C (mmol/L)	3.27 (0.74)*†	2.79 (0.59)	2.58 (0.72)	3.28 (0.65)*†	2.88 (0.55)†	2.21 (0.76)
HDL-C (mmol/L)	1.34 (0.23)	1.36 (0.25)	1.27 (0.19)	1.25 (0.27)	1.39 (0.26)	1.39 (0.29)
TG (mmol/L)	1.05 (0.76, 1.70)	1.04 (0.65, 1.32)	1.13 (0.86, 1.56)	1.17 (0.90, 1.49)	0.99 (0.67, 1.29)	0.88 (0.49, 1.49)
ApoE (mg/L)	39 (32, 44)*†	44 (40, 52)†	50 (45, 58)	41 (38, 47)†	44 (40, 50)†	57 (50, 65)
RLP-C (mg/L)	38 (19, 69)	34 (18, 52)	37 (30, 52)	33 (21, 56)	37 (21, 48)	29 (15, 64)
Adiponectin (mg/L)	6.1 (4.7, 8.6)	6.6 (5.3, 8.9)	5.1 (4.5, 6.7)	5.9 (4.3, 8.3)	6.2 (4.6, 7.7)	5.5 (4.0, 8.8)
FPG (mmol/L)	5.30 (4.95, 5.48)	5.11 (4.88, 5.38)	5.16 (4.94, 53.00)	5.05 (4.72, 5.27)	4.94 (4.77, 5.27)	4.94 (4.55, 5.27)
Insulin (mU/L)	17.8 (11.1, 23.4)	17.8 (11.8, 26.7)	21.5 (13.8, 30.3)	24.6 (16.8, 43.3)*	19.0 (12.9, 29.7)	14.0 (10.8, 17.7)
HOMA-IR	4.12 (2.6, 5.4)	3.9 (2.7, 6.1)	4.9 (3.1, 6.8)	5.6 (3.5, 10.4)	3.9 (2.9, 6.6)	2.7 (2.3, 4.1)

POW, a percentage of the standard weight for age, height and sex; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglycerides; apoE, apolipoprotein E; RLP-C, remnant-like particle cholesterol; FPG, fasting blood plasma; HOMA-IR, homeostasis model assessment of insulin resistance. Data are presented as mean (SD) or median (25th, 75th percentiles)

*$P < 0.05$ vs. E3/3 children

†$P < 0.05$ vs. E3/2 children

Table 4 Effects of adiponectin concentrations on anthropometric and lipoprotein profiles, and homeostasis model assessment of insulin resistance values in obese children with apoE3/3

Parameters	Boys (n = 79)			Girls (n = 39)		
	Low (n = 26)	Middle (n = 26)	High (n = 27)	Low (n = 13)	Middle (n = 13)	High (n = 13)
Age (years)	11.2 (2.0)	10.2 (2.5)	10.3 (2.0)	10.7 (1.6)*	9.2 (1.9)	10.1 (2.6)
Body height (m)	1.48 (0.11)*	1.41 (0.15)	1.45 (0.14)	1.46 (0.09)	1.39 (0.10)	1.38 (0.16)
Body weight (kg)	63.2 (16.2)*	52.1 (16.3)	58.2 (16.8)	61.0 (14.6)	50.6 (11.3)	51.4 (18.3)
POW (%)	56.1 (16.7)	50.4 (12.6)	53.4 (11.9)	57.0 (17.4)	53.1 (20.9)	51.3 (10.1)
SBP (mmHg)	122 (13)*,†	114 (13)	114 (12)	117 (9)	114 (13)	115 (13)
DBP (mmHg)	59 (7)*,†	55 (8)	54 (6)	57 (9)	53 (8)	55 (7)
TC (mmol/L)	4.68 (0.70)	4.49 (0.63)	4.47 (0.67)	4.59 (0.66)	4.73 (0.69)	4.75 (0.55)
TG (mmol/L)	1.31 (1.00, 1.65)*,†	0.91 (0.62, 1.23)	0.84 (0.62, 1.22)	0.87 (0.66, 1.17)	1.22 (0.78, 1.65)	0.90 (0.62, 1.11)
ApoE (mg/L)	46 (38, 52)	43 (40, 46)	46 (42, 55)	42 (40, 47)	44 (40, 53)	46 (35, 51)
Adiponectin (mg/L)	4.6 (3.9, 5.3)*,†	6.5 (5.9, 7.0)†	9.5 (8.7, 11.5)	4.1 (3.3, 4.7)*,†	6.2 (5.5, 6.3)†	9.7 (7.8, 11.2)
FPG (mmol/L)	5.16 (4.83, 5.49)	5.19 (4.99, 5.45)†	4.99 (4.77, 5.33)	4.88 (4.63, 5.41)	5.05 (4.91, 5.36)†	4.83 (4.72, 5.06)
Insulin (mU/L)	22.9 (17.8, 30.9)*	12.7 (9.8, 17.7)	16.6 (11.3, 28.3)	22.8 (18.9, 33.7)†	20.4 (13.3, 9.14)	13.7 (9.17, 18.0)

POW, a percentage of the standard weight for age, height and sex; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; apoE, apolipoprotein E; FPG, fasting plasma glucose. Data are presented as the mean (SD) or median (25th, 75th percentiles). Subjects are divided into tertiles (low, middle and high groups) according to adiponectin concentrations; cut-off values of adiponectin concentrations were 5.6 and 7.6 μg/mL in boys and 5.0 and 6.5 μg/mL in girls

*$P < 0.05$ vs. middle adiponectin groups

†$P < 0.05$ vs. high adiponectin groups

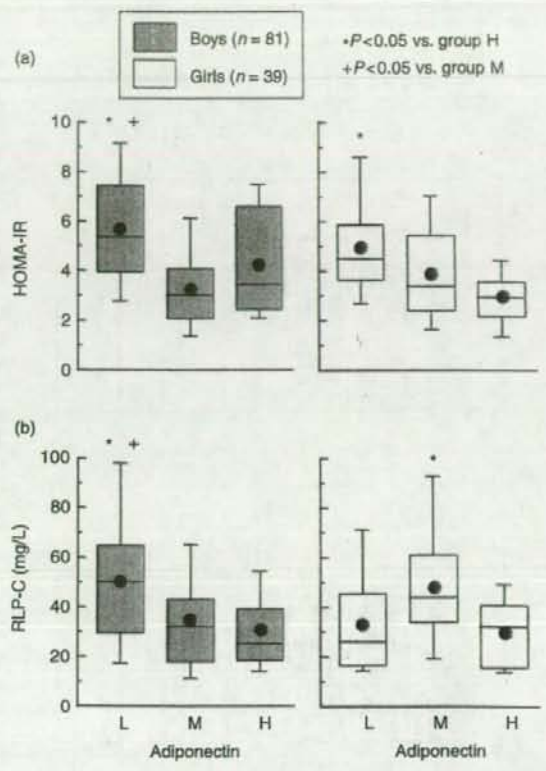


Figure 1 Homeostasis model assessment of insulin resistance (HOMA-IR) and remnant-like particle cholesterol (RLP-C) in obese E3/3 children according to sex and serum adiponectin concentration. Obese boys and girls with E3/3 were classified into tertiles according to their fasting adiponectin concentrations. HOMA-IR (a) was calculated from fasting plasma glucose and insulin concentrations. Fasting RLP-C concentrations (b) were measured by the detergent-based method as described in the Subjects and Methods. Upper and lower edges of the boxes indicate the 75th and 25th percentiles, respectively. The lines and closed circles in the boxes denote the median and mean values of the data. Upper and lower ends of the vertical lines indicate the 90th and 10th percentiles, respectively

in obese boys than in obese girls. In obese boys with E3/3, the low adiponectin group had slightly but significantly higher SBP and DBP concentrations compared with the middle and high adiponectin groups (Table 4). However, no significant difference was observed in BP among the three groups of obese girls. In obese boys, the low adiponectin group had the highest LDL-C (Figure 2a) and the lowest HDL-C concentrations (Figure 2b) of the three groups. The respective median values of TG and RLP-C were 43 and 69% higher in the low adiponectin group compared with those in the middle adiponectin group. In addition, the respective median values of TG and RLP-C were 56 and 96% higher in the low adiponectin group compared with those in the high adiponectin group (Table 4; Figure 1b). However, the median apoE concentration was very similar among the three groups (Table 4). In contrast, in obese girls with E3/3, a lower adiponectin concentration showed

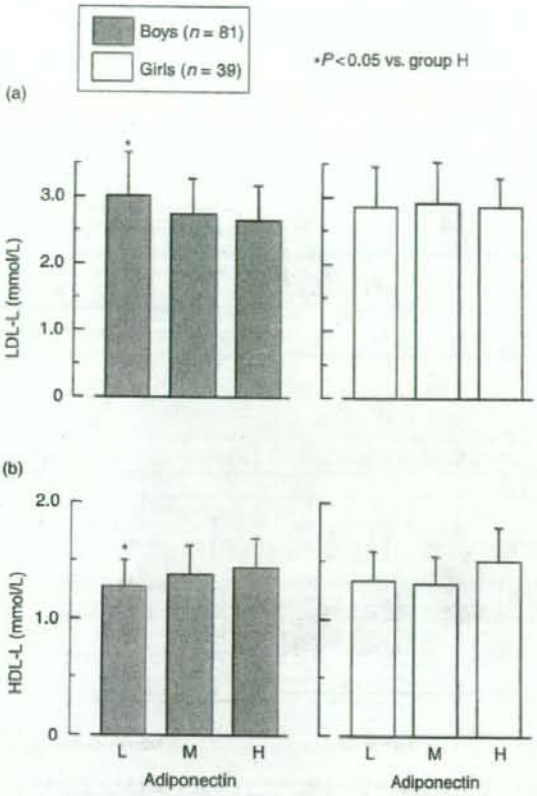


Figure 2 LDL-C and HDL-C concentrations in obese E3/3 children according to sex and serum adiponectin concentration. Obese boys and girls with E3/3 were classified into tertiles according to their fasting adiponectin concentrations. L, M, and H represent the low, middle, and high adiponectin groups, respectively. Fasting LDL-C (a) and HDL-C (b) concentrations were measured by homogenous assays. Data are presented as the mean ± SD

no significant association with any lipoprotein parameter (Table 4, Figures 1 and 2).

Multiple stepwise regression analysis was performed in obese boys and girls separately, using HOMA-IR as a dependent variable, and using age, adiponectin, apoE phenotype, POW, SBP and TG as independent variables. On account of collinearity, insulin, glucose, HDL-C, apoE, and RLP-C were excluded from the independent variables. These models yielded sufficient correlations in obese boys ($R = 0.660$, $P < 0.00001$) and obese girls ($R = 0.815$, $P < 0.00001$). In the obese children, adiponectin concentration was a significant determinant of HOMA-IR (Table 5). Age, TG and POW were also selected as independent variables for HOMA-IR.

Discussion

Our findings demonstrate that low adiponectin concentrations are associated with insulin resistance and downstream metabolic abnormalities in obese children. This association seems to be more evident in obese boys than girls. We found that the low adiponectin groups had greater HOMA-IR values than the middle and high adiponectin groups in obese children, independent of sex (Figure 1a; Table 5). Obese boys with E3/3 in the low adiponectin group had high blood pressure, LDL-C, TG and RLP-C, as well as low HDL-C; however, the corresponding obese girls demonstrated fewer clinical manifestations (Table 4; Figures 1 and 2).

Many studies have shown that the apoE phenotype is an important regulator of lipoprotein profiles.[13-18] Three common apoE alleles, ε2, ε3 and ε4, result in six major apoE phenotypes (E2/2, E3/2, E4/2, E3/3, E4/3 and E4/4). In addition, many apoE variants, including apoE5 and E7, have been identified.[25-28] Among them, E3/3 is the most frequent phenotype reported in every country. The prevalence of E3/3 in Japan is reported to be between 63.6 and 74.2%,[29-31] which is in accordance with our data (Table 1). E4 is associated with high LDL-C and low apoE concentrations, whereas E2 is associated with low LDL-C and high apoE concentrations.[13,15,17,18,29,32] E5 and E7 were first discovered in Japan by Yamamura *et al.*,[25,26] who found that both apoE variants were frequently associated with coronary artery disease (CAD) and hypercholesterolaemia. In our studies, one E5 carrier and four E7 carriers were detected in non-obese and obese children, respectively, and they tended to have hypercholesterolaemia.

Note that apoE phenotypes affected the lipoprotein profiles of obese children, but not non-obese children (Tables 2 and 3). Such inconsistencies are most likely attributable to differences in dietary fat and carbohydrate intake between the two groups. In young Finns aged 9–24 years,

Table 5 Multiple stepwise regression analysis for homeostasis model assessment of insulin resistance

Selected variables	Obese boys (n = 123)			Obese girls (n = 57)		
	Standardized regression coefficient	F value	P value	Standardized regression coefficient	F value	P value
Age	0.269	13.81	0.01	0.390	18.16	<0.001
Adiponectin	−0.174	5.65	0.02	−0.183	4.07	0.02
TG	0.141	3.85	0.1	0.390	18.99	0.02
POW	0.438	38.33	<0.001	0.300	10.98	<0.01

Multiple stepwise regression analysis was performed in obese boys and girls separately.
The F value to enter was set at 2.0 at each step. Sex was excluded from independent variables in this model
POW, a percentage of the standard weight for age, height and sex; TG, triglyceride; SBP, systolic blood pressure; apoE, apolipoprotein E