Investigation of polymorphisms in non-coding region of human mitochondrial DNA in 31 Iranian Hypertrophic Cardiomyopathy (HCM) patients

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Abstract
The D-loop region is a hot spot for mitochondrial DNA (mtDNA) alterations, containing two hypervariable segments, HVS-I and HVS-II. In order to identify polymorphic sites and potential genetic background accounting for Hypertrophic CardioMyopathy (HCM) disease, the complete non-coding region of mtDNA from 31 unrelated HCM patients and 45 normal controls were sequenced. The sequences were aligned upon the revised Cambridge Reference Sequence (rCRS) and any incompatibilities were recorded as numerical changes in homoPolymeric C Tract (PCT), single base substitutions, insertions and deletions (Indels). Nucleotide substitutions were found to make up the majority of the mutations, rather than indels. We drew significantly high transition rate (81.8%) versus lower frequency of transversions (18.2%). 12 polymorphisms were identified in this study which had not been published in the MitoMap database. PCT changes at position 303-309 were detected in 83% of our samples. Our results suggest that an increased level of HVS-I and HVS-II substitutions may be an indicator of mitochondrial DNA instability. Furthermore, mtDNA mutations may play an important role in pathogenesis of cardiac arrest which has remained unexplained for long.
Keywords*: MtDNA; Hypertrophic Cardiomyopathy (HCM); D-loop; HVS-I; HVS-II*

INTRODUCTION

Human mitochondrial DNA (mtDNA) is a double-stranded closed circular molecule found at 10^2-10^5 copies per cell and the vast majority of these copies are identical (homoplasmic) at birth (Lightowlers *et al*., 1997). The mutation rate of mtDNA is about 10 times higher than that of nuclear DNA (Wallace, 1994). The mitochondrial genome is rather small (16.5 kb) and encodes 13 respiratory chain subunits, 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs). Expression of the entire complement of mitochondrial genes is required to maintain proper function of the organelle, suggesting that even slight alterations in DNA sequences could have profound effects (Mitomap database). It is generally accepted that mtDNA mutations are generated during oxidative phosphorylation through pathways involving reactive oxygen species (ROS).

Displacement Loop (D-loop), which is 1124 bp in size (Position 16024-576), is located between the tRNA genes for proline ($tRNA^{Pro}$) and phenylalanine ($tRNA^{Phe}$), and is a non-coding region acting as a promoter for both the heavy and light strands of the mtDNA. It contains essential transcription and replication elements. Despite its functional importance, this region is believed to be the most rapidly evolving part of the molecule (Upholt and David, 1997). The D-loop region is a hot spot for mtDNA alterations, which contains two hypervariable Segments: HVS-I at position 16024-16383 and HVS-II at position 57-372 (Anderson *et al*., 1981). Nucleotide substitutions accumulate in the mitochondrial genome with a considerably higher rate than for single-copy nuclear DNA (Brown *et al*., 1979). This is most probably due to the lower efficiency of DNA repair, lack of protective histones as well as higher frequency of DNA replication errors in mtDNA (Wilson *et al*., 1993; Croteau and Bohr, 1997).

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Consequently, mtDNA and in particular the non-coding region, is highly polymorphic. The alterations consist of two major categories: one is numerical changes in homoPolymeric C Tract (PCT), and the other Single Base Substitutions (SBS), insertions and deletions (Indels). Within the HVS-II, a region of microsatellite-like sequence can be found (position 208-315). These short tandem repeats, particularly a C-mononucleotide track interrupted by a single thymidine at position 310, has been shown to exhibit length polymorphism among individuals, as well as variation within an individual, which accompany the process of aging and cancer (Michikawa *et al*., 1999; Liu *et al*., 2003).

Hypertrophic CardioMyopathy (HCM) is the most common cause of Sudden Cardiac Death (SCD) also known as cardiac arrest in the young. It is characterized by left and/or right ventricular hypertrophy that is usually asymmetric and involves the intraventricular septum, in the absence of other loading conditions such as hypertension or hyperthyroidism (Semsarian and Maron, 2002; Roberts and Sigwart, 2001; Bonne *et al*., 1998; Maron *et al*., 1995). The disease occurrences are approximately 1 in 500 (Spirito *et al*., 1997; Maron *et al*., 1995). HCM is clinically heterogeneous and most patients have few or no symptoms while others develop serious complications including heart failure, arrhythmias and sudden death (Van Driest *et al*., 2002; Maron *et al*., 2000; Spirito *et al*., 1997). The most common clinical symptoms of HCM are dyspnea and chest pain. Other manifestations include lightheadedness, presyncope, syncope, tiredness, palpitation, orthopnea and SCD (Roberts and Sigwart, 2001, Elliott *et al*., 2000). In most patients there are systolic murmurs due to left ventricular outflow tract obstruction and mitral valve incompetence. The ECG is often abnormal, showing the feature of left ventricular hypertrophy and non-specific ST changes and arrhythmias (Roberts and Sigwart, 2001; Bonne *et al*., 1998). Diagnosis is made by echocardiography showing hypertrophy with the septum or ventricular wall thickness of at least 13 mm without other cause. (Erdmann *et al*., 2003; Roberts and Sigwart; 2001) The heart is a highly ATP-dependent organ and mitochondria constitute about one-third of the total cytoplasmic volume of cardiomyocytes. It has long been speculated that inadequate energy production may be an important factor contributing to heart failure. Ventricular hypertrophy is an important compensatory response to increased load. Interestingly, hypertrophy is accompanied by increased amounts of mitochondria (Braunwald, 1997), which makes it likely that upregulation of cardiac energy production is a mechanism allowing increased cardiac work. The importance of cardiac energy production is further underscored by the finding of cardiomyopathy in patients with mitochondrial diseases caused by mtDNA mutations. Several different point mutations of mtDNA are associated with maternally inherited and sporadic cases of hypertrophic and dilated cardiomyopathy (Antozzi and Zeviani, 1997); Mitochondrial dysfunction has also been suggested to have a role in heart failure and age-associated decline in heart function. (Hattori *et al*., 1991; Melov *et al*., 1995) The importance of mtDNA mutations and deficient oxidative phosphorylation in age-associated heart disease remains to be proven and presently only circumstantial evidence exists. To investigate the association between mtDNA haplotypes and HCM mutations and further our knowledge about alterations in HVS-I and HVS-II, the nucleotide sequence in the D-loop region was inspected in an Iranian population suffering from HCM. Taking the D-loop background into account may contribute to a better understanding of the molecular functioning of phenotypic modifiers in HCM and also the haplotype diversity among patients.

MATERIALS AND METHODS

To address the question whether HCM is preferentially associated with a distinct haplotype defined by D-loop variations, we sequenced the hypervariable D-loop region from 31 HCM patients. We also chose 45 healthy controls matched for age, sex and ethnicity. Control subjects had no significant signs of HCM when enrolled in the study. All of the patients and control group were informed on the aims of the study and gave their informed consents to the genetic analysis. Peripheral blood samples were obtained and the total DNA was isolated from white blood cells, using DNA extraction kit (Diatom DNA Extraction Kit, Gen fanavaran, Iran). PCR amplification was carried out in a final volume of 50 µl containing 200-300 ng total DNA, 10 pmol each primer, 2.5 mM $MgCl_2$ 200 µM each dNTP and 2 Units Taq DNA polymerase (Roche Applied Science, Germany). The specific primers used for the reaction were as follows: primer pair 1: ONPF 38 (1-20 nt) 5´-GAT CAC AGG TCT ATC ACC CT-3´ and ONPR 79 (780-761 nt) 5´-GAG CTG CAT TGC TGC GTG CT-3´ and primer pair 2: ONPF206 (15340-15360 nt) 5´-ATC CTT GCA CGA AAC GGG ATC-3´ and ONPR 77 (110-91 nt) 5´-GCT CGG GCT CCA GCG CTC CG-3´. These primers amplified a 780 bp and 1366 bp length respectively, encompassing two HVS_s in the D-loop of the mtDNA to fetch the 359 bp sequence (16024-

16383 nt) and 315 bp sequence (57-372 nt) for HVS I and HVS II. PCR (5 min initial incubation at 94°C, 35 cycles of one minute at 94°C, one minute at 60ºC and 35 seconds at 72°C, 5 min final extension at 72ºC) was performed in a Techne PCR device (Techne Ltd., UK). The nucleotide sequence of the amplicon was directly determined by automated sequencing 3700 ABI machine, using primer ONPF38 and ONPR77 (Macrogene, Seoul, Korea).

The obtained mtDNA sequences were aligned in the multiple sequence alignment interface, CLUSTALX, against the revised Cambridge Reference Sequence (rCRS).

RESULTS

Comparing with the revised Cambridge Reference Sequence (rCRS), our samples showed 40 mitotypes within the control region of which 85 were observed in single individual. All samples contained mutations apparently different from the reference sequence. D-loop mutation rate in HCM samples was higher than normal controls. (Table 1) Most of the mutations were located in HVS-I including single base substitutions. Majority of mutations in HVS-II were transitions (66.6%) rather than transversions (33.4%). Mutations in HVS-I were transitional (90.4%) and transversional (9.6%) substitutions. We also found an adenine base deletion in np249 in two patients (6.45%). One patient showed C-del in np459. Our results showed A575CCCCCCC, A575CCC and C61TCG insertions each in one samples. Another patient showed a C61TCG variation.

12 polymorphisms (T60C, C61T, C61G, G62T, C320T, T321G, C324G, A432C, C517A, C519A, A16318C, and C16327A) were newly identified in this population study and not recorded previously in the human genome database (Mitomap database); PCT changes were present in 26 patients (83%). Correspondence of this microsatellite marker in HCM patients to that of the published Cambridge Sequence revealed that mutations were exclusively found on a microsatellite base in a poly-cytidine sequence (position 303-315 of the Cambridge notation), interrupted by a single T. The 3´-c-repeat of the poly-cytidine microsatellite of all our samples contained six cytosines instead of five as the database suggests (hence C_7TC_6). In 26 patients with PCT changes, C_8TC_6, C_9TC_6 and $C_{10}TC_6$ have been observed in nineteen (73%), two (7.7%) and one (4%) respectively. The remaining 4 individuals had a sequence of C_7TC_6.

DISCUSSION

This work is part of an effort to determine if common mutations in mitochondrial D-loop exist in HCM specimens or not. The long-term goal of this work is to determine the quantity or nature of the mutations which may play role in sudden cardiac death that was unexplained before. There has already been considerable interest in the possible role of mtDNA background on the phenotype expression of mitochondrial genetic disorders (Torroni *et al*., 1997). The non-coding D-loop was found to be a mutational hot spot in bladder, lung, head and neck neoplasms (Fliss *et al*., 2000). Mutations in this region may alter the function of the D-loop, as this represents a regulatory site for both replication and expression of the mitochondrial genome. A large number of mitochondrial polymorphisms identified here likely reflect the high mutation rate of mtDNA, which is thought to be caused mainly by high levels of ROS (De Grey, 2005; Wei, 1998). In agreement with this, our data imply that constitutive hypervariable areas such as the D-loop region represent somatic mutational hot spots.

DNA-based gene analysis has shown that more than half of HCM cases are the outcome of sarcomeric disease (Solomon *et al*., 1990; Spirito *et al*., 1997). However, in the remaining cases of HCM and in cases of dilated cardiomyopathy (DCM) and of variable phenotype expression among members of the same family with a particular gene mutation the pathogenesis remains unknown (Graham and Owens, 1999). Meanwhile, mtDNA mutation has emerged as a cause of hereditary HCM (Wallace, 1992). We examined 31 HCM patients and found that all the samples showed at least one mutation within the region of HVS-I and HVS-II. There was a substantial difference in the frequency of mutations between patients in our study. Some of the samples displayed similar levels of mutations. We were unable to draw any correlations of mutation level and clinical characteristics with the data that was available to us (dyspnea, chest pain, lightheadedness, presyncope, syncope, tiredness, palpitation, orthopnea and SCD). However, the study does indicate that mitochondrial mutations are indeed frequent in HCM and mutation rate in D-loop of patient group was higher than control . The distribution of SBS was not random across the entire length of the D-loop but was concentrated in hypervariable regions HVR-I and HVR-II. To our knowledge this is the first report that SBS in the D-loop of HCM patients occur in a non-random distribution. In addition PCT changes were frequent (%83) in our HCM patients. In contrast to the published rCRS, the 3´-c-repeat of the poly-cyti-

Table 1. Instabilities and variations of mitochondrial HVS-I and HVS-II in 31 Iranian patients with HCM and 45 normal controls.

Map Locus	Shorthand	Map position (np)	Description	Variation position	Variation	Patients with variation (%)	Control with variation (%)
MTHV2	HVS-II	57-372	Hypervariable Sequence-2	57	T to C	6.45	-
				60	T to C[a]	86.00	-
				61	C to T[a]	86.00	-
				61	C to G[a]	80.60	-
				62	G to T[a]	87.00	-
				63	C to T	67.00	-
				73	A to G	67.00	18.6
MTOHR	OH	110-441	H-Strand Origin	189	A to G	13.00	-
MTCSB1	CSB1	213-235	Conserved Sequence Block 1	-	-[b]	-	-
MTTFX	TFX	233-260	mtTF1 binding site	-	-[b]	-	-
MTTFY	TFY	276-303	MtDNA binding site	263	A to G	96.00	50.6
MTCSB2	CSB2	299-315	Conserved Sequence Block 2	-	-[b]	-	-
MTHRR	HPR	317-321	Replication Primer	320	C to T[a]	3.2.0	-
				321	T to G[a]	3.20	
MTCSB3	CSB3	346-363	Conserved Sequence Block 3	324	C to G[a]	9.67	-
MTMT4H	Mt4H	371-379	mt4-H-Strand Control Element	-	-[b]		
MTMT3H	Mt3H	384-391	mt3-H-strand Control Element	-	-[b]		
MTLSP	PL	392-445	L-Strand Promotor	-	-[b]		
MTTFL	--	418-445	Mt TF1 binding site	432	A to C[a]	3.20	-
MTTFH	--	523-550	MtTF1 binding site	462	C to T	9.67	-
				489	T to C	19.35	-
				497	C to T	6.45	-
				517	C to A[a]	3.22	4.0
				519	C to A[a]	3.22	
MTHSP1	PH1	545-567	Major H-Strand Promotor	-	-[b]		
MTHV1	HVS-I	16024-16383	Hypervariable Sequence 1	-	-[b]		
MT7sDNA	7s DNA	16106-16191	7s DNA	16069	C to T	12.90	-
				16111	C to T	6.45	-
				16126	T to C	19.30	2.6
				16129	G to A	9.67	
				16145	G to A	9.67	
				16153	G to A	6.45	
MTTAS	TAS	16157-16172	Termination Associated sequence	16189	T to C	3.00	-
				16192	C to T	6.45	-
MTMT5	mt5	16194-16208	Control Element	16222	C to T	3.22	-
				16223	C to T	22.58	-
				16224	T to C	6.45	-
				16261	C to T	9.67	-
				16266	C to T	6.45	-
				16292	C to T	6.45	-
				16294	C to T	6.45	-
				16296	C to T	6.45	-
				16304	T to C	6.45	12.0
				16311	T to C	22.58	-
				16318	A to C[a]	3.22	-
				16325	T to C	6.45	-
				16327	C to A[a]	3.22	-
MTMT3L	mt3L	16499-16506	L-Strand Control Element	16524	A to Ca	3.22	-

Notes: [a]: Variations not found previously, [b]: Relatively stable and no variation of base

dine microsatellite of all our samples contained six cytosines and not five as the database suggests (hence C_7TC_6). We considered this discrepancy as a error in the database. Substitutions in the non-coding HVR1 sequence of the mtDNA D-loop alone is not responsible for causation of disease. Some may however, be part of a haplotype, which may indicate deleterious mutation elsewhere in mtDNA (Marchington *et al*., 1996).

It was shown that a unit increase in the number of HVR1 substitution increased the odds of a potentially pathogenic mutation by 30%. In patients with known mtDNA disease, a large number of sequence variants have been found in the D-loop region (Marchington *et al*., 1996). The D-loop region, which is a control region, holds the origin of replication for the heavy strand of the coding mtDNA genome, as well as promoters for the transcription of the whole of the mtDNA genome (Wallace, 1994). Hofmann *et al.* (1997) found that the distribution pattern of D-loop variants among patients with different mtDNA diseases differed considerably from that of a control population. This supports a theory that a high substitution rate in the D-loop might be a marker for mtDNA instability as it was shown in the present study. This is not surprising, if we consider that over 1,000 different mtDNA polymorphisms have been reported, and that the greatest polymorphic degree occurs in the mtDNA noncoding region (Mitomap database). In general, D-loop fragments with many heteroplasmic sites show high levels of polymorphism (Jazin *et al*., 1996). Mutations in HVS-I and HVS-II could account for the reduction in mitochondrial proteins and copy number, because these elements have been proposed to provide the primer for initiating mtDNA H-strand synthesis at OH_1 and OH_2. (H-strand origin) It seems likely that somatic mtDNA control region mutations accumulate with age in all individuals (Wang *et al*., 2001), but that the mutation rate of certain individuals is much higher. These later individuals, in turn, have a higher probability for occur in one of the HCM-specific mutation, thus enhancing the probability of sudden death. HVS-I and II are areas of mtDNA with a high substitution rate. A mechanism that could affect the entire mtDNA is mutations occurring in nuclear genes coding for mitochondrial proteins involved in mtDNA replication. Such mutations might increase the error rate during mtDNA replication (Lauber *et al*., 1991). It has been proposed that a 16189 polymorphism reflects a predisposition to the formation or fixation of mutations in the $tRNA^{leu}$ gene (Hofmann *et al*., 1997), and this may also be true for other D-loop polymorphisms. This mutation may predispose individuals to mtDNA rearrangements (Torroni *et al*., 1994).

The significance of the high intrinsic rate of alterations in mitochondria is not fully understood, the finding of high mutation rates in HCM suggests that they play a role in cardiomyopathy pathogenesis. In fact, we are not sure whether some special variations and increased variant frequency of mtDNA are the causes of HCM or just the results of cardiomyopathy. It is possible that mtDNA mutations are just the results of clonal expansion of spontaneous somatic mutations that occur at a very low frequency during previous replication precursor cells, and become apparent both by clonal expansion of the cells and by predominant selection and later become homoplasmic or at least somewhat predominant within the cell. It also indicates that frequencies and types of mtDNA mutations reflect hidden genetic and environmental actions.

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