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Forensic Evidence for Cytochrome b Gene SNPs in Obese and Non Obese Saudi Arabians

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Abstract: Oral swabs from obese and non obese Saudi Arabians from both sexes with an average age of 35 years old were collected and their DNAs were extracted. PCR for 1000 bp of the mitochondrial cytochrome b (cytb) gene was conducted and the amplified products were sequenced in order to determine the possible forensic or obesity-related SNPs. Alignment of the obtained sequences with its counterparts of 100 healthy Afro-Asians deposited in the Genbank was undertaken and the polymorphic sites were compared. Sixteen Single Nucleotide Polymorphic sites (SNPs) and 26 variations were noted. From the 26 variations, sixteen were synonymous and the other 10 were non-synonymous. Four common haplogroups were determined using Mitomaster software (H2a, JT, U5a and R0a). Most of SNPs were related to tribes more than to obesity and the major SNP (C15452A) was recorded in both obese and non obese haplotypes. Two non-synonymous amino acid changes were found in 2 obese males (H15 and H27; A15043G) and 2 obese females (H48 and H51; C15677A) indicating that both SNPs could be obesity markers. In conclusion, cytb gene is reasonably applicable in forensic purpose while it was unclear to be used as an obesity marker. It needs to be examined for hundreds of unrelated obese and non obese people.

Keywords: Cytb, Haplotypes, SNPs, Forensic, Obesity, Saudi Arabia

Introduction

The hypervariable fragments of mtDNA control region were commonly applied in forensic studies (Monsalve and Hagelberg, 1997; Harihara *et al*., 1998; Al-Zahery *et al*., 2011; Hameed *et al*., 2015) when Short Tandem Repeats (STR) of the nuclear genome are unavailable due to DNA fragmentation and the impossibility of the two-copy DNA markers to persist over time (Gabriel *et al*., 2001; Tsai *et al*., 2009).

Cytb gene was used for identification of obese and non obese humans (Demir *et al*., 2014). The gene has become valuable key for species identification in routine forensic practice too (Lopez-Oceja *et al*., 2016). Lee *et al*. (2002) found 30 polymorphic sites distributed along cytochrome b gene in 98 unrelated Koreans. Cytb gene has been also applied in screening polymorphic sites for different human haplotypes based on environmental and geographic implications (Kong *et al*., 2003; Mishmar *et al*., 2003). The gene was reported recently to elevate its expression in diabetic people who are possibly non-obese (Carlos *et al*., 2017). Besides, the cytb gene, the whole mtDNA is also used in identifying mother relatives (Ma *et al*., 2018) and other forensic caseworks (Eduardoff *et al*., 2017) which are useful in identifying unknown persons.

On the other hands, obesity induces an increase in the mtDNA content rather than inducing mutation (Alé *et al*., 2017). Meanwhile, Veronese *et al*. (2018) correlated the obesity prevalence to certain ethnic Caucasian groups by using the mtDNA data and a recent study used cytb gene in diagnosing obesity (Demir *et al*., 2014).

Saudi Arabia hosts different human haplogroups among them Saudi population comprising different Arabian tribes. The other haplogroups include the immigrating Asian and African haplotypes. In this study, random individuals from different tribes were collected and the cytb SNPs were recorded to examine variations between the different haplotypes. Unique SNPs for obesity or forensic purposes could be also recorded.

Materials and Methods

Samples

Sixty-six healthy Saudi males (39 obese and 7 non obese) and females (10 obese and 10 non obese) were randomly chosen for this study based on their Body Mass Index (BMI). The ratio of males to females was 69.7 to 30.3%. Criteria for inclusion and exclusion were based on that all samples were healthy with no back or family history disease. Samples with diabetic and blood pressure or cardiovascular diseases were excluded. The BMI of all obese samples were above 30 and samples with BMI between 25 and 30 were excluded. All samples were collected from people who are living in the same geographic region at the western Saudi Arabia. Samples were taken after the agreement of the donors. BMI of the healthy samples was below 25 and above 18. All samples were within the range between 25 to 50 years old. Obese males were numbered from H1 to H39 and the healthy ones were numbered from H40 to H46. Obese Females were labeled from H47 to H56 while healthy females were numbered from H57 to H66.

DNA Extraction

Wizard® genomic DNA extraction kit (Promega Corporation, Madison, USA) was used for extracting genomic DNA from swab samples according to manufacturer's instructions (www.promega.com/protocols). The concentration of extracted DNA and the extract purity were measured by UV-Vis spectrophotometer at 260/280 nm and DNA was stored in 4°C for further use.

Cytb Gene Amplification

The revised Cambridge reference sequence (Andrews *et al*., 999) was used to design the forward cytbF: 5'- CCCCAATACGCAAAATTAACCC -3' and the reverse cytbR: 5'- GTATAGTACGGATGCTACTTGTC -3' primers in order to amplify 1000 bp of cytb gene (nt14747-15887). PCR tube was prepared with a volume of 50 µL of 2 µL DNA, 2 µL of 10 picomolar from the forward and the reverse primers, 25 µL PCR master mix (Promega Corporation, Madison, WI) and 19 µL autoclaved dd H_2O. PCR was run 5 min with primary denaturation at 94°C, followed by 35 cycles of denaturation at 94°C, annealing at 56°C and extension at 72°C (each for 1 min) and finally with post-extension at 72°C for 5 min. Amplified products were visualized in gel electrophoresis (1 g agarose, 1X TAE buffer, ethidium bromide and 100 bp Biolabs DNA ladder). PCR products were purified by spin column (BioFlux, Tokyo, Japan) kit based on the manufacturer's procedure.

Cytb Sequencing

Sequencing of the purified products was conducted in Applied Biosystems Sequencer (ABI PRISM ABI3730xl) by BigDyeTM Terminator Kits including AmpliTaq-DNA polymerase (FS enzyme). Mitomaster search tool in MitoWeb program (http://mitomap.org/MITOMAP) was used to compare the obtained data with the mtDNA rCRS (Andrews *et al*., 1999) to search for SNPs and to for haplogroup identification.

Results and Discussion

Approximately, 1 Kbp of cytb gene was amplified and sequenced in the present study and the data were deposited in the NCBI Genbank database (accession numbers: KT215436-KT215473, KT248511- KT248517 and MG988054-MG988073). Polymorphic sites were determined by comparing all the obtained sequences to the revised Cambridge reference sequence (Andrews *et al*., 1999). Sixteen SNPs that were noted in at least 2 haplotypes and 26 substitutions were recorded (Table 1). Similar to the finding of Farghadani and Babadi (2015) in Southeast Asian ethnic groups, frequently mutable sites were shown (A15326G, G15301A, G15043A and C15452A). As cytb gene is a protein-coding, mutations were only base substitutions with no deletion or insertion and this substitutions were transitions more than transversion (transition:transversion ratio = 26:3). Several studies (Tzen *et al*., 2001; Lee *et al*., 2009; Farghadani and Babadi, 2015) have revealed similar findings in other human groups.

From 26 changes found in the sequenced fragment among the different haplotypes, 16 were found in the third position of the codon (Table 2) keeping the amino acids unchanged (synonymous) while 10 changes were either in the first or in the second codon positions (non-synonymous) and thus amino acids changed. The sequences of H9, H17 and H34 were different from rCRS (Andrews *et al*., 1999) in A15326G which could be considered as an obesity marker. The haplotype H19 was different from the reference sequence just in one nucleotide at a position G15148A which was synonymous. Two obese female haplotypes (H48 and H51) showed a unique non-synonymous position (A15677C, Gln to Lys) which could be considered as an obesity marker since it was recorded only in these two obese females (Table 2). H2a, JT, U5a and R0a were the common recorded haplogroups (Table 3) according to MITOMAP (2018). Each different individual sequence could be assigned to a spate haplogroup since, to the best of our knowledge, no standard for haplogroup identification could be found and thus, 20 haplogroups were recorded.

Table 1: Sequence variations in cytb gene compared to Andrews sequence. The variations are listed when they were polymorphic (found in more than one individual). The reference stands in bold for Andrews sequence. Numbers on the top indicate nucleotide position in whole mitochondrial genome and dots (.) represent matches with the reference sequence. Haplotypes sharing the same nucleotide at a certain position are listed together

Sharing haplotypes	14905	15043	15218	15235	15257	15262	15301	15326	15431	15452	15466	15607	15674	15677	15679	15784
rCRS	**G**	**G**	**A**	**A**	**G**	**T**	**G**	**A**	**G**	**C**	**G**	**A**	**T**	**A**	**A**	**T**
H6, H33, H65	A	.	.	.	.	.	.	G	.	A	.	G	.	.	.	.
H11, H15, H53, H54	.	A	.	.	.	.	A	G	.	.	.	.	.	.	.	.
H8, H47	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.
H15, H27, H49	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.
H26, H32	.	.	G	.	.	.	.	G	.	.	.	.	.	.	.	.
H10, H16, H48, H51, H61, H66	.	.	.	.	A	.	.	G	.	A	.	.	.	.	G	.
H29, H46	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
H1-H3, H5, H8, H11, H14, H15, H18, H29, H35-H39, H40-H42, H46, H53, H54	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.
H4, H6, H10, H16, H20-H22, H31, H33, H40, H43-H45, H48, H51, H55-H57, H61-H63, H65- H66	.	.	.	.	.	.	.	G	.	**A**	.	.	.	.	.	.
H31, H40, H55, H57, H60	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H24, H25, H28, H60	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	.
H48, H51	.	.	.	.	.	.	.	G	.	**A**	.	.	.	**C**	.	.
H14, H26, H41, H42	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C

Table 2: Amino acid changes as a result of 26 nucleotide substitutions

Reference nucleotide position	Substitution	Individuals	Amino acid	Position in the codon	Synonymous
14905	G-A	H6, H33, H65	Met-met	3	+
14981	A-C	H27	Ile-Leu	1	-
15043	G-A	H11, H15, H53, H54	Gly-Gly	3	+
15110	G-A	H18	Ala-Thr	1	-
15136	C-T	H27	Gly-Gly	3	+
15148	G-A	H19	Pro-Pro	3	+
15217	G-A	H18	Gly-Gly	3	+
15218	A-G	H26, H32	Thr-Ala	1	-
15229	T-C	H32	Val-Val	3	+
15235	A-G	H8, H47	Trp-Trp	3	+
15257	G-A	H10, H16, H48, H51, H61, H66	Asp-Asn	1	-
15262	T-C	H29, H46	Ser-Ser	3	+
15301	G-A	H1-H3, H5, H8, H11, H14, H15, H18, H29, H35-H39, H40-H42, H46, H53, H54	Leu-Leu	3	+
15326	A-G	all except H19	Thr-Ala	1	-
15358	A-G	H5	Gly-Gly	3	+
15388	T-C	H29, H46	His-His	3	+
15431	G-A	H15, H27, H49	Ala-Thr	1	-
15452	C-A	H4, H6, H10, H16, H20-H22, H31, H33, H40, H43-H45, H47, H50-H53, H55-H56, H58, H61-H63, H66	Leu-Ile	1	-
15466	G-A	H31, H40, H55, H57, H60	Met-met	3	+
15514	T-C	H8	Tyr-Tyr	3	+
15607	A-G	H6, H33, H65	Lys-Lys	3	+
15674	T-C	H24, H25, H28, H60	Ser-Pro	1	-
15677	A-C	H48, H51	Gln -Lys	1	-
15679	A-G	H10, H16, H48, H51, H61, H66	Lys-Lys	3	+
15746	A-G	H13	Ile-Val	1	-
15784	T-C	H14, H26, H41, H42	Pro-Pro	3	+

Table 3: Haplogroups and their frequencies in obese samples (n = 49). The genetic diversity was calculated according to the formula D = (1- Σp^2) (Tsai *et al*., 2009)

Haplogroup	Number of individuals/haplogroup	Haplogroup frequency
H2a	9	0.1840
JT	7	0.1430
U5a	5	0.1020
R0a	4	0.0820
J2a	3	0.0610
T	3	0.0610
L3	3	0.0610
HV1a	2	0.0410
J1b	2	0.0410
L0a	1	0.0200
L3i	1	0.0200
L3f	1	0.0200
L2b	1	0.0200
L2a	1	0.0200
M30	1	0.0200
M	1	0.0200
R30b	1	0.0200
B4a	1	0.0200
X2i	1	0.0200
J2b	1	0.0200
Genetic diversity	$1-\Sigma p^2$	0.9105

Application of cytb gene in human identification for forensic purposes is questionable since debates between forensic researchers on this marker are found. Farghadani and Babadi (2015) revealed lower efficiency of cytb gene in forensic caseworks than hyper variable regions of the mtDNA control region (Van der Walt *et al*., 2003; Wong *et al*., 2007). The authors concluded that the gene is probably not appropriate for routine forensic caseworks. On the other hands, other investigators (Tsai *et al*., 2009; Hwa *et al*., 2010; Ablimit *et al*., 2013) approved forensic efficiency of cytb gene. The present study disagreed with Farghadani and Babadi (2015) since the authors based their conclusion on a small fragment of cytb gene (402 bp), however ours and that of Tsai *et al*. (2009), Hwa *et al*. (2010) and Ablimit *et al*. (2013) were based on nearly the entire gene sequence. Haplogroup frequency in this study ranged between 0.184 and 0.02 (Table 3). This frequency is small as the sampling size was small. It could be increased as the sampling size increased. Further study on hundreds of samples is, thus, necessary for wide different Saudi Arabian tribes. Haplogroup frequencies (Table 3) were used to calculate the genetic diversity (Jones, 1972) among the studied haplogroups (D = 0.9105). It was comparable to the genetic distance of the d-loop regions (D = 0.964) calculated by Hameed *et al*. (2015) and this finding approved the efficiency of cytochrome b gene in forensic investigations.

Regarding obesity, cytb gene diagnosed 2 obese male haplotypes at G15431A. At this position, alanine (Ala) was changed to thrionine (Thr) (non-synonymous) in H15 and H27. Meanwhile, two obese females H48 and H51 acquired a change of glutamine (Gln) to lysine (Lys) at C15677A. Kobayashi *et al*. (2011) found similar mutation in different ages Japanese groups suffering from obesity-induced cardiomyopathy. BMI of the abovementioned four obese individuals ranged between 33 and 44.8. We may consider these two positions weak obesity markers since other haplotypes with higher BMI did not exhibit such mutation. Meanwhile, the maternally-related haplotypes, either obese or not, exhibited similar forensic SNPs as shown for H8 and H41, H43 and H44, H29 and H46 and H20, H21, H22 and H45 (Table 2). We cannot, therefore, neglect the possibility of applying cytb gene in obesity discrimination since very recent study (Veronese *et al*., 2018) have strongly found a relation between obesity and a certain ethnic group via mtDNA data including cytb gene.

Conclusion

In conclusion, cytb gene could be used as a forensic marker for Saudi Arabian tribes with a limited resolution; however its application in diagnosing obesity is questionable. The application of this gene in both trends needs more studies on thousands of unrelated obese and non obese humans.

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Author's Contributions

Sayed Amin Mohamed Amer: Constructed the idea, conducted the practical part, wrote the manuscript and followed its publication.

Bandar Raddat Allah AlHothali: Collected the male samples, extracted their DNAs and checked the manuscript writing.

Monira Hmoud Alotaibi: Collected the female samples, extracted their DNAs and conducted PCR experiments.

Salah Mohamed Tubaigy: Supported the work financially, followed the practical part, shared in revising the manuscript and its publication.

Ethics

The authors declare that they have no competing interests in this study.

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