Table 6. Genetic combinations of HLA-DRB1-DQB1 haplotypes in Japanese children with Type 1A diabetes and their siblings

Genetic combination of HLA-DRB1-DQB1 haplotype	Type 1A n = 66	%	Siblings n = 83	%	Type 1A vs. Siblings p	Pc	OR	(95% CI)
I. Two susceptible haplotypes in homozygote	16	24.24	11	13.25	NS			
DR4/4 (*04:05-*04:01)	4	6.06	3	3.61	NS			
DR4/4 (*04:05-*03:02)	3	4.55	2	2.41	NS			
DR9/9 (*09:01-*03:03)	9	13.64	6	7.23	NS			
DR8/8 (*08:02-*03:02)	0	0.00	0	0.00	NS			
II. Two susceptible haplotypes in heterozygote	19	28.79	17	20.48	NS			
DR4/9	9	13.64	13	15.66	NS			
DR4/8	8	12.12	2	2.41	2.31E-02	NS		
DR9/8	2	3.03	2	2.41	NS			
III. One susceptible haplotype and no protective allele	20	30.30	15	18.07	NS			
DR4/X	9	13.64	5	6.02	NS			
DR9/X	10	15.15	7	8.43	NS			
DR8/X	1	1.52	3	3.61	NS			
IV. One susceptible haplotype and a protective allele	5	7.58	16	19.28	NS			
V. No susceptible haplotype and no protective allele	4	6.06	3	3.61	NS			
VI. No susceptible haplotype and a protective allele	2	3.03	21	25.30	1.50E-04	$<10^{-2}$	0.09	(0.02–0.41)

CI, confidence interval; OR, odds ratio.
Susceptible haplotype: *04:05-*04:01, *09:01-*03:03, *08:02-*03:02, *04:05-*03:02.
Protective allele in DRB1: *08:03, *15:01, *15:02, *04:06.
Protective allele in DQB1: *06:01, *06:02, *03:01.
X in DRB1: *01:01, *03:01, *04:01, *04:03,*04:04,*04:07, *04:10, *07:01,*10:01, *11:01, *11:06, *12:01, *12:02, *13:02, *16:02.
X in DQB1: *02:01, *04:02, *05:01, *05:02, *06:04, *06:09.

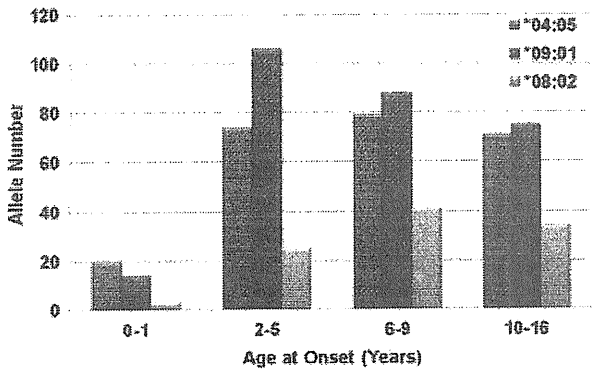

Fig. 1. DRB1 allele frequencies in four age groups of Japanese children with Type 1A diabetes according to the age at onset. The frequency of DRB1*09:01 was higher (Pc < 0.01) in the 2–5-year onset group, while the frequency of DRB1*08:02 tended to be higher in the 6–16-year onset group, although the difference was not significant. The distribution of the DRB1*04:05 allele frequency was not different among the four age groups.

childhood-onset type 1 diabetes mellitus in Japan. In the study, a TDT was performed for the first time in a population of Japanese children with Type 1A diabetes; the results confirmed the identities of susceptible and protective DRB1, DQB1, DPB1, A, C, and B, alleles.

We confirmed previously reported HLA-DRB1-DQB1 susceptible and protective haplotypes and obtained new findings regarding the DPB1 allele. DPB1*02:01 and DPB1*03:01 were identified as susceptible alleles among Japanese children with Type 1A diabetes (Table 1). DPB1*02:01 is unique to the Japanese population, while DPB1*03:01 is observed in multiple ethnic groups (4, 5, 16–19). This finding is noteworthy because neither the susceptible DRB1 allele nor the DQB1 allele is common to both Japanese and Caucasian populations. Moreover, the DPB1*02:01 and DPB1*03:01 alleles were not specifically associated with any susceptible DRB1-DQB1 haplotype. DPB1*04:02 was identified as a protective allele among Japanese children with Type 1A diabetes, similar to cases in multiple ethnic groups (4, 5, 16–19). Moreover, the DPB1*04:02 allele was not associated with any protective DRB1-DQB1 haplotypes. Of note, however, the association of DPB1*02:01, DPB1*03:01, and DPB1*04:02 with Type 1A diabetes was relatively weak in Japanese children, as the association was significant in the case-control study but not in the TDT. However, DPB1*09:01 was identified as a protective allele in both the case-control study and the TDT,

and DPB1*09:01 appeared to be associated with the protective DRB1*15:02-DQB1*06:01 haplotype, which is a major protective haplotype in the Japanese population but is rare in Caucasian populations.

The independent effects of HLA-A and B have been demonstrated in Caucasian populations (2, 4, 5). Following adjustment for LD to haplotypes at the DR-DQ region, both susceptible and protective alleles were found at HLA-A (e.g., A*24:02, susceptible allele; A*11:01, protective allele) and HLA-B (e.g., B*39:06, susceptible allele; B*57:01, protective allele) (4, 5). A*24:02 was a susceptible allele independent of the susceptible DRB1-DQB1 haplotypes among Japanese children with Type 1A diabetes. A*11:01 was also a protective allele among Japanese children with Type 1A diabetes. However, the association of A*24:02 and A*11:01 with Type 1A diabetes was relatively weak in the Japanese children, as the association was significant in the case-control study but not in the TDT (Tables 3 and 4). Of note, the B*39:06 and B*57:01 alleles were not observed in this study.

The analysis of LD between DRB1-DQB1 haplotypes and DPB1, A, C, or B alleles demonstrated both susceptible (C*08:01-B*40:06-DRB1*09:01-DQB1*03:03 and C*01:02-B*54:01-DRB1*04:05-DQB1*04:01) as well as protective (C*12:02-B*52:01-DRB1*15:02-DQB1*06:01-DPB1*09:01 and A*33:03-C*14:03-B*44:03) haplotypes among Japanese children with Type 1A diabetes.

In terms of genomic imprinting of the HLA-class II gene, several studies have been reported (26–29). In a Caucasian population, a striking feature of the data was that HLA-DR3/DR4 patients inherit their DR3 allele from their mother and the DR4 allele from their father more often than vice versa. Margaritte-Jeannin et al. (27) proposed that parental imprinting for a specific allelic combination may explain the HLA genotypes observed in the patients and their relatives. Sadauskaite-Kuehne et al. (28) also studied diabetes-associated allelic transmission rates from mothers and fathers to children with diabetes in 125 families in Lithuania, an area with a low incidence of type 1 diabetes. They reported that the DR4-DQB1*03:02-DQA1*03:01 haplotype was transmitted significantly more frequently from both parents, but that the DR3-DQB1*02:01-DQA1*05:01 haplotype was transmitted more frequently from only mothers. In Japan, Sasaki et al. (29) reported that maternal alleles in a susceptible DQA1*03:01-DQB1*03:02 haplotype showed a strong transmission disequilibrium with GADAb-positive type 1 diabetes, while paternal alleles in the same haplotype did not in 28 nuclear families, supporting the hypothesis that an epigenetic mechanism including genomic imprinting at the HLA-DQ region is involved in the pathogenesis and the genetic complexity of Japanese type 1 diabetes. However, none of the DRB1, DQB1, DPB1, A, C, or B alleles were preferentially transmitted from the mother or the father to the children with Type 1A diabetes in this study (Table 3). Our study suggests that the genomic imprinting of HLA-class II and class I genes is not involved in the pathogenesis of Type 1A diabetes in Japanese patients.

The frequency of subjects with two susceptible DRB1-DQB1 haplotypes was significantly higher among the children with Type 1A diabetes than among their parents. Of note, the frequencies of homozygosity for DRB1*09:01-DQB1*03:03 and of heterozygosity for DRB1*04:05-DQB1*04:01 and DRB1*08:02-DQB1*03:02 were significantly higher among children with Type IA diabetes, while the frequency of subjects with one susceptible haplotype and without a protective allele (group III) was not different between children with Type 1A diabetes and their parents. The frequencies of subjects with one susceptible haplotype and a protective allele (group IV) and with no susceptible haplotype and a protective allele (group VI) were lower among the children with Type 1A diabetes than among their parents (Table 5). These results suggest a dose effect of susceptible DRB1-DQB1 haplotypes and the effect of protective alleles.

The siblings of children with Type 1A diabetes may also represent a high-risk group for type 1 diabetes in the Japanese population, as the high prevalence (about 4%) of diabetes among Japanese siblings is comparable with that among Caucasian siblings (about 6%) (7, 8). The prevalences of the susceptible DRB1 and DQB1 alleles were similar between the children with Type 1A diabetes and their siblings. However, the prevalence of the protective DQB1*06:01 allele was higher among non-diabetic siblings. The frequency of group IV (no susceptible haplotype and a protective allele) was higher among the siblings than among the children with Type 1A diabetes. These results suggest the role of the protective allele among the siblings.

Only the allele frequency of DRB1*09:01 was significantly different among four age groups of Japanese children with Type IA diabetes determined according to the age at the time of onset (0–1, 2–5, 6–9, and 10–16 years). DRB1*09:01 may be strongly associated with an early onset in preschool children, whereas DRB1*08:02 may be weakly associated with a later onset in school-age children. Murao et al. (15) focused on the differences in the contributions of HLA-DR and -DQ haplotypes to the susceptibility to Type 1 diabetes during adulthood (later than 20 years of age) and childhood (1.0–18 years of age) in Japanese patients. They reported that the DRB1*09:01-DQB1*03:03 (DR9) frequency/DRB1*04:05-DQB1*04:01 (DR4) frequency increased with an increasing age of onset, and that another susceptible haplotype, DRB1*08:02-DQB1*03:02 (DR8), was involved only in the childhood-onset group. They did not mention any

difference among childhood-onset type 1 diabetes, and our results complement the data reported by Murao et al. The present results are also compatible with and complementary to our previous report, in which the frequency of the DR9 genotype was found to be significantly higher among a younger age group (0–10 years) than among an older age group (11–16 years) at the time of onset, and the frequency of DR4-DQ4 was higher in the older age group (11–16 years) (13).

Kawabata et al. (30) reported the age-related association of the MHC class I chain-related gene A and a marker in the class I C region with Japanese type 1 diabetes. However, this study did not show an association of susceptible class I A*24:02, C*01:02, C*08:01, or B*54:01 alleles with age at the time of onset in children with Type 1A diabetes (data not shown).

The amino acid residue at position 57 of the DQβ chain has been shown to play a key role in genetic susceptibility to type 1 diabetes. The lack of aspartic acid at this position at both DQ alleles is strongly associated with type 1 diabetes in Caucasian populations (31, 32). However, this Asp57 hypothesis is not tenable for Japanese type 1 diabetic patients (33). The influence of the HLA-DR and HLA-DQ molecules on the risk of type 1 diabetes is probably related to their central role in antigen presentation and the activation of a helper T cell-mediated immune response (2, 32). The HLA-class II and class I pocket structure is critical to the etiology of autoimmunity, as different pocket variants may have different affinities to the antigenic peptides of specific proteins from pancreatic β cells, including insulin and GAD; therefore, certain variants are more likely to present autoantigenic peptides to T cells than others (32, 34). In a future study, an analysis of how variations in amino acids, especially those found within the peptide-binding domains, are correlated with changes in disease risk would be valuable, providing a possible link between genetic association studies and the causal mechanism(s) of Type 1A diabetes.

In conclusion, this study demonstrated the characteristic association, which was mostly different but partly the same as that in Caucasian populations, of HLA-DRB1, DQB1, DPB1, and A, C, B, genes with Type 1A diabetes among Japanese children. A TDT did not reveal the genomic imprinting of HLA-class II and class I genes in Type 1A diabetes in the present population. A comparison of children with Type 1A diabetes and their parents and siblings suggested a dose effect of susceptible DRB1-DQB1 haplotypes and the effect of protective alleles on the immunological pathogenesis of Type 1A diabetes. These results may provide fundamental data for further genetic studies examining other immune-related and insulin resistance or beta cell function-related genes in Japanese patients with type 1 diabetes.

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Conflict of interest

No potential conflicts of interest relevant to this article were present.

References

1. Todd J, Bell JI, MacDevitt HO. HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 1987: 329: 599–604.
2. Pociot F, Akolkar B, Concannon P et al. Genetics of type 1 diabetes: What's next? Diabetes 2010: 50: 1561–1571.
3. Cox NJ, Wapelhorst B, Morrison VA et al. Seven regions of the genome show evidence of linkage to type 1 diabetes in a consensus analysis of 767 multiplex families. Am J Hum Genet 2001: 69: 820–830.
4. Howson JM, Walker NM, Clayton D, Todd JA. Confirmation of HLA class II independent type 1 diabetes associations in the major histocompatibility complex including HLA-B and HLA-A. Diabetes Obes Metab 2009: 11 (Suppl. 1): 31–45.
5. Nejentsev S, Howson JM, Walker NM et al. Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A. Nature 2007: 450: 887–892.
6. Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care 2000: 23: 1516–1526.
7. Thomson G, Robinson WP, Kuhner MK et al. Genetic heterogeneity, modes of inheritance, and risk estimates for a joint study of Caucasians with insulin-dependent diabetes mellitus. Am J Hum Genet 1988: 43: 799–816.
8. Ikegami H, Ogihara T. Genetics of insulin-dependent diabetes mellitus. Endocr J 1996: 43: 605–613.
9. Aparicio JMR, Wakisaka A, Takada A, Matsuura N, Aizawa M. HLA-DQ system and insulin-dependent diabetes mellitus in Japanese: does it contribute to the development of IDDM as it does in Caucasians? Immunogenetics 1988: 28: 240–246.
10. Kida K, Mimura G, Kobayashi T et al. Immunogenetic heterogeneity in type 1 (insulin-dependent) diabetes among Japanese HLA antigens and organ-specific autoantibodies. Diabetologia 1989: 32: 34–39.
11. Awata T, Kuzuya T, Matsuda A, Iwamoto Y, Kanazaw Y. Genetic analysis of HLA class II alleles and susceptibility to type 1 (insulin-dependent) diabetes

mellitus in Japanese subjects. Diabetologia 1992: 35: 419–424.
12. Ikegami H, Kawaguchi Y, Yamato E et al. Analysis by the polymerase chain reaction of histocompatibility leukocyte antigen-DR9-linked susceptibility to insulin dependent diabetes mellitus. J Clin Endocrinol Metab 1992: 75: 1381–1385.
13. Sugihara S, Sakamaki T, Konda S et al. Association of HLA-DR, DQ genotype with different β-cell function at IDDM diagnosis in Japanese children. Diabetes 1997: 46: 1893–1897.
14. Kawabata Y, Ikegami H, Kawaguchi Y et al. Asian-specific HLA haplotypes reveal heterogeneity of the contribution of HLA-DR and -DQ haplotypes to susceptibility to type 1 diabetes. Diabetes 2002: 51: 545–551.
15. Murao S, Makino H, Kaino Y et al. Differences in the contribution of HLA-DR and -DQ haplotypes to susceptibility to adult- and childhood-onset type 1 diabetes in Japanese patients. Diabetes 2004: 53: 2684–2690.
16. Erlich HA, Rotter JI, Chang JD et al. Association of HLA-DPB1*0301 with insulin dependent diabetes mellitus in Mexican-Americans. Diabetes 1996: 45: 610–614.
17. Cucca F, Dudbridge F, Loddo M et al. The HLA-DPB1–associated component of the IDDM1 and its relationship to the major loci HLA-DQB1, -DQA1, and -DRB1. Diabetes 2001: 50: 1200–1205.
18. Cruz TD, Valdes AM, Santiago A et al. DPB1allele are associated with type 1 diabetes susceptibility in multiple ethnic groups. Diabetes 2004: 53: 2158–2163.
19. Baschal EE, Aly TA, Babu SR et al. HLA-DPB1*0402 protects against type 1A diabetes autoimmunity in the highest risk DR3-DQB1*0201/DR4-DQB1 *0302 DAISY population. Diabetes 2007: 56: 2405–2409.
20. Nishimaki K, Kawamura T, Inada H et al. HLA DPB1*0201 gene confers disease susceptibility in Japanese with childhood onset type I ' diabetes, independent of HLA-DR and DQ genotypes. Diabetes Res Clin Pract 2000: 47: 49–55.
21. Akaza T, Imanishi T, Fujiwara K et al. HLA allele and haplotype frequencies in Japanese (The report of the 11th Japan HLA workshop). Transplantation Now Suppl 1994: 7: 87–101 (in Japanese).
22. Nakajima F, Nakamura J, Yokota T. Analysis of HLA haplotypes in Japanese, using high resolution allele typing. MHC 2002: 8: 1–32 (in Japanese).
23. Keicho N, Itoyama S, Kashiwase K et al. Association of human leukocyte antigen class II alleles with severe acute respiratory syndrome in the Vietnamese population. Human Immunology 2009: 70: 527–531.
24. Imanishi T, Akaza T, Kimura A et al. Estimation of Allele and Haplotype Frequencies for HLA and Complement Loci. In: Tsuji K et al. eds., HLA 1991. Oxford University Press, Tokyo, 1992, pp 76–79.
25. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 2001: 68: 978–989.
26. Deschamps I, Hors J, Clerget-Darpoux F et al. Excess of maternal HLA-DR3 antigens in HLA DR3, 4 positive type 1 (insulin-dependent) diabetic patients. Diabetologia 1990: 33: 425–430.
27. Margaritte-Jeannin P, Clerget-Darpoux F, Hors J, Deschamps I. Testing parental imprinting in insulin-dependent diabetes Mellitus by the marker-association-segregation-chi^2 method. Am J Hum Genet 1995: 56: 1080–1087.
28. Sadauskaite-Kuehne V, Veys K, Ludvigsson J, Padaiga ZV, Sanjeevi CB. Inheritance of MHC class II genes in Lithuanian families with type 1 diabetes. Ann NY Acad Sci 2003: 1005: 295–300.
29. Sasaki T, Nemoto M, Yamasaki K, Tajima N. Preferential transmission of maternal allele with DQA1*0301-DQB1*0302 haplotype to affected offspring in families with type 1 diabetes. J Hum Genet 1999: 44: 318–322.
30. Kawabata Y, Ikegami H, Kawaguchi Y et al. Age-related association of MHC class I chain-related gene A (MICA) with type 1 (insulin-dependent) diabetes mellitus. Hum Immunol 2000: 61: 624–629.
31. Morel PA, Dorman JS, Todd JA et al. Aspartic acid at position 57 of the HLA-DQ β chain protects against type I diabetes: A family study. Proc Natl Acad Sci USA 1988: 85: 8111–8115.
32. Kelly MA, Rayner ML, Mijovic CH et al. Molecular aspects of type 1 diabetes. J Clin Pathol: Mol Pathol 2003: 56: 1–10.
33. Awata T, Kuzuya T, Matsuda A et al. High frequency of aspartic acid at position 57 of HLA-DQ beta-chain in Japanese IDDM patients and nondiabetic subjects. Diabetes 1990: 39: 266–269.
34. Skowera A, Ellis RJ, Varela-Calvino R et al. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 2008: 118: 3390–3402.

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JCEM ONLINE. BRIEF REPORTS

Identification and Functional Analysis of Novel Human Growth Hormone Secretagogue Receptor (*GHSR*) Gene Mutations in Japanese Subjects with Short Stature

Hiroshi Inoue, Natsumi Kangawa, Atsuko Kinouchi, Yukiko Sakamoto, Chizuko Kimura, Reiko Horikawa, Yosuke Shigematsu, Mitsuo Itakura, Tsutomu Ogata, Kenji Fujieda† and on behalf of the Japan Growth Genome Consortium

Author Affiliations

Diabetes Therapeutics and Research Center (H.I.), The University of Tokushima, Tokushima 770-8503, Japan; Division of Genetic Information (H.I., N.K., A.K., Y.S., C.K., M.I.), Institute for Genome Research, The University of Tokushima, Tokushima 770-8503, Japan; Division of Endocrinology (R.H.), National Medical Center for Children and Mothers, Tokyo 157-8535, Japan; Fukui University Hospital (Y.S.), Fukui 910-8507, Japan; Department of Endocrinology and Metabolism (T.O.), National Research Institute for Child Health and Development, Tokyo 157-8535, Japan; and Department of Pediatrics (K.F.), Asahikawa Medical Collage, Asahikawa 078-8510, Japan

Address all correspondence and requests for reprints to: Dr. H. Inoue, Division of Genetic Information, Institute for Genome Research, The University of Tokushima, Kuramoto 3-18-15, Tokushima 770-8503, Japan. E-mail: hinoue@genome.tokushima-u.ac.jp.

Abstract

Context: Short stature (SS) is a multifactorial developmental condition with a significant genetic component. Recent studies have revealed that rare deleterious mutations in the GH-secretagogue receptor type 1A (*GHSR1A*) gene could be a cause of familial SS or GH deficiency.

Objective: The aim of this study was to evaluate the contribution of *GHSR1A* mutations to the molecular mechanism underlying SS in Japanese subjects.

Methods: We performed mutational screening of the *GHSR1A* gene in 127 unrelated Japanese SS patients diagnosed with either isolated GH deficiency or idiopathic SS. Identified mutations were analyzed in 188 control subjects, and their functional properties were examined in a heterologous expression system.

Results: Four novel heterozygous *GHSR1A* mutations were identified (ΔQ36, P108L, C173R, and D246A). Expression studies demonstrated that these mutations had varying functional consequences: 1) all mutations showed a loss-of-function effect on the constitutive signaling activity of GHSR1A, but the degree of loss varied widely; 2) C173R caused intracellular retention of the

mutated protein, resulting in total loss of receptor function; 3) P108L resulted in a large decrease in binding affinity to ghrelin, without affecting its surface expression; 4) D246A uniquely impaired agonist- and inverse agonist-stimulated receptor signaling; and 5) ΔQ36 showed only a subtle reduction in constitutive activity. The cumulative frequency of these putative functional mutations was significantly higher in the patient group than in controls (4.72 *vs.* 0.53%; $P = 0.019$; odds ratio = 9.28; 95% confidence interval, 1.10–78.0).

Conclusions: Our results suggest that *GHSR1A* mutations contribute to the genetic etiology of SS in the Japanese population.

Ghrelin exerts pleiotropic effects, including stimulation of GH secretion and enhancement of appetite, through binding and activation of the G protein-coupled GH-secretagogue receptor (GHSR) (1, 2). Two GHSR isoforms have been identified (3, 4); the primary GHSR1A product contains seven-transmembrane domains, whereas GHSR1B is an inactive form with five-transmembrane domains. In view of the ghrelin/GHSR pathway contributing to pituitary GH release, *GHSR1A* is a biological candidate for influencing/modulating height. However, recent genome-wide association studies (5, 6) as well as studies using selected haplotype-tagging single nucleotide polymorphisms (7, 8) did not provide evidence for association between common *GHSR1A* variants and adult or childhood height.

On the other hand, rare but functionally significant *GHSR1A* mutations were discovered in patients with familial short stature (SS) (9–11), thus shedding new light on the physiological importance of the ghrelin/GHSR system in somatic growth. Initially, two missense mutations, A204E and F279L, were identified in an obese patient and a SS child, respectively (9). A204E was subsequently found in two unrelated pedigrees with familial SS, showing a codominant mode of inheritance with incomplete penetrance and variable phenotypic expressivity (10). Functional characterization demonstrated that both mutant receptors had diminished or significantly reduced constitutive activities (CA), although they showed preserved ability to respond to ghrelin (10, 12) [a high constitutive ligand-independent signaling activity, up to ~50% of ligand-stimulated signaling, has been proven for GHSR1A in an *in vitro* setting (13, 14)]. More recently, the first case of compound heterozygosity for W2X and R237W was identified in an SS patient with partial GH deficiency (GHD) (11). In this case, transmission of the GHD phenotype suggested a recessive mode of inheritance. Expression studies showed that the nonsense W2X mutation favored complete loss-of-function, whereas R237W caused only a partial, but potentially important, loss of CA.

In this study, to facilitate elucidation of the molecular etiology of familial/genetic SS, we screened for *GHSR* mutations in a cohort of Japanese patients (n = 127) with isolated GHD or idiopathic SS.

Patients and Methods

All methods are described in more detail in the Supplemental Data (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Subjects

This study was approved by the Ethics Committee for Human Genome/Gene Research of the University of Tokushima. A total of 127 unrelated Japanese individuals, diagnosed with either isolated GHD (n = 14) or idiopathic SS (n = 113) according to established clinical criteria (10, 15), were recruited by the Japan Growth Genome Consortium, a research network of Japanese pediatric endocrinologists. Written informed consent was obtained from all participants. DNA from unrelated healthy Japanese individuals (n = 188) was used as the control.

Mutational analysis

The two *GHSR1A* coding exons were screened for mutations by sequencing (Supplemental Table 1). Frequencies of variant alleles in control subjects were determined by PCR-restriction fragment length polymorphism (Supplemental Table 2).

Transfection studies

Human *GHSR1A* cDNA was used to create either N-terminal hemagglutinin (HA)-tagged or C-terminal enhanced green fluorescent protein (EGFP)-tagged expression constructs (Supplemental Table 1). Mutations were introduced by site-directed mutagenesis. Receptor-mediated luciferase (*luc*) reporter gene assays were performed on transiently transfected HEK293A cells using either serum-responsive element (SRE)-*luc* or cAMP-responsive element-*luc* reporter (12). Whole-cell receptor binding assays were conducted using ^{125}I-labeled ghrelin. Both cell-surface and total protein expression levels of the HA-tagged receptor were determined by a cell-based ELISA (12). Immunoblotting was performed with a horseradish peroxidase-conjugated anti-HA antibody. For deglycosylation experiments, lysates were treated with endoglycosidase H or protein N-glycosidase F (PNGase F). The subcellular distribution of EGFP-tagged receptors was monitored by fluorescence microscopy. Indirect immunofluorescence was performed with an antibody against the endoplasmic reticulum (ER) marker calnexin.

Statistics

Data are presented as mean ± SD. Statistical significance was analyzed using Student's *t* test and Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Novel *GHSR* mutations

Eight *GHSR* sequence variants were identified (Table 1), which included: 1) four novel variants affecting amino acid residues common to both the 1A and 1B isoforms [ΔQ36 (a 3-bp in-frame deletion), P108L, C173R, and D246A; Supplemental Fig. 1, A and B]; 2) a missense substitution of the 1B-specific residue (A277P); and 3) three silent changes (G57G, L118L, and R159R). Heterozygous ΔQ36 was detected in three patients as well as one control. P108L, C173R, and D246A were rare, being found in only a single patient each, all in a heterozygous condition. A277P was detected in one patient and three controls. According to the SIFT and PolyPhen results, P108L and C173R were predicted as having a potentially damaging effect on protein function (Table 1).

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Table 1.

Summary information on *GHSR* variants detected in Japanese population

On the basis of these observations, we decided to focus our secondary efforts on the four novel *GHSR1A* mutations, ΔQ36, P108L, C173R, and D246A. The pedigrees of the six families carrying one of the selected mutations are shown in Supplemental Fig. 2. Little information was available regarding clinical and auxological variables for the propositi, their parents, and other family members. In the present cohort, the cumulative number of these alleles was six in 127 (4.72%) patients and one in 188 (0.53%) controls [$P = 0.019$ by two-tailed Fisher's exact test; odds ratio = 9.28; 95% confidence interval, 1.10–78.0; population attributable risk = 4.21%; 95% confidence interval, 0.37–8.06].

Functional characterization of *GHSR1A* mutations

Expression constructs encoding wild-type (WT) or mutant GHSR1A were used for transient expression in HEK293A cells and subsequent functional evaluation. In a SRE-*luc* reporter assay, when compared with WT or the two previously characterized mutant receptors [A204E and F279L (10, 12)], novel *GHSR1A* mutations displayed different signaling properties (Fig. 1A): 1) ΔQ36 displayed a partial but significant decrease in CA (67.5%) but showed comparable ghrelin (agonist)-induced activation and [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P(SPA) (inverse agonist)-induced inhibition; 2) P108L showed a significant decrease in CA (32.0%) and a normal response against ghrelin, but had less sensitivity to SPA; 3) C173R was devoid of CA with a complete lack of response to ghrelin; and 4) D246A had reduced but significant CA (58.8%) while displaying a significantly lower response to ghrelin (even at 1 μM) and a nearly normal response to SPA. We obtained essentially the same findings in the cAMP-responsive element-*luc* reporter assay (data not shown).

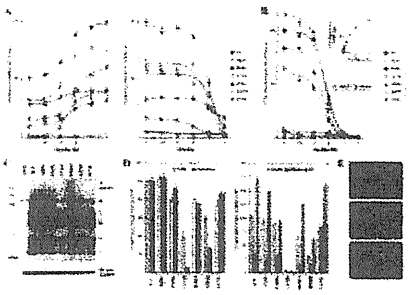

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Fig. 1.

Functional characterization of GHSR1A mutants. A, SRE-*luc* reporter gene assay. HEK293A cells were cotransfected with the WT or a mutant GHSR1A expression construct, pSRE-*luc* and pRL-TK plasmids, and subsequently stimulated with increasing concentrations of ghrelin (agonist; *left*) or SPA (inverse agonist; *right*). The WT receptor (*filled circles*) showed significant basal, ligand-independent CA, and treatment with ghrelin resulted in a concentration-dependent activity increase, whereas SPA inhibited CA. The two previously characterized mutant receptors, A204E (*open triangles*) and F279L (*filled inverted triangles*), showed greatly reduced CA (4.41 and 17.7%, respectively, compared with WT) but retained their ability to respond to ghrelin, these observations being consistent with previous findings (10, 12). The novel GHSR1A mutants displayed different signaling property patterns, as described in the text (ΔQ36, *open circles*; P108L, *filled squares*; C173R, *open squares*; and D246A, *filled triangles*). Transfection of vector alone resulted in no significant *luc* activity (data not shown). Results as compared with WT activity [arbitrarily set at 100, either treated with 1 μM ghrelin (*left*) or not treated (*right*)] are mean ± SD for at least four determinations. B, Whole-cell radioligand binding assay. In HEK293A cells transiently expressing the WT receptor, saturation receptor binding analysis using [^{125}I]ghrelin demonstrated a single class of high affinity, saturable binding sites with Kd of 0.43 nM, comparable to those reported in previous studies (depicted in the *inset*), whereas no specific binding was observed in cells transfected with vector alone (data not shown). Competition binding studies using 100 pM [^{125}I]ghrelin showed high affinity binding of ghrelin (IC_{50} = 7.07 nM) with the WT receptor (*filled circles*), whereas A204E (*open triangles*) displayed no detectable specific binding and F279L (*filled inverted triangles*) showed a small decrease in binding, by approximately 80%, but comparable affinity for ghrelin (IC_{50} = 3.34 nM). Newly identified GHSR1A mutants displayed variable competition binding results as described in the text (ΔQ36, *open circles*; P108L, *filled squares*; C173R, *open squares*; D246A, *filled triangles*). Results as compared with WT binding (arbitrarily set at 100), in the absence of unlabeled ghrelin, are mean ± SD for at least four determinations. C, Immunoblot (IB) analysis. N-terminally HA-tagged WT or mutated GHSR1A was transiently expressed in HEK293A cells, and whole-cell lysates were prepared. Equal protein amounts were resolved in SDS-PAGE, blotted, and probed with anti-HA antibody (*upper panel*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

expression was evaluated as the loading control (*lower panel*). Size markers (in kilodaltons) are to the *left of the blots*. Results are representative of at least three separate independent transfection experiments yielding similar results. *Arrows* indicate major protein bands of approximately 34, 46, and 62 kDa, and *bracket* indicates a broad high molecular-weight protein band migrating between 60 and 90 kDa. *Filled triangles*, nonspecific band. D, Cell-based ELISA. *Left*, HEK293A cells were transiently transfected with N-terminally HA-tagged WT or mutated GHSR1A expression constructs. The receptor amount was measured by whole-cell ELISA assays, either in permeabilized (for total receptors; *open columns*) or nonpermeabilized (for cell-surface receptors; *black columns*) cells. Data were normalized to the WT receptor expression value and are presented as mean ± SD from three independent experiments, each performed in quadruplicate. *Right*, To evaluate the effects of agonist and inverse agonist treatment on cell-surface receptor expression, at 24 h after transfection, the medium was replaced with serum-free medium (*open columns*) and medium containing either ghrelin (1 μM; *black columns*) or SPA (1 μM; *gray columns*), and the cells were then incubated for an additional 18 h. The cell-surface receptor amounts were quantified by whole-cell ELISA assays. Data were normalized to the WT receptor expression value under the nonstimulated condition and are mean ± SD of three independent experiments, each performed in quadruplicate. Note that ghrelin treatment of WT-expressing cells resulted in significant down-regulation of cell-surface receptor expression (to <40% of that in corresponding nonstimulated cells), whereas, as opposed to ghrelin treatment, surface expression of the WT receptor was significantly increased when cells were exposed to SPA (by approximately 1.7-fold). E, Double immunofluorescent staining. HEK293A cells were transiently transfected with C-terminally EGFP-tagged WT (*top panel*) or mutated GHSR1A construct, either C173R (*middle panel*) or A204E (*lower panel*). The cells were fixed, permeabilized, and processed for indirect immunofluorescent staining with an antibody against calnexin, an ER marker protein. *Green* fluorescence corresponds to EGFP-GHSR1A, and *red* corresponds to calnexin. *Yellow* represents colocalization of green and red. Nuclei stained with DAPI (4′,6-diamino-2-phenylindole) are shown in *blue*.

In whole-cell [^{125}I]ghrelin binding assays, *GHSR1A* mutations displayed variable competition binding results (Fig. 1B): 1) ΔQ36 showed a slight increase in binding (about 1.15-fold) with similar binding affinity to WT (IC_{50} = 4.38 and 7.07 nM for ΔQ36 and WT, respectively); 2) specific binding was virtually undetectable for P108L and C173R; and 3) D246A showed reduced binding (~60%), with comparable binding affinity (IC_{50} = 3.38 nM).

Western blot analysis of WT-expressing cells showed the existence of intense, multiple immunoreactive bands (Fig. 1C). The immunoblot patterns from endoglycosidase H- and PNGase F -digested samples (Supplemental Fig. 3) suggested that: 1) the approximately 34- and 62-kDa bands most likely represent the nonglycosylated monomeric and dimeric forms, respectively; 2) the approximately 46-kDa species is the core-glycosylated monomeric form located in the ER; and 3) the broad band migrating at 60–90 kDa corresponds to the mature, terminally glycosylated, dimeric, or oligomeric forms. The ΔQ36, P108L, D246A, and F279L receptors showed a distribution and intensity of immunoreactive bands essentially similar to WT. In contrast, both C173R and A204E exhibited a selective and profound loss of intensity of 60- to 90-kDa bands, with the former being more severely affected, whereas their approximately 46-kDa species were preserved to some extent (Fig. 1C). These changes appeared to be not associated with an alteration of mRNA levels (*e.g.* decreased transcription, reduced mRNA stability), because the *GHSR1A* transgene levels of transfected cells, as assessed by quantitative RT-PCR, were not significantly different from that of WT-expressing cells (data not shown).

The total protein expression levels of WT and mutant receptors, as assessed by a cell-based ELISA, were largely consistent with immunoblot results (Fig. 1D): 1) total cellular expression of ΔQ36 was equivalent to that of WT; 2) P108L, D246A, and F279L were expressed at a slightly reduced level (60–80%); 3) A204E exhibited significantly lower expression (<60%); and 4) C173R was expressed at the lowest level (<40%). Quantification of cell-surface receptors showed that, with respect to their total expression levels, ΔQ36, P108L, D246A, and F279L were expressed at levels approximately equal to that of WT. In contrast, C173R and A204E displayed either almost complete loss or significantly decreased surface expression (5.4 and 25.6%, respectively, compared with WT). In addition, ghrelin-induced down-regulation of ΔQ36, P108L, and D246A was comparable to that of WT, but was of a lesser degree for C173R and A204E (Fig. 1D). In contrast, the cell-surface expression of F279L did not change in response to ghrelin. The SPA-induced increase in cell surface expression of ΔQ36, D246A, A204E, and F279L, but not P108L and C173R, was comparable with that of WT.

The subcellular distributions of ΔQ36, P108L, D246A, and F279L, as monitored by fluorescence microscopy, were similar to that of WT (Supplemental Fig. 4). In contrast, both C173R and A204E displayed a fine reticular pattern of fluorescence extending from the perinuclear area and distributed throughout the cytoplasm. Double-immunofluorescent staining confirmed that the signals of C173R and A204E overlapped exclusively with that of the ER-marker, calnexin (Fig. 1E).

Discussion

We report herein the identification of four novel *GHSR1A* mutations. Functional characterization, as summarized in Supplemental Table 3, indicates that all the mutations are associated with a loss of CA, thus being consistent with a previous notion that reduced CA is responsible for SS phenotypes (10, 16). On the other hand, we found that the degree of loss could vary greatly, from only modest impairment to complete loss. Our results also highlight that *GHSR1A* mutations can have varying functional characteristics attributable to differences in their mutational mechanisms, *i.e.*: 1) P108L results in a large decrease in binding affinity to ghrelin; 2) C173R likely causes misfolding and aberrant ER retention; and 3) D246A leads to impaired agonist- and inverse agonist-stimulated receptor signaling. Notably, ΔQ36 showed only a subtle reduction in CA, thus raising the possibility that ΔQ36 may be a benign polymorphic variant. However, we cannot rule out the possibility of ΔQ36 having pathological significance, because this situation resembles the case of R237W (11), whose phenotype involves only partial loss of CA. We also provided additional information on previously identified mutations (9, 10, 12), *i.e.* A204E most likely interferes with normal intracellular trafficking resulting in ER retention, but to a much lesser extent than that of C173R, whereas F279L has impaired ability to undergo agonist-mediated receptor down-regulation, a control mechanism determining receptor responsiveness.

It should be mentioned that the *GHSR1A* mutations identified in this study occurred rarely and were each, except for ΔQ36, found only in one patient or a single family, all in a heterozygous condition, and thus their pathological significance in individual patients and families may not be sufficiently elucidated. We found the cumulative frequency of these mutations to be significantly higher in the patient group (4.72 *vs.* 0.53% in controls; $P = 0.019$), supporting that these account for a significant fraction of patients. However, obviously our sample size was small and this preliminary finding also needs to be replicated in a large and genetically homogeneous sample.

In summary, our data emphasize the importance of detailed characterization of the mutational mechanisms and functional consequences of individual *GHSR1A* mutations. Because the ghrelin/GHSR system exerts multiple biological actions in different cell types, the variability in the functional consequences of *GHSR1A* mutations may be associated with variable clinical phenotypes in patients.

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Footnotes

† This paper is dedicated to Professor Kenji Fujieda, who sadly passed away on March 19, 2010, during the completion of this work.

Abbreviations:

CA	Constitutive activity or activities
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
GHD	GH deficiency
GHSR	GH secretagogue receptor
HA	hemagglutinin
luc	luciferase
SPA	[D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P
SRE	serum-responsive element
SS	short stature
WT	wild-type.

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References

1. Kojima M, Kangawa K2005 Ghrelin: structure and function. *Physiol Rev* **85**:495–522
Abstract/FREE Full Text

2. Kojima M, Kangawa K2006 Drug insight: the functions of ghrelin and its potential as a multitherapeutic hormone. *Nat Clin Pract Endocrinol Metab* **2**:80–88 CrossRef Medline

3. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273**:974–977 Abstract

4. Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M2002 The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* **87**:2988 Abstract/FREE Full Text

5. Weedon MN, Lango H, Lindgren CM, Wallace C, Evans DM, Mangino M, Freathy RM, Perry JR, Stevens S, Hall AS, Samani NJ, Shields B, Prokopenko I, Farrall M, Dominiczak A, Johnson T, Bergmann S, Beckmann JS, Vollenweider P, Waterworth DM, Mooser V, Palmer CN, Morris AD, Ouwehand WH, Zhao JH, Li S, Loos RJ, Barroso I, Deloukas P, Sandhu MS, Wheeler E, Soranzo N, Inouye M, Wareham NJ, Caulfield M, Munroe PB, Hattersley AT, McCarthy MI, Frayling TM2008 Genome-wide association analysis identifies 20 loci that influence adult height. *Nat Genet* **40**:575–583 CrossRef Medline

6. Sanna S, Jackson AU, Nagaraja R, Willer CJ, Chen WM, Bonnycastle LL, Shen H, Timpson N, Lettre G, Usala G, Chines PS, Stringham HM, Scott LJ, Dei M, Lai S, Albai G, Crisponi L, Naitza S, Doheny KF, Pugh EW, Ben-Shlomo Y, Ebrahim S, Lawlor DA, Bergman RN, Watanabe RM, Uda M, Tuomilehto J, Coresh J, Hirschhorn JN, Shuldiner AR, Schlessinger D, Collins FS, Davey Smith G, Boerwinkle E, Cao A, Boehnke M, Abecasis GR, Mohlke KL2008 Common variants in the GDF5-UQCC region are associated with variation in human height. *Nat Genet* **40**:198–203 CrossRef Medline

7. Garcia EA, Heude B, Petry CJ, Gueorguiev M, Hassan-Smith ZK, Spanou A, Ring SM, Dunger DB, Wareham N, Sandhu MS, Ong KK, Korbonits M2008 Ghrelin receptor gene polymorphisms and body size in children and adults. *J Clin Endocrinol Metab* **93**:4158–4161 Abstract/FREE Full Text

8. Gueorguiev M, Lecoeur C, Benzinou M, Mein CA, Meyre D, Vatin V, Weill J, Heude B, Grossman AB, Froguel P, Korbonits M2009 A genetic study of the ghrelin and growth hormone secretagogue receptor (GHSR) genes and stature. *Ann Hum Genet* **73**:1–9 CrossRef Medline

9. Wang HJ, Geller F, Dempfle A, Schäuble N, Friedel S, Lichtner P, Fontenla-Horro F, Wudy S, Hagemann S, Gortner L, Huse K, Remschmidt H, Bettecken T, Meitinger T, Schäfer H, Hebebrand J, Hinney A2004 Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight and underweight students, and children with short normal stature. *J Clin Endocrinol Metab* **89**:157–162 Abstract/FREE Full Text

10. Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M, Kadiri A, Epelbaum J, Le Bouc Y, Amselem S2006 Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J Clin Invest* **116**:760–768 CrossRef Medline

11. Pantel J, Legendre M, Nivot S, Morisset S, Vie-Luton MP, le Bouc Y, Epelbaum J, Amselem S2009 Recessive isolated growth hormone deficiency and mutations in the ghrelin receptor. *J Clin Endocrinol Metab* **94**:4334–4341 Abstract/FREE Full Text

12. Liu G, Fortin JP, Beinborn M, Kopin AS2007 Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities. *J Pharmacol Exp Ther* **322**:1036–1043 Abstract/FREE Full Text

13. Holst B, Cygankiewicz A, Jensen TH, Ankersen M, Schwartz TW2003 High constitutive signaling of the ghrelin receptor—identification of a potent inverse agonist. *Mol Endocrinol* **17**:2201–2210 Abstract/FREE Full Text

14. Holst B, Holliday ND, Bach A, Elling CE, Cox HM, Schwartz TW2004 Common structural basis for constitutive activity of the ghrelin receptor family. *J Biol Chem* **279**:53806–53817 Abstract/FREE Full Text

15. Ranke MB1996 Towards a consensus on the definition of idiopathic short stature. *Horm Res* **45**(Suppl 2):64–66 **Medline**

16. Holst B, Schwartz TW2006 Ghrelin receptor mutations—too little height and too much hunger. *J Clin Invest* **116**:637–641 **CrossRef** **Medline**

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ORIGINAL ARTICLE

Prediction of pregnancy-induced hypertension by a shift of blood pressure class according to the JSH 2009 guidelines

Seung Chik Jwa[1,2], Naoko Arata[3], Naoko Sakamoto[2], Noriyoshi Watanabe[1], Hiroaki Aoki[1], Asako Kurauchi-Mito[3], Qiu Dongmei[2], Yukihiro Ohya[4], Atsuhiro Ichihara[5,6] and Michihiro Kitagawa[1,6]

Elevated blood pressure (BP) at early or mid pregnancy is a known risk factor for pregnancy-induced hypertension (PIH). However, the association between BP changes during the first half of pregnancy and subsequent PIH development is unknown. We used changes in maternal BP between 16 and 20 weeks of gestation to evaluate the risk of PIH. A total of 976 pregnant women with BP estimations recorded before 16 weeks and at 20 weeks of gestation participated in this study. BPs were classified by the Japanese Society of Hypertension 2009 Hypertension Treatment Guidelines (JSH 2009). There was a significant trend for future PIH in women whose JSH 2009 BP class increased between 16 and 20 weeks of gestation, and the risk of PIH was highest among women whose BP was Class IV Hypertension (systolic BP ⩾ 140 mm Hg and/or diastolic BP ⩾ 90 mm Hg). The risk of PIH increased in women whose BPs shifted from Classes I Optimal (systolic BP < 120 mm Hg and diastolic BP < 80 mm Hg) and II Normal (systolic BP 120–129 mm Hg and/or diastolic BP 80–84 mm Hg) before 16 weeks to Class III High-Normal (systolic BP 130–139 mm Hg and/or diastolic BP 85–89 mm Hg) at 20 weeks of gestation. These shifts in BP class were significantly correlated with the risk of PIH after adjustments for variables (*P*-value for trend < 0.05). Within JSH 2009 Classes I, II and III, a shift in BP from a low to a high class between 16 and 20 weeks of gestation predicts the subsequent development of PIH.
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Keywords: blood pressure; prediction; pregnancy-induced hypertension; risk factor

INTRODUCTION

Pregnancy-induced hypertension (PIH) refers to high blood pressure (BP) during pregnancy. PIH affects 3–10% of all pregnancies[1–3] and is associated with high levels of maternal, fetal, and neonatal morbidity and mortality.[1,4,5] Furthermore, the long-term prognosis of women with a history of PIH includes increased risks of cerebrovascular disease, ischemic heart disease and renal disease.[6–12] These data indicate that the early identification, and subsequent monitoring and management of PIH are critical for maternal and fetal well-being.

In normotensive women, BP in early pregnancy decreases up to 20 weeks of gestation, and gradually increases to normal or higher than pre-pregnancy levels before delivery.[13,14] A diagnosis of PIH includes a BP > 140/90 mm Hg in the late second or third trimester. Previous studies described successful screening for PIH development following a single estimation of maternal BP. However, the false positive rate and sensitivity of these studies varied widely, from 7 to 52% and 8 to 93%,[15–19] respectively, indicating that this method is not sufficient for effective PIH prediction. In contrast, systematic monitoring of changes in BP during the early to mid stages of pregnancy may predict the development of PIH more exactly. Systematic sampling with 48-h ambulatory BP monitoring indicated that PIH was associated with a stable BP in the first half of gestation and a greater increase to delivery than in healthy pregnancies.[20] In addition, the development of PIH in women with low education levels was related to the absence of a significant fall in diastolic BP at mid pregnancy compared with healthy pregnancies in women with higher education.[21] These data indicate that information describing the changes in BP during early to mid pregnancy may be more predictive of subsequent PIH development than data recorded at a single measurement.

In the present study, we examined whether BP changes from early to mid gestational age are capable of predicting the development of PIH. BP was classified according to the JSH 2009 for easy clinical use.[22]

[1]Department of Maternal-Fetal and Neonatal Medicine, National Center for Child Health and Development, Tokyo, Japan; [2]Department of Social Medicine, National Research Institute for Child Health and Development, National Center for Child Health and Development, Tokyo, Japan; [3]Department of Women's Health, National Center for Child Health and Development, Tokyo, Japan; [4]Department of Medical Specialties, National Center for Child Health and Development, Tokyo, Japan and [5]Department of Medicine II, Institute of Endocrinology and Hypertension, Tokyo Women's Medical University, Tokyo, Japan
[6]These authors contributed equally to this paper
Correspondence: Professor A Ichihara, Department of Medicine II, Institute of Endocrinology and Hypertension, Tokyo Women's Medical University, Tokyo 162-8666, Japan.
E-mail: atzichi@endm.twmu.ac.jp
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METHODS

Study population and design

Our investigations are part of the Tokyo-Children's Health, Illness and Development Study. This is a unicenter, longitudinal, prospective observational birth cohort study conducted at the National Center for Child Health and Development (Tokyo, Japan). The aims of this cohort study are: (1) to investigate the influence of maternal weight gain and nutrition during pregnancy on the birth weight, growth and development of infants, (2) to identify the influence of maternal environment on the development of childhood allergies, (3) to study the influence of the prenatal and perinatal environment (fetus) on the psychological and psychiatric development of the infant, (4) to study the influence of parental attitude and knowledge about child rearing on the parent–child relationship and the development of children and (5) to determine the feasibility of using an electronic medical record system to conduct a birth cohort study. The participants in the Tokyo-Children's Health, Illness and Development Study were recruited at their first antenatal visit, before 16 weeks of gestation, from October 2003 to December 2005. Institutional review boards at the National Center for Child Health and Development approved our investigations.

We used the cohort data to analyze the relationship between BP changes during the first half of pregnancy and the onset of PIH. Our inclusion criteria accepted only participants with BP estimations recorded before 16 weeks and at 20 weeks of gestation (18–22 weeks of gestation), and who delivered at our institution after 22 weeks. Subjects with multiple gestations, pre-existing hypertension and pre-existing proteinuria were excluded. A total of 1019 women were initially included, from which 43 cases were excluded because of mismatched selection criteria and loss of information; therefore, data from 976 women were used in our analyses (Figure 1).

Measurement and classification of BP

After 5 min rest, BP was measured in the sitting position with the right arm held at heart level, using an automated sphygmomanometer (Omron BP-203RVIII oscillometer; Nippon Colin, Tokyo, Japan). BP monitoring was performed at two time points: before 16 weeks and between 18 and 22 weeks of gestation. If BP was measured on several occasions before 16 weeks, the average systolic and diastolic values were evaluated.

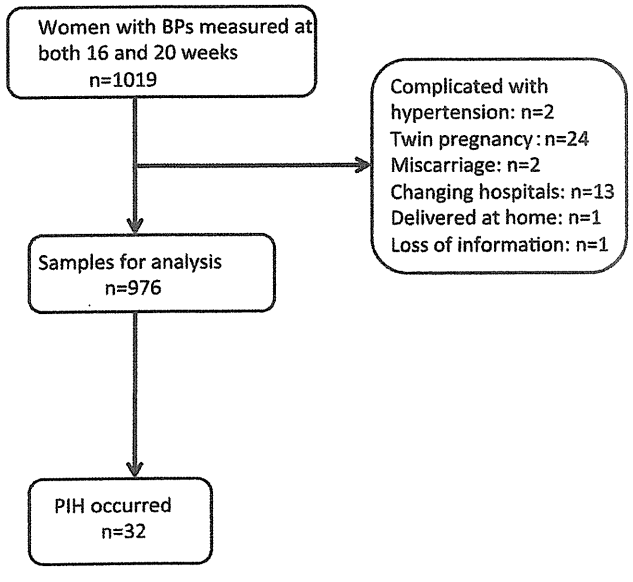

Figure 1 Flow diagram showing sample selection for our analyses. A total of 1019 pregnant women with BP estimations recorded before 16 weeks and at 20 weeks of gestation (18–22 weeks of gestation) were initially included in the study; 976 subjects were enrolled because of mismatched selection criteria and loss of information. Pregnancy-induced hypertension occurred in 32 women. BP, blood pressure; PIH, pregnancy-induced hypertension.

BPs were stratified into four groups based on Japanese Society of Hypertension 2009 Hypertension Treatment Guidelines (JSH 2009):[22] Class I (Optimal), systolic BP < 120 mm Hg and diastolic BP < 80 mm Hg; Class II (Normal), systolic BP 120–129 mm Hg and/or diastolic BP 80–84 mm Hg; Class III (High-Normal), systolic BP 130–139 mm Hg and/or diastolic BP 85–89 mm Hg; Class IV (Hypertension), systolic BP ⩾ 140 or diastolic BP ⩾ 90 mm Hg.

Definition of PIH

PIH was defined according to Guideline 2009 for care and treatment of hypertension in pregnancy by the Japan Society of the Study of Hypertension in Pregnancy[23] as: 'hypertension with or without proteinuria occurring after 20 weeks of gestation but resolving by twelve weeks postpartum.'. We excluded superimposed PIH, defined as: 'pre-existing hypertension with new onset of proteinuria after 20 weeks of gestation, or pre-existing proteinuria with new onset of hypertension.'

Other baseline data

Information describing sociodemographic, medical and behavioral data, past medical history, previous pregnancy complications, family history of hypertension or diabetes mellitus, smoking, education, family income and delivery were collected from the database of the cohort study.

Statistical analysis

Student's *t*-test and Mann–Whitney *U*-test were performed for analysis between two continuous variables, and χ^2-test or Fisher's exact test was used for discrete variables. The influence of BP class on the development of PIH was assessed by multiple logistic regression analysis. The probability of PIH occurrence was determined from the shifts in BP classes between 16 and 20 weeks of gestation and evaluated with odds ratios and *P*-values. All analyses were performed with the SPSS software (version 18 for Windows; SPSS, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Baseline patient characteristics are shown in Table 1. In 976 participants, 32 of the index pregnancies (3.3%) were eventually complicated with PIH. There were no significant differences in the gestational ages of participants at the times of BP monitoring. Maternal age, maternal pre-existing diabetes mellitus and previous history of PIH were significantly different between the PIH and non-PIH groups. Other variables (pre-pregnancy body mass index, rate of nulliparity, maternal pre-existing renal disease, previous pregnancy history of fetal growth restriction and placental abruption, rate of smoking, educational levels, distribution of family income, family history of diabetes mellitus, hypertension, ischemic heart disease, cerebrovascular stroke, chronic renal disease) were similar.

Pregnancy and delivery outcomes

Pregnancy and delivery outcomes in the non-PIH and PIH groups are shown in Table 2. The gestational age of delivery was significantly lower and the rate of preterm delivery was higher in the PIH group compared with non-PIH subjects. The rate of normal vaginal delivery was significantly lower and the rate of instrumental delivery was higher in the PIH group. The frequency of cesarean section (both planned and emergency) was similar between both groups. Placental weight and neonatal birth weight were significantly lower in the PIH group, and the number of neonates with an Apgar score of ⩽7 at 5 min was significantly higher.

Analysis for PIH risk based on BP classification

Table 3 shows the crude and adjusted odds ratios, and confidence intervals of PIH occurrence according to class of BP (based on JSH

Table 1 Baseline characteristics of the PIH and non-PIH groups

Characteristics		*All (n=976)*	*PIH group (n=32)*	*non-PIH group (n=944)*	*P-value*
Maternal age (years)	mean (s.d.)	33.6 (4.1)	35.3 (4.5)	33.5 (4.1)	0.028
Maternal height (cm)	mean (s.d.)	159.4 (5.1)	158.7 (5.5)	159.4 (5.1)	NS
Maternal pre-pregnancy body weight (kg)	mean (s.d.)	51.3 (6.5)	52.0 (8.0)	51.3 (6.5)	NS
Pre-pregnancy BMI ($kg\,m^{-2}$)	mean (s.d.)	20.2 (2.4)	20.6 (2.4)	20.2 (2.4)	NS
Parity					
0	*n* (%)	489 (50.1)	19 (59.4)	470 (49.8)	NS
≧1	*n* (%)	487 (49.9)	13 (40.6)	474 (50.2)	
Mean gestational age before 16 weeks blood pressure	mean (s.d.)	14.3 (1.0)	14.3 (1.0)	14.2 (1.1)	NS
Mean gestational age at 20 weeks blood pressure	mean (s.d.)	20.0 (1.2)	20.0 (1.2)	20.1 (1.2)	NS
Maternal pre-pregnancy complications					
Diabetes mellitus	*n* (%)	6 (0.6)	2 (6.3)	4 (0.4)	0.014
Renal disease	*n* (%)	2 (0.2)	1 (3.1)	1 (0.1)	NS
Previous pregnancy complications					
PIH	*n* (%)	12 (1.2)	4 (12.5)	8 (0.8)	<0.001
Fetal growth restriction	*n* (%)	5 (0.5)	1 (3.1)	4 (0.4)	NS
Placental abruption	*n* (%)	2 (0.2)	1 (3.1)	1 (0.1)	NS
Smoking					
Never or former	*n* (%)	944 (96.9)	31 (96.9)	913 (96.9)	NS
Current	*n* (%)	30 (3.1)	1 (3.1)	29 (3.1)	
Education (high school or less)	*n* (%)	94 (10.1)	4 (12.9)	90 (10.0)	NS
Income (per year)					
<4 million yen	*n* (%)	55 (6.2)	3 (10.0)	52 (6.0)	NS
<6 million yen	*n* (%)	202 (22.6)	6 (20.0)	196 (22.7)	
<8 million yen	*n* (%)	198 (22.2)	9 (30.0)	189 (21.9)	
<10 million yen	*n* (%)	192 (21.5)	4 (13.3)	188 (21.8)	
over 10 million yen	*n* (%)	246 (27.5)	8 (26.7)	238 (27.6)	
Family History					
Diabetes mellitus	*n* (%)	73 (7.5)	0 (0.0)	73 (7.7)	NS
Hypertension	*n* (%)	72 (7.4)	4 (12.5)	68 (7.2)	NS
Ischemic heart disease	*n* (%)	38 (3.9)	1 (3.1)	37 (3.9)	NS
Cerebrovascular stroke	*n* (%)	18 (1.8)	0 (0.0)	18 (1.9)	NS
Chronic renal disease	*n* (%)	8 (0.8)	0 (0.0)	8 (0.8)	NS

Abbreviations: BMI, body mass index; NS, not significant; PIH, pregnancy-induced hypertension; s.d., standard deviation.

2009) before 16 weeks of gestation. Although the risk of PIH was significantly higher in Class III and IV subjects without adjustments for any variables, these risks became insignificant after variables were accounted for. However, the trend of PIH occurrence was statistically significant, regardless of any adjustments.

Table 4 demonstrates the crude and adjusted odds ratios of PIH occurrence based on BPs at 20 weeks of gestation. The risk of PIH was significantly greater in all Class II, III and IV subjects with or without adjustments for variables.

BP class shift from 16 weeks to 20 weeks gestation and the risk of PIH occurrence

Table 5 shows the shifts in BP classes between 16 and 20 weeks of gestation, and their associations with PIH occurrence. Odds ratios and 95% confidence intervals were calculated based on each BP class before 16 weeks of gestation. Women with Class IV (Hypertension) BPs before 16 or at 20 weeks of gestation were excluded, because they were already considered high risk for PIH as indicated in Tables 3 and 4.

The subjects with BPs that did not shift class between 16 and 20 weeks of gestation were referred to as baseline. The risk of PIH occurrence was significantly higher in subjects whose BP shifted from Class I at 16 weeks to Class III at 20 weeks of gestation. The risk of PIH occurrence was not statistically significant in subjects whose BP shifted from Class I to II at 16 and 20 weeks of gestation, respectively; however, the trend for PIH risk was significant (*P*-value for trend <0.05) and remained significant even after adjustments for all variables. When comparing two groups, one in which the BP class elevated to Class II or III at 20 weeks gestation and the other in which the BP class did not change, the sensitivity, false positive rate and positive predictive value of BP class elevation between 16 and 20 weeks of gestation were 33.3, 10.8 and 7.4%, respectively. The risk of PIH occurrence was significantly higher in subjects whose BP shifted from Class II at 16 weeks to Class III at 20 weeks of gestation, although this

Table 2 Pregnancy and delivery outcomes of the PIH and non-PIH groups

		All (n=976)	*PIH group (n=32)*	*non-PIH group (n=944)*	P-*value*
Gestational age (weeks)	mean (s.d.)	39.1 (1.8)	37.8 (1.9)	39.1 (1.7)	<0.001
<37	*n* (%)	59 (6.0)	11 (34.4)	48 (5.1)	<0.001
≧37	*n* (%)	917 (94.0)	21 (65.6)	896 (94.9)	
Stillbirth	*n* (%)	2 (0.2)	1 (3.1)	1 (0.1)	NS
Delivery mode					
Normal vaginal delivery	*n* (%)	614 (62.9)	11 (34.4)	603 (63.9)	<0.001
Instrumental delivery	*n* (%)	152 (15.6)	11 (34.4)	141 (14.9)	0.01
Total Cesarean section	*n* (%)	208 (21.3)	10 (31.3)	198 (21.0)	NS
Planned cesarean section	*n* (%)	124 (12.7)	6 (18.8)	118 (12.5)	NS
Emergency cesarean section	*n* (%)	84 (8.6)	4 (12.5)	80 (8.5)	NS
Placental weight (g)	mean (s.d.)	558.4 (104.6)	513.0 (100.9)	560.0 (104.4)	0.022
Birth weight (g)	mean (s.d.)	3003.6 (421.8)	2618.7 (538.7)	3016.6 (411.3)	<0.001
Head circumference (cm)	mean (s.d.)	33.1 (1.4)	32.4 (1.7)	33.2 (1.4)	0.007
Chest circumference (cm)	mean (s.d.)	31.5 (1.7)	30.0 (2.0)	31.6 (1.7)	<0.001
Apgar score at 5 min of ≦7	*n* (%)	18 (1.8)	3 (9.4)	15 (1.6)	0.02

Abbreviations: NS, not significant; PIH, pregnancy-induced hypertension; s.d., standard deviation.

Table 3 Unadjusted and multivariable adjusted ORs (95% CIs) of PIH occurrence classified by blood pressure before 16 weeks of gestation

	<16 weeks blood pressure OR (95% CI)				
Classification	*I*	*II*	*III*	*IV*	
n	*713*	*189*	*59*	*15*	
PIH	*18*	*7*	*5*	*2*	P *for trend*
Unadjusted	1	1.49 (0.61–3.61)	3.58 (1.28–10.00)	5.94 (1.25–28.28)	0.003
Age adjusted	1	1.51 (0.62–3.68)	3.25 (1.15–9.19)	5.15 (1.07–24.90)	0.006
Age+BMI adjusted	1	1.52 (0.62–3.71)	3.26 (1.12–9.54)	5.17 (1.03–25.94)	0.008
age+BMI+parity adjusted	1	1.51 (0.62–3.71)	3.13 (1.06–9.21)	5.59 (1.09–28.60)	0.009
Age+BMI+pre-existing DM adjusted	1	1.55 (0.63–3.83)	3.25 (1.08–9.77)	5.81 (1.16–29.16)	0.007
Age+BMI+pre-existing renal disease adjusted	1	1.62 (0.66–3.99)	3.49 (1.19–10.28)	5.56 (1.10–27.99)	0.005
Age+BMI+family history of HTN adjusted	1	1.51 (0.61–3.69)	3.22 (1.10–9.49)	5.53 (1.10–27.89)	0.008
Age +BMI+previous history of PIH adjusted	1	1.38 (0.55–3.45)	2.80 (0.92–8.47)	3.86 (0.70–21.36)	0.032
Fully adjusted (all above)	1	1.50 (0.59–3.82)	2.71 (0.85–8.63)	5.41 (0.90–32.50)	0.025

Abbreviations: BMI, body mass index; CI, confidence interval; DM, diabetes mellitus; HTN, hypertension; OR, odds ratio; PIH, pregnancy-induced hypertension.

Table 4 Unadjusted and multivariable adjusted ORs (95% CIs) of PIH occurrence classified by blood pressure at 20 weeks of gestation

	20 weeks blood pressure OR (95% CI)				
Classification	*I*	*II*	*III*	*IV*	
n	*782*	*143*	*44*	*7*	
PIH	*14*	*7*	*9*	*2*	P *for trend*
Unadjusted	1	2.82 (1.12–7.12)	14.11 (5.72–34.81)	21.94 (3.92–122.90)	<0.001
Age adjusted	1	2.78 (1.10–7.05)	13.57 (5.45–33.78)	19.10 (3.32–109.76)	<0.001
Age+BMI adjusted	1	2.89 (1.14–7.36)	14.84 (5.76–38.37)	21.02 (3.57–123.61)	<0.001
Age+BMI+parity adjusted	1	2.84 (1.11–7.24)	14.66 (5.68–37.84)	19.34 (3.23–115.71)	<0.001
Age+BMI+pre-existing DM adjusted	1	2.94 (1.15–7.50)	13.94 (5.29–36.73)	22.76 (3.85–134.50)	<0.001
Age +BMI+pre-existing renal disease adjusted	1	2.95 (1.16–7.54)	13.94 (5.31–36.62)	21.63 (3.66–127.85)	<0.001
Age+BMI+family history of HTN adjusted	1	2.88 (1.13–7.34)	14.40 (5.53–37.46)	21.58 (3.66–127.38)	<0.001
Age +BMI+previous history of PIH	1	2.98 (1.15–7.76)	13.96 (5.22–37.31)	23.50 (3.95–140.02)	<0.001
Fully adjusted (all above)	1	3.01 (1.14–7.98)	11.72 (4.13–33.26)	24.14 (3.81–152.99)	<0.001

Abbreviations: BMI, body mass index; CI, confidence interval; DM, diabetes mellitus; HTN, hypertension; OR, odds ratio; PIH, pregnancy-induced hypertension.

Table 5 Blood pressure class shift from 16 weeks to 20 weeks of gestation and the risk of PIH occurrence

	20 weeks (no. of PIH(+)/PIH(−))			
16 weeks	*I*	*II*	*III*	P *for trend*
I	12/619	3/65	3/10	<0.001
II	2/120	2/52	3/9	0.001
III	0/26	2/15	2/13	0.081
I	*12/619*	*3/65*	*3/10*	P*<0.001*
BP Class I before 16 weeks gestation (OR (95% CI))				
Unadjusted	1	2.38 (0.65–8.65)	15.47 (3.77–63.45)	0.001
Age adjusted	1	2.40 (0.66–8.74)	16.44 (3.96–68.34)	0.001
Age+BMI adjusted	1	2.40 (0.66–8.79)	16.47 (3.85–70.56)	0.001
Age+BMI+parity adjusted	1	2.43 (0.66–8.97)	14.78 (3.38–64.73)	0.002
Age+BMI+pre-existing DM adjusted	1	2.26 (0.604–8.44)	17.06 (3.97–73.33)	0.001
Age+BMI+pre-existing renal disease adjusted	1	2.47 (0.67–9.08)	12.98 (2.70–62.32)	0.009
Age+BMI+family history of HTN adjusted	1	2.40 (0.65–8.80)	16.63 (3.84–71.99)	0.001
Age +BMI+previous history of PIH adjusted	1	2.60 (0.70–9.63)	17.81 (4.11–77.08)	0.001
Fully adjusted (all above)	1	2.35 (0.58–9.47)	13.01 (2.64–64.16)	0.010
II	*2/120*	*2/52*	*4/9*	P*<0.001*
BP Class II before 16 weeks of gestation (OR (95% CI))				
Unadjusted	0.43 (0.06–3.16)	1	8.67 (1.27–159.35)	0.002
Age adjusted	0.45 (0.06–3.28)	1	7.40 (1.04–52.35)	0.003
Age+BMI adjusted	0.43 (0.06–3.18)	1	7.73 (1.08–55.54)	0.002
Age+BMI+parity adjusted	0.38 (0.05–2.82)	1	8.13 (1.06–62.26)	0.002
Age+BMI+pre-existing DM adjusted	0.4 (0.05–3.00)	1	4.78 (0.54–42.00)	0.008
Age+BMI+pre-existing renal disease adjusted	NA	NA	NA	NA
Age+BMI+family history of HTN adjusted	0.42 (0.06–3.15)	1	13.65 (1.64–113.53)	0.001
Age +BMI+previous history of PIH adjusted	0.27 (0.03–2.55)	1	8.21 (1.12–60.07)	0.001
Fully adjusted (all above)	0.22 (0.02–2.25)	1	8.44 (0.81–87.72)	0.008
III	*0/26*	*2/15*	*2/13*	P*=0.081*
BP Class III before 16 weeks of gestation (OR (95% CI))				
Unadjusted	NA	0.87 (0.11–7.05)	1	0.114
Age adjusted	NA	0.64 (0.06–6.28)	1	0.066
Age+BMI adjusted	NA	0.71 (0.07–7.62)	1	0.079
Age+BMI+parity adjusted	NA	0.72 (0.07–7.78)	1	0.080
Age+BMI+pre-existing DM adjusted	NA	0.43 (0.04–5.33)	1	0.053
Age+BMI+pre-existing renal disease adjusted	NA	NA	NA	NA
Age+BMI+family history of HTN adjusted	NA	0.44 (0.03–6.41)	1	0.086
Age +BMI+previous history of PIH adjusted	NA	0.74 (0.07–8.08)	1	0.083
Fully adjusted (all above)	NA	NA	NA	NA

Abbreviations: BMI, body mass index; BP, blood pressure; CI, confidence interval; DM, diabetes mellitus; HTN, hypertension; NA, not available; OR, odds ratio; PIH, pregnancy-induced hypertension.

risk became insignificant after adjustment for pre-existing diabetes mellitus. The risk of PIH occurrence was not statistically significant in subjects whose BP shifted from Class II at 16 weeks to Class I at 20 weeks of gestation. However, the trend for PIH risk was significant ($P<0.05$) and remained significant even after adjustments for all variables. When comparing two groups, one in which the BP class elevated to Class III at 20 weeks gestation and the other in which the BP class did not change or decreased to Class I, the sensitivity, false positive rate and positive predictive value of BP class elevation between 16 and 20 weeks of gestation were 50.0, 5.0 and 30.8%, respectively. Subjects whose BP shifted from Class III at 16 weeks to Classes I and II at 20 weeks of gestation were not associated with a significant risk reduction of PIH (Table 5).

DISCUSSION

In this study, we classified BP in pregnant women according to JSH 2009. Our results suggested that the risk of PIH could be predicted from BP class shifts between 16 and 20 weeks of gestation. The elevation of BP over the course of pregnancy was associated with a significant risk for future development of PIH even among women with Class I (Optimal) and II (Normal) BPs before 16 weeks of gestation, who are normally recognized as low risk for PIH.

In a previous study, Hermida *et al.*[20] compared the time course of BP changes during pregnancy in normotensive and PIH women. The normotensive women had a steady decrease in BP toward 20 weeks; this was absent in patients with gestational hypertension and pre-eclampsia. Silva *et al.*[21] investigated the effect of maternal education levels on BP alterations in pregnancy. They found the absence of a mid-pregnancy fall in diastolic BP in low educational groups with a high occurrence of PIH. However, both studies failed to analyze the direct association between changes in BP during pregnancy and the risk of PIH. In this study, we clearly demonstrated a significant association between an increased BP during the first half of pregnancy and the risk of PIH.

Our results are in accordance with those previously reported, which identified normal and high-normal BPs at early and mid pregnancy as predictors of subsequent PIH development.[5,23] We used the JSH 2009 classification to show a significant trend for risk of future PIH in women whose BP class increased both before 16 (Table 3) and 20 weeks (Table 4) of gestation, even after adjustment for all variables. Thus, JSH 2009 may be a novel, more accurate system for PIH prediction based on a single estimation of maternal BP during pregnancy, particularly if the measurement is taken at early-mid pregnancy, and especially if performed at 20 weeks.

In a previous report, Duckitt *et al.*[16] conducted a meta-analysis to evaluate patient characteristics recorded at antenatal booking as risk factors of PIH. These included age, body mass index, nulliparity, previous history of PIH and diabetes mellitus, multiple gestations, family history of PIH and antiphospholipid syndrome. In our study population, age, pre-existing diabetes mellitus and previous history of PIH were also identified as significant risk factors for PIH.

There were some limitations to our study, including the retrospective design allowing the possibility of some bias and the small size of the study population.

In conclusion, the JSH 2009 classification may be used early in pregnancy to identify women at risk of PIH even in those with optimal or normal BPs. A shift in BP class at 20 weeks of gestation is predictive of subsequent PIH development. JSH 2009 has potential widespread clinical application.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Rao AK, Cheng YW, Caughey AB. Perinatal complications among different Asian-American subgroups. *Am J Obstet Gynecol* 2006; **194**: e39–e41.
2 Geographic variation in the incidence of hypertension in pregnancy. World Health Organization International Collaborative Study of Hypertensive Disorders of Pregnancy. *Am J Obstet Gynecol* 1988; **158**: 80–83.
3 Cunningham FG, Leveno KJ, Bloom SL, Hauth JC, Rouse DJ, Spong CY. *Williams Obstetrics*, 23rd edn. McGraw-Hill, New York, NY, 2009, pp. 706.
4 Schroeder BM. ACOG practice bulletin on diagnosing and managing preeclampsia and eclampsia. American College of Obstetricians and Gynecologists. *Am Fam Physician* 2002; **66**: 330–331.
5 Ohkuchi A, Iwasaki R, Suzuki H, Hirashima C, Takahashi K, Usui R, Matsubara S, Minakami H, Suzuki M. Normal and high-normal blood pressures, but not body mass index, are risk factors for the subsequent occurrence of both preeclampsia and gestational hypertension: a retrospective cohort study. *Hypertens Res* 2006; **29**: 161–167.
6 Irgens HU, Reisaeter L, Irgens LM, Lie RT. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study. *BMJ* 2001; **323**: 1213–1217.
7 Norden Lindeberg S, Hanson U. Hypertension and factors associated with metabolic syndrome at follow-up at 15 years in women with hypertensive disease during first pregnancy. *Hypertens Pregnancy* 2000; **19**: 191–198.
8 Wolf M, Hubel CA, Lam C, Sampson M, Ecker JL, Ness RB, Rajakumar A, Daftary A, Shakir AS, Seely EW, Roberts JM, Sukhatme VP, Karumanchi SA, Thadhani R. Preeclampsia and future cardiovascular disease: potential role of altered angiogenesis and insulin resistance. *J Clin Endocrinol Metab* 2004; **89**: 6239–6243.
9 Arnadottir GA, Geirsson RT, Arngrimsson R, Jonsdottir LS, Olafsson O. Cardiovascular death in women who had hypertension in pregnancy: a case-control study. *BJOG* 2005; **112**: 286–292.
10 Vikse BE, Irgens LM, Leivestad T, Skjaerven R, Iversen BM. Preeclampsia and the risk of end-stage renal disease. 2008; **359**: 800–809.
11 Aukes AM, de Groot JC, Aarnoudse JG, Zeeman GG. Brain lesions several years after eclampsia. *Am J Obstet Gynecol* 2009; **200**: 504 e1–504 e15.
12 Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ* 2007; **335**: 974.
13 Metoki H, Ohkubo T, Watanabe Y, Nishimura M, Sato Y, Kawaguchi M, Hara A, Hirose T, Obara T, Asayama K, Kikuya M, Yagihashi K, Matsubara Y, Okamura K, Mori S, Suzuki M, Imai Y. Seasonal trends of blood pressure during pregnancy in Japan: the babies and their parents' longitudinal observation in Suzuki Memorial Hospital in Intrauterine Period Study. *J Hypertens* 2008; **26**: 2406–2413.
14 Denolle T, Daniel JC, Calvez C, Ottavioli JN, Esnault V, Herpin D. Home blood pressure during normal pregnancy. *Am J Hypertens* 2005; **18**(9 Part 1): 1178–1180.
15 Poon LC, Kametas NA, Pandeva I, Valencia C, Nicolaides KH. Mean arterial pressure at 11(+0) to 13(+6) weeks in the prediction of preeclampsia. *Hypertension* 2008; **51**: 1027–1033.
16 Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ* 2005; **330**: 565.
17 Paynter NP, Cook NR, Everett BM, Sesso HD, Buring JE, Ridker PM. Prediction of incident hypertension risk in women with currently normal blood pressure. *Am J Med* 2009; **122**: 464–471.
18 Dekker GA, Sibai BM. Early detection of preeclampsia. *Am J Obstet Gynecol* 1991; **165**: 160–172.
19 Chesley LC, Sibai BM. Blood pressure in the midtrimester and future eclampsia. *Am J Obstet Gynecol* 1987; **157**: 1258–1261.
20 Hermida RC, Ayala DE, Iglesias M. Predictable blood pressure variability in healthy and complicated pregnancies. *Hypertension* 2001; **38**(3 Part 2): 736–741.
21 Silva LM, Steegers EA, Burdorf A, Jaddoe VW, Arends LR, Hofman A, Mackenbach JP, Raat H. No midpregnancy fall in diastolic blood pressure in women with a low educational level: the Generation R Study. *Hypertension* 2008; **52**: 645–651.
22 Ogihara T, Kikuchi K, Matsuoka H, Fujita T, Higaki J, Horiuchi M, Imai Y, Imaizumi T, Ito S, Iwao H, Kario K, Kawano Y, Kim-Mitsuyama S, Kimura G, Matsubara H, Matsuura H, Naruse M, Saito I, Shimada K, Shimamoto K, Suzuki H, Takishita S, Tanahashi N, Tsuchihashi T, Uchiyama M, Ueda S, Ueshima H, Umemura S, Ishimitsu T, Rakugi H. The Japanese Society of Hypertension guidelines for the management of hypertension (JSH 2009). *Hypertens Res* 2009; **32**: 3–107.
23 Japan Society for study of HYPERTENSION IN PREGNANCY. *Guideline 2009 for Care and Treatment of Hypertension in Pregnancy: MEDICAL VIEW* 2009 pp. 17.

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CASE REPORT

Successful completion of pregnancy in a woman with chronic granulomatous disease

Michi Hisano MD*, **Shinichi Kobayashi** MD†, **Naoko Arata** MD*‡, **Atsuko Murashima** MD*‡ and **Koushi Yamaguchi** MD*‡

*National Center for Child Health and Development, Tokyo; †Kobayashi Kids Clinic, Kanagawa; ‡Japan Drug Information Institute in Pregnancy, Tokyo, Japan

Summary: Chronic granulomatous disease (CGD) used to be a fatal illness of childhood and patients rarely survived past the first decade. Although antimicrobial prophylaxis has dramatically reduced mortality and morbidity in recent years, CGD remains a life-threatening condition. We present the successful obstetric course of a patient with CGD.

Keywords: chronic granulomatous disease, pregnancy, antimicrobial prophylaxis, neutrophil oxidative burst activity

INTRODUCTION

Chronic granulomatous disease (CGD) is an inherited immunodeficiency disorder caused by defects in NADPH oxidase subunits (gp91phox, p47phox, p22phox, p67phox and p40phox) expressed in phagocytes (neutrophils, monocytes and macrophages). These enzymatic defects result in an inability to kill catalase-positive bacteria and fungi and render the patient susceptible to recurrent life-threatening infections, such as sepsis, pneumonia and meningitis. Patients with an X-linked form of CGD caused by a defect in the gene encoding gp91phox appear to have a more serious clinical phenotype than those with autosomal-recessive forms caused by defects in the other four genes. Antimicrobial prophylaxis and aggressive management of these infections have dramatically reduced infectious complications, but CGD remains a life-threatening condition.

A patient with CGD was referred to us from an attending pediatrician because of her desire to become pregnant. However, little is known of the clinical course, management and risk of infection in such cases. We present the successful obstetric course of a patient with CGD and document the management, *immunological laboratory changes*, and neutrophil oxidative burst activity during pregnancy.

CASE REPORT

The patient had a mutation in the *CYBA* gene encoding p22phox, a small subunit of *membrane-bound flavocytochrome b558*. She had been hospitalized several times for recurrent bacterial infections, such as pneumonia, lymphadenitis, subcutaneous abscess and blepharitis. She had been in relatively good health taking cotrimoxazole (trimethoprim–sulfamethoxasole) prophylaxis into adulthood, except for a severe infection in her first pregnancy at 29 years of age. Then, following a uterine infection with *Burkholderia cepacia* complex, clinical findings of haemophagocytic syndrome (HPS) and septic pulmonary embolism appeared. She underwent aggressive treatment with multiple antibiotic combinations and immunosuppressant agents (prednisolone and cyclosporin A). Although her pregnancy ended in a spontaneous miscarriage at seven weeks of gestation, she recovered completely from the serious infectious complications of HPS two months later.[1]

The patient became pregnant again at 31 years of age. Based on her previous miscarriage caused by infectious complications, it was clear that the pregnancy was potentially high risk and it was essential to devise a management strategy for the CGD. Cotrimoxazole prophylaxis was continued at the same dosages used before pregnancy (160 mg of trimethoprim and 800 mg of sulfamethoxasole per day). Folate was replenished in the first trimester. At every obstetric visit, inflammatory markers such as the white blood cell count, serum C-reactive protein and blood 1,3-beta-D glucan were measured to ensure an early recognition of any occult bacterial and fungal infections. To evaluate whether pregnancy affected neutrophil function, we performed dihydrorhodamine 123 (DHR)-staining flow cytometry analysis of the patient's neutrophil oxidative burst activity. Whole leukocytes were incubated with DHR after being treated with catalase. The leukocytes were then stimulated with phorbol 12-myristate 13-acetate and analysed by flow cytometry, with gating based on forward- and side-scatter properties.

The course of pregnancy was uneventful. No significant increases in the levels of blood inflammatory markers or 1,3-beta-D glucan were observed during pregnancy (Table 1). To investigate the immunological changes during pregnancy, we performed immunologic tests, such as evaluating lymphocyte subsets, natural killer (NK) cell activity and lymphocyte blastoid transformation by phytohemagglutinin (PHA) in each trimester of pregnancy. The proportions of lymphocyte

Correspondence to: Michi Hisano Department of Women's Health, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
Email: hisano-m@ncchd.go.jp

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