



Research article

Evaluation of D-loop hypervariable region I variations, haplogroups and copy number of mitochondrial DNA in Bangladeshi population with type 2 diabetes

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ABSTRACT

The profound impact of mitochondrion in cellular metabolism has been well documented. Since type 2 diabetes (T2D) is a metabolic disorder, mitochondrial dysfunction is intricately linked with the disease pathogenesis. Mitochondrial DNA (mtDNA) variants are involved with functional dysfunction of mitochondrion and play a pivotal role in the susceptibility to T2D. In this study, we opted to find the association of mtDNA variants within the D-loop hypervariable region I (HVI), haplogroups and mtDNA copy number with T2D in Bangladeshi population. A total of 300 unrelated Bangladeshi individuals (150 healthy and 150 patients with T2D) were recruited in the present study, their HVI regions were amplified and sequenced using Sanger chemistry. Haplogrep2 and Phylotree17 tools were employed to determine the haplogroups. MtDNA copy number was measured using primers of mitochondrial tRNA^{Leu} (UUR) gene and nuclear β 2-microglobulin gene. Variants G16048A (OR:0.12, $p = 0.04$) and G16129A (OR: 0.42, $p = 0.007$) were found to confer protective role against T2D according to logistic regression analysis. However along with G16129A, two new variants C16294T and T16325C demonstrated protective role against T2D when age and gender were adjusted. Haplogroups A and H showed significant association with the risk of T2D after adjustments out of total 19 major haplogroups identified. The mtDNA copy numbers were stratified into 4 groups according to the quartiles (groups with lower, medium, upper and higher mtDNA copy numbers were respectively designated as LCN, MCN, UCN and HCN). Patients with T2D had significantly lower mtDNA copy number compared to their healthy counterparts in HCN group. Moreover, six mtDNA variants were significantly associated with mtDNA copy number in the participants. Thus, our study confers that certain haplogroups and novel variants of mtDNA are significantly associated with T2D while decreased mtDNA copy number (though not significant) has been observed in patients with T2D. However, largescale studies are warranted to establish association of novel variants and haplogroup with type 2 diabetes.

1. Introduction

Mitochondrion is intricately involved in cellular metabolism that supplies energy for function and growth of cells. On the other hand, mitochondrial metabolism has also been implicated as one of the harmful effects as it causes oxidative stress by generating free radical and mediating apoptosis. Free radicals can also exert impact on DNA leading to

mutation. It is now well documented that mutation in mitochondrial DNA (mtDNA) contributes potentially to the pathogenesis of type 2 diabetes (T2D) and insulin resistance (Gordon et al., 2015; Jiang et al., 2017; Liou et al., 2012; Martin and McGee, 2014; Szendroedi et al., 2012; Ye et al., 2013). T2D is a multifactorial polygenic disease which is considered as global health burden. Prevalence of T2D is increasing gradually among world population and about 500 million individuals

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confirmed with T2D have been reported in 2018 (Kaiser et al., 2018) and by 2045 about 10.9% of the world population may suffer from diabetes (IDF Diabetes Atlas 9th Edition 2019, n.d.). On the other hand, early diagnosis and proper management provide effective health benefits to the patients with T2D.

Variations within mtDNA have been shown to augment the production of reactive oxygen species which in turn further deteriorate the pathological conditions in patients with T2D (Rösen et al., 2001). Many of these variants have been identified within the coding regions of mitochondrial genome with incompatible results (Jiang et al., 2021; Saha et al., 2019; Sun et al., 2019). On the other hand, polymorphisms in the non-coding D loop region have important contribution to the proper functioning of mitochondria (Liou et al., 2010, 2012). The non-coding control region D-loop from 16024 – 16576 nucleotide position comprising of 1124 base pairs contains three hypervariable regions (HV1:16024-16383; HV2:57-372 and HV3:438-574). The hypervariable regions are hotspots for mtDNA variations (Stoneking, 2000; Tipirissetti et al., 2014). In previous studies, different variants in hypervariable region I (HV1) were found to be associated with T2D. Variant G16390A was weakly associated with T2D in Tunisian population (Hsouna et al., 2015). C16270T and C16320T was found to be significantly associated with increased risk of T2D in Moroccan population (Charoute et al., 2018). The poly C tract (16184-16193) of HV1 had been the prime focus of many association studies (Liao et al., 2008; Meiloud et al., 2013; Mueller et al., 2011; Palmieri et al., 2011; Saldaña-Rivera et al., 2018). T16189C polymorphism, one of the most widely studied D loop region variants, is reported to be associated with the regulation of reactive oxygen species production and mitochondrial DNA copy number (Lin et al. 2005; Liou et al., 2010). Both T16519C and T16189C were found to be associated with T2D in Italian population (Navaglia et al., 2006). A meta-analysis revealed association of T16189C with T2D and cancer (Kumari et al., 2018). D loop region is very important as it is essential for the replication and regulation of mitochondrial DNA copy number. MtDNA copy number, indirect marker of mitochondrial dysfunction (Malik and Czajka, 2013), alterations have been found in several diseases (Filograna et al., 2020). Reduced mtDNA copy number in patients with type 2 diabetes is now well evident (Al-Kafaji et al., 2018; Fazzini et al., 2021; Latini et al., 2020; Xu et al., 2012). Busnelli et al. (2019) demonstrated indirect relation between reduced mtDNA copy number and oxidative stress along with inflammation. Variants within mtDNA are population specific and are clustered in lineages that in turn define haplogroups. Haplogroup J has been reported to be significantly associated with the increased risk of T2D (Crispim et al., 2006) while European population did not show such association (Chinnery et al., 2007). Thus, studies to find out the association between haplogroups and T2D had produced conflicting results.

Our recent finding demonstrated protective role of G10398A polymorphism (within NADH dehydrogenase subunit 3 or ND3 gene) while C5178A variant (within NADH dehydrogenase subunit 2 or ND2 gene) was found to be associated with the risk of T2D (Saha et al., 2019). However, data regarding the association of hypervariable region of mtDNA with T2D with respect to Bangladeshi population are completely lacking. Thus, in the present study, we opted to analyze the HVI segment within the D-loop region of mtDNA to i) investigate the frequency of highly frequent variants and their probable association with T2D, ii) determine and compare haplogroups between T2D and healthy individuals and iii) determine and compare mtDNA copy number between T2D and healthy individuals with respect to Bangladeshi population.

2. Methodologies

2.1. Study participants

A total of 300 individuals participated in the current study. Out of them, 150 were healthy individuals and the rest of the 150 were patients with T2D. The study was approved by the ethical review committee of

the Department of Biochemistry and Molecular Biology, University of Dhaka. Type 2 diabetic patients were diagnosed by determining the levels of fasting blood glucose (>6.9 mmol/L) and HbA_{1c} (>6.5%) according to the criteria set by the World Health Organization (WHO). Healthy participants were selected who had no symptoms of infectious disease, liver and kidney disorders along with other noncommunicable diseases. Pregnant women and children were also excluded from this study. Anthropometric and demographic data were also recorded. After obtaining consent from the participants, five mL of blood was collected from each and stored at -80 °C for further analysis. This is to mention here that the case and control participants in the present study were not age matched as they were randomly selected. The statistical analyses performed by adjusting the age in regression models account for the age difference between patients and controls.

2.2. Extraction of genomic and mitochondrial DNA

Cellular fraction of blood was used for the extraction of genomic and mtDNA. Genomic DNA was extracted using the organic phenol chloroform extraction methods as described in our previous studies (Huda et al., 2018; Goswami et al., 2021) while mtDNA was extracted and confirmed according to the protocol described by Saha et al. (2019).

2.3. Determination of mitochondrial DNA copy number

MtDNA copy number was determined using qPCR (quantitative polymerase chain reaction). Primers specific to mitochondrial tRNA^{Leu} (UUR) gene were: forward primer: 5'-tgctgtctccatgtttgatgtatct-3' and reverse primer: 5'-tctctgtctcccaccctctaagt-3'. On the other hand, primers specific to the single copy nuclear gene beta-2 microglobulin (β2M) were: forward primer: 5'-caccaagaacagggtttg-3' and reverse primer: 5'-tggccatgggtatgttga-3'. Each 10 μL of reaction mixture contained 1 μL of 5 μM forward primer, 1 μL of 5 μM reverse primer, 5 μL of 2x SYBR Green SuperMix and 3 μL of 20 ng/μL of template DNA. The PCR reactions were measured in triplicate. The following program was used in the StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., USA): 1 cycle of 50°C for 2 minutes and 1 cycle of 95°C for 20 seconds followed by 40 cycles of denaturation at 95°C for 15 seconds and 40 cycles of annealing/extension at 56°C for 30 seconds. The mtDNA copy number was calculated using the equation $2 \times 2^{\Delta Ct}$, where ΔCt is the difference in Ct values of β2M and tRNA^{Leu} (UUR) gene.

2.4. Amplification of mitochondrial hypervariable region I and Sanger sequencing

The hypervariable region I of mtDNA was amplified using forward primer 5'-accagtctgttaaaccggag-3' and reverse primer 5'-gtggctatttagctttat-3' that amplified a stretch of mtDNA from 15911 to 16540 nucleotides. To perform polymerase chain reaction, a total volume of 30 μL was prepared and the condition was set at initial denaturation step of 5 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 48°C and 40 seconds at 72°C, and a final extension step of 5 minutes at 72°C. All the PCR amplicons were verified using agarose gel electrophoresis (2.5%) and visualized via ethidium bromide. Bands of 630 bp confirmed amplification of our desired sequence of mtDNA. Each amplicon was purified by Wizard® SV Gel and PCR Clean-Up System (Promega, USA) followed by sequencing using Sanger chemistry. It is to mention here that after sequencing, 11 chromatograms were excluded as they generated noise with lower strength of baseline signals. Thus, a total of 289 chromatograms (145 of healthy individuals and 144 of patients with T2D) were analyzed in the present study.

2.5. Analysis of sequence data and allele frequencies

Sequences without ambiguities were obtained between 16017 to 16519 and they were aligned to relate with rCRS (reference, please

provide the NCBI reference ID) with the help of Geneious software (Version 2021.0.3). Haplogrep2 was employed to determine the haplogroups. Allele frequencies were obtained using mitomap (<http://www.mitomap.org/MITOMAP>). Variation in distribution of allele frequencies between healthy individuals and patients with T2D were performed using data from the Human mitochondrial DNA Genome Polymorphism Database available at http://mitsnp.tmg.or.jp/mitsnp/index_e.shtml.

2.6. Statistical analysis

Demographic data was obtained from structured questionnaire and the quantitative data were compared between patients with T2D and healthy individuals using SPSS v21.0. The results were expressed as mean \pm SD for continuous variables and % for categorical variables. Odds ratios with p-value were calculated using epitools (Aragon, 2020) package in R to find probable association of mtDNA variants as well as individual haplogroups with T2D. Haplogroup M was used as reference during odds ratio calculation since it was the predominant haplogroup. Statistical analyses were also performed to find out relationships of SNPs and haplogroups with T2D after adjusting the confounding factors i.e., age and gender.

The quartiles of the whole study participants were measured. The mtDNA copy number was stratified according to the quartiles. Group with low mtDNA copy number (LCN) represents those individuals with mtDNA copy number less than lower quartile, group with medium mtDNA copy number (MCN) represents those with mtDNA copy number less than median but greater than or equal to lower quartile, group with upper mtDNA copy number (UCN) represents those individuals with copy number greater than or equal to median but lower than upper quartile and finally, group with higher mtDNA copy number (HCN) represents those individuals with copy number greater than or equal to upper quartile. Normality of mtDNA copy number was determined using Shapiro-Wilk test. Variables not normally distributed were analyzed using Wilcoxon test. Both Shapiro-Wilk test and Wilcoxon test were done using R language. Graphs were plotted in R programming language using the package ggplot2 (Wickham, 2016). Association of the mtDNA copy number with mtDNA variants was analyzed using R language. The differences of the mean mtDNA copy numbers were calculated between the mutant allele and the rCRS allele. For this particular association analyses, conditions i.e., case and control were also considered as confounding factors and hence along with age and gender, conditions were also adjusted. The rationale for adjusting disease condition was due to the decreased mtDNA copy number in patients with T2D compared to healthy individuals.

3. Results

3.1. General characteristics of the study participants

Among the T2D patients, 73 (48.67%) of them were males and 77 (51.33%) were females. The average BMI of the T2D patients was 26.23

\pm 3.37 kg/m² and the average age was 52.42 \pm 9.77 years. The mean systolic blood pressure for T2D patients was 126.03 \pm 6.61 mmHg while the mean diastolic blood pressure was 84.75 \pm 6.80 mmHg. In case of healthy individuals, there were 87 males (58%) and 63 females (42%). The average BMI of the healthy individuals was 24.07 \pm 2.80 kg/m² and the average age was 38.17 \pm 12.31 years. The mean systolic blood pressure for the healthy individuals was 120.75 \pm 8.20 mmHg while the mean diastolic blood pressure was 80.64 \pm 7.89 mmHg. The anthropometric and demographic data of male and female T2D patients and healthy individuals is shown in Table 1. In T2D patients, the estimated mean value of glycated hemoglobin (HBA1c in %) was 8.57 \pm 1.50 while in controls this value was 5.51 \pm 0.35. The biochemical parameters has also been presented in Table 1. Statistical analysis showed that all the parameters (both demographic and biochemical) were significantly different between healthy controls and T2D patients.

3.2. Frequency distribution of variants within the D-loop region of mtDNA sequence

A total of 147 variants were identified within the D-loop region of mtDNA located within 16017–16525 that also harbors the hypervariable region I or HV I (16024–16383). The Manhattan plot presented in Figure 1 symbolizes the variants in the Hypervariable region I (HV I) of mtDNA D-loop. The plot was constructed using the qqman (Turner, 2018) package in R. The variants were found in 131 nucleotide positions. Among the identified variants and positions, 32 were unique with respect to T2D and 37 were unique to the healthy controls while rest 78 were found in both the groups of participants. These variants were classified into three major groups on the basis of the minor allele frequency: common variants ($>/ = 10\%$); variants with low frequency ($>/ = 5\%$ to $<10\%$) and rare variants ($<5\%$). Supplementary Table 1 represents frequency distribution and association analysis of each variants with T2D. Out of 147 variants 132 (89.80%) were grouped as rare variants, 6 (4.08%) were grouped into low frequency and 9 (6.12%) were grouped as common variants. When frequency of the variants were analyzed independently in the healthy controls and patients with T2D, it was observed that out of 115 variants found in healthy controls, 4 (3.48%) were common variants, four were variants with lower frequency while rest of the 107 (93.04%) were rare variants. On the other hand, out of total 110 variants identified in patients with T2D, 2 (1.82%) were common, 7 (6.36%) were less frequent while rest 101 (91.82%) were rare variants.

Further investigation revealed that 15 positions harbor two or more nucleotide changes. Among them, three different types of variants were identified at positions 16093 and 16318 that include transition (T-C and A-G) and transversion (T-G, A-C, A-T and T-A) in a total of 41 individuals (Supplementary Table 1), while rest of the 13 positions contain two different types of variations. Interestingly, further observation revealed that out of these nucleotide positions 5 (33.33%) were within the continuous stretch of 10 nucleotide (C enriched) from 16256 to 16265 (¹⁶²⁵⁶CCACCCCTC¹⁶²⁶⁵ according to rCRS). This stretch harbors a total of 14 variants (8 unique) present in 36 individuals. Among them, 21 were

Table 1. Demographic and biochemical attributes of the subject participants.

Parameters	HI (n = 150) Male: 87; Female: 63	T2D (n = 150) Male: 73; Female: 77	p (HI vs T2D)
Age (years)	38.17 \pm 12.31	52.42 \pm 9.77	<0.00001
BMI(kg/m ²)	24.07 \pm 2.80	26.23 \pm 3.37	<0.00001
Systolic BP(mmHg)	120.75 \pm 8.20	126.03 \pm 6.61	<0.00001
Diastolic BP(mmHg)	80.64 \pm 7.89	84.75 \pm 6.80	<0.00001
Creatinine (mg/dL)	0.74 \pm 0.15	0.81 \pm 0.21	<0.001
HBA1c (%)	5.51 \pm 0.35	8.57 \pm 1.50	<0.00001

Values are presented as mean \pm SD. HI: Healthy individuals; T2D: patients with Type 2 Diabetes.

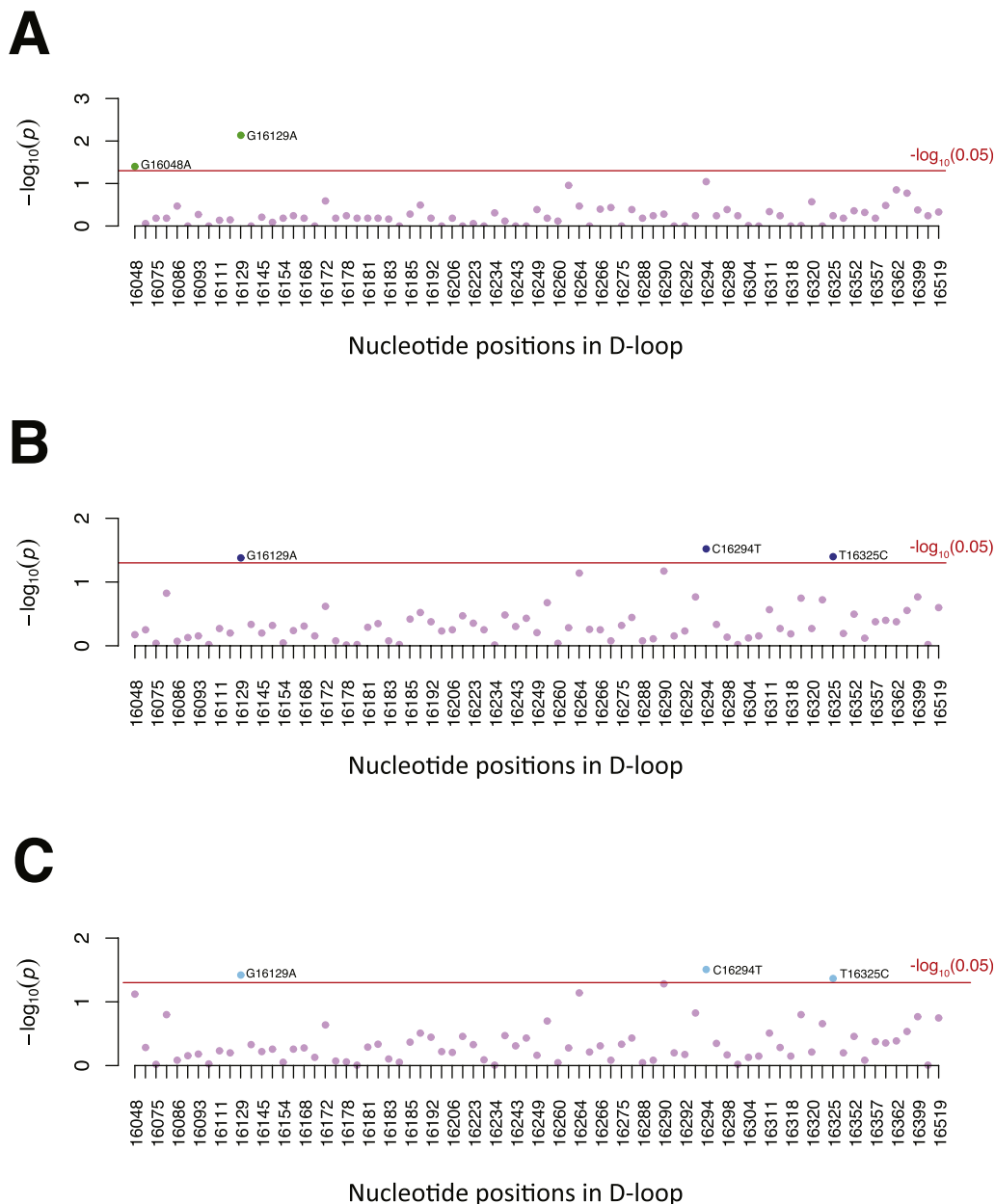


Figure 1. A modified Manhattan plot presenting the variants in the hypervariable region I of mtDNA loop. The y-axis shows the $-\log_{10}$ of p-values and the x-axis shows nucleotide positions in the D-loop. The red line is for $-\log_{10}(0.05)$. Any points above or on the line are statistically significant (p-value ≤ 0.05). A) Modified Manhattan plot without any adjustment (crude). The green points represent statistically significant SNPs. The plum color points represent SNPs which are not statistically significant. B) Modified Manhattan plot after age adjustment. The dark blue points represent statistically significant SNPs. The plum color points represent SNPs which are not statistically significant. C) Modified Manhattan plot after age and gender adjustments. The turquoise color points represent statistically significant SNPs. The plum color points represent SNPs which are not statistically significant.

healthy individuals and 15 were patients with T2D. However, statistical analysis revealed that none of the variants in these positions had significant association with T2D (Supplementary Table 1).

3.3. Association of various variants with type 2 diabetes

Out of total 147 variants identified within the HVI region of mtDNA, highest frequency was observed at positions 16519 (T-C, 69.55%) followed by position 16223 (C-T, 59.52%), 16311 (T-C, 18.34%), 16129 (G-A, 17.99%), 16362 (T-C, 14.19%), 16126 (T-C, 12.46%), 16051 (A-G, 10.73%), 16319 (G-A, 10.38%), 16189 (T-C, 10.03%). Distribution of allele frequency in the present study participants was almost similar to that of reported frequencies found in the mitomap database comprising of 51,836 full length mtDNA sequences. However, it was observed that the frequency of A allele at position 16319 was two-fold, G allele at position 16051 was five-fold and T allele at position 16223 was 1.5-fold higher in Bangladeshi population compared to that reported in mitomap allele frequency database. On the other hand, frequency of C allele at

position 16189 was found to be 2.5-fold lower in the present study population compare to that of C allele frequency reported in mitomap. Out of the common variants, presence of A alleles at positions 16129 and 16048 instead of G alleles showed a protective role against T2D (OR: 0.42, $p = 0.007$ and OR: 0.12, $p = 0.04$, respectively) without adjusting the confounding factors i.e., age and gender. On the other hand, after adjusting age and gender out of two reported associated SNPs, only G16129A showed significant protective role against developing T2D (Table 2). Moreover, after age adjustment we identified association of two new variants (C16294T and T16325C) conferring protective role against T2D. These three SNPs G16129A, C16294T and T16325C also conferred protective role against T2D even after adjusting both age and gender (Table 2).

3.4. Poly-C 16184-16193 tract

Analysis of poly-cysteine(C) 16184-16193 tract within the D-loop of mtDNA sequence revealed that 29 (10.03%) of the total individuals had C

Table 2. DNA variants within the hypervariable region I of mtDNA having significant association with type 2 diabetes before and after adjusting confounding factors.

Nucleotide Positions	Types of changes	Variants in all participants	Total frequency of the alleles	Variants in HI	Frequency in HI	Variants in T2D	Frequency in T2D	OR (95% CI)	P-value	Frequency in mitomap database	OR ^a (95% CI)	P-value ^a	OR ^{a+g} (95% CI)	p-value ^{a+g}
16048	G-A	8	2.77	7	2.42	1	0.35	0.12 (0.01–0.97)	0.04	0.264	0.16 (0.02–1.58)	0.67	0.16 (0.02–1.65)	0.076
16129	G-A	52	17.99	35	12.11	17	5.88	0.42 (0.22–0.79)	0.007	13.429	0.45 (0.20–0.99)	0.042	0.44 (0.20–0.97)	0.038
16294	C-T	7	2.42	6	2.08	1	0.35	0.16 (0.02–1.36)	0.09	8.922	0.09 (0.01–1.03)	0.03	0.10 (0.01–1.06)	0.031
16325	T-C	7	2.42	4	1.38	3	1.04	0.76 (0.17–3.46)	0.72	3.077	0.16 (0.03–0.91)	0.04	0.17 (0.03–0.95)	0.043

a = after age adjustment.

a+g = after age and gender adjustment.

HI: Healthy individuals; T2D: Type 2 diabetes; OR: Odds ratio; CI: Confidence interval.

nucleotide instead of T at position 16189. Among them, 12 (4.15%) were found in healthy individuals while 17 (5.88%) were obtained in patients with T2D (Table 3). Further scanning of this region identified substitution of C nucleotide by T in 2 individuals (1 from healthy and 1 from T2D) at position 16184, substitution of C nucleotide by T in ten individuals (6 from healthy individuals and 4 from T2D) at position 16185, substitution of C nucleotide by T at 16187 (2 from healthy individuals), 1 healthy individual had T instead of C at position 16188, five individuals had T instead of C (3 from healthy and 2 from T2D) at position 16192 while 2 individuals (1 healthy and 1 T2D) had T nucleotide instead of C at position 16193. Nucleotides at positions 16186, 16190 and 16191 was found to be conserved in all individuals who took part in the present study. It is evident from Table 3 that the poly-C tract is almost evenly distributed between the two groups of study participants and no significant association of C16189T was observed. Even after adjusting the confounding factors (age alone along with both age and gender), the frequencies of variants within the poly-C tract did not show any significant association with T2D.

3.5. Haplogroup analysis

After analyzing D-loop region, a total of 19 haplogroups (A, B, D, E, F, H, J, L, M, N, O, P, R, S, T, U, X, W, Z) were identified. Among these, haplogroups B, E, J and O were only detected in case of healthy individuals while haplogroups P, S and X were found in patients with T2D. The frequencies of the haplogroups found in study participants have been presented in Table 4. Among these, 40.48% of the study participants belong to M macrohaplogroup followed by 14.18%, 10.73%, and 7.2% which belong to H, U and R haplogroups, respectively. Further analysis revealed that other than haplogroups unique to healthy individuals or to patients with T2D frequency of common haplogroups were almost evenly distributed. However, frequencies of N and T haplogroups were found to be 3-fold higher in healthy individuals while haplogroups A, D and L were found to be 6-fold, 3-fold and >2-fold higher in patients with T2D compared to their healthy counterparts, respectively. Logistic regression analysis demonstrated that haplogroup A is significantly associated with the risk of T2D in our population (OR: 5.61, CI: 0.89–148.49, $p = 0.05$). After adjusting age and gender, both haplogroup A and H showed association with the risk of T2D (Table 4).

3.6. mtDNA copy number in healthy individuals and patients with type 2 diabetes

The median mtDNA copy number of healthy individuals was 334.37 (Inter Quartile Range or IQR = 506.24) that did not differ significantly from that of T2D which was 287.73 (IQR = 421.70) while computed by Wilcoxon test ($p = 0.16$). More variations of the mtDNA copy number was observed in case of healthy individuals compared to T2D as indicated by the IQR (Figure 2). The mtDNA copy number of the study participants was not normally distributed ($p < 2.2 \times 10^{-16}$ according to Shapiro Wilk test). The normality was checked using Q-Q plot (Supplementary Figure 1).

The lower quartile, median and upper quartile of mtDNA copy number for the study population were 147.37, 314.24 and 603.65, respectively. LCN group had $X < 147.3732$, group MCN had $147.3732 \leq X < 314.2396$, group UCN had $314.2396 \leq X < 603.6459$ and group HCN had $X \geq 603.6459$ (where X represents mtDNA copy number). The distributions of mtDNA copy numbers of healthy individuals and patients with T2D in different groups have been portrayed in Figure 3. Even when median mtDNA copy number of healthy individuals and patients with T2D were stratified into Groups L, M and U, no significant variation was observed according to Wilcoxon test ($p = 0.08, 0.80$ and 0.55 , respectively). However, the median mtDNA copy number between the two groups of study participants varied significantly in Group H ($p = 0.03$). The mtDNA copy number of healthy individuals in group H (IQR =

Table 3. Frequency distribution of mitochondrial DNA variants within the Poly-C 16184-16193 tract of D-loop.

Position of Nucleotides	Types of changes	Variables in all participants	Total frequency of the alleles	Frequency in HI	Frequency in T2D	OR (95% CI)	p-value	Frequency in mitomap database	OR ^a (95% CI)	p-value ^b	OR ^{a+g} (95% CI)	p-value ^{a+g}
16184	C-T	2	0.69	0.35	1	1.00 (0.06–16.25)	0.99	0.735	1.08 (0.04–27.91)	0.96	1.26 (0.05–33.07)	0.89
16185	C-T	10	3.46	2.08	4	0.66 (0.18–2.39)	0.53	1.086	0.52 (0.12–2.20)	0.38	0.56 (0.13–2.34)	0.43
16187	C-T	2	0.69	0.69				4.676				
16188	C-T	1	0.35	0.35				0.558				
16189	T-C	29	10.03	4.15	17	1.48 (0.68–3.23)	0.32	25.367	1.63 (0.65–4.08)	0.3	1.61 (0.64–4.04)	0.31
16192	C-T	5	1.73	1.04	2	0.67 (0.11–4.10)	0.66	4.265	0.42 (0.05–3.74)	0.42	0.37 (0.04–3.44)	0.36
16193	C-T	2	0.69	0.35	1			1.304	2.20 (0.13–36.34)	0.59	2.17 (0.13–37.05)	0.6

a = after age adjustment.

a+g = after age and gender adjustment.

HI: Healthy individuals; T2D: Type 2 diabetes; OR: Odds ratio; CI: Confidence interval.

380.01) varied more compared to that of patients with T2D in that group (IQR = 228.99).

Variants G16048A and G16129A had significantly increased mtDNA copy number without adjusting age gender and conditions of the participants (Table 5). On the other hand, variants T16126C, C16234T, T16311C and T16519C were found to have reduced mtDNA copy number before adjustments. After adjusting the age, gender and conditions, variants G16048A, T16126C, C16234T, T16311C and T16519C remained significantly associated with mtDNA copy number. A new variant C16291T was found to have increased copy number after adjusting the confounders as shown in Table 5.

4. Discussion

Mitochondrial DNA is an excellent tool in forensic and geneological studies owing to its high stability, high copy number, uniparental maternal inheritance and high mutational rates (particularly in the hypervariable regions). Many studies confirmed the association of T16189C of HV1 with the risk of T2D in different population (Bhat et al., 2007; Khogali et al., 2001; Kumari et al., 2018; Liao et al., 2008; Mueller et al., 2011; Palmieri et al., 2011; Park et al., 2008; Saldaña-Rivera et al., 2018; Tang et al., 2006). However, the T16189C variant was not significantly associated with T2D in our study though the variant was prevalent among the patients compared to healthy individuals (OR:1.48, Table 3). This finding is in concordance with other previous studies containing large number of cases (Chen et al., 2009; Chinnery et al., 2005; Hsouna et al., 2015; Meiloud et al., 2013; Mohlke et al., 2005; Saxena et al., 2006). A meta-analysis performed on Asian population conferred association of T16189C with the increased risk of T2D while such association was not manifested in European Finnish and British populations (Mohlke et al., 2005; Soini et al., 2012) as well as in North African Tunisian and Mauritanian populations (Hsouna et al., 2015; Meiloud et al., 2013). Interestingly, the frequency distribution of T16189C was similar in Asian and North African populations (Hsouna et al., 2016). It clearly indicates that genetic make-up of Asian, North African and European populations may contribute to such incompatible association of the T16189C variant with T2D. Two variants G16048A (OR: 0.12, p = 0.04) and G16129A (OR: 0.42, p = 0.007) were found to play protective role in T2D in

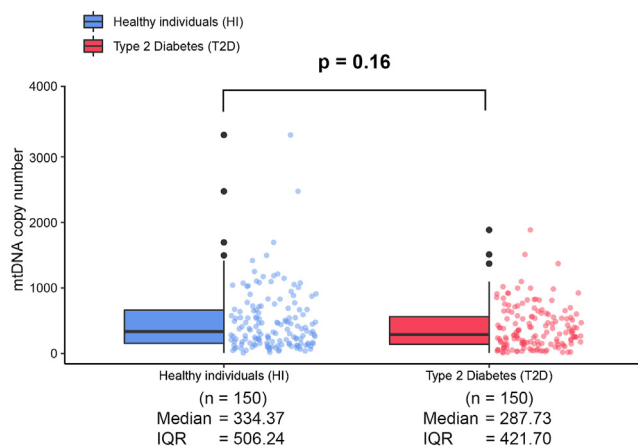


Figure 2. The hybrid boxplot representing the mtDNA copy number after stratifying according to quartiles. LCN: mtDNA copy number less than lower quartile, MCN: mtDNA copy number less than median but greater than or equal to lower quartile, UCN: mtDNA copy number greater than or equal to median but lower than upper quartile, HCN: higher mtDNA copy number greater than or equal to upper quartile. Corn flower blue and crimson respectively represent healthy individuals (HI) and represents patients with type 2 diabetes (T2D). Statistical analyses revealed that mtDNA copy number was significantly lower (p = 0.03) in patients with T2D compared to that of HI only in case of HCN. The median and intra quartile ranges (IQRs) of each group have been presented. The black circles column of HCN represent outliers of that group.

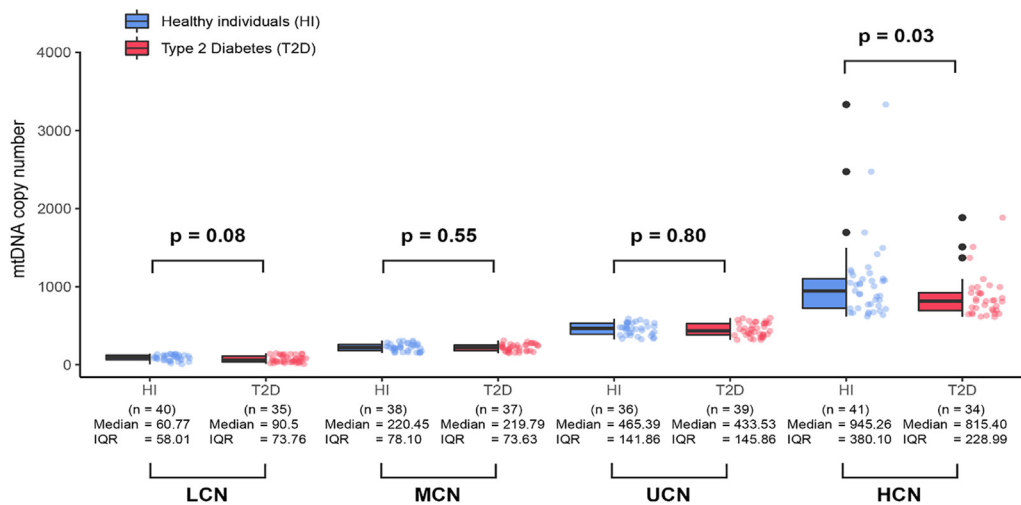


Figure 3. The hybrid boxplot of the mtDNA copy number in healthy individuals (HI) and patients with Type 2 diabetes (T2D). Corn flower blue and crimson respectively represent HI and patients with T2D. The median mtDNA copy number 334.37 of healthy individuals (Inter Quartile Range or IQR = 506.24) did not differ significantly (p = 0.16) from that of T2D (median: 287.73; IQR = 421.70). More variations of the mtDNA copy number was observed in case of healthy individuals compared to T2D as indicated by the IQR. The black circles indicate outliers of mtDNA copy number of the two groups of study participants.

Table 4. Association between mtDNA haplogroups and type 2 diabetes.

Haplogroups	HI	T2D	OR (95% CI)	p-value	OR ^a (95% CI)	p-value ^a	OR ^{a+g} (95% CI)	p-value ^{a+g}
M	60	57	Reference	-	Reference	-	Reference	-
B	2	-	-	-	-	-	-	-
D	6	2	2.99 (0.63–23.32)	0.15	0.18 (0.02–1.33)	0.10	0.2 (0.02–1.46)	0.12
E	2	-	-	-	-	-	-	-
F	3	3	1.05 (0.17–6.35)	0.95	0.59 (0.07–4.76)	0.61	0.62 (0.08–4.75)	0.64
H	16	25	1.63 (0.79–3.44)	0.17	2.56 (1.09–6.22)	0.03	2.43 (1.02–5.98)	0.05
J	2	-	-	-	-	-	-	-
L	5	11	2.26 (0.76–7.76)	0.13	2.26 (0.53–10.60)	0.28	2.05 (0.49–9.47)	0.33
A	1	6	5.6 (0.89–148.49)	0.05	20.13 (2.31–448.49)	0.01	23.56 (2.61–533.35)	0.01
N	7	2	0.31 (0.04–1.43)	0.12	0.41 (0.04–2.67)	0.39	0.44 (0.04–2.85)	0.43
O	1	-	-	-	-	-	-	-
P	-	1	-	-	-	-	-	-
R	12	10	0.87 (0.34–2.22)	0.77	1.1 (0.35–3.41)	0.86	1.05 (0.33–3.29)	0.93
S	-	3	-	-	-	-	-	-
T	3	1	0.38 (0.01–3.41)	0.35	0.12 (0.01–1.49)	0.10	0.13 (0.01–1.54)	0.12
U	16	15	0.98 (0.44–2.2)	0.97	1.31 (0.47–3.61)	0.60	1.36 (0.49–3.73)	0.55
W	4	4	1.05 (0.23–4.87)	0.94	1.03 (0.21–5.33)	0.97	0.97 (0.20–4.99)	0.97
X	-	1	-	-	-	-	-	-
Z	2	3	1.53 (0.23–13.64)	0.62	1.54 (0.16–27)	0.72	1.67 (0.17–28.24)	0.68

Alphabets represent haplogroups; HI: Healthy individuals; T2D: Type 2 diabetes, OR: Odds ratio; CI: Confidence interval.

Bangladeshi population. The variant G16048A (3.11%) was more frequent in the current study population compared to that reported in Mitomap (0.264%). G16129A is one of the ancestral SNPs ('RSRS50') as reported by Mitomap. None of these two variants were previously reported to confer risk or play protective role in T2D.

Certain variations were found to be more frequent in our population compared to that reported in Mitomap. Variations G16319A, A16051G and C16223T were more prevalent in our population. In a previous study involving Bangladeshi population, variants G16319A, G16129A and C16223T were more frequent which concurs with our findings (Sultana

Table 5. DNA variants having significant association with mitochondrial DNA copy number before and after adjusting the confounding factors.

Nucleotide Positions	Types of changes	*Mean Difference (95% CI)	p-value	*Mean Difference ^{a+c+g} (95% CI)	p-value ^{a+c+g}
16048	G-A	376.69 (104.78–648.6)	0.00	356.55 (82.40–630.69)	0.01
16126	T-C	-147.28 (-283.02 to -11.53)	0.03	-139.49 (-275.26 to -3.73)	0.04
16129	G-A	130.09 (12.54–247.64)	0.03	116.14 (-2.63 to 234.90)	0.06
16234	C-T	-193.00 (-369.61 to -16.40)	0.03	-185.82 (-362.42 to -9.22)	0.04
16291	C-T	529.50 (-12.03 to 1071.02)	0.06	543.41 (0.87–1085.95)	0.05
16311	T-C	-170.87 (-286.81 to -54.93)	0.00	-170.65 (-286.62 to -54.67)	0.00
16519	T-C	-146.00 (-242.70 to -49.29)	0.00	-147.37 (-245.31 to -49.43)	0.00

a+c+g = after adjusting age, condition (healthy individuals or T2D) and gender.

* Difference = mean mtDNA copy number with respect to mutant allele – mean mtDNA copy number with respect to rCRS allele.

et al., 2014). T16519C was the most frequent variant according to this study. This variant was found in 69.55% of the study population, which is nearly similar to the frequency reported in the Mitomap (62.94%). This change was slightly higher in patients with T2D (OR: 1.2) than healthy individuals but the association was not significant ($p = 0.47$). The variation T16189C was less frequent in Bangladeshi population (10.03%) compared to the frequency in the database (25.37%). A total of 147 variations were found in this study of which 120 were transitions, 20 were transversions, 2 were deletions and 5 were insertions. According to Supplementary Table 1, 38.3% of the transitions were C-T (46 out of 120) and 40% of the transversion were A-C (8 out of 20). However, G-A and C-G were the most frequent transition and transversion, respectively reported by Sultana et al. (2014). The frequency of a particular insertion C16151CC (an insertion of C at 16151) was within the low frequency group (5.88%) while insertion of C at 16083 (C16083CC) was among the rare variant (1.79%) found in our study population. No such insertions have been reported in the mitomap database. Surprisingly, a stretch of 10 nucleotides (16256-16265 in rCRS) was found to harbor 14 variants of whom 8 were unique (Supplementary Table 1). This region seemed to be quite flexible to accumulate mutations in Bangladeshi population.

The variants G16048A, G16129A, T16189C, C16294T and T16325C were further compared to the data in HmtVar (<https://www.hmtvar.uniba.it/>). The variant G16048A was only found in 0.31% of healthy Asians according to HmtVar while in our population the prevalence is 3.11%. The prevalence of G16129A in Asians is 0.38% (0.21% for healthy individuals, 0.17% for diseased individuals) according to HmtVar. However, this particular variant was found to be more frequent (17.99%) in our study population (12.11% in healthy and 5.88% in T2D). The highly studied variant T16189C has a frequency of 49.3% in Asians (17.6% for healthy and 31.7% for diseased) according to HmtVar. In our study, the frequency of this variant is much less compared to HmtVar (10.03%; healthy = 4.15% and T2D = 5.88%). The frequency of the variant C16294T is 5.9% for Asians in HmtVar (4.6% for healthy and 1.3% for T2D). Whereas, in our study the particular variant had a frequency of 2.43% (healthy = 2.08% and T2D = 0.35%). The variant T16325C had a frequency of 6.1% for Asians according to HmtVar (healthy = 1.5%, T2D = 4.6%). The same variant had a frequency of 2.42% (healthy = 1.38%, T2D = 1.04%) in our study participants. Our results concord with HmtVar for the multiple variant bearing stretch 16256-16265 except for the variant C16261T. HmtVar has a frequency of 13.7% for this particular variant in Asians (5.8% for healthy and 7.9% for diseased) while we found the variant in 3.11% of the study population (2.42% for healthy and 0.69% for T2D).

We retrieved rsID of 79 variants out of 147 using the VCF file of mitochondria DNA variants in gnomAD v.3.1.1 (<https://gnomad.broadinstitute.org/>). Out of this 79 variant, none were reported in ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) except one rs201864830 (T16017C: reported as benign). 9 variants out of 79 were reported in LitVar (Allot et al., 2018). C16278T variant (rs41458645) was found to significantly reduce the survival length in patients with esophageal squamous cell carcinoma (Zhang et al., 2010). Variants T16093C, C16222T, T16262C, T16304C, A16309G and T16311C (rs2853511, rs386829282, rs386829294, rs386829305, rs373517769 and rs34799580, respectively) were found to have differential response to the anti-cancer drug cisplatin (Patel et al., 2019).

The involvement of haplogroups to the pathogenesis of type 2 diabetes is rather controversial. No association between diabetes and European mtDNA haplogroups was reported (Chinnery et al., 2007; Hsouna et al., 2015) while haplogroup N9a was found to be associated with T2D in Southern Chinese population (Fang et al., 2018). On the other hand, the same N9a haplogroup was reported to play a protective role in Japanese population (Fuku et al., 2007). Haplogroup J and T indicated association with T2D in Caucasians-Brazilian population of South Brazil (Crispim et al., 2006). Also, haplogroup J was found to confer risk of T2D in Finnish population (Mohlke et al., 2005). In another study involving Chinese population, Haplogroup M9 was found to confer risk to T2D

(Liao et al., 2008). We found a total of 19 haplogroups in our study of which haplogroup M was the most frequent (40.48%) followed by H (14.18%), U (10.73%) and R (7.2%), respectively. The haplogroup frequency pattern is similar to that reported previously involving the Bangladeshi population (Sultana et al., 2014). Haplogroup A was found to be marginally associated with the risk of T2D in this study (OR = 5.60, $p = 0.05$) before considering confounding factors (age and gender) while after adjustment along with haplogroup A, a new haplogroup H was found to be associated with the risk of T2D as shown in Table 5. Africa specific haplogroup L (all were L3) was also found in 16 individuals (5.53%) of which 11 were patients with T2D (OR: 2.27). However, the result was not significant ($p = 0.13$). Haplogroup L3 is the immediate ancestor of haplogroups M and N. Soares et al. (2011) and Cabrera et al. (2018) suggested that L3 most likely expanded from East Africa into Eurasia. Eurasian-distributed M and N derivative clades are considered to be originated from L3 leading to "Out of Africa" migration (Soares et al., 2011). On the other hand, Cabrera et al. (2018) suggested back migration of females carrying L3 from Eurasia to East Africa. Thus, genetic admixtures due to forth and back migration could be the reason of having the presence of haplogroup L in Bangladeshi population though Sultana et al. (2014) as well as Rishishwar and Jordan (2017) did not report haplogroup L while analyzing mtDNA sequences of 108 and 86 Bengali speaking Bangladeshi population, respectively. Further investigation of the maternal lineage of those 16 individuals could reveal the reason behind the presence of such haplogroup. Also, haplogroups B, E, J and O were only detected in case of healthy individuals while haplogroups P, S and X were found in patients with T2D (Table 4).

The hypervariable regions are the non-coding control regions which constitutes the D-loop. This region plays a crucial role in mitochondrial DNA replication. Certain mutations in the hypervariable regions can lead to imbalance in regulation of mtDNA replication resulting in mtDNA copy number alterations. In the present study, mtDNA copy number was stratified into 4 groups based on the quartile of mtDNA copy number. In all the 4 groups, median mtDNA copy number of the healthy individuals were greater than that of patients with T2D. However, only for group with higher mtDNA copy number, the difference of median varied significantly ($p = 0.03$) between T2D and healthy individuals. The variation of mtDNA copy number in patients with T2D (IQR = 421.70) was less than that of healthy individuals (IQR = 506.24). All the groups had similar number of T2D patients and healthy individuals. MtDNA copy number was also found to be decreased in Bahrain and Italian populations (Al-Kafaji et al., 2018; Latini et al., 2020). A cohort study in Korean population also reported decreased mtDNA copy number (Lee et al., 1998; Song et al., 2001). Decreased mtDNA was found to be associated with metabolic syndrome and T2D in Italian and German populations (Fazzini et al., 2021). Mitochondrial dysfunctions in T2D were reported to be linked with mtDNA copy number reduction (Rolo and Palmeira, 2006). On the other hand, a study with Bangladeshi population found elevated mtDNA copy number in T2D patients with nephropathy (Malik et al., 2009). However, this study was conducted only in 65 Bangladeshi individuals. When these individuals were stratified into diabetic nephropathy, healthy control and diabetics without nephropathy for comparative analysis with respect to mtDNA copy number, the statistical inference became rather weaker. Also, increased mtDNA was found in patients with T2D in a Mexican population (Cataño Cañizales et al., 2018). In our study, variants G16048A and C16291T had significantly increased while variants C16234T, T16311C and T16519C had significantly decreased mtDNA copy number after adjusting the confounders i.e., age, gender and conditions. The increased copy number associated with the variants G16048A and G16129A (Table 5) may be one of the reasons behind the protective role of these variants against the development of T2D as demonstrated in Table 2.

In conclusion, our study revealed protective role of three novel variants against the development of T2D, association of haplogroups A and H with the risk of T2D in Bangladeshi population. Mitochondrial DNA copy number was found to be significantly lower in patients with T2D

compared to healthy individuals in HCN group and six mtDNA variants were recognized to be significantly associated with mtDNA copy number in the participants. Also, unique insertion of C was observed at positions 16083 and 16151 not reported in any other population yet. In this study, inclusion of HV2 and HV3 regions could have generated a comprehensive variant landscape of the D-loop region in Bangladeshi population. The control and cases randomly included in this study were not age matched though adjustments of the age in regression models account for the age difference between the two groups. Thus, the replication of this study in the form of a larger cohort using age matched control and cases can give further insight into the variations in T2D and validate our findings. Whether inheritance of these three variants would confer or delay the onset of T2D will be a great interest of further research.

Declarations

Author contribution statement

Sajoy Kanti Saha: Performed the experiments; Analyzed and interpreted the data; Contributed analysis tools or data; Wrote the paper.

Abdullah Al Saba and Md. Hasib: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Razoan Al Rimon and Imrur Hasan: Performed the experiments.

Md. Sohrab Alam and Ishtiaq Mahmud: Contributed reagents, materials, analysis tools or data.

A.H.M. Nurun Nabi: Conceived and designed the experiments, Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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