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The *Arg121Trp* variant in *PAX4* gene is associated with beta-cell dysfunction in Japanese subjects with type 2 diabetes mellitus

Yoshiharu Tokuyama[a,*], Kana Matsui[b], Toshiharu Ishizuka[a], Toru Egashira[b], Azuma Kanatsuka[a]

[a]*Diabetes Center, Chiba Central Medical Center, Chiba 264-0017, Japan*
[b]*Development of Clinical Genomics, R&D Center, BML Inc, Kawagoe 350-1101, Japan*

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Abstract

Mutations in PAX4, a transcription factor involved in the beta-cell differentiation, could predispose to the development of type 2 diabetes mellitus. To clarify the role of *PAX4 Arg121Trp* mutation on the development of type 2 diabetes mellitus, we try to determine the clinical phenotype in diabetic subjects with this mutation. Study subjects consisted of 793 type 2 diabetic patients and 318 control subjects. Genotyping for *Arg121Trp* polymorphism was performed by Invader assay. Clinical phenotype was determined in diabetic subjects including 20 *Trp121* carriers and 142 wild-type subjects using a combination of 2-compartment model of C-peptide kinetics and minimal model analysis during intravenous glucose tolerance test. We detected 3 *Trp/Trp*, 51 *Arg/Trp*, and 739 *Arg/Arg* in diabetic subjects, and 16 *Arg/Trp* and 302 *Arg/Arg* in control subjects. The frequency of *Trp121* allele was 3.59% and 2.51% in diabetic and control groups, respectively ($P = .19$). Rate of insulin users was higher in *Trp121* carriers compared with the wild-type group (42.5% vs 25.0%, $P = .0046$). First-phase C-peptide secretion was significantly decreased in the diabetic subjects with *Trp121* allele compared with the patients with wild type ($P = .0048$), whereas there were no significant differences in insulin sensitivity and glucose effectiveness between the groups. *Arg121Trp* mutation in *PAX4* gene could be associated with beta-cell dysfunction in Japanese subjects with type 2 diabetes mellitus.

1. Introduction

Type 2 diabetes mellitus usually results from an inadequate mass of functional pancreatic beta cells because of the lack of compensation to overcome insulin resistance. Thus, mutations in genes responsible for the beta-cell differentiation may predispose to the development of diabetes. PAX4, a transcription factor belonging to the Pax family, is reported to be essential for the differentiation of beta cells [1-3]. In Japanese population with type 2 diabetes mellitus, *Arg121Trp* mutation in *PAX4* gene was found and demonstrated to lack the inhibitory effect on transcription activity induced by Pax6 in vitro [4]. Recently, we reported that patients with homozygous mutation of *Arg121Trp* in *PAX4* gene showed severe defects in the first-phase insulin secretion, suggesting that this genetic alteration resulted in beta-cell dysfunction [5]. Population-based study in Okinawa district in Japan showed that the onset age of diabetes was earlier and the rate of insulin user was higher in the subjects with this heterozygous mutation [6]. To clarify the effect of the *Trp121* allele on the development of type 2 diabetes mellitus, we compared insulin secretion, insulin sensitivity, and insulin-independent glucose disposal during intravenous glucose tolerance test between Japanese type 2 diabetic patients with *Trp121* allele and patients with the wild type, using a combined method of 2-compartment model of C-peptide kinetics and minimal model analysis [7-9], and demonstrated that *Arg121Trp* in *PAX4* gene accelerates the decline in beta-cell function once diabetes has set in.

2. Subjects and methods

2.1. Subjects

We recruited 793 subjects (men, 541; women, 252) with type 2 diabetes mellitus and 318 control subjects (men, 145; women, 173) at the Diabetes Center, Chiba Central Medical Center, Chiba, Japan. All the subjects enrolled in this study were ethnic Japanese. Diabetes mellitus was diagnosed

* Corresponding author. Tel.: +81 43 232 3691; fax: +81 43 232 9100.
E-mail address: yt4486yt@yahoo.co.jp (Y. Tokuyama).

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according to the 1985 World Health Organization criteria [10]. All patients tested were negative for anti–glutamic acid decarboxylase and anti–tyrosine phosphatase–related protein (IA2) antibodies. Their present age, onset age of diabetes, body mass index (BMI), and HbA_{1c} were 61.5 ± 12.0 years, 51.3 ± 12.4 years, 24.9 ± 4.06 kg/m^2, and 7.04% ± 1.32%, respectively. Control subjects had to meet all of the following 3 criteria: (1) age of older than 60 years; (2) HbA_{1c} of less than 5.8% (reference range, 4.3%-5.7%); and (3) no family history of type 2 diabetes mellitus. Their present age, BMI, and HbA_{1c} were 73.3 ± 9.04 years, 23.2 ± 3.05 kg/m^2, and 4.91% ± 0.34%, respectively. Before participation, the purpose and risk of the study were explained, and informed consent was obtained from all the participants. The protocol was approved by the ethics committee of Chiba Central Medical Center.

2.2. Detection of Arg121Trp variant in PAX4 gene by Invader assay

Genomic DNA was extracted from the peripheral blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). Invader assay was carried out according to the standard method [11]. Extracted DNA was denatured at 95°C for 10 minutes and applied to the 384-well plate. In each well, 50 to 100 ng genomic DNA, 0.07 μmol/L Invader oligonucleotide probe (5′-GGTAGTCCCTGGTCCTCC-TGTAATGCCCT-3′), 0.7 μmol/L signal oligonucleotide probes (5′-CGCGCCGAGGACAGGACTCGGTTGAT-3′, 5′-ATGACGTGGCAGACGCAGGACTCGGTTGA-3′; flap sequences are underlined) for mutant and wild-type DNA, 5 pmol/L fluorescence resonance energy transfer probes for mutant and wild-type DNA, 10 mmol/L MOPS, 7.5 mmol/L $MgCl_2$, 2.7% PEG 8000, and 100 ng per well of Cleavase enzyme were reacted at 63°C for 2 to 4 hours. Fluorescence signal was detected using a standard fluorescence plate reader (CytoFluor4000, Applied Biosystems, Tokyo, Japan). The *PAX4 Arg121Trp* variant in 118 DNA samples was also determined by polymerase chain reaction (PCR)–restriction fragment length polymorphism, as described before [8], to confirm the results of Invader assay. Briefly, PCR was carried out using the forward primer: 5′-GGGTTGTTGTGAGGGT GATCCAA-3′(nt 29898-29920, GenBank accession number AC000359) and the reverse primer: 5′-TAGGTGGAGA CAGATGGGAAAAAG-3′ (nt 30158-30181). The *PAX4 Arg121Trp* variant results in a loss of *Aci*I site (New England Biolabs, Beverly, MA), and therefore, *Aci*I digestion of the PCR product could determine the polymorphism.

Table 1
Comparison of clinical features of type 2 diabetic patients with *Arg121Trp* mutation

	Genotype		*P*
	Arg/Arg	*Arg/Trp* or *Trp/Trp*	
n	739	54	
Male/female	506/233	35/19	
BMI (kg/m^2)	24.9 ± 4.09	24.5 ± 3.56	.41
Age (y)	61.5 ± 11.9	61.4 ± 12.6	.97
Onset age (y)	51.3 ± 12.3	50.2 ± 13.9	.57
HbA_{1c} (%)	7.02 ± 1.29	7.23 ± 1.70	.37
Treatment			
Diet/OHA/insulin	214/340/185	13/18/23	

Data are presented as means ± SD. OHA indicates oral hypoglycemic agents.

Table 2
Comparison of clinical features between type 2 diabetic patients with *Arg121Trp* mutation who participated in FSIGT

	Genotype		*P*
	Arg/Arg	*Arg/Trp* or *Trp/Trp*	
n	142	20	
Male/female	96/46	12/8	
BMI (kg/m^2)	25.3 ± 4.03	25.1 ± 3.75	.89
Age (y)	55.1 ± 13.3	58.5 ± 16.5	.39
Onset age (y)	48.3 ± 13.0	48.8 ± 15.0	.90
HbA_{1c} (%)	7.49 ± 1.74	7.73 ± 1.63	.54
Treatment			
Diet/OHA/insulin	48/53/41	1/9/10	

Data are presented as means ± SD.

2.3. C-peptide secretion rate and minimal model analysis

We randomly selected 20 diabetic patients carrying the *Trp121* allele in *PAX4* (17 *Arg/Trp* and 3 *Trp/Trp*) and 142 diabetic patients with *Arg/Arg* genotype, who underwent the frequently sampled intravenous glucose tolerance test (FSIGT). A combined method of 2-compartment model of C-peptide kinetics and minimal model approach in FSIGT was performed according to the protocol described before to examine the reserve of insulin secretion, insulin sensitivity, and glucose effectiveness [9]. Briefly, after 10 to 12 hours of overnight fast, a bolus of 50% glucose (25 g) was injected over 1 minute from the antecubital vein at time 0. Regular human insulin (0.05 U/kg; Humulin R, Lilly, Indianapolis, IN) dissolved in 5 mL of 0.9% normal saline was infused over 30 seconds at the 20-minute period. Blood samples were collected at −5, 0, 2, 3, 5, 7, 10, 15, 20, 22, 23, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, and 150 minutes for the determination of plasma glucose and insulin concentrations. C-peptide concentrations were measured at −5, 0, 2, 3, 5, 7, 10, 15, and 20 minutes. C-peptide secretion rate was mathematically estimated from serum C-peptide levels by deconvolution with a 2-compartment model for C-peptide disappearance kinetics. The first-phase C-peptide secretion rate (CS1) was determined by the sum of the C-peptide secretion rate from 0 to 5 minutes after intravenous glucose load. CS1 is 6.8 to 18.5 ng/mL per 5 minutes (mean ± SD, 10.8 ± 3.9 ng/mL per 5 minutes) in subjects with normal glucose tolerance without diabetic patients in their family. Parameters Si (insulin sensitivity) and Sg (glucose effectiveness) were calculated using the glucose and insulin concentrations by the minimal model software program, which we developed according to the algorithm previously described by Pacini et al. Si and Sg are 2.6×10^{-4}/min/(μU · mL) to 7.6×10^{-4}/min/(μU/mL) ($4.59 \pm 1.76 \times 10^{-4}$) and 1.15×10^{-2}/min to 4.1×10^{-2}/min ($2.56 \pm 0.92 \times 10^{-2}$),

Table 3
Comparison of the first-phase C-peptide secretion and minimal model analysis in FSIGT of type 2 diabetic patients with *Arg121Trp* mutation

	Genotype		*P*
	Arg/Arg	*Arg/Trp* or *Trp/Trp*	
CS1 (ng/mL per 5 min)	1.38 ± 1.46	0.79 ± 0.69	.0048
Si ($\times 10^{-4}$/min/[μU/mL])	1.75 ± 1.94	1.45 ± 2.14	.55
Sg ($\times 10^{-2}$/min)	2.19 ± 1.58	2.07 ± 1.34	.73

Data are presented as means ± SD.

respectively, in subjects with normal glucose tolerance without diabetic patients in their family.

2.4. Statistical analysis

Data are means ± SD. The statistical significance of the differences in mean values and frequencies was determined by the Student *t* test and simple χ^2 test, respectively.

3. Results

3.1. Determination of Arg121Trp variant in PAX4 gene

Complete concordance was observed when 121 DNA samples were genotyped by both the Invader assay and by allele-specific PCR. Using the Invader assay, we detected 3 homozygous (*Trp/Trp*), 51 heterozygous (*Arg/Trp*), and 739 wild-type (*Arg/Arg*) subjects for *PAX4 Arg121Trp* polymorphism in diabetic group. On the other hand, 16 heterozygous and 302 wild-type subjects were detected in the control group. The frequency of *Trp121* allele was 3.59% and 2.51% in diabetic and control groups, respectively. Diabetic subjects tended to have more *Trp121* allele than control group, although it did not reach statistical significance ($P = .19$). Clinical features in diabetic subjects were compared between *Trp121* carriers (*Arg/Trp* or *Trp/Trp*) and wild type (*Arg/Arg*). There were no significant differences in age, onset age, BMI, and HbA_{1c} between the 2 groups (Table 1). The rate of insulin users was higher in the *Trp121* carriers than in the wild-type group (42.5% vs 25.0%, $P = .0046$).

3.2. C-peptide secretion rate and minimal model analysis

Clinical features in the patients who participated in FSIGT were compared (Table 2). There were no significant differences in age, onset age, BMI, and HbA_{1c} between the 2 groups. Importantly, *Trp121* carriers exhibited significant decrease in CS1 after glucose infusion compared with the wild-type (*Arg/Arg*) group, although there was no significant difference in Si and Sg between the 2 groups (Table 3).

4. Discussion

In this study, we successfully developed the design of a serial Invader assay for analyzing the *Arg121Trp* mutation in *PAX4* gene. The results of this assay were completely identical to the results of PCR–restriction fragment length polymorphism. This assay is promised to genotype directly from genomic DNA without the requirement of PCR amplification, providing rapid, accurate, and cost-effective technique for the SNP genotyping in a large scale of samples.

There were several previous studies about the allele frequency of *Arg121Trp* in the *PAX4* gene [4-6]. Shimajiri et al [4] reported that it was 2% and 0% in diabetic and nondiabetic subjects who resided in Wakayama prefecture in Japan, respectively, although the genotype data were not in Hardy-Weinberg equilibrium in the cases. They also reported that the allele frequency of *Arg121Trp* was 3.1% and 1.6% in 193 diabetic and 372 healthy control subjects, respectively, in Okinawa district in Japan, showing no significant difference [6]. We compared the allele frequencies of *Arg121Trp* in *PAX4* gene between the 793 diabetic and 318 control subjects, resulting in no significant difference (3.58% and 2.51%). The required sample size in case-control study is determined by the frequency of the susceptibility allele of the target gene in the population (P), the penetrance for mutant genotype to the disease (*f*), mode of inheritance (recessive, dominant, multiplicative, or additive), and the power to be achieved [12]. In case-control study for *Arg121Trp* in *PAX4* gene, if we assume $P = .02$ and $f = 0.1$, under a multiplicative model, at least 700 subjects of each group would be necessary to get the power 0.8. Thus, a larger study would be needed to detect the association of *Arg121Trp* allele with type 2 diabetes mellitus. Combining the data presented in this article with that in the study on Okinawans [6] (ie, pooled analysis with 986 cases and 690 controls), we found a significant difference in the frequency of the *Arg121Trp* polymorphism between cases and controls (3.49% and 2.02%, $P = .012$), suggesting that this polymorphism would increase risk of type 2 diabetes mellitus.

The clinical features including present age, onset age of diabetes, BMI, and HbA_{1c} in the subjects who participated in FSIGT did not differ between *PAX4 Trp121* carriers and the wild-type subjects, indicating that it would be proper to compare insulin secretory reserve and minimal model parameters between the 2 groups. The combined method of 2-compartment model of C-peptide kinetics and minimal model analysis revealed that CS1 was markedly decreased in *PAX4 Trp121* carriers, whereas Si and Sg did not differ between the groups. These findings strongly suggest that the *Arg121Trp* mutation in *PAX4* gene is associated with beta-cell dysfunction in diabetic patients, not with insulin sensitivity or glucose effectiveness. We previously reported that patients with homozygous mutation of *Arg121Trp* in *PAX4* gene showed severe defects in the first-phase insulin secretion [5]. The present study confirmed earlier study and also demonstrated that not only homozygous mutation, but also heterozygous mutation of *Arg121Trp* in *PAX4* gene is involved in the impaired insulin secretion in the development of type 2 diabetes mellitus. Thus, the patients carrying

Arg121Trp mutation, who are 6.8% of Japanese type 2 diabetic subjects (54/793 subjects), would have a risk of further deterioration of beta-cell function.

The mutation of *Arg121Trp* is located in the paired domain, which is important as a DNA binding site, and the *Arg121Trp* mutant Pax4 lacked the inhibitory effect on transcription activity induced by Pax6 in COS7 cells [4]. Recently, new missense mutations in *PAX4* gene, *Arg133Trp* and *Arg37Trp*, were identified in the group of ketosis-prone diabetes, in people of West African ancestry [13]. In vitro, the *Arg133Trp* and *Arg37Trp* mutations had decreased transcriptional repression of target gene promoter in α-TC cells. The patients with homozygous *Arg133Trp* and heterozygous *Arg37Trp* mutations showed more severe alteration in insulin secretory reserve, during glucagon-stimulation test. Because the *Arg133Trp* and *Arg37Trp* mutations have not been detected in Japanese population [4], we did not examine those mutations in this study. Ketosis-prone diabetes is increasingly frequent in Japan. Its clinical phenotype is characterized by fulminant initial insulin dependence, without immunologic markers observed in classic type 1 diabetes, followed by absence of insulin requirements for years as observed in type 2 diabetes mellitus [14]. Interestingly, in the present study, 2 patients carrying heterozygous *Trp121* were also ketosis-prone. Although the *PAX4* variant may contribute to the predisposition to transient ketosis with severe insulin deficiency in these cases, the relationship between *Arg121Trp* polymorphism in *PAX4* gene and ketosis-prone diabetes remains to be elucidated.

Because the insufficiency of insulin secretion is thought to be a primary cause of type 2 diabetes mellitus in Japanese patients [15], the genetic defects in transcription factors involved in the development of endocrine pancreas could play a role in the development of diabetes. So far, mutations in hepatocyte nuclear factor (HNF) 4α, HNF-1α, insulin promoter factor 1, HNF-1β, and NeuroD1, which are transcription factors involved in the differentiation of beta cell, have been identified in maturity-onset diabetes of youth 1, 3, 4, 5, and 6, respectively [16]. We previously reported that the diabetic subjects with homozygous mutation of *Ala45Thr* in *NEUROD1* gene also showed a severe defect in the first-phase insulin secretion [5]. In the present study, both homozygote and heterozygote of *Trp121* carriers with diabetes in *PAX4* gene exhibited a severe defect in insulin secretion. The rate of insulin users was higher in the *Trp121* carriers compared with the wild-type diabetic patients, consistent with the recent population study in Okinawans in Japan [6]. These findings suggest that beta-cell function progressively deteriorates in the patients with *Trp121* allele in the *PAX4* gene, resulting in requirement of insulin therapy. Thus, *Trp121* allele in the *PAX4* gene could be a marker of severe insulin deficiency in Japanese type 2 diabetic populations. Therefore, it is important to assess pancreatic insulin reserve in the patients carrying the genetic defects in transcription factors involved in the development of endocrine pancreas, including *Arg121Trp* polymorphism in *PAX4* gene, who has not yet receive insulin therapy.

References

[1] Sosa-Pineda B, Chowdhury K, Torres M, et al. The *Pax4* gene is essential for differentiation of insulin-producing β cells in the mammalian pancreas. Nature 1997;386:399-402.
[2] Dohrmann C, Gruss P, Lemaire L. Pax genes and the differentiation of hormone-producing endocrine cells in the pancreas. Mech Dev 2000;92:47-54.
[3] Sosa-Pineda B. The gene Pax4 is an essential regulator of pancreatic β-cell development. Mol Cells 2004;18:289-94.
[4] Shimajiri Y, Sanke T, Furuta H, et al. A missense mutation of *Pax4* gene (R121W) is associated with type 2 diabetes in Japanese. Diabetes 2001;50:2864-9.
[5] Kanatsuka A, Tokuyama Y, Nozaki O, et al. β cell dysfunction in late-onset diabetic subjects carrying homozygous mutation in transcription factors NeuroD1 and Pax4. Metabolism 2002;151:1161-5.
[6] Shimajiri Y, Shimabukuro M, Tomoyose T, et al. PAX4 mutation (R121W) as a prodiabetic variant in Okinawans. Biochem Biophys Res Commun 2003;302:342-4.
[7] Bergman RN, Watanabe R, Rebrin K, et al. Toward an integrated phenotype in pre-NIDDM. Diabet Med 1996;13:S67-S77.
[8] Kanatsuka A, Makino H, Sakurada M, et al. First-phase insulin response to glucose in nonobese or obese subjects with glucose intolerance: analysis by C-peptide secretion rate. Metabolism 1988;37:878-84.
[9] Tokuyama Y, Sakurai K, Yagui K, et al. Pathophysiologic phenotypes of Japanese subjects with various degrees of glucose tolerance: using the combination of C-peptide secretion rate and minimal model analysis. Metabolism 2001;50:812-8.
[10] World Health Organization. Diabetes mellitus: report of a WHO study group, WHO technical report series, no. 727, Geneva, Switzerland; 1985.
[11] Lyamichev V, Neri B. Invader assay for SNP genotyping. Methods Mol Biol 2003;212:229-40.
[12] Ohashi J, Yamamoto S, Tsuchiya N, et al. Comparison of statistical power between 2 × 2 allele frequency and allele positivity tables in case-control studies of complex disease genes. Ann Hum Genet 2001;65:197-206.
[13] Mauvais-Jarvis F, Smith SB, Le May C, et al. *PAX4* gene variations predispose to ketosis-prone diabetes. Hum Mol Genet 2004;13:3151-9.
[14] Mauvais-Jarvis F, Sobngwi E, Porcher R, et al. Ketosis-prone type 2 diabetes in patients of sub-Saharan African origin: clinical pathophysiology and natural history of β-cell dysfunction and insulin resistance. Diabetes 2004;53:645-53.
[15] Kosaka K, Kuzuya T, Hagura R, et al. Insulin response to oral glucose load is consistently decreased in established non–insulin-dependent diabetes mellitus: the usefulness of decreased early insulin response as a predictor of non–insulin-dependent diabetes. Diabet Med 1996;13:S109-19.
[16] Fajans SS, Bell GI, Polonsky KS. Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. N Engl J Med 2001;345:971-80.