RESEARCH ARTICLE

Mitochondrial D-Loop Polymorphism and Microsatellite Instability in Prostate Cancer and Benign Hyperplasia Patients

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Abstract

In this study mitochondrial D-Loop variations in Iranian prostate cancer and benign prostatic hyperplasia (BPH) patients were investigated. Tumour samples and corresponding non-cancerous prostate tissue from 40 prostate cancer patients and 40 age-matched BPH patients were collected. The entire mtD-loop region (16024-576) was amplified using the PCR method and products were gel-purified and subjected to direct nucleotide sequencing. A total of 129 variations were found, the most frequent being 263A→G and 310T→C among both BPH and prostate cancer patients. Variation of 309 C→T was significantly more frequent in prostate cancer patients (P value<0.05). Four novel variations were observed on comparison with the MITOMAP database. Novel variations were np16154delT, np366G→A, np389G→A and 56insT. There was no correspondence between the different variations and the age of subjects. Considering that D-loop variations were frequent in both BPH and prostate cancer patients in our study, the fact that both groups had high average age can be a possible contributing factor. D-loop polymorphisms and microsatellite instability can influence cell physiology and result in a benign or malignant phenotype. Significantly higher frequency of 309 C→T variation in cancer patients is a notable finding and must be a focus of attention in future studies.

Keywords: Prostate cancer - mitochondrial DNA - D-Loop - benign prostatic hyperplasia (BPH)

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Introduction

Prostate cancer is one of the most common malignancies among men over the age of 55 and is responsible for about 6% of all male cancer deaths (Parkin et al., 2001). The causes of prostate cancer, which is a heterogeneous disease, are unknown but are thought to include environmental, nutritional, hormonal, and genetic factors. Molecular mechanism of prostate cancer is poorly understood; however, several molecular abnormalities such as chromosomal loss or gain, gene amplification, mutations leading to increase, decrease or induction of gene expression, mutations leading to changes in function of the proteins and some polymorphisms have been described (Roylance et al., 1997; Ousati Ashtiani et al., 2009; Ghafouri-Fard et al., 2010; Ashtiani et al., 2011).

During last two decades, association between mtDNA mutations and many human diseases including neurodegenerative diseases, metabolic diseases, and various types of cancer has been reported (Fliss et al., 2000; Penta et al., 2001; Wallace, 2005; Lu et al., 2009). Mitochondrion plays an essential role in different pathways in the cell such as energy production, reactive oxygen species (ROS) generation, and also in a fundamental biological process in which cells die in a controlled manner called apoptosis. All these processes have been associated with tumorigenesis (Gomez-Zaera et al., 2006). Due to critical role of apoptosis in cancer development, understanding the molecular biology of mitochondria and its contribution to tumorigenesis is very important(Wang, 2001). It is postulated that mitochondrial dysfunction induced by accumulation of somatic mutations in mtDNA, plays a central role in a wide range of age-related disorders and various forms of cancers (Wallace, 2005).

The human mitochondrial DNA, which is inherited maternally and is present in thousands of copies per cell, is a 16.5 kb circular double-stranded DNA molecule with no introns, protective histones and has limited DNA repair mechanism. It encodes 13 polypeptides involved in respiration and oxidative phosphorylation, 22 tRNAs for protein synthesis in mitochondria and 2 rRNAs. The mitochondrial non-coding displacement loop (D-Loop) region contains crucial elements for DNA replication and transcription. Mutations in mtDNA can cause impairment of oxidative phosphorylation and increase ROS production which in turn accelerates the DNA mutation rate (Pang et al., 2008). It is reported that the mutation frequency in mtDNA is about 10- to 20-fold greater as compared

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to nuclear DNA (Copeland et al., 2002). Mitochondrial DNA mutations are present in both coding and non-coding regions and most of them appeared to be homoplasmic in nature(Chatterjee et al., 2006). There is growing evidence that mitochondrial genetic variation has a role in carcinogenesis (Booker et al., 2006). In human malignancies majority of mtDNA mutations occur in D-Loop region, which has been shown to be a "hot spot" for point mutations in many human cancers (Fliss et al., 2000; Penta et al., 2001; Pang et al., 2008; Sharawat et al., 2010). D-Loop region is highly polymorphic and has two hypervariable segments: HVS1 (16093-16362) and HVS2 (150-372). Point mutations and mitochondrial microsatellite instability (mtMSI) occur frequently in these segments; moreover, the incidence of somatic mutations in this region in some cancers increases with the cancer stage (Lee et al., 2005). In contrast in another study there was no association between mitochondrial haplogroups and control region polymorphisms with prostate cancer (Mueller et al., 2009).

In spite of a limited number of publication data on mitochondrial DNA, there are controversial results of investigating mtDNA variations in prostate cancer. One study observed mtDNA variations in 20% of patients (Jeronimo et al., 2001) and two other studies stated variations in 90% (Petros et al., 2005) and 100% (Gomez-Zaera et al., 2006) of their patients. Data regarding the role of mtDNA variations in prostate cancer and BPH are still insufficient. Therefore, in this study we aimed to investigate mtDNA variations in the D-Loop region of prostate cancer patients and benign prostatic hyperplasia (BPH) patients.

Materials and Methods

Patient samples and DNA Extraction

Prostate tumour samples and corresponding non-cancerous prostate tissue from 40 prostate cancer patients with known histological information and from 40 age-matched untreated BPH patients were collected. In addition pathologic paraffin embedded tumour tissues from the same set of individual were also used to cross-check the DNA changes. Diagnosis of prostate cancer and BPH was made by an elevated serum level of prostate specific antigen (PSA) and transrectal ultrasound-guided biopsies (TRUS). The cancerous, benign hyperplasia and non-tumor tissue were confirmed by pathological examination. For all patients, relevant clinical and pathological data including: PSA level, prostate weight and Gleason Score were collected. Written informed consents were obtained from all participants in this research. Approval was obtained through the Investigation Review Board at the Tehran University of Medical Sciences. This study was also approved by the ethics and clinical studies committee of Tehran University of Medical Sciences. DNA from samples and paraffin-embedded tumor tissue sections were extracted by Qiagen DNA extraction kits (QIAamp DNA Mini Kit & QIAamp DNA FFPE, Qiagen, Hilden, Germany) using manufactures' instructions. The quality and quantity of obtained DNAs were assessed by spectrophotometry.

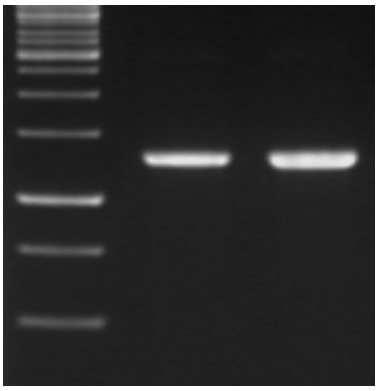

Figure 1. Purified PCR Products; lane 1: marker; lanes 2 and 3: cancer samples

Molecular analysis of mtD-Loop

The D-loop region of mitochondria (16024-576) was amplified using the following primers, forward: 5´-CTAATACACCAGTCTTGTAAACC - 3´; reverse: 5´-GGTGATGTGAGCCCGTCTAAAC - 3´. Total volume of 25 μl reaction mixture contained about 100 ng DNA, PCR buffer, 1.5 mM MgCl2, 200 μmol deoxynucleotide triphosphate, 10 pmol of each primer and 2 U SmarTaq polymerase (Cinnagen, Iran). Touchdown PCR was used and thermocycling conditions were as follows: 4 min in 95 °C for initiation denaturation and 15 cycles with following parameters, 95 °C, 57-50 °C 30 s (annealing temperatures was decreased by 0.5 °C every cycle), 72 °C 1min, and 20 cycles with following specifications, 95 °C 30 s, 50 °C 30 s, 72 °C 1 min. Amplification was performed in an ABI thermal cycler 2720 (Applied Biosystems, Foster City, CA, USA). PCR products were examined by electrophoresis on 1% agarose gel. PCR products were gel-purified using Accuprep™ gel purification kit (Bioneer Co., Korea) (Figure 1).

All purified products which contained mtD-Loop region were subjected to direct nucleotide sequencing (Applied Biosystems 3730/3730 xl; BigDye V3.1 USA) by using three primers. Because the size of PCR product was 1309 bp, a third overlapping primer was designed for performing sequencing and better analysis, forward 2: 5´-TAAAGCCTAAATAGCCCACAC- 3´. By having overlapping segments, the entire region of the D-Loop was sequenced. Sequences and chromatograms obtained were examined by chromas software version 2.13 and aligned by BLAST (www.ncbi.nlm.nih.gov/blast). All sequences were compared with last version of Revised Cambridge Reference Sequence (rCRS) of the Human Mitochondrial DNA (NC_012920) and MITOMAP: mtDNA Control Region Sequence Polymorphisms (www.mitomap.org) for new variations.

Statistical Analysis

SPSS version 16 was used for statistical analysis. Patients' characteristics are presented as Mean± Standard Deviation. Analysis of difference between frequencies of each variation in different groups was performed using Chi-square and Fischer's exact test. Analysis of difference of patients' characteristics in two groups was performed using independent T test. P values < 0.05 were considered significant.

Table 1. Cancer and Benign Prostatic Hyperplasia (BPH) Patients' Characteristics

Characteristics	Age (Mean±SD)	PSA level	Prostate weight	Gleason Score
Prostate cancer patients	68.1±6.5	20.2±24.3	51.5±26.3	6.6±1.3
BPH patients	65.1±6.2	15.2±25.6	68.0±34.8	-

Table 2. Frequency of Each Identified Nucleotide Variation in Cancer and Benign Prostatic Hyperplasia (BPH) Patients

Nucleotide position	Base variation	Cancer	BPH	Nucleotide position	Base variation	Cancer	BPH	Nucleotide position	Base variation	Cancer	BPH
16010	T→C	1	0	16270	C→T	0	2	215	A→G	2	0
16048	G→A	1	0	16289	A→G	1	0	220	T→G	1	0
16051	A→G	0	1	16290	C→T	0	1	227	A→G	1	0
16069	C→T	1	2	16292	C→T	1	0	239	T→C	1	1
16071	C→T	2	0	16294	C→T	3	4	249	A→G	1	0
16086	T→C	1	1	16296	C→T	1	1	257	A→G	1	0
16091	A→G	1	0	16300	A→G	0	1	263	A→G	32	25
16092	T→C	0	1	16304	T→C	1	1	271	C→T	0	2
16104	C→T	1	2	16309	A→G	3	0	272	A→G	0	1
16114	C→T	2	1	16311	T→C	4	3	285	C→T	1	0
16115	C→T	1	0	16318	A→T	4	0	295	C→T	2	1
16126	T→C	10	7	16325	T→C	1	1	303	INS→C	12	6
16127	T→C	1	1	16342	T→C	1	0	303	INS→CC	12	12
16129	G→A	1	3	16343	A→G	0	2	309	C→T	24	9
16129	G→C	0	1	16352	T→C	1	1	310	T→C	25	15
16145	G→A	0	2	16355	C→T	1	1	311	C→T	1	1
16148	C→T	1	0	16356	T→C	0	1	315	INS→C	2	0
16154	DEL→T	1	0	16357	T→C	1	1	315	INS→CC	1	0
16162	A→G	1	0	16362	T→C	5	5	324	C→G	1	0
16163	A→G	1	2	16384	G→A	1	0	366	G→A	1	0
16166	A→G	0	1	16390	G→A	1	0	385	A→G	1	0
16172	T→C	0	1	16399	A→G	0	1	389	G→A	1	0
16173	C→T	0	1	16456	G→A	1	0	456	C→T	0	1
16183	A→C	1	0	16482	A→G	1	0	462	C→T	2	2
16186	T→C	1	2	16519	T→C	16	10	466	T→C	0	1
16188	C→T	1	0	56	INS→T	1	0	480	T→C	4	1
16189	T→C	5	2	60	INS→T	1	0	482	T→C	1	1
16193	C→T	2	0	64	C→T	3	1	488	T→C	1	0
16218	T→C	1	0	65	INS→G	1	0	489	T→C	3	5
16223	C→T	4	2	73	A→G	20	20	497	C→T	0	2
16224	T→C	0	2	103	G→A	0	1	506	C→T	1	1
16235	A→G	0	1	117	T→C	0	1	508	T→C	0	1
16239	C→T	2	0	146	T→C	5	5	514	DEL→C	5	6
16240	A→G	1	0	150	C→T	5	3	515	DEL→A	6	6
16243	T→C	1	0	151	C→T	3	0	516	DEL→C	1	0
16246	A→T	0	1	152	T→C	18	7	518	DEL→C	2	1
16256	C→T	2	2	153	A→G	1	1	519	DEL→A	2	1
16260	C→T	0	1	195	T→C	4	5	564	G→A	1	0
16261	C→T	0	1	198	C→T	1	0	584	DEL→T	3	1
16264	C→T	1	0	200	A→G	0	1	588	DEL→T	10	6
16265	A→G	0	1	204	T→C	0	1	589	DEL→T	1	0
16265	A→C	1	0	206	T→G	1	0	592	C→T	0	1
16266	C→A	0	1	207	G→A	2	1				

INS, insertion; DEL, deletion

Results

Forty prostate cancer samples and 40 BPH samples were analyzed in this study. Patients' demographic data and characteristics are displayed in Table 1. Total 129 variations were found compared to rCRS. Four novel variations were observed in comparison to MITOMAP database including (np16154delT, np366G→A, and np389G→A) and (56insT) variations (Figure 2). Among these variations the most frequent nucleotide substitutions were transitions.. Total alterations are displayed in Table 2. No mutation was observed by comparing nucleotides sequences from cancerous and corresponding non-cancerous tissues.

The most frequent variations among patients are shown in Table 3. In addition to these frequent changes, a number of transition variations such as 16126T→C, 16189T→C, 16362T→C, 146T →C, 150C→T, and also deletions of C and A (CA) at nucleotide positions (np) 514 and 515 were relatively higher than the other variations (Figure 2). Variation of 309 C→T was significantly more frequent in prostate cancer patients as compare to BPH group (P

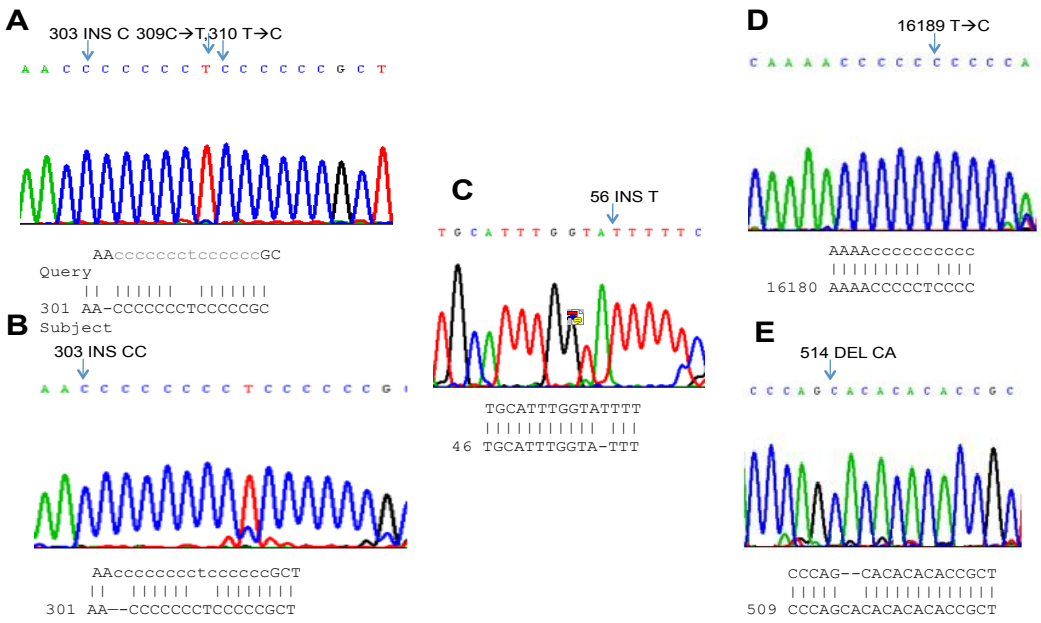

Figure 2. Detection of Nucleotide Alterations and Microsatellite Instability by DNA Sequencing. A and B: insertion of C and CC respectively; C: insertion of T which was novel; D, one of common nucleotide alterations; and E: CA deletion

Table 3. Most Frequent Variations in Cancer and Benign Prostatic Hyperplasia (BPH) Patients

Prostate cancer			BPH		
Variations	N	%	Variations	N	%
263A→G	33	82.5	263A→G	36	90
310T→C	26	65	310T→C	21	52.5
309C→T	24	60	73A→G	20	50
73A→G	20	50	303INS→CC	18	45
152T→C	18	45	16519T→C	14	35
16519T→C	16	40	309C→T	13	32.5
303INS→C	12	30	152T→C	10	25
303INS→CC	12	30	303INS→C	9	22.5

INS, insertion

value<0.05; odds ratio: 3.6, CI 95% 1.2-10.1). Alteration in poly C tract (np303-315) was observed by nucleotide number variation. In order to determine whether mtD-Loop variations have any relationship with age, gleason score and prostate size, they were analyzed but there was no correspondence between the frequency of different variants and mentioned factors.

Discussion

The mtD-loop is responsible for regulation of replication and transcription of mtDNA. D-loop mutations and altrations are frequently found in various human cancers; therefore, it is known as a "hot spot" for point mutations in cancers. These mutations are considered to be the result of high concentrations of ROS and incompetent mtDNA repair system (Lee et al., 2005). Mitochondrial DNA polymorphism can act as risk modifier in late onset diseases such as cancers (Gomez-Zaera et al., 2006). Both homoplasmic and heteroplasmic variations of D-loop are reported in previous studies. Homoplasmic alterations, which were most frequent in our study, may have a significant role in the development of neoplasia (Pang et al., 2008).

In present investigation mitochondrial DNA D-loop region variations were found among all patients of both cancer and BPH groups. The prevalences of D-loop variations are in compatibility with other reports (Chen et al., 2002; Gomez-Zaera et al., 2006). Chen et al have found point mutations in 90% of their prostate cancer patients (Chen et al., 2002). All prostate cancer patients carried D-loop variations in Gomez-Zaera et al's study (Gomez-Zaera et al., 2006). In addition, various frequencies of the mtDNA alterations were identified in several types of malignancies including: glioma 35% (Kirches et al., 2001; Chatterjee et al., 2006), colorectal cancer 38%, (Lievre et al., 2005) hepatocellular carcinoma 39%, (Lee et al., 2005) osteosarcoma, 70% (Guo, 2006) and AML 79%, (Sharawat et al., 2010).

It was reported that polymorphisms 73A→G and 263A→G were two of the most common variations in European population and may act as discriminating factors of 5 major mtDNA clusters (Pang et al., 2008). However, our results also showed that these variations are among the most common in both prostate cancer and BPH patients. These variations were found in all patients with nasopharyngeal carcinoma in the study of Pang et al (Pang et al., 2008). In contrast lower prevalence (19%) of these variations were observed in prostate cancer patients in Chen et al's study (Chen et al., 2002). A number of other studies had low prevalence of 73A→G and 263A→G variations or did not encounter one in their cancer affected patients (Sharawat et al., 2010, Shen et al., 2011). Although, we did not examined normal individuals in our research; it is possible that these variations are prevalent in Iranian population similar to European population.

Mitochondrial D-Loop includes two Hypervariable segments (HVSI-HVSII). Most frequent variations in our study were present in HVS II. Majority of these variations were transitions, deletions, insertion of nucleotides. One of the most common instabilities was observed at the first polymorphic C track (np303-315, named D310). In this

research we also found insertion of 1 or 2 C at np303, and transitions of 309 C→T and 310 T →C. However, Shen et al have observed heteroplasmy at D310 C repeat in breast cancer tumor. In present work C track alterations have been observed in both cancer and BPH groups; however, cancer patients had higher frequency of these alterations. Variation of 309 C→T was significantly more prevalent in prostate cancer group. As in our study both prostate cancer and BPH tumors were analyzed, significantly higher prevalence of 309 C→T is notable and should be more emphasized in future studies. Our findings confirm that the first polymorphic C track is a hotspot for alterations. In another CCCCCTCCCC sequence which is highly polymorphic and located at np16184-16193 in HVS I; we have observed 4 transition alterations, two C→T and two T →C. Czarnecka et al also reported T16189C polymorphism in breast cancer in 2009 (Czarnecka et al., 2009). According to last version of rCRS, D-Loop contains 5 CA repeats at np514-523 but our result showed dinucleotide microsatellite instability in this region. About 20% of cancer patients and 15% of BPH patients had CA deletion at this site which is about ten fold greater than endometrial carcinoma (Liu et al., 2003). Thus, it is likely that there are different mechanisms of mtDNA alterations and repair in different tissues during tumorigenesis.

Mitochondrial mutations are observed to be associated with older age in normal subjects, particularly 189A→G alteration (Chatterjee et al., 2006). The role of age as an important risk factor for prostate cancer has been established and more than 75% cases occur in men over the age of 65 (Gomez-Zaera et al., 2006). In present study we did not observe any correspondence between the different variations and the age of subjects. Considering that D-Loop variations were frequent in both BPH and prostate cancer patients in our study, the fact that both groups had high average age can be a possible contributing factor. Repeated exposure to ROS over the time may be a possible cause. D-Loop variations might be individually neutral but the accumulation of these neutral variations may cause synergistic effects and contribute to cancer development.

Undoubtedly nucleotide change in D-loop region can affect mitochondrial function, because non-coding sequence of mitochondria controls the initiation of mtDNA replication and transcription. With respect to high alterations in D-Loop in cancer and BPH patients it can be concluded that D-Loop polymorphisms and microsatellite instability can influence cell physiology and result in benign or malignant phenotype. Although a specific variation in D-Loop region has not been established as a biomarker helping to identify individuals at high risk for developing specific cancer types and to develop screening approaches for early diagnosis of cancer; higher prevalence of 309 C→T found in our study is a notable finding and must be focused in future. Because of some limitations, we were not able to use more samples to show mutations that probably exist between cancerous and corresponding non-cancerous prostate tissue. Further studies are suggested to analyze the synergistic effects of D-Loop mutations with larger sample size.

Acknowledgements

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