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ORIGINAL ARTICLE

PAX4 R192H and P321H polymorphisms in type 2 diabetes and their functional defects

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We have previously identified *PAX4* mutations causing MODY9 and a recent genome-wide association study reported a susceptibility locus of type 2 diabetes (T2D) near *PAX4*. In this study, we aim to investigate the association between *PAX4* polymorphisms and T2D in Thai patients and examine functions of PAX4 variant proteins. *PAX4* rs2233580 (R192H) and rs712701 (P321H) were genotyped in 746 patients with T2D and 562 healthy normal control subjects by PCR and restriction-fragment length polymorphism method. PAX4 variant proteins were investigated for repressor function on human *insulin* and *glucagon* promoters and for cell viability and apoptosis upon high glucose exposure. Genotype and allele frequencies of *PAX4* rs2233580 were more frequent in patients with T2D than in control subjects ($P = 0.001$ and 0.0006, respectively) with odds ratio of 1.66 ($P = 0.001$; 95% confidence interval, 1.22–2.27). *PAX4* rs712701 was not associated with T2D but it was in linkage disequilibrium with rs2233580. The 192H/321H (A/A) haplotype was more frequent in T2D patients than in controls (9.5% vs 6.6%; $P = 0.009$). PAX4 R192H, but not PAX4 P321H, impaired repression activities on *insulin* and *glucagon* promoters and decreased transcript levels of genes required to maintain β-cell function, proliferation and survival. Viability of β-cell was reduced under glucotoxic stress condition for the cells overexpressing either PAX4 R192H or PAX4 P321H or both. Thus these *PAX4* polymorphisms may increase T2D risk by defective transcription regulation of target genes and/or decreased β-cell survival in high glucose condition.
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INTRODUCTION

Paired box 4 (PAX4) has pivotal roles in β-cell development, differentiation and survival.[1,2] A well-defined role of Pax4 in promoting β-cell development was demonstrated in the knockout mice model.[3] *Pax4*-deficient mice lacks insulin-producing β-cells and somatostatin-producing δ-cells, whereas increases the glucagon-producing α-cells in the pancreas.[3] During pancreas development, Pax4 functions as a strong repressor of several target genes, including *glucagon*, *insulin*, *somatostatin*, *IAPP* and *ghrelin*, resulting in promoting the development and differentiation of insulin-producing β-cell. Variations of these genes lead to susceptibility to type 2 diabetes (T2D).[4] Furthermore, PAX4 has important roles in adult β-cells by stimulating transcription of genes that promote islets proliferation and/or protect cells from stress-induced apoptosis.[5,6] Interestingly, *PAX4* gene expression is increased in islets isolated from T2D patients, an effect that is most likely mediated by high blood glucose level.[2] These data indicated that *PAX4* may function as a survival and/or proliferation gene allowing mature islets to adapt in response to a high glucose condition. Decreased β-cell proliferation and/or excessive loss of β-cells through apoptosis contributes to the development of diabetes. Several evidences support the role of PAX4 in maintaining pancreatic β-cell mass; therefore, genetic defects of *PAX4* affecting β-cell development, differentiation and survival may lead to predisposition of diabetes. Mutations of *PAX4* cause maturity-onset diabetes of the young, type 9 (MODY9).[7–10] Single-nucleotide polymorphisms (SNPs) and mutations of *PAX4* were linked to diabetes in various ethnic groups, including German and Swiss populations[11] as well as Japanese[12,13] and West Africans and African-Americans.[14] The differences of susceptibility loci associated with *PAX4* for T2D between Asians and Europeans were demonstrated, which may reflect ethnic diversities and effect of *PAX4* variations. *PAX4* variations seem to increase diabetes risk in Asian populations. The A allele of *PAX4* rs712701 (1168C>A; P321H) is frequent in the Asians, including Japanese[12] and Han Chinese[15] but is less frequent in the Europeans.[16] A recent genome-wide association study in a Chinese population[17] and a large meta-analysis in East Asian population[18] identified

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susceptibility loci for T2D near the *PAX4*. Our group reported that *PAX4* mutations cause MODY9 and *PAX4* rs2233580 (781G>A; R192H) genotype and allele frequencies were higher in MODY probands and patients with T2D than in patients without diabetes.[7] The patients who carried the A allele of this SNP (781A) developed diabetes at earlier ages.[7] Furthermore, Thai children who survived acute lymphoblastic leukemia and carried *PAX4* rs2233580 (781G>A; R192H) had higher prevalence of impaired glucose tolerance,[19] implying the significance of this variation as a predisposing genetic risk to diabetes. Remarkably, all Thai MODY probands and non-diabetic controls carrying *PAX4* rs2233580 (781G>A; R192H) also carried *PAX4* rs712701 (1168C>A; P321H). We hypothesize that either one or two of these *PAX4* (R192H and P321H) variations increase T2D risk. We therefore investigate the association between *PAX4* (R192H and P321H) and T2D in larger Thai cohorts and also examine their impacts on PAX4 protein functions.

MATERIALS AND METHODS

Study subjects

This study was approved by the Independent Ethics Committee, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Patients with T2D and healthy control subjects were Thai ethnic, who resided in central Thailand, mainly in Bangkok and nearby provinces. All subjects gave their informed consent before enrollment into the study project. We randomly recruited 746 unrelated patients with T2D attending to Diabetic Clinic of Siriraj Hospital, located in the middle part of Thailand. Patients' first- and second-degree relatives were not accessible. The diagnosis of T2D was according to the American Diabetes Association 1997 diagnostic criteria. We enrolled 562 non-diabetic healthy subjects without family history of diabetes, hemoglobin A1C <5.7% and age >40 years, from the health check-up unit of Siriraj Hospital. The biochemical data of patients with T2D and non-diabetic controls are shown in Supplementary Table S1.

Genotyping

Genotyping was performed by PCR and restriction fragment-length polymorphism method. To genotype *PAX4* rs2233580 and rs712701, PCR products of exons 5 and 9 were digested with *Nla*III (New England Biolab, MA, USA) and *Sec*I (Fermentas Inc., Hanover, MD, USA), respectively. The digested PCR products were detected by agarose-gel electrophoresis and ethidium bromide staining. All genotyping calls were confirmed by automated DNA sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA, USA).

Cell culture

Mouse βTC3 and αTC1.9 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 15 mM HEPES, 0.1 mM non-essential amino acids and 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. For αTC1.9 cell, cultured media was additionally supplemented with 4 mM L-glutamine and 0.02% bovine serum albumin. These cells were maintained in 37 °C incubator with a humidified air containing 5% CO_2. INS-1 832/13 cells, rat insulinoma cell line, were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 10 mM HEPES, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C in humidified air containing 5% CO_2. INS-1 832/13 cells were normally maintained in the medium containing 11.1 mM glucose (basal level), and they were cultured in medium containing high glucose (40 mM) for testing cell viability under glucotoxic condition. These cells were used for PAX4 protein expression and functional studies after transfections with recombinant plasmids.

Plasmid construction

The full-length human wild-type *PAX4* cDNA was amplified from cDNA of normal human placenta (BioChain Institute, Inc., Singapore, Singapore) by PCR using platinum *Pfx* DNA polymerase (Invitrogen, Leek, The Netherlands) and cloned into pcDNA3.1-hisB expression vector to generate pcDNA3.1hisB-*PAX4* WT. The plasmid constructs containing *PAX4* variants, including R192H, P321H or R192H/P321H, were generated from the pcDNA3.1-hisB-*PAX4* WT construct by site-directed mutagenesis using the Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA, USA), as previously described.[20]

Western blotting analysis

The βTC3 cells were transfected with plasmid constructs containing either wild-type or variant *PAX4* (500 ng) by using Fugene 6 transfection reagent (Roche, Indianapolis, IN, USA). After transfection and culture for 48 h, the transfected βTC3 cells were lysed in a RIPA buffer (Pierce, Rockford, IL, USA) and were quantified for total protein by a Bradford assay (Bio-Rad Laboratories Inc., Philadelphia, PA, USA). Thirty micrograms of total protein were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane and the membrane was blocked with 5% skim milk. Then the membrane was incubated with mouse anti-Xpress antibody (Invitrogen, Carlsbad, CA, USA) or mouse anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark) at room temperature for 1 h. Protein bands were detected by the enhanced cheminescence system (Pierce, Rockford, IL, USA) and exposed on X-ray films.

Immunofluorescence microscopy

The transfected βTC3 cells were fixed in 4% paraformaldehyde/phosphate-buffered saline for 15 min at room temperature. After washing, the cells were permeabilized with a solution of 0.2% Triton X-100/phosphate-buffered saline for 15 min. Non-specific binding proteins were blocked with fetal bovine serum, and the cells were incubated with mouse anti-Xpress antibody (Invitrogen, Carlsbad, CA, USA) to detect Xpress-epitope tagged to PAX4 protein. After washing, the cells were incubated with Alexa488-labeled goat anti-mouse antibody (Abcam, Cambridge, MA, USA). Nuclei were counter-stained with Hoechst33342 dye. The cells were then mounted onto slides with antifade reagents (Invitrogen, Carlsbad, CA, USA) and visualized by laser scanning confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany).

Luciferase reporter assay

βTC3 and αTC1.9 cells were transfected with plasmid constructs containing either wild-type or variant *PAX4* (500 ng) by using Fugene 6 transfection reagent (Roche, Indianapolis, IN, USA) together with 100 ng of pGL3-human *insulin* promoter or pGL3-human *glucagon* promoter and 10 ng of pRL-SV40 (control). After transfection and culture for 24 h, cell lysates were collected and assayed for luciferase activities of wild-type and mutant PAX4 proteins by the Dual Luciferase Reporter Assay System (Promega Corp., Fitchburg, WI, USA). The firefly luciferase activity was normalized to that of *Renilla* luciferase to control for differences in transfection efficiency.

Assessment of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

INS-1 832/13 cells were transfected with plasmids containing either wild-type or variant *PAX4* by using X-tremeGENE 9 DNA transfection reagent (Roche). After transfection and culture for 24 h, the cultured media were replaced with basal medium (11.1 mM glucose) or high glucose medium (40 mM glucose) and incubated for 72 h. Cells were then incubated with MTT solution (final concentration was 5 mg ml^{-1} in RPMI640) at 37 °C for 4 h. Then the media was removed and the reaction was stopped by adding acidic isopropanol and incubated at 37 °C for 1 h. The absorbance was measured at OD570 and OD650 nm using PowerWave microplate scanning spectrophotometer (BIO-TEK, Winooski, VT, USA).

Analysis of cellular apoptosis by using AnnexinV/propidium iodide (PI) staining

Transfected INS-1 832/13 cells were cultured under high glucose condition medium for 72 h. Cells were then collected after trypsinization. Cells (4×10^5)

Table 1 Genotype and allele frequencies of *PAX4* rs2233580 and rs712701

				Genotype frequencies, n *(%)*					*Allele frequencies (%)*		
SNP ID	*Location*	*Position (base change)*	*Genotypes*	*T2D*	*Controls*	P-*value*	*OR (95% CI)*	P-*value*	*T2D*	*Controls*	P-*value*
rs2233580	Exon 5	781G>A	GG	601 (80.6)	491 (87.4)	0.0010[a]	1.66	0.0011	G: 89.7	G: 93.5	0.0006
			GA	136 (18.2)	69 (12.3)	0.003	(1.22–2.27)		A: 10.3	A: 6.5	0.0012
			AA	9 (1.2)	2 (0.3)						
			Total	746	562						
rs712701	Exon 9	1168C>A	CC	120 (16.1)	93 (16.6)	0.9436[b]	1.03	0.8282	C: 40.9	C: 41.2	0.8719
			CA	368 (49.5)	276 (49.2)		(0.78–1.38)		A: 59.1	A: 58.8	
			AA	256 (34.4)	192 (35.2)						
			Total	744	561						

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism; T2D, type 2 diabetes.
ORs were analyzed by using MedCalc program.
Corrected *P* after Bonferroni correction are underlined.
[a]Dominance.
[b]Co-dominance.

Table 2 Frequencies of the *PAX4* rs2233580 and rs712701 haplotypes

		Haplotype frequency (%)		
Index	*Haplotypes*	*T2D*	*Controls*	P-*value*
1	G–C	40.4	41.2	0.821
2	G–A	49.2	52.2	0.153
3	A–A	9.5	6.6	0.009
4	A–C	0.9	0	–

Abbreviation: T2D, type 2 diabetes.

were incubated with AnnexinV-fluorescein isothiocyanate at 4 °C for 15 min and then incubated with PI for 2–3 min. Stained cells were analyzed by flow cytometry (FACsort, BD Biosciences, USA). The apoptotic cells were analyzed with a FACSort flow cytometer by the CellQuest software (BD Biosciences, San Jose, CA, USA).

Quantitative real-time reverse transcriptase-PCR (qRT-PCR)

Total RNA was extracted from INS-1 832/13 cells by using TRIzol reagent (Life Technologies Co., Carlsbad, CA, USA). Total RNA (2.5 μg) was reverse transcribed into complementary DNA (cDNA) by using the SuperScript III First-Strand Synthesis Kit (Invitrogen, Leek, The Netherlands) and oligo$(dT)_{18}$ primers, following the manufacturer's instructions. cDNA was diluted three-folds in sterile water. An aliquot of 1 μl of cDNA was then added into 9 μl of PCR master mixture (SYBR Green I, Roche) containing a pair of specific primers at a concentration of 300 nM each. cDNA amplification was carried out by PCR using Light Cycler 480 (Roche). The qPCR mixture was preincubated at 95 °C for 5 min and then followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 20 s and extension at 72 °C for 10 s. The calculation of the relative mRNA expression was determined by normalization to β-actin mRNA level, according to the $2^{-\Delta\Delta Ct}$ method.[21] Sequences of all primers are available in Supplementary Table S2.

Statistical analysis

Statistical analysis was performed by using the SPSS version 21.0 software (SPSS, Chicago, IL, USA). Odds ratio (OR) were calculated along with 95% confidence interval (CI) using the MedCalc program. Genotype and allele distributions between two groups were compared using the chi-squared (χ^2) test. Significant differences of the means between two or three genotypes were analyzed by Student's *t*-test or one-way analysis of variance, respectively. Linkage disequilibrium (LD) analysis of the two SNPs was performed by using SNPStats program (http://bioinfo.iconcologia.net/SNPstats). The estimation of haplotype frequencies were conducted using CHAPLIN program under a dominant model of the haplotype effect. A $P<0.05$ was considered to be statistically significant. The statistical power for association testing of the two SNPs was analyzed by using the Quanto program (http://hydra.usc.edu/gxe).

RESULTS

Genotype and allele frequencies

Genotype and allele frequencies of *PAX4* rs2233580 and rs712701 are shown in Table 1. Genotype distributions of these two SNPs were well fitted in the Hardy–Weinberg equilibrium in both groups (data not shown). For rs2233580, GA and AA genotypes were more common in patients with T2D than in those of the control subjects (GA+AA vs GG, $P=0.001$) and the OR was 1.66 (95% CI: 1.22–2.27; $P=0.0011$). The minor A allele was more frequent in patients with T2D than that of the control subjects (10.3% vs 6.5%, $P=0.0006$; Table 1). The association between this SNP and T2D was still significant after the Bonferroni correction (corrected $P=0.003$ and 0.0012 for genotype and allele frequencies, respectively) (Table 1). For *PAX4* rs712701, there was no difference in genotypes and allele distribution between the two groups. We observed that *PAX4* rs712701 A allele is more frequent in Thais and other Asian populations[12,15] in contrast to those reported in Europeans[11] and African Americans[14] (Supplementary Table S3). LD of the two SNPs was 0.85 for D' and 0.04 for R^2. D' at 0.85 indicated a high LD of these two SNPs. The low R^2 indicated that the two SNPs could not substitute each other. The low R^2 value was most likely due to SNP rs2233580 being much rarer than SNP rs712701. Haplotype analysis revealed three common haplotypes, including R192/P321 (G-C), R192/H321 (G-A) and H192/H321 (A-A). The 192H/321H (A-A) haplotype was more frequent in patients with T2D than in the control subjects (9.5% vs 6.6%; $P=0.009$; Table 2).

Protein expression level and nuclear translocation

To gain insight into expression and function of PAX4 variant proteins, we expressed the wild-type PAX4 and PAX4 R192H, P321H or R192H/P321H variant proteins in βTC3 cells and examined the protein expression level and nuclear translocation in this cell line. Although *PAX4* R192H functions had previously been investigated,[20] this variant was included in the present study as a control and for comparing to the combined effects of *PAX4* R192H/P321H variant. Upon transfection of these plasmids into βTC3 cells, the expression of wild-type PAX4 and PAX4 variant proteins could be detected in whole-cell lysates by using western blotting analysis. Figure 1a showed

that all the PAX4 variant proteins were expressed in the βTC3 cells at similar levels to that of the wild-type PAX4. In addition, wild-type PAX4 and all PAX4 variant proteins could translocate into the cellular nuclei (Figure 1b). These data indicate that neither protein expression of PAX4 R192H, P321H and R192H/P321H variant proteins nor their nuclear translocation was altered.

Transcriptional repressor activities on *insulin* and *glucagon* promoters

PAX4 protein has two DNA-binding domains: paired-domain and homeodomain, and a repressor domain at the carboxy-terminus of protein. *PAX4* R192H and P321H variations are located on homeodomain and repressor domains, respectively. To study the functions of PAX4 R192H, P321H and R192H/P321H variant proteins, we assessed repressor activities of wild-type PAX4 and PAX4 variant proteins on human *insulin* and *glucagon* promoters in mouse insulinoma (βTC3) and glucagonoma (αTC1.9) cells, respectively (Figures 2a and b). In animal model, the repression activity of PAX4 protein is apparently observed in early pancreas development through the competition with PAX6 protein on the same binding-site on the target gene promoters.[22] The wild-type PAX4 protein repressed *insulin* and *glucagon* promoter activities by 60% and 70% of the empty vector, respectively (Figures 2a and b). However, PAX4 R192H variant protein repressed the promoter activity by 50% of the empty vector. The same result was obtained in cells over-expressing PAX4 R192H/P321H variant protein while repressor activity of PAX4 P321H protein alone was similar to that of the wild-type PAX4 protein.

INS-1 cell viability under hyperglycemic stress condition

As *PAX4* gene expression could be activated in hyperglycemic condition,[1] we examined the effect of PAX4 variant protein on β-cell survival in a high glucose condition. To investigate the effect of high glucose condition on β-cell viability and apoptosis, INS-1 832/13 cells overexpressing wild-type PAX4 and PAX4 variant proteins were cultured for 72 h in the medium containing basal glucose or high glucose concentrations. Cell viability and apoptosis were examined by using the MTT assay and AnnexinV/PI staining, respectively. Overexpression of human wild-type PAX4 in INS-1 832/13 cells significantly increased cell viability (Figure 3a) and decreased cellular

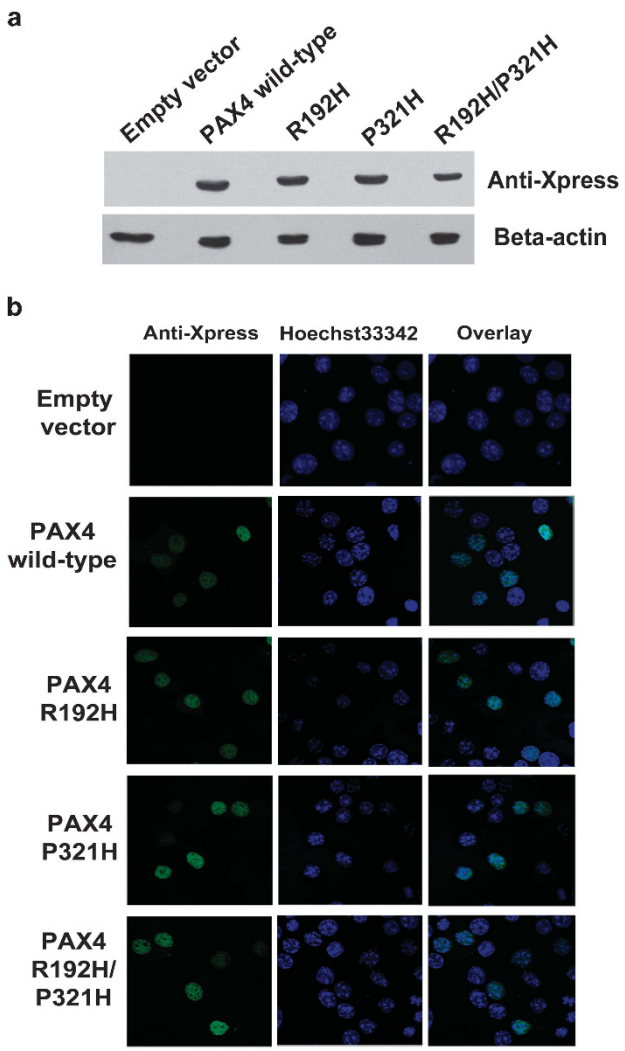

Figure 1 Expression and nuclear translocation of wild-type PAX4 and PAX4 variant proteins in βTC3 cells. (**a**) βTC3 cells overexpressing wild-type PAX4 (lane 2) or R192H (lane 3) or P321H (lane 4) or R192H/P321H (lane 5) variant proteins were equally produced after normalization with β-actin protein (lower panel). (**b**) Nuclear translocation of βTC3 cells overexpressing PAX4 variant proteins were similar to the wild-type PAX4 determined by using immunofluorescence staining and monitored under confocal microscope. Wild-type PAX4 or PAX4 variant protein was immunolabeled with anti-Xpress (green color) antibody. Nuclei were counterstained with Hoechst33242 dye (blue color).

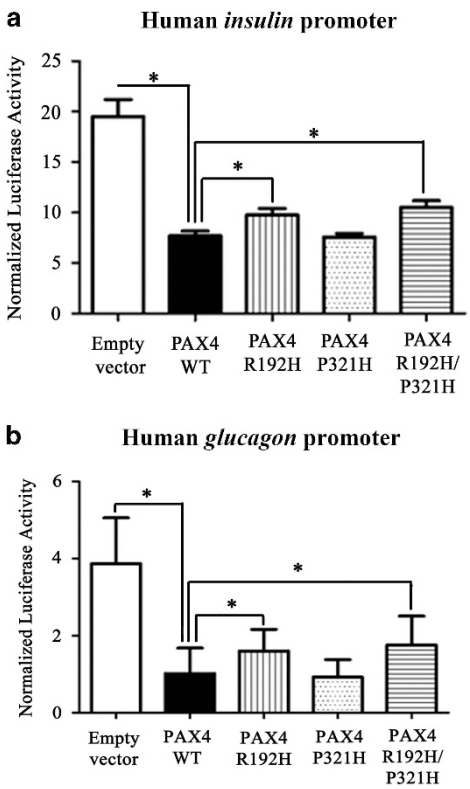

Figure 2 Functional analyses of wild-type PAX4 and PAX4 variant proteins on transcriptional repression activity. βTC3 cells (**a**) or αTC1.9 (**b**) were transfected with pcDNA3.1/hisB-empty vector or pcDNA3.1/hisB-*PAX4* wild-type or the pcDNA3.1/hisB-*PAX4* variants together with pGL3-*insulin* vector (**a**) or pGL3-*glucagon* vector (**b**) and pRL-SV40. Luciferase activity was normalized by the activity of the internal control pRL-SV40. Data were presented as mean ± s.e.m. ($N=3$). *$P<0.05$ compared with wild-type PAX4 by one-way ANOVA followed by Scheffe *post-hoc* test.

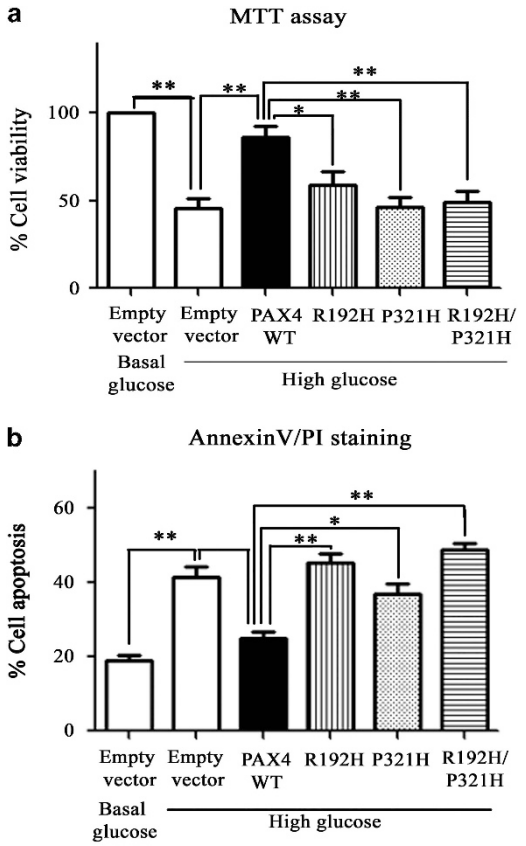

Figure 3 Functional analyses of wild-type PAX4 and PAX4 variant proteins on β-cell survival upon chronic high glucose exposure. Cell viability (**a**) and apoptosis (**b**) of INS-1 832/13 cells overexpressing wild-type PAX4 or PAX4 variant proteins culturing in a basal medium (11 mM glucose) or a high glucose medium (40 mM glucose) for 72 h were determined by using MTT assay and AnnexinV/PI staining, respectively. Cell viability of INS-1 832/13 cells transfected with pcDNA3.1/hisB empty vector (white bar) culturing in basal medium was expressed as 100%. The data represent the means of at least five independent experiments, and the error bars represent s.e.m. The differences of means between the results of wild-type PAX4 and PAX4 variant proteins were analyzed by using Student's *t*-test; $^{*}P<0.05$, $^{**}P<0.01$.

apoptosis (Figure 3b) under a prolonged culture in a high glucose medium. In contrast, the cell viability was significantly decreased (Figure 3a) and the cellular apoptosis was increased (Figure 3b) when the INS-1 832/13 cells overexpressing PAX4 R192H, P321H or R192H/P321H variant proteins were cultured in a high glucose media.

mRNA expression of genes in INS-1 832/13 cells overexpressing wild-type PAX4 and PAX4 variant proteins

PAX4 has important role in stimulating the expression of genes implicating in β-cell function, proliferation and survival.[5,23] We thus assessed the mRNA expression of some genes involving in those functions, including *TCF7L2*, *Cdk4*,[6] *Lgals9*[24] and *Calr*[24] in INS1 832/13 cells overexpressing wild-type PAX4 and PAX4 variant proteins by using qRT-PCR. Our results showed that mRNA expression levels of these four genes were increased in INS1 832/13 cells overexpressing wild-type PAX4 protein (Figure 4). In contrast, their expression levels were decreased in the cells overexpressing PAX4 R192H but not PAX4 P321H variant protein (Figure 4). In consistence with the previous report,[24] we also observed that PAX4 R121W, a diabetes-linked variant

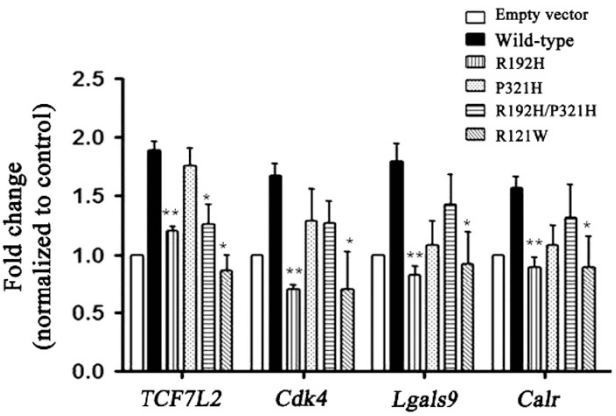

Figure 4 mRNA expression levels of genes in INS-1 832/13 cells overexpressing wild-type PAX4 and PAX4 variant proteins. mRNA expression of *TCF7L2*, *Cdk4*, *Lgals9* and *Calr* genes were examined in INS-1 832/13 cells overexpressing wild-type PAX4 and PAX4 variant proteins by using qRT-PCR. Relative mRNA levels of the genes were normalized to the mRNA level of *β-actin*. Data are calculated as fold change compared with that of the cells transfected with pcDNA3.1/hisB empty vector and they are expressed as mean ± s.e.m. from three independent experiments. The differences of mean between the results of wild-type PAX4 and PAX4 variant proteins were analyzed by using the Student's *t*-test; $^{*}P<0.05$, $^{**}P<0.01$.

reported in Japanese with T2D,[12] was less efficient to activate the expression of *Lgals9* and *Calr* genes than the wild-type PAX4 protein (Figure 4). These data illustrated that PAX4 R192H variant protein impaired transcription activation on target genes that required to maintain β-cell function, proliferation and survival.

DISCUSSION

In this study, we showed that genotype and allele frequencies of *PAX4* rs2233580 (781G>A; R192H) were more frequent in T2D patients than in the controls (corrected $P=0.003$ and 0.0012, respectively) with OR of 1.66 ($P=0.001$; 95% CI, 1.22–2.27). *PAX4* rs712701 (1168C>A; P321H) was not associated with T2D but it was in LD with *PAX4* rs2233580. The 192H/321H (A/A) haplotype was more frequent in T2D patients than in the controls (9.5% vs 6.6%; $P=0.009$). *PAX4* rs2233580 (781G>A; R192H) results in the change of amino acid at the highly conserved position among mammalian species, located in the homeodomain that is required for DNA-binding to target gene promoters. *PAX4* R192H may disrupt its binding on target DNA sequences and exerts a reduction of transcriptional activity of target genes. During pancreas development, PAX4 shows a strong repressor function that will compete with PAX6 on *glucagon* promoter and allows the PAX6 to activate *insulin* gene transcription. This event may promote the development and differentiation of β-cells. In our study, we found that PAX4 R192H variant protein impaired repressor activities on *insulin* and *glucagon* promoters; thus PAX4 R192H variant protein might not be able to compete with PAX6 protein for transactivation of genes involving in β-cell development and differentiation.[22] In mature β-cells, PAX4 has a critical role in promoting β-cell proliferation after exposure to toxic conditions, such as high glucose or cytokines. Our results revealed that overexpression of wild-type PAX4 in INS-1 832/13 cells increased cell viability and decreased cell apoptosis under prolonged culture in a high glucose medium while those cells overexpressing PAX4 R192H variant protein had defect in both transcription repression activities on target gene promoters and decreased cell survival under the high glucose condition. These findings were similar to the results from

another study showing that mouse PAX4 R129W mutant protein attenuated protection against cytokine-induced cell death by reduced islet cells proliferation.[5]

Furthermore, we found that wild-type PAX4 protein stimulates the expression of the investigated genes involved in β-cell function, proliferation and survival, including *TCF7L2*, *Cdk4*, *Lgals9* and *Calr*, encoding for TCF7L2, Cdk4 galectin-9 and calreticulin, respectively. It is well defined that TCF7L2 is required for maintaining glucose-stimulated insulin secretion and β-cell survival.[25] Depletion of *TCF7L2* results in impaired β-cell function while its overexpression protects the cells from glucotoxicity and cytokine toxicity in isolated mouse and human islets.[25] In addition, overexpression of Cdk4 increased human and rodent β-cell replication.[26] Thus decreased expression of either *TCF7L2* or *Cdk4* due to the PAX4 R192H variant protein may cause a reduction of β-cell proliferation. A recent study showed that wild-type PAX4 protein regulates endoplasmic reticulum homeostasis to prevent cell degeneration and to maintain cell survival by increased transcription levels of *Lgals9* and *Calr*, whereas the PAX4 R129W variant protein did not. Our data demonstrated that transcription levels of these two later genes were increased in the INS-1 832/13 cells overexpressing wild-type PAX4 protein, whereas their expression levels were decreased in those cells overexpressing PAX4 R192H or PAX4 R121W variant protein. Although PAX4 P321H variant protein did not have any effects on transcription-repressor activity and mRNA expression levels of the investigated genes, it significantly decreased cell viability in the high glucose condition. This indicates that PAX4 P321H variant protein may only have effect in a high glucose-stressed condition. PAX4 R192H variant protein exhibits dual defects in transcription repression, which reduces β-cell mass, and in transactivation of anti-apoptotic genes, which decreased mature β-cell survival under the high glucose condition, while the PAX4 P321H variant protein may only have an effect on the reduction of cell viability after exposure to glucotoxic condition. It is possible that the defect caused by *PAX4* P321H alone is insufficient to affect β-cell function in the physiological condition. These support our genetic analysis showing that individuals who carried *PAX4* R192H variation had increased T2D risk but not when they carried only P321H. The results from the previous and our studies indicate that *PAX4* SNP rs2233580 (781G>A; R192H) and rs712701 (1168C>A; P321H), which are in LD and common in Asian (Thai, Japanese and Han Chinese) populations,[8,15] associate with increased risk to T2D development, whereas these SNPs might not have the similar role in European populations.[10,11,14] This may suggest that specific *PAX4* variations are responsible for diabetes susceptibility in Asian, especially Thai, populations.

In conclusion, we demonstrated that *PAX4* rs2233580 (R192H) influences T2D risk. The mechanisms are likely involved in the reduced functions of PAX4 R192H variant protein to repress target genes promoting β-cell development and to activate genes maintaining β-cell function, proliferation and survival in both physiological and pathophysiological conditions. The results of this work provide more insight into the implication of *PAX4* polymorphisms in T2D risk.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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