

Evaluation of SNPs in the Mitochondrial DNA Using NanoChip Microarrays in the Turkish Population

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ABSTRACT The single nucleotide polymorphism (SNP) genotyping technology was developed to analyze the currently increasing number of genetic variations associated with diseases and for genetic identification of individuals. This paper describes a novel assay for genotyping of SNPs at the displacement loop (D-loop) in mitochondrial DNA using the Nanogen, NanoChip Technology. The researchers selected the most common four SNPs in the mitochondrial DNA from population data around the world. The researchers developed assay conditions for SNPs including, 73 A→G, 16304 T→C, 16311 T→C, and 16362 T→C. This paper carried out SNP genotyping including the first and second hypervariable regions of unrelated 72 samples of the Turkish population. All the identified genotypes were compared with results obtained previously from DNA sequencing of the same individuals. These findings demonstrated that the assay was accurate, reliable and reproducible for individual identification in the forensic and anthropology fields and to determine the risk of various diseases.

INTRODUCTION

Single nucleotide polymorphisms (SNPs), classified as non-coding SNPs and coding SNPs are the most common DNA sequence variations in the human genome. SNPs are two alleles, with DNA sequence differences occurring in a single nucleotide at a defined site in the human genome (Schork et al. 2000; Gill 2001; Sachidanandam et al. 2001).

In recent years, rapid developments in the microelectronic array technology have especially proven their value for SNP genotyping in the human genome. Many SNPs have been genotyped using different genotyping technologies such as NanoChip, Affymetrix and Illumina. The genotyping arrays have contributed extensively to structural variation analysis, as genetic markers in genome wide association studies (GWAS) and population studies (Sigurdsson et al. 2006). Besides, DNA microarray is also used

to determine antibiotic resistance and virulence in pathogens and for accurate identification of microorganisms rather than culture identification (Walsh et al. 2010; Patro et al. 2015).

The single nucleotide polymorphism arrays (SNPa) have recently been developed to identify copy number variants (CNVs) in the human genome. Some researchers determined variations of the new leukemia genes such as RNASEL, ARHGEF12, LYL1 and SMARCA4, which are found on chromosome 1, 11 and 19 in acute lymphoblastic leukemia (ALL), using the SNP array technology (Dirse et al. 2015). Furthermore, the cDNA microarray analysis has been used for the detection of RNA expression levels of the genes in skin lesions of dermatology patients in the past years (Mitsui et al. 2012). SNP arrays provide the understanding of structure and function of human genes on chromosomes (Luo et al. 2014).

Each mitochondrion, numbers of which range from a few hundred to thousands in a human cell, contains numerous mtDNA. D-loop is a non-coding region at positions 16024-576 in the mtDNA (Anderson et al. 1981). D-loop that involves two hypervariable regions (HVI and HVII) has important roles for regulating transcription and replication of mitochondria.

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HVI and HVII in D-loop of mtDNA provide a high degree of information for discriminating among unrelated individuals. The hypervariable regions are studied primarily by forensic science, anthropology and molecular evolution (Stoneking 2001; Jankova-Ajanovska et al. 2014; Oh et al. 2015; Quintans et al. 2004).

The analysis of mtDNA has become a powerful tool in forensic casework, mainly due to its high copy number per cell, its stability against degradation and its maternal mode of inheritance (Brandstatter et al. 2003; Coble et al. 2004). In addition, mtDNA variants, including the entire mtDNA, especially HVI and/or HVII regions are implicated in several human diseases (Hudson et al. 2013).

Identification of mtDNA SNPs can be performed using several techniques. The DNA sequence analysis of HVI and HVII is very robust but time consuming and labor intensive, so several technologies that are easier to perform have been developed, such as microarrays, chromosomal microarrays, mini-sequencing by arrayed primer extension, SNP by pyrosequencing, TaqMan allelic discrimination, and SNPlex (Ahmadian et al. 2000; De La Vega et al. 2005; Divan and Allen 2005; Tebbutt 2007; Riggs et al. 2013).

The NanoChip[®] microelectronic array technology provides many advantages to identify SNPs and mutation detection (Thistlethwaite et al. 2003). Using this technique, angiotensin converting enzyme (ACE) genotyping have also become easier and more efficient for determination of predisposition to cardiovascular diseases, without the hassle of using conventional PCR analysis with the risk of mistyping (Bolli et al. 2011).

The NanoChip Molecular Biology Workstation (Nanogen, Inc., San Diego, CA) employs a two-color assay, based on thermal discrimination and electronic hybridization. Biotinylated PCR products of the gene of interest are loaded onto streptavidin-coated electronic pads. The PCR products were hybridized with probes specific to the wild type and variant sequences that were labeled with Cy-3 and Cy-5, respectively. Data analysis is done according to fluorescence signals.

Advances in genotyping NanoChip microarray technology offer suitable and flexible methods for the evaluation of mtDNA SNPs of various SNPs-related disease and research fields. The researchers concluded that the NanoChip

microelectronic array technology provides an accurate and reliable assay platform.

Objectives

The researchers aimed to develop a method for detection of SNPs in mtDNA using the NanoChip[®] technology that could be implemented in various fields such as population genetics, anthropology and medicine. The objectives to achieve this goal were to:

1. Determine common mtDNA SNPs in human populations.
2. Design probes for four different SNPs in mtDNA.
3. Generate assay conditions for selected SNPs in mtDNA (73 A→G, 16304 T→C, 16311 T→C, and 16362 T→C selected from HV1 and HV2 regions).
4. Determine genotypes of unrelated individuals to test the validity of the method.
5. Compare with previous DNA sequencing results of the same individuals.

MATERIAL AND METHODS

Sample Preparation

Blood samples and dried blood spots were collected from a total of 72 unrelated individuals living in Turkey. All the genomic DNA samples were isolated from leucocytes of fresh blood and dried blood spots using the QIAamp[®] DNA kit (QIAGEN[®], GmbH, Germany). The DNA samples were stored at -20°C until the genotype analysis was performed.

Primers, Stabilizer, Reporters and PCR Amplification

Novel forward and reverse primer sequences, flanking the HV1/HV2 were designed using the Primer3 software (<http://workbench.sdsc.edu/primer3.0>). The forward primers were synthesized with biotin on the 5' end. The sequences of primers covering SNPs at 73, 16304, 16311 and 16362 positions, optimum annealing temperature and targeted amplicon sizes are listed in Table 1.

DNA was amplified in reaction mixtures of 50 µl, containing 1.5 mmol/L MgCl₂, 100 µM of each dNTP, 20 pmol of each primer, 100 ng of a

Table 1: PCR primers and annealing temperatures for the amplification of SNPs in the mtDNA

SNP	Primer name	Primer sequences	Location of 3' base	PCR product length	$t(C^{a\%})^*$
73A→G	Set1-F	5'- Biotin-TCACAGGTCTATCACCCCTATTAACC-3'	L27	174 bp	56°C
	Set1-R	5'- TTGAACGTAGGTGCGATAAAAT-3'	H156		
16304T→C	Set2-F	5'- Biotin-CACCCCTACCCACTAGGAT-3'	L16278	172 bp	56°C
16311T→C	Set2-R	5'- CGGGATATTGATTTACGGA-3'	H16411		

* Annealing temperature

genomic DNA, 1×PCR buffer and 1 U Taq DNA polymerase. The PCR conditions consist of an initial denaturation at 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds. The PCR was completed by final extension incubation at 72°C for 10 minutes. After amplification, 5 µl PCR products were visualized on two percent agarose to check the quality and quantity of the products.

Stabilizer, wild-type reporter (5'-Cy3) and mutant reporter (5'-Cy5) labeled with fluorophore for each SNPs were used in the NanoChip assay. The SNP was at the 3' terminal base of the reporters. Discrimination temperatures, sequences of wild or mutant reporters and stabilizer of each mtDNA SNPs used for genotyping in this paper were shown in Table 2.

Sample Preparation for NanoChip

Amplicons were desalted using the QIAquick PCR purification kit (QIAGEN®, GmbH, Germany) prior to addressing to the NanoChip cartridge and amplicons were re-suspended in 60 µl of 50 mmol/L histidine.

Loading the NanoChip Cartridge and Reporting

Desalted amplicons were transferred into a 96-well plate. The NanoChip cartridge and the plate were placed in the Loader for the loading step. A loader map file was created. All amplicons for each assay were electronically addressed to defined test sites at 2 V for 120 seconds and bound to the layer with streptavidin. Then, a 50-mmol/L histidine as background control and a heterozygous control for each assay was addressed. The cartridge was washed with a 50 mmol/L histidine solution without amplicons. The amplicons were denatured using 0.1 mol/L NaOH for 3 minutes.

Probe Hybridization

Stabilizer and Cy3- (wild type) and Cy5- (mutant) labeled reporters were prepared in 100 µL of high salt buffer (50 mmol/L sodium phosphate, 500 mmol/L NaCl, pH 7.4) and hybridized to the chip for 5 minutes at room temperature. The chip was washed with 150 µL of high salt buffer and the hybridization stopped.

Sample Detection and Analysis

After reporter hybridization, the cartridge was placed in the NanoChip Reader for scanning the specific fluorescence and heated to the appropriate discrimination temperature. The specific discrimination temperatures were set as 31°C for 73 A→G, 39°C for 16304 T→C, 29°C for 16311 T→C, and 32°C for 16362 T→C in this paper (Table 2). The detection was performed using differences in hybridization energies of two fluorescently labeled reporters to each SNP. The cartridge was washed with low salt buffer (50 mmol/L sodium phosphate, pH 7.0) to remove unbound reporters/stabilizer and scanned using the two-laser system.

RESULTS

The researchers worked on the development of the Nanochip electronic DNA microarray. The researchers selected frequently encountered SNPs, three at HVI and one at HVII region. The researchers designed primers, stabilizers and reporters for these four SNPs. The researchers optimized experimental conditions for 72 unrelated individuals living in Turkey.

Assay Optimization

HV1 and HV2 segments of mtDNA were amplified in two separate fragments covering four

Table 2: The sequences of reporters, stabilizer and analysis conditions used for SNP genotyping in mtDNA

PCR product	SNP	Oligo name	Oligo sequences(5'-3')	<i>t</i> (C ^{0%})**
Set1	73 A→G	WT ratio	ACGGGAGCTCTCCATGCATTTGGTATTTTCGTCT	31°C
		reference	GGGGGTCTGGGGGGT a TGCACGCGATAGCATT	
		MUT	ACGGGAGCTCTCCATGCATTTGGTATTTTCGTCT	
		ratio	GGGGGGT g TGCACGCGATAGCATT	
		reference	Cy3-TATCGCGTGC a t	
		WT		
	reporter			
	MUT	Cy5-TATCGCGTGC a c		
	reporter			
	Stabilizer	ACCCCCAGACGAAAATACCAAATGCAT		
	WT	TCACCCACTAGGATACCAACAAACCTACCCACCC	39°C	
	Set2	16304 T→C		ratio
reference				
MUT				TCACCCACTAGGATACCAACAAACCTACCC
ratio				ACCCTTAACAG c ACATAGTACATAAAGG
reference				
WT			Cy3-ATGTACTATGT a	
reporter				
MUT	Cy5-ATGTACTATGT g			
reporter				
Stabilizer	CTGTTAAGGGTGGGTAGGTTTGTGGT	29°C		
Set2	16311 T→C		WT	CTAGGATACCAACAAACCTACCCACCCCTAA
			ratio	CAGTACATAG t ACATAAAGCCATTAC
			reference	
			MUT	CTAGGATACCAACAAACCTACCCACCCCTAA
			ratio	CAGTACATAG c ACATAAAGCCATTAC
		reference		
WT	Cy3-TGGCTTTATGT a			
reporter				
MUT	Cy5-TGGCTTTATGT g			
reporter				
Stabilizer	CTATGTAAGGGTGGGTAGGTT	32°C		
Set2	16362 T→C		WT	CATTTACCGTACATAGCACATTACAGTCAAAA
			ratio	TCCCTTCTCG t CCCCATGGATGACCCC
			reference	
			MUT	CATTTACCGTACATAGCACATTACAGTCAAAA
			ratio	TCCCTTCTCG c CCCCATGGATGACCCC
		reference		
WT	Cy3-CATCCATGGGG a			
reporter				
MUT	Cy5-CATCCATGGGG g			
reporter				
Stabilizer	CGAGAAGGGATTTGACTGTAATGTGCT			

** discrimination temperature

different SNPs. The success of PCR conditions, which can affect PCR specificity and efficiency, were increased by optimizing primer concentrations and cycling conditions. The researchers tested annealing temperatures from 50°C to 62°C and obtained the best results with NanoChip® Molecular Biology Workstation by using 56°C for Set 1 for A73G and Set 2 for T16304C, T16311C and T16362C (Table 1).

Hybridization conditions were optimized for four SNPs positions: 73 A→G, 16304 T→C, 16311 T→C, and 16362 T→C variations. The researchers tested known DNA samples, which had pre-

viously been genotyped by DNA sequencing and observed complete concordance between methods.

Data Analysis

The NanoChip® Molecular Biology Workstation (Nanogen, San Diego, CA, USA) was used as an electronic DNA microarray. The ratio of two fluorescent signals (red to green) was calculated. Genotypes were normalized to known heterozygote control.

Allelic discrimination was made to determine the polymorphism using ratios of fluorescently

labeled allele specific reporter. Bi-allelic fluorescence intensity ratios (Cy5: Cy3) of greater than 5:1 are defined as homozygous for the wild type and mutant alleles. The results with intensity ratios of 2:1 or less are defined as heterozygous.

The results with ratios between 5:1 and 2:1 were designated as indeterminate and repetitive. The signal ratios for wild type alleles against mutants are 1:85.9 – 48.8:1 [73 A→G], 1:66.2 – 24.4:1 [16304 T→C], 1:43.7 – 79.5:1 [16311 T→C], 1:110.3 – 31.2:1 [16362 T→C]. A representative genotype calling was shown in Figure 1.

Analysis of DNA Samples

In this paper, the researchers used the NanoChip® technology to detect SNPs in mtDNA. The researchers chose four different SNPs in mtDNA (three from HV1, one from HV2 region). Detection of four SNPs was made owing to reporters and stabilizer providing base-stacking inter-

actions between the 3' end of the reporter and the 5' end of the stabilizer.

The researchers investigated 72 samples for analysis of four SNPs (73 A→G, 16304 T→C, 16311 T→C, and 16362 T→C). Successful amplification was observed for all the samples (including dried blood spots) and genotyped successfully (genotype call rate is >99%).

All the identified genotypes were identical to those obtained previously from DNA sequencing. According to this paper, 47 (65.3%) individuals have G at nucleotide 73, 3 (4.2%) individuals have C at nucleotide 16304, 10 (13.9%) individuals have C at nucleotide 16311, and 4 (5.6%) individuals have C at nucleotide 16362.

DISCUSSION

Conventional methods for SNP detection involve DNA sequencing, single-strand conformation, PCR-restriction fragment length polymorphism (RFLP) and denaturing gradient-gel

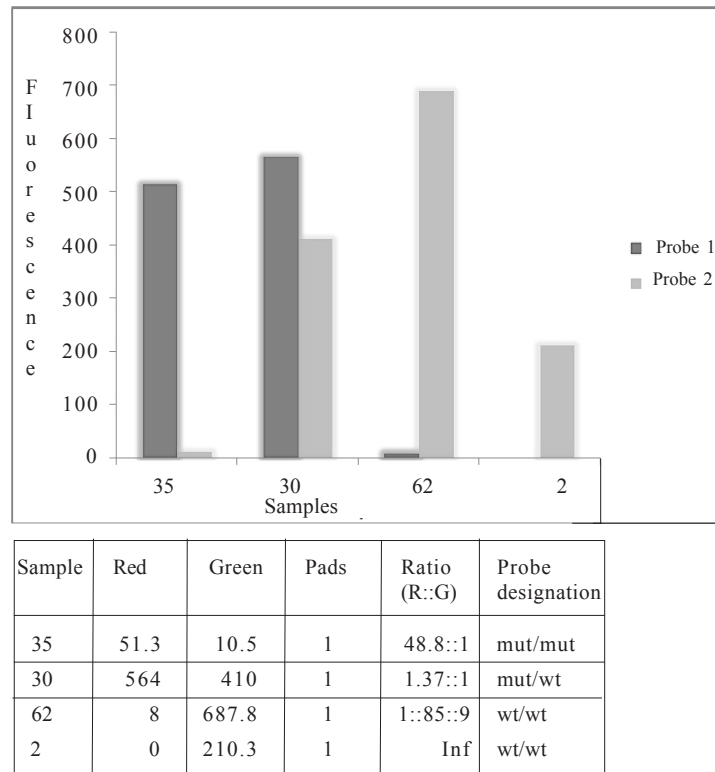


Fig. 1. Representative results showing the fluorescent signals obtained for genotyping of nucleotide 73

electrophoresis analysis (Tully et al. 2000; Valian and Nassiri 2010; Aral et al. 2011).

Microarray based DNA methods allow diagnosis of biochemical abnormalities, genetic diseases such as cystic fibrosis, progression of cancer and rapidly genotyping of known SNPs for individual identification (Molinario et al. 2015).

mtDNA SNPs at coding sequences lead to many outcomes including cytochrome c inactivation, which may predispose individuals to common metabolic diseases. The relationship between metabolic diseases such as high body mass index (BMI) and mtSNPs in cytochrome c subunits and NADH dehydrogenase subunits was investigated using different microarrays such as Affymetrix and Illumina. This paper suggests that mtSNPs may be related to high BMI in adults due to altered function of mitochondria (Flaquer et al. 2014).

Besides, other SNPs in mtDNA may be the cause of important changes in the mitochondria. These changes imply risk of breast, prostate and colorectal cancers. Mohideen et al. (2015) investigated an association between progression of colorectal cancer and mtSNPs located in the D-loop mtDNA region.

Effects of 10398 (A/G) polymorphism found in the mitochondrial NADH dehydrogenase 3 gene were investigated in 50 patients with breast cancer and this mtDNA variant provides resistance to apoptosis and has an influence on the metastasis condition in mice (Kulawiec et al. 2009).

Furthermore, the 16189 (T/C) SNP in the D-loop region gives rise to length heteroplasmy at 16180-16195 positions. This situation results in decreased mtDNA copy number in mitochondria due to the problems that occur during mtDNA replication and increased susceptibility to diseases in humans (Liou et al. 2010).

Hybridization-based microarrays have recently been developed to identify links between mitochondrial dysfunction and mechanisms of mitochondrial diseases, aging and cancer progression (Du et al. 2009).

The single nucleotide polymorphism array (SNPa) is especially applied in cancer research (Alkan et al. 2011). Identification of specific genetic mutations by SNP array provides understanding of cancer development and novel therapeutic targets for cancer. The detection ratio of

genetic changes with SNP array technique in acute lymphoblastic leukemia (ALL) and myelodysplastic syndrome (MDS) is higher than karyotyping (Noronha et al. 2015).

Numerous researches are needed to explain if there is an association between mtDNA SNPs with pathogenesis of neurodegenerative diseases such as Alzheimer's and Parkinson's. Several studies investigated that mtDNA SNPs had effects on Parkinson's and Alzheimer's disease (Hudson et al. 2012; Chen et al. 2015).

Conventional DNA fingerprinting is used to investigate any possible matches between suspect DNA and that retrieved, from evidence in a number of criminal cases. A paper on genotyping of 34 SNPs was carried out on 109 individuals from the Chinese Han population using oligonucleotide microarray for individual identification. The results of this research showed a high discrimination power for SNPs at forensic studies (Li et al. 2006).

A research group developed a method for detection of SNPs within the human FVII, β -globin and RET genes by NanoChip technology based on hybridization to allele-specific oligonucleotide probes that can screen known mutations (Santacroce et al. 2002).

LDLR mutations in familial hypercholesterolemia patients were analyzed using the same technology. It was showed that NanoChip analysis was suitable and rapid for detecting a small number of mutations and known mutations (Laios and Drogari 2006).

Previous reports demonstrated that NanoChip microelectronic array technology was a promising, fast and low cost method when compared with other techniques (Frusconi et al. 2004; Sethi et al. 2004).

There are also published reports determining mutations in Turkish glycogen storage disease type Ia and Gaucher patients (Ezgu et al. 2008; Eminoglu et al. 2013). Another group showed no association between 16189 T>C SNP in the D-loop region of mtDNA and type II diabetes mellitus using PCR and RFLP methods in Turkish population (Aral et al. 2011). Using the NanoChip microelectronic array technology for investigation of mtDNA SNPs in type II diabetes mellitus offers many advantages rather than PCR and RFLP methods.

The NanoChip technology detects the relationship between 16189 T>C SNP and metabolic disorders relatively rapidly, with ninety-nine

percent accuracy in a single assay with a large number of samples. The NanoChip technique for identification of mitochondrial DNA mutations could be used for early detection and monitoring the progression of some types of cancer in humans. It would provide advantages in understanding the pathogenesis of particular human cancers.

In this paper, the researchers reported a novel assay by using NanoChip technology for SNP genotyping in mtDNA. A total of 72 different genomic DNA samples were amplified and correctly genotyped for four SNPs in mtDNA on a single electronic microarray without any contamination between samples.

The selected SNPs in this paper are the most common sequence changes of mtDNA in the Turkish population (Calafell et al. 1996; Comas et al. 1996; Mergen et al. 2004). The results of SNPs in mtDNA determined by the NanoChip microelectronic array technology were confirmed by comparison with results obtained previously by sequencing. These results showed one hundred percent concordance between these two methods.

CONCLUSION

The NanoChip technology is a rapid screening technique to detect known SNPs in mitochondrial DNA. This technology can be utilized with great accuracy to confirm SNPs that contribute to occurrence and progression of many conditions such as diabetes, cancer and aging. Furthermore, it could allow typing of mtDNA SNPs successfully in small amounts and degraded archaeological samples. It would be beneficial, especially in the evaluation of highly degraded evidence for forensic events.

RECOMMENDATIONS

The NanoChip microelectronic array technology will provide a rapid and powerful tool for genotyping a large number of known sites of SNPs at forensic studies in future. This method would be a useful alternative tool for individual genotyping at large epidemiological studies. NanoChip microelectronic array technology could also be used to genotype SNPs related to aging, diabetes, and other metabolic disorders. NanoChip technology may be a beneficial tech-

nique for detection of known point mutations in mitochondrial diseases.

FOR FUTURE STUDIES

In order to enhance the discrimination power of this technique, it is planned to develop more primers and probes for a large number of new SNPs in mtDNA in the future.

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