ORIGINAL ARTICLE

Mitochondrial haplogroups have a better correlation to insulin requirement than nuclear genetic variants for type 2 diabetes mellitus in Taiwanese individuals

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ABSTRACT

Aims/Introduction: Identifying diabetes-susceptible genetic variants will help to provide personalized therapy for the management of type 2 diabetes. Previous studies have reported a genetic risk score (GRS), computed by the sum of nuclear DNA (nDNA) risk alleles, that may predict the future requirement for insulin therapy. Although mitochondrial dysfunction has a close association with insulin resistance (IR), there are few studies investigating whether genetic variants of mitochondrial DNA (mtDNA) will affect the clinical characteristics of type 2 diabetes.

Materials and Methods: Mitochondrial haplogroups were determined using mtDNA whole genome next generation sequencing and 13 single nucleotide polymorphisms (SNPs) in nDNA susceptibility loci of 13 genes in 604 Taiwanese subjects with type 2 diabetes. A GRS of nDNA was computed by summation of the number of risk alleles. The correlation between the mtDNA haplogroup and the clinical characteristics of type 2 diabetes was assessed by logistic regression analysis. The results were compared with the GRS subgroups for the risk of insulin requirement.

Results: Mitochondrial haplogroups modulate the clinical characteristics of type 2 diabetes, in which patients harboring haplogroup D4, compared with those harboring non-D4 haplotypes, were less prone to require insulin treatment, after adjusting for age, gender, and diabetes duration. However, there was no association between insulin requirement and GRS calculated from nuclear genetic variants.

Conclusions: Mitochondrial haplogroups, but not nuclear genetic variants, have a better association with the insulin requirement. The results highlight the role of mitochondria in the management of common metabolic diseases.

INTRODUCTION

Type 2 diabetes is a heterogenous disease characterized by dysfunction of insulin secretion and insulin resistance (IR), and both are affected by genetic and/or environmental factors

contributing to the pathogenesis of the disease[1,2] The relative contributions of these two factors has been debated extensively. Some studies have suggested that beta cell dysfunction has a critical role in the progression of diabetes, whereas others proposed that insulin resistance is the primary contributor that induces beta cell dysfunction[3,4] In spite of previous studies of

nuclear DNA (nDNA) genetic variants associated with type 2 diabetes, the genetic and physiological bases remain obscure[5,6] Mitochondrial dysfunction and insulin resistance in classical target organs such as liver, fat, and muscle of individuals with type 2 diabetes have been clearly demonstrated[7–9] Mitochondrial DNA (mtDNA) mutations accumulated along the pathway of human migration out of Africa and around the world. Those mutations that were functionally beneficial in a particular environment were selected regionally, and defined the related mtDNA haplogroups[10] In Chinese populations, our group have demonstrated that the diabetes-susceptible haplogroup (B4) harbors 10398A and the poly C tract, while the diabetes-resistant haplogroups (D4, N9) harbor 10398G without the poly C tract[11] By the generation of a trans-mitochondrial cybrid cell, we verified the influence of haplogroup B4 on chronic inflammation and cellular insulin resistance. On the other hand, cybrid N9 and D4 harboring the diabetes-protective haplogroups were more insulin sensitive and less prone to inflammation[12]

This study explored whether there is a correlation between mtDNA haplogroups and the clinical features of type 2 diabetes in a cohort of Taiwanese patients. In addition, a list of single nucleotide polymorphisms (SNPs) in nDNA susceptibility loci found to be associated with type 2 diabetes in an Asian population (CDKAL1, CDKN2A, FGF21, FTO, IRS1, PPAR-ɤ, KCNJ11, HHEX, IGF2BP2, TCF7L2, KCNQ1, C2CD4A/B, SLC30A8, PSMD6) were compared. A genetic risk score (GRS) calculated according to the number of risk alleles was used to assess the association between nuclear SNPs and clinical features in patients with type 2 diabetes.

MATERIALS AND METHODS
Study populations
Between January 2018 and December 2019, all patients with type 2 diabetes and aged 30 years or older, who regularly attended the endocrinology and metabolism outpatient clinics in a tertiary referral center in South Taiwan (Kaohsiung Chang Gung Memorial Hospital) were asked to participate in this study. Type 2 diabetes was diagnosed according to the American Diabetes Association criteria[13] and type 1 diabetes patients were excluded from this study. A total of 604 patients were recruited and informed consent was obtained from all study subjects. Each patient received a detailed interview regarding his or her personal disease. Demographic data, anthropometric measurements, laboratory tests results, and the use of antidiabetic drugs were also obtained. The Human Research Ethics Committee of Chang Gung Memorial Hospital approved this study.

Clinical and serum biochemical analyses
Waist circumference was measured at the mid-point between the lowest rib and the iliac crest. Measurements of body height and weight were taken from study patients to calculate the body mass index (BMI), which was defined as the weight divided by the square of the height (kg/m^2). Venous samplings were done for analyzing glycosylated hemoglobin (HbA1c), fasting blood glucose (FPG), serum creatinine, total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglycerides (TG). The estimated glomerular filtration rate (eGFR) was calculated with the abbreviated Modification of Diet in Renal Disease (MDRD) Study Group equation [eGFR ($mL/min/1.73\ m^2$) = 186.3 × (serum creatinine$^{-1.154}$) × (age$^{-0.203}$) × 0.742 (if female)]. Urine albumin concentrations were determined by immunonephelometry (Dade-Behring). Insulin resistance was assessed using the homeostasis model assessment estimate (HOMA) of insulin resistance [HOM-IR = fasting insulin levels (μIU/mL) × fasting glucose levels (mM)/22.5].[14] Insulin secretion capacity was represented by HOMA-β, which was calculated as (20 × fasting insulin levels)/(fasting glucose levels − 3.5)[14] Insulin requirement is defined as the use of insulin, either alone or in combination with an oral hypoglycemic agent, at enrolment to the study.

Selection of SNPs and genotyping
A list of single nucleotide polymorphisms found previously to be associated with type 2 diabetes were selected in our study with the following considerations: (1) SNPs found to be associated with type 2 diabetes in an Asian/Han population; or (2) SNPs found to be associated with drug treatment responses. Finally, we genotyped 17 SNPs located in or near 14 candidate genes. The following SNPs were examined: CDKAL1 (rs10946398), CDKN2A (rs10811661), FTO (rs8050136), FGF21 (rs838147), HHEX (rs1111875), IGF2BP2 (rs4402960), IGF2BP2 (rs7651090), IRS1 (rs1801278), IRS1 (rs2943641), KCNJ11 (rs5219), PPAR-ɤ (rs1801282), SLC30A8 (rs13266634), TCF7L2 (rs12255372), TCF7L2 (rs7901695), KCNQ1 (rs2237892), C2CD4A/B (rs7172432), and PSMD6 (rs831571).

DNA was extracted using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA). The SNPs genotyping TaqMan assay (Thermofisher Scientific) of 96-well format real-time PCR was used to detect the polymorphisms of the indicated genes above.

Construction of genetic risk score
Two methods were used to create the GRS: an unweighted method (u-GRS) and a weighted method (w-GRS). The SNPs in IGF2BP2, rs4402960, and rs7651090, were located in the same linkage disequilibrium block. Thus, only rs7651090 was used to calculate the GRS in the analysis. Also, only the TCF7L2 (rs12255372) was used to calculate GRS because the selected two SNPs (rs7901695 and rs12255372) in TCF7L2 were in the same linkage disequilibrium block. The SNPs in FGF21 (rs838147) had a minor allele frequency of 0.2%, and IRS1 (rs1801278) was monomorphic in the study group, therefore were not included in the GRS calculation.

The unweighted total GRS (uT-GRS) was calculated by summing the number of risk alleles (0/1/2) of all 13 selected SNPs

in each individual. We also created a weighted total GRS (wT-GRS) that was calculated by summation of weighting each risk allele with the effect size (the natural log of the odds ratios) (Table S1) for risk of type 2 diabetes mellitus reported in Asian populations[15,16] These selected 13 genetic variants were further classified into two categories: (1) 10 β-cell function–related SNPs including CDKAL1 (rs10946398), CDKN2A (rs10811661), HHEX (rs1111875), IGF2BP2 (rs7651090), KCNJ11 (rs5219), TCF7L2 (rs12255372), KCNQ1 (rs2237892), C2CD4A/B (rs7172432), SLC30A8 (rs13266634), and PSMD6 (rs831571) and (2) three insulin resistance/obesity-related SNPs including FTO (rs8050136), IRS1 (rs2943641), and PPAR-ɣ (rs1801282). We then calculated the weighted GRS of β-cell function-related SNPs (wβ-GRS) and insulin resistance and obesity-related SNPs (wR-GRS), respectively. The results of the analysis did not show a difference between the u-GRS and w-GRS so that only the results from the w-GRS are presented in this study.

Determination of mitochondrial haplogroup using whole mitochondrial sequencing

Genomic DNA was extracted from venous blood by using a Puregene DNA purification kit (Gentra, Minneapolis, MN, USA). The mitochondrial genome was prepared in two runs of polymerase chain reaction (PCR), first by six primer pairs to amplify the mitochondrial genome followed by a 64 overlapping primer pairs by multiplex PCR using ExTaq DNA polymerase (Takara Bio Inc., Otsu, Japan). The barcoded PCR libraries were pooled and then sequenced by Illumina MiSeq 2×300 bp paired-end runs. The raw reads data were processed with FastQC, FastQ groomer, Trimmomatics to remove primer sequences and then mapped to the revised Cambridge Reference Sequence (rCRS) with Burrows-Wheeler Aligner (BWA-MEM). The aligned BAM files were then analyzed using mtDNA-Server (https://mtdna-server.uibk.ac.at)[17] The haplogroup calling was classified by the HaploGrep2[18]

Statistical analysis

Continuous data that are normally distributed are expressed as the mean ± standard deviation (SD) and those variables that are non-normally distributed are presented as the median and interquartile range. The comparisons between patients with haplogroup carrier and non-carrier were conducted using the unpaired Student's t-test for comparing normally distributed continuous variables and the Mann-Whitney U-test for variables not normally distributed. Study subjects were divided into three groups according to wT-GRS (low, medium, and high) and differences in baseline characteristics were determined using ANOVA. Categorical data are expressed as numbers and percentages and the differences between categorical variables were tested by the Chi-square test. To test if the association between insulin requirement and haplogroup D4 was significant, we further performed multiple testing by using the false discovery rate (FDR)

approach with the Benjamini and Hochberg method. Additionally, logistic regression analysis was used to analyze the interaction between the risk of insulin requirement and the mitochondrial haplogroup or w-GRS groups. $P < 0.05$ was considered to be significant.

RESULTS
Clinical information of the patients
The characteristics of total and subgroups of the study populations are shown in Table 1. The mean age of the study subjects was 61.3 years and the duration of diabetes was 13.2 years. The mean BMI of the study subjects was 26.8 kg/m^2 and the mean level of HbA1c was 7.5%. The proportion of subjects who received insulin therapy was 21.3% in our study. The median of HOMA-IR and HOMA-β was 2.34 and 37.4, respectively.

Association of nuclear SNPs with patients' clinical features
All the enrolled participants were divided into three approximately equally sized groups according to the levels of wT-GRS (Table 1). There were no significant differences of sex, age, duration of diabetes, age of diagnosis, BMI, waist circumference, HbA1c, and lipid profiles among the three groups. The levels of HOMA-β in the medium wT-GRS group were higher than those in the low wT-GRS group. The levels of HOMA-IR, however, were not different among these three groups. Also, the ratio of insulin therapy did not have a significant difference in individuals divided according to wT-GRS. Logistic regression analysis for risk of insulin requirement associated with w-GRS, adjusted for sex, age, and duration of diabetes is shown in Table 2. The nuclear genetic variants did not show a significant correlation to insulin requirement in the unadjusted model or adjusting other parameters. We further tried to assess the association between the insulin requirement and wT-GRS, wβ-GRS, or wR-GRS. The results, however, did not reveal an association between the wT-, w β-, or wR-GRS and insulin therapy in unadjusted or adjusted models (Table 2). The comparisons of subgroups of patients, divided by GRS counted in simple methods are described in Table S2. There was no significant difference in all parameters between these three groups.

Association of mitochondrial haplogroup with patients' clinical features
We then divided all the patients into subgroups with mtDNA haplogroups B, D, F, M as well as the group called "others", which included minor mtDNA haplogroups such as A, C, E, G, H, N, R, Y, or Z. Table 3 summarizes the clinical characteristics of these five groups. Patients with haplogroup B had the youngest age of diagnosis (45.9 ± 1l.0 years) in these five groups. The other characteristics and clinical parameters of the study populations did not differ statistically between the different haplogroups analyzed. The patients were then divided into two groups and compared, according to each haplogroup carrier (such as B, D, F, M) and non-carrier (non-B, non-D, non-F, non-M). The age of diagnosis was younger in patients with

Table 1 | Clinical characteristics of the three groups according to the weighted genetic risk score (w-GRS) of 13 SNPs

wT-GRS	Total	Low	Medium	High	P (ANOVA)
M/F	303/271	89/101	102/87	112/83	0.106
Age at enrolment (years)	61.3 ± 9.8	60.9 ± 9.3	60.9 ± 10.3	62.2 ± 9.8	0.345
Duration of diabetes (years)	13.2 ± 8.0	13.4 ± 8.0	12.9 ± 8.6	13.3 ± 7.5	0.820
Age of diagnosis (years)	48.7 ± 9.9	48.1 ± 9.5	48.6 ± 10.4	49.5 ± 9.8	0.371
BMI (kg/m^2)	26.8 ± 4.4	26.8 ± 4.1	26.7 ± 4.8	26.9 ± 4.3	0.950
Waist circumference (cm)	91.0 ± 10.7	90.8 ± 10.5	90.2 ± 10.8	91.9 ± 10.7	0.297
FPG (mg/dL)	135.5 ± 36.1	137.9 ± 35.2	130.7 ± 37.3	137.6 ± 35.4	0.114
HbA1c (%)	7.5 ± 1.0	7.6 ± 1.1	7.4 ± 0.9	7.4 ± 1.1	0.249
HbA1c (mmol/mol)	58.6 ± 11.5	59.2 ± 11.7	57.3 ± 10.2	57.8 ± 12.4	0.249
Creatinine (mg/dL)	0.86 (0.70-1.15)	0.85 (0.69-1.09)	0.84 (0.68-1.02)	0.90 (0.72-1.20)	0.097
eGFR (mL/min/1.73 m^2)	78.7 ± 26.3	79.0 ± 25.6	81.8 ± 25.2	75.7 ± 27.9	0.082
Total cholesterol (mg/dL)	168.0 ± 31.4	171.1 ± 29.6	163.9 ± 30.4	169.1 ± 33.7	0.071
HDL cholesterol (mg/dL)	47.0 ± 12.8	46.0 ± 10.5	46.8 ± 14.2	48.4 ± 13.5	0.176
LDL cholesterol (mg/dL)	90.0 ± 24.1	92.6 ± 22.3	87.6 ± 24.6	89.7 ± 25.1	0.127
Triglyceride (mg/dL)	118.0 (82.0-174.0)	123.0 (84.5-170.0)	108.0 (76.0-163.0)	115.5 (81.0-175.0)	0.215
HOMA-IR	2.34 (1.70-3.10)	2.44 (1.83-3.14)	2.23 (1.55-3.08)	2.46 (1.73-3.07)	0.519
HOMA-β	37.4 (27.2-60.5)	34.9 (26.7-56.7)	40.5 (28.3-66.0)*	37.4 (26.2-58.4)	0.031
Insulin requirement	122 (21.3)	44 (23.2)	36 (19.0)	42 (21.5)	0.723

Data are presented as *n* (%), mean ± standard deviation or mean (interquartile range). *P < 0.05 vs. low GRS. BMI, body mass index; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; wT-GRS, weighted total genetic risk score.

Table 2 | Adjusted odds ratio for weighted genetic risk scores (w-GRS) and haplogroups for insulin use

	Unadjusted	Model 1
Haplogroup		
Non-D4	1	1
D4	0.41 (0.18-0.92)*	0.41 (0.17-0.97)*
w-GRS		
wT-GRS	1.08 (0.48-2.46)	1.13 (0.48-2.66)
wβ-GRS	1.32 (0.59-2.96)	1.30 (0.61-3.04)
wR-GRS	0.61 (0.10-3.72)	0.80 (0.12-5.47)
wT-GRS		
Low	1	1
Medium	0.78 (0.48-1.28)	0.78 (0.46-1.33)
High	0.91 (056-1.47)	0.96 (0.58-1.60)

Values represent odd ratios and confidence intervals. Model 1 adjusted for sex, age and duration of diabetes. *P < 0.05. wT-GRS, weighted total genetic risk score; wβ-GRS, weighted β-cell function-related genetic risk score; wR-GRS, weighted insulin resistance and obesity-related genetic risk score.

mtDNA haplogroup B compared with non-B haplogroups (Table S3). The glycemic control was better in patients with mtDNA haplogroup D compared with non-D (7.3 ± 0.9 vs. 7.5 ± 1.0, P = 0.046) (Table S4). No significant differences were noted between the subjects haplogroup F vs non-F, and haplogroup M vs non-M (Tables S5, S6).

Our previous study with a Chinese population demonstrated that patients with haplogroup B4 were insulin resistant and more prone to chronic inflammation, while patients with cybrid D4 and N9 were more insulin sensitive and less prone to inflammation[12] So, we compared patients with haplogroup B4 and those with non-B4 haplotypes (Table 4). The age of diagnosis was younger in subjects with haplogroup B4. In addition, the subjects with haplogroup B4 had higher levels of HOMA-IR and FPG compared with non-B4 carriers, respectively. We also compared patients with haplogroup D4 and those with non-D4 haplotypes (Table 4). The haplogroup D4 group had a lower HbA1c level than the non-D4 group (7.2 ± 0.9 vs. 7.5 ± 1.0, P = 0.021). Furthermore, the proportions of patients requiring insulin therapy was lower in the D4 group than in the non-D4 group (11.3 vs. 23.8%, P = 0.033). To test if the association between D4 and insulin requirement was significant, we further performed multiple testing by using the false discovery rate (FDR) approach with the Benjamini and Hochberg method. We divided the study populations into insulin users and non-insulin users (Table S7). The significant parameters between these two groups are the duration of diabetes, age of diagnosis, fasting plasma glucose, HbA1c, creatinine, eGFR, and patients harboring haplogroup D. Then after putting the probabilities of duration of diabetes, fasting plasma glucose, HbA1c, eGFR, and patients harboring haplogroup D for analysis, the corrected probabilities of the association between D4 and insulin requirement was 0.036. Thus, we thought the association between D4 and insulin requirement was significant, although the significance was very modest. Finally, logistic regression analysis showed that carriers with haplogroup D4 had a lower risk of receiving insulin therapy than those with non-D4 haplogroup after adjustment of sex, age, and duration of diabetes (OR 0.41, P < 0.05) (Table 2).

Table 3 | Clinical characteristics of the five groups according to the mitochondrial haplogroups in patients with type 2 diabetes

Haplogroup	B	D	F	M	Others[†]	P
M/F	46/46	43/53	55/41	72/47	66/61	0.152
Age at enrolment (years)	59.3 ± 10.4	62.0 ± 10.8	62.7 ± 9.7	62.6 ± 8.8	60.6 ± 9.7	0.063
Duration of diabetes (years)	14.1 ± 8.9	13.2 ± 8.4	14.0 ± 9.0	13.5 ± 6.9	12.1 ± 7.5	0.391
Age of diagnosis (years)	45.9 ± 11.0	49.3 ± 9.6	49.4 ± 9.8	49.7 ± 9.3*	48.9 ± 9.5	0.045
BMI (kg/m^2)	27.1 ± 5.2	27.4 ± 4.7	26.6 ± 3.9	26.5 ± 3.8	26.5 ± 4.0	0.394
Waist circumference (cm)	91.1 ± 11.1	91.3 ± 11.9	90.8 ± 10.2	91.5 ± 10.1	90.0 ± 10.3	0.833
FPG (mg/dL)	143.7 ± 44.0	133.2 ± 32.2	134.3 ± 36.7	135.3 ± 36.8	135.2 ± 32.8	0.282
HbA1c (%)	7.6 ± 1.0	7.3 ± 0.9	7.4 ± 1.0	7.6 ± 1.0	7.5 ± 1.2	0.302
HbA1c (mmol/mol)	59.1 ± 11.2	56.2 ± 10.0	57.6 ± 10.7	59.1 ± 10.9	58.6 ± 12.7	0.302
Creatinine (mg/dL)	0.81 (0.68-1.00)	0.84 (0.69-1.16)	0.84 (0.68-1.06)	0.90 (0.72-1.13)	0.91 (0.70-1.13)	0.880
eGFR (mL/min/1.73 m^2)	82.9 ± 26.5	78.7 ± 26.1	80.9 ± 26.8	79.3 ± 24.4	75.8 ± 25.6	0.388
Total cholesterol (mg/dL)	170.5 ± 27.9	163.1 ± 30.6	167.6 ± 31.9	164.6 ± 31.3	172.9 ± 35.0	0.119
HDL cholesterol (mg/dL)	46.4 ± 11.5	46.1 ± 11.7	47.5 ± 13.3	46.1 ± 10.8	49.1 ± 16.6	0.239
LDL cholesterol (mg/dL)	93.5 ± 22.2	85.9 ± 23.2	88.6 ± 23.9	87.9 ± 24.4	91.4 ± 26.3	0.215
Triglyceride (mg/dL)	111.0 (83.0-173.0)	120.0 (81.0-187.0)	113.5 (74.3-156.0)	118.0 (84.0-169.5)	117.5 (79.0-178.8)	0.762
HOMA-IR	2.45 (1.92-3.30)	2.17 (1.58-2.89)	2.40 (1.75-3.14)	2.47 (1.71-3.05)	2.29 (1.69-3.15)	0.363
HOMA-β	34.1 (24.1-60.5)	39.5 (26.9-64.4)	37.4 (28.2-61.8)	37.6 (29.6-61.4)	37.2 (27.6-58.4)	0.572
Insulin requirement (%)	21 (22.8)	15 (15.8)	25 (26.0)	33 (27.7)	24 (18.9)	0.200

Data are presented as *n* (%), mean ± standard deviation or mean (interquartile range). BMI, body mass index; FPG, fasting plasma glucose; eGFR, estimated glomerular filtration rate. *$P < 0.05$ vs. haplogroup B. [†]Including minor mtDNA haplogroups like A, C, E, G, H, N, R, Y, or Z.

Table 4 | Clinical characteristics of haplogroup B4 vs. non-B4 and haplogroup D4 vs. non-D4 in patients with type 2 diabetes

Haplogroup	B4	Non-B4	P	D4	Non-D4	P
M/F	30/32	252/215	0.420	26/36	256/211	0.059
Age at enrolment (years)	59.3 ± 9.9	61.7 ± 9.8	0.081	61.8 ± 10.4	61.3 ± 9.8	0.733
Duration of diabetes (years)	14.1 ± 9.3	13.2 ± 7.9	0.432	12.6 ± 8.3	13.4 ± 8.1	0.454
Age of diagnosis (years)	45.9 ± 11.3	49.1 ± 9.6	0.037	49.9 ± 8.9	48.6 ± 10.0	0.328
BMI (kg/m^2)	26.4 ± 5.0	26.8 ± 4.2	0.444	26.7 ± 4.4	26.8 ± 4.3	0.914
Waist circumference (cm)	90.3 ± 10.4	91.0 ± 10.7	0.630	89.4 ± 12.1	91.1 ± 10.4	0.238
FPG (mg/dL)	147.2 ± 45.3	134.5 ± 35.8	0.044	129.1 ± 30.2	136.9 ± 37.1	0.138
HbA1c (%)	7.7 ± 1.1	7.4 ± 1.0	0.113	7.2 ± 0.9	7.5 ± 1.0	0.021
HbA1c (mmol/mol)	60.3 ± 12.1	57.9 ± 11.1	0.113	55.1 ± 9.9	58.6 ± 11.3	0.021
Creatinine (mg/dL)	0.82 (0.68-1.00)	0.86 (0.70-1.13)	0.149	0.81 (0.55-1.09)	0.86 (0.70-1.10)	0.432
eGFR (mL/min/1.73 m^2)	83.7 ± 26.7	78.8 ± 25.7	0.157	80.7 ± 26.9	79.2 ± 25.7	0.658
Total cholesterol (mg/dL)	179.8 ± 26.3	167.4 ± 32.4	0.441	164.8 ± 31.2	168.2 ± 31.9	0.423
HDL cholesterol (mg/dL)	46.8 ± 12.1	47.1 ± 13.1	0.857	46.5 ± 10.7	47.1 ± 13.3	0.714
LDL cholesterol (mg/dL)	93.0 ± 21.6	89.0 ± 24.6	0.224	88.0 ± 23.2	89.7 ± 24.4	0.606
Triglyceride (mg/dL)	113.0 (82.5-175.0)	116.0 (80.3-171.0)	0.828	110.0 (69.8-186.3)	116.0 (81.0-169.0)	0.688
HOMA-IR	2.50 (2.06-3.35)	2.35 (1.70-3.07)	0.021	2.39 (1.52-2.98)	2.43 (1.75-3.14)	0.280
HOMA-β	34.6 (24.3-61.8)	37.7 (27.7-60.5)	0.485	40.9 (28.4-66.7)	37.0 (27.2-60.3)	0.438
wT-GRS	1.37 ± 0.27	1.34 ± 0.26	0.385	1.36 ± 0.25	1.34 ± 0.27	0.568
Insulin requirement	16 (25.8)	102 (21.8)	0.516	7 (11.3)	111 (23.8)	0.033

Data are presented as *n* (%), mean ± standard deviation or mean (interquartile range). BMI, body mass index; FPG, fasting plasma glucose; eGFR, estimated glomerular filtration rate; wT-GRS, weighted total genetic risk score.

DISCUSSION

In the present study, we first demonstrated that mitochondrial haplogroups modulate the clinical feature of type 2 diabetes, in which patients harboring haplogroup D4, compared with those harboring non-D4 haplotypes, had a better glycemic control and were less prone to require insulin treatment during the disease course, after adjusting for age, sex, and diabetes duration. On the contrary, patients with haplogroup B4, compared with those with non-B4 haplotypes had a younger age of diagnosis, higher levels of HOMA-IR,

and FPG. Our study revealed that the mitochondria play an important role in the management of common metabolic diseases. However, there was no correlation between GRS derived from nuclear genetic variants investigated in our study and the clinical features of patients with type 2 diabetes.

Type 2 diabetes has a genetic predisposition[19] Identifying the diabetes-susceptible genetic variants in humans has been challenging. Since the first landmark genome-wide association study (GWAS) on type 2 diabetes was conducted in 2007[20] the progress of genetic association studies have identified at least 75 independent genetic loci for type 2 diabetes in nDNA[6] These findings underline the contribution of multiple modest effect variants, explaining only 20% of the type 2 diabetes heritability[21,22] and the causality of identified common variants in type 2 diabetes is largely unknown. Iwata *et al.* reported that the generation of GRS calculated by summing the number of 14 nDNA risk alleles related to the pathogenesis of diabetes may predict the future insulin requirements in Japanese people with type 2 diabetes[23] In addition, Jiang *et al.* also have reported that the glycemic progression can be predicted by a polygenic risk score including 123 known nDNA risk alleles in 7,091 Chinese patients with type 2 diabetes[24] However, our findings in Chinese patients with 13 susceptibility nDNA alleles are not consistent with them. Moreover, Zhou *et al.* reported that high GRS derived from 61 risk alleles for diabetes was associated with a younger age of initiating insulin therapy but was associated with glycemic progression in a study including 5,250 patients with type 2 diabetes in Scotland[25]

Mitochondria, as the cellular power plant, can function as signaling platforms to regulate cellular growth and death[26] Mitochondrial genes preferentially affect organs with a high energy demand, such as endocrine systems, muscle, brain and heart. In addition to the bioenergetic and biosynthetic function, mitochondria are increasingly recognized as being a central hub triggering innate immunity that highly influences the pathogenesis of age-related "inflammaging"[27,28]

The analysis of mitochondrial haplogroups can be used to track global migrations of human populations, and there is substantial evidence that they are drivers to produce both the susceptibility to disease and the adaptation of evolution[10] In a meta-analysis, there was a significant correlation of haplogroup J subjects with longevity, haplogroup H with the risk of developing Alzheimer's disease, and haplogroup cluster HV with developing Parkinson's disease in Caucasian populations.[29] Chinese subjects harboring the mitochondrial haplogroup B5 have been found to be resistant to Parkinson's disease[30] and haplogroup B4 was diabetes-susceptible, while haplogroup D4 was diabetes-resistant[11] In addition, individuals who harbor haplogroup N9a were significantly correlated with reduced susceptibility to type 2 diabetes in Japanese and Korean populations[31] More recently, mtDNA haplogroups have been suggested to contribute to the development and modulation of the radiographic progression of osteoarthritis[32]

The underlying molecular mechanisms for the protective role of haplogroup D4 in type 2 diabetes in our study may be related to the findings of our *in vitro* trans-mitochondrial cybrid cell studies[12,33-35] The use of cytoplasmic hybrid (cybrid) model offers the advantage to study the genetic effects of mitochondria because the cells have the same background of nuclear genome. Cybrid D4 *in vitro* showed significantly lower oxidative stress than cybrid B4 in baseline conditions and after treatment with insulin[12] Further, cybrid D4 revealed a more elongated mitochondrial network, enhanced mitochondrial biogenesis and bioenergetics, and a high expression of fusion-related mitochondrial dynamic molecules. Under the circumstance of insulin stimulation, the response of increasing network formation and the production of ATP were clearly observed in cybrid D4[34] Mitochondrial dynamics controls their quality and has been related to mtDNA stability, respiratory capacity, and cell response to stress[36,37] In fact, mitochondrial dynamics has been proposed as a bridge between the insulin resistance and a defect in mitochondrial function[33,38-40] We later discovered the causal role of mitochondrial dynamics in regulating IR and pro-inflammation in diabetes-susceptible cybrids. A bidirectional interaction between mitochondrial dynamics and mitochondrial ROS plays an important role[33,35]

Our study has some limitations. First, the phenotype of nDNA variants can be modulated by the mtDNA haplogroups[41,42] which was not analyzed in our study. Second, mitochondrial oxidative phosphorylation is exquisitely sensitive to environmental toxins. Environmental factors, which may modulate gene expression via epigenetic mechanisms, also have a crucial role in the development of type 2 diabetes. The genetic and environmental interaction was not investigated. Third, the association between D4 and insulin requirement was confirmed only after adjustment for sex, age, and the duration of diabetes without multiple testing. We consider the most important factor for insulin requirement is the duration of disease, and these three factors do not change during the study. Other variables, such as BMI and HbA1c, are more changeable during the disease course. Weight gain and HbA1c reduction associated with insulin therapy are significantly larger than genetic effects, which will lead to a limitation to disclose the genetic effect through statistical manipulation. Actually if we add BMI or HbA1c into the analysis, the association will disappear.

In conclusion, we have shown that mitochondrial haplogroups, but not GRS constructed by nuclear genetic variants, have a better association with the requirement for insulin among Taiwanese subjects with type 2 diabetes. Haplogroup screening in patients with type 2 diabetes might be suggested as a way of choosing the most adequate antidiabetic treatment in clinical practice. Future studies with a larger sample size would help to confirm our findings.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Information of selected genotyped genetic variants in the present study

Table S2 | Clinical characteristics of the three groups according to the total genetic risk score (T-GRS) of 13 SNPs in patients with type 2 diabetes

Table S3 | Clinical characteristics of haplogroup B and non-B in patients with type 2 diabetes

Table S4 | Clinical characteristics of haplogroup D and non-D in patients with type 2 diabetes

Table S5 | Clinical characteristics of haplogroup F and non-F in patients with type 2 diabetes

Table S6 | Clinical characteristics of haplogroup M and non-M in patients with type 2 diabetes

Table S7 | Clinical characteristics of insulin user vs. non-insulin user in patients with type 2 diabetes