RESEARCH ARTICLE

New Haplotypes of the ATP Synthase Subunit 6 Gene of Mitochondrial DNA are Associated with Acute Lymphoblastic Leukemia in Saudi Arabia

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children and represents approximately 25% of cancer diagnoses among those younger than 15 years of age. Aim and Objectives: This study investigated substitutions in the ATP synthase subunit 6 gene of mitochondrial DNA (mtDNA) as a potential diagnostic biomarker for early detection and diagnosis of acute lymphoblastic leukemia. Based on mtDNA from 23 subjects diagnosed with acute lymphoblastic leukemia, approximately 465 bp of the ATP synthase subunit 6 gene were amplified and sequenced. Results: The sequencing revealed thirty-one mutations at 14 locations in ATP synthase subunit 6 of mtDNA in the ALL subjects. All were identified as single nucleotide polymorphisms (SNPs) with a homoplasmic pattern. The mutations were distributed between males and females. Novel haplotypes were identified in this investigation: haplotype (G) was recorded in 34% in diagnosed subjects; the second haplotype was (C) with frequency of 13% in ALL subjects. Neither of these were observed in control samples. Conclusions: These haplotypes were identified for the first time in acute lymphoblastic leukemia patients. Five mutations able to change amino acid synthesis for the ATP synthase subunit 6 were associated with acute lymphoblastic leukemia. This investigation could be used to provide an overview of incidence frequency of acute lyphoblastic leukemia (ALL) in Saudi patients based on molecular events.

Keywords: Acute lymphoblastic leukemia - ATP synthase subunit 6 gene - mtDNA - biomarker

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children and represents approximately 25% of cancer diagnoses among children younger than 15 years of age (National Cancer Institute, 2012 a; b). ALL occurs at an annual rate of 35 to 40 cases per 1 million people in the United States (Smith et al., 1999; National Cancer Institute, 2012a; b). Among children and adolescents younger than 20 years of age, 2,900 are diagnosed with ALL each year in the United States (Smith et al., 1999; Dores et al., 2012). Over the past 25 years, there has been a gradual increase in the incidence of ALL (Shah and Coleman, 2007; National Cancer Institute, 2012 a; b).

A sharp peak in the occurrence of ALL is observed among children of 2 to 3 years of age (more than 90 cases per 1 million per year), with rates decreasing to fewer than 30 cases per 1 million by the age of 8. The incidence of ALL among children of 2 to 3 years of age is approximately four times greater than that for infants and is four to five times greater than that for children of 10 years of age and older (National Cancer Institute, 2012 a; b). The incidence of ALL appears to be highest in Hispanic children (43 cases per 1 million), and the incidence is substantially higher in white children than in black children, with a nearly three times higher incidence from age 2 to 3 in white children than in black children (Smith et al., 1999; National Cancer Institute, 2012 a;b).

Awan et al. (2012) reported that the frequency of BCR-ABL FO in pediatric ALL, associated with poor overall survival. Their data indicated that an immediate need for incorporation of tyrosine kinase inhibitors in the treatment of BCR-ABL+ pediatric ALL in this population and the

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Table 1. Primer Pairs for Amplification of ATP Synthase Subunit 6 of mtDNA

Pairs	Fragment name	Forward Primer Sequence	Reverse Primer Sequence
1	ATPase 6 (Leiven et al., 1999)	CTGTTCGCTTCATTCATTGCC	GTGGCGCTTCCAATTAGGTG

development of facilities for stem cell transplantation.

Mitochondria are most important organelles that produce ATP through a vital pathway that is well known as oxidative phosphorylation. This pathway is accomplished by a group of protein complexes and mitochondrial respiratory chains (MRC) which are controlled by both of nuclear and mitochondrial genomes (Higuchi, 2012). Several studies demonstrated that mtDNA mutation is common in cancer (Lu et al., 2009; Cook and Higuchi, 2011). Mitochondria play vital functions in ATP metabolism, free radical generation, and regulation of apoptosis, subsequently changes in mitochondrial DNA would affect cellular energy capacities, increase oxidative stress, trigger ROS-mediated damage to DNA, and alter the cellular response to apoptosis induction by anticancer agents (Carew et al., 2003).

Many mutations in mitochondrial DNA have been characterized in different types of human cancer. Mutations in mitochondrial DNA have been appeared in different regions within a certain genome and most of these mutations have been reported as homoplasmic in nature (Chatterjee et al., 2006). Previous studies reported that bladder cancer exhibited base transitions from T to C and G to A in ND3, ND4, mitochondrial Cytochrome b, 16SrRNA and D-loop region (Fliss et al., 2000), and deletion and insertion in the D310 region (Parrella et al., 2003).

It is well know that the mutational changes in the mitochondrial genome could be used as a diagnostic biomarker for early detection of cancer and as a potential target in the development of new therapeutic approaches is discussed. These findings strongly indicate that mtDNA mutations exert a crucial role in the pathogenic mechanisms of tumor development, but continued investigations are definitely required to further elucidate the functional significance of specific mtDNA mutations in the etiology of human cancers (Yu, 2012).

The aims of this investigation are to use the alterations at ATP synthase subunit 6 gene of mtDNA as a risk factor and diagnostic biomarker for early detection and diagnosis of acute lymphoblastic leukemia. To apply the mitochondrial DNA mutations as a prognostic markers in a certain disease. This may make it possible to apply mitochondrial DNA mutations as a prognostic marker for the disease. This type of study has not been applied before on Saudi patients diagnosed with acute lymphoblastic leukemia, and is considered the first such report in the Kingdom of Saudi Arabia.

Materials and Methods

Subjects and acute lymphoblastic leukemia profile

Twenty-three subjects diagnosed between July 2009 and May 2013 with acute lymphoblastic leukemia (n=23; 16 males and 7 females) were involved in this study. Approval and consent were obtained from the Center of Excellence in Genomic Medicine Research (CEGMR) at King Abdulaziz University, in the Kingdom of Saudi Arabia.

Genomic DNA isolation

Genomic DNA samples were isolated from the subjects. Samples were deposited and stored in the biobank of the CEGMR at King Abdulaziz University.

D-loop amplification of human mitochondrial DNA

The primers designed by (Leiven et al., 1999) was used to amplify approximately 465 bp of ATP synthase subunit 6 of human mitochondrial DNA as listed in (Table 1). PCR amplification reactions were performed in 50µl total volume consisting of 50 ng of template DNA, 10pmol of each primer, 0.25 U of Taq DNA polymerase, 250µM of dNTPs mix , 10µM of Tris-HCl (pH.9.0), 30µM of KCl, 1.5µM of MgCl2 and sterile water free nuclease to make 50µl as a maximum volume. PCR amplification were applied in a Labnet international Inc thermocycler with the following cycling conditions: pre-denaturation at 94ºC for 5 min, denaturing at 94ºC for 30 sec, annealing at 55ºC for 30 sec , and extension at 72ºC for 30 sec for 35 cycles followed by a final extension at 72ºC for 10 min. The amplified fragments of PCR reactions were analyzed by applying gel electrophoresis using a DNA ladder in order to assess the size of the amplificon product. The images were obtained from Gel documentation system (Ultra-Violet Products Ltd. UVP,LLC Upland, CA). The size of the amplicons was determined using software available with the gel documentation system.

Sequencing performance and sequencing analysis

The PCR products were purified and sequenced at (Bioneer Inc, Daejeon, and Republic of Korea). The obtained sequences were aligned with GenBank Accession number (NC_012920, GI 251831106) using nucleotide-nucleotide BLAST (blastn) software in htt://www.ncbi.nlm.nih.gov/blast/ and CLUSTALW 2.0.12.

Homoplasmic/ Heteroplasmic identification

The Homoplasmic and Heteroplasmic variations were evaluated from obtained sequences chromatograms.

Results and Discussion

All PCR fragments of ATP synthase subunit 6 of mtDNA from ALL subjecets were successfully sequenced, and deposited in GenBank databases (KM821411-KM821433) . The blast results from ALL patients was achieved as shown in Figure.1 using nucleotide-nucleotide BLAST (blastn) software in htt://www.ncbi.nlm.nih.gov/blast/. A total 23 patients were used in the study who diagnosed with acute lymphoblastic leukemia (ALL). The patient's age from 2 to 43 years with a mean of 14.2 years old and male: female ratio was 2:1.

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Control.1   GCAGTACTGATCATTCTATTTCCCCCTCTATTGATCCCCACCTCCAAATATCTCATCAAC 60
19          GCAGTACTGATCATTCTATCTCCCCCTCTATTGATCCCCACCTCCAAATATCTCATCAAC 60
Control.4   GCAGTACTGATCATTCTATTTCCCCCTCTATTGATCCCCACCTCCAAATATCTCATCAAC 60
11          GCAGTACTGATCATTCTATTTCCCCCTCTATTGACCCCCACCTCCAAATATCTCATCAAC 60
2           GCAGTACTGATCATTCTATTTCCCCCTCTATTGACCCCCACCTCCAAATATCTCATCAAC 60

Control.1   AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAACC 120
5           AACCGACTAATCACCGCCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAACC 120
Control.3   AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAACC 120
12          AACCGACTAATCACCACCCAACAATGACTAATCAAACTAATCTCAAAACAAATGATAACC 120
15          AACCGACTAATCACCACCCAACAATGACTAATCAAACTAATCTCAAAACAAATGATAACC 120
Control.4   AACCGACTAATCACCACCCAACAATGACTAATCAAACTAATCTCAAAACAAATGATAACC 120
3           AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120
7           AACCGACTAATTACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120
4           AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120
20          AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120
10          AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120
11          AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120
18          AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120
2           AACCGACTAATCACCACCCAACAATGACTAATCAAACTAACCTCAAAACAAATGATAGCC 120

Control.4   GCCACAACTAACCTCCTCGGACTCCTGCCTCACTCATTTACACCAACCACCCAACTATCT 240
20          GCCACAACTAACCTCCTCGGACTCCTGCCCCACTCATTTACACCAACCACCCAACTATCT 240

Control.1   ATAAACCTAGCCATGGCCATCCCCTTATGAGCGGGCGCAGTGATTATAGGCTTTCGCTCT 300
8           ATAAACCTAGCCATGGCCATCCCCCTATGAGCGGGCGCAGTGATTATAGGCTTTCGCTCT 300

Control.4   ATACTAGTTATTATCGAAACCATCAGCCTACTCATTCAACCAATAGCCCTGGCCGTACGC 420
1           ATACTAGTTATTATCGAAACCATCAGCCTACTCATTCAACCAATAGCCCGGGCCGTACGC 420

Control.4   CTAACCGCTAACATTACTGCAGG--CCCCCTACTCATGCACCTAAT 464
18          CTAACCGCTAACATTAGTGCAGG--CCCCCTACTCATGCACCTAAT 464
2           CTAACCGCTAACATTACTGGGGGGGCCCCCTACTCATGCACCTAAT 465
1           CTAACCGATAACATTGGTGGGGGGCCCCCTACTCATGCACCTAAT 465
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Figure.1. Clastal W 2.1 Multiple Sequence Alignment among Acute Lymphoblastic Leukemia and Control Subjects. Changes are marked bold, underlined in red

Thirty-one mutant sites were exploited at fourteen location in ATP synthase subunit 6 of ALL subjects as shown in (Table 2). All variation in ATP synthase subunit 6 of mtDNA were identified as single nucleotide polymorphisms (SNPs) with homoplasmic pattern. The excited mutations were distributed in between male and female.

Allele frequencies in ATP synthase subunit 6 of mtDNA in acute lymphoblastic leukemia patients

The normal and mutant allele frequencies of ATP synthase subunit 6 of mtDNA in ALL patients were shown in (Table.2). The results show that fourteen mutant site at ATP synthase subunit 6 gene were observed in this investigation. The results showed that the mutant allele at ATP synthase subunit 6 of mtDNA in ALL subjects had different frequency up to 34% and most of mutants' alleles have not been conducted before in any other studies.

Novel haplotype in acute lymphoblastic leukemia patients

Novel haplotypes were identified in ATP synthase subunit 6 gene of mtDNA in ALL subjects in this investigation as shown in Figure 1. Haplotype (G) was recorded 34% in diagnosed patients and it was not shown in control ones. The second haplotype was (C) with frequency of 13% in ALL subjects and it was not observed in control samples as well. These haplotypes were identified at the first time in acute lymphoblastic leukemia patients and have not been conducted before in any other studies.

Amino acids substitutions in acute lymphoblastic leukemia subjects

Thirty-one mutant sites were observed at ATP synthase subunit 6 of ALL subjects in this study and some of these mutations were led to substitutions in amino acids synthesis as shown in (Table 3). Five mutations were be able to change amino acids synthesis at ATP synthase subunit 6 and are associated with acute lymphoblastic

Table 2. Allele Frequency at ATP Synthase Subunit 6 of mtDNA in Acute Lymphoblastic Leukemia (ALL) Subjects

SNP	Normal allele		Mutant allele	
T/C	T	0.96	C	0.04
T/C	T	0.92	C	0.08
A/G	A	0.96	G	0.04
C/T	C	0.95	T	0.05
C/T	C	0.92	T	0.08
A/G	A	0.66	G	0.34
T/C	T	0.96	C	0.04
T/C	T	0.96	C	0.04
T/G	T	0.96	G	0.04
C/G	C	0.96	G	0.04
A/G	G	0.96	A	0.04
-/G	-	0.92	G	0.08
A/C	A	0.87	C	0.13
A/G	A	0.96	G	0.04
C/G	C	0.92	G	0.08

Table 3. Amino Acids Conversion at ATP Synthase Subunit 6 of mtDNA in Acute Lymphoblastic Leukemia (ALL) Patients

Effected Mutation	Amino acid change	Mutation frequency
A/G	Methionine to Isoleucine	0.34
C/G	Alanine to Aspartic Acid	0.04
A/G	Threonine to Glycine	0.04
A/C	Histidine to proline	0.13
C/G	Alanine to Glycine	0.08

leukemia patients. These alterations in amino acids were associated with the novel haplotypes that excited in this investigation especially for (G) haplotypes, which made a conversion to isoleucine from methionine. The same note was recoded for (C) haplotype which led to convert histidine to amino acid proline

Many studies report that alterations in mitochondrial DNA play a fundamental role in diseases such as Leber's hereditary optic neuropathy, maternally inherited diabetes mellitus, and Leigh's syndrome (DiMauro and Schon, 2001). While these diseases are due to germline mutations, somatic mutations have been observed in other diseases, especially cancer. The accumulation of somatic mutations is greater in mtDNA than in nuclear DNA because DNA replicates only at the time of cell division and undergoes proofreading by DNA polymerase. However, turnover of mtDNA is high, as degradation and replication is a continuous process in mitochondria, even within a single cell cycle, and mtDNA polymerase γ does not have the ability to proofread (Shadel and Clayton, 1997).

Shaikh et al. (2014) concluded that the relative lack of good prognostic cytogenetic abnormalities like t(12;21) (p13;q22) and hyperdiploidy (47-57 chromosomes) in Pakistani children with ALL. Prevalence of poor prognostic cytogenetic aberrations like t(9;22) (q34;q11.2) is comparable to available international literature.

Saber et al. (2012) reported that the frequency of 5 fusion oncogenes in adult ALL patients, and their association with clinical features, treatment response and outcome. Frequencies of some of the oncogenes were different from those reported elsewhere and they appear to be associated with distinct clinical characteristics and treatment outcome.

The most relevant difference between the mitochondrial and nuclear genomes is therefore their inherent susceptibility to damage (Carew and Huang, 2002). In fact, it is well established that mtDNA is much more susceptible to alterations than nuclear DNA, due to its lack of histone protection, limited repair capacity, and close proximity to the electron transport chain, which constantly generates superoxide radicals that also cause genetic damage. Since mtDNA lacks introns, most mutations also occur in coding sequences and are thus likely to be of biological consequence (Zastawny et al., 1998).

Mitochondria are most important organelles that produce ATP through a vital pathway, which is well known as oxidative phosphorylation. This pathway is accomplished by a group of protein complexes and mitochondrial respiratory chains (MRC) which are controlled by both of nuclear and mitochondrial genomes (Higuchi, 2012).

The mitochondrial genome of human is appearing with a high copy number for each cell, and this number is greatly differed based on kind or cell (Chatterjee et al., 2006). Mitochondria also have fundamental functions in energy metabolism, production of reactive oxygen species (ROS), and apoptosis (Carew et al., 2003). Apoptosis performs a vital function in cancer growth and in the cellular response to anticancer agents. The oxidative phosphorylation and cellular respiration pathways are responsible for generation of Reactive oxygen species (ROS) which is a free radical of oxygen metabolism. The tightly relationships between mitochondrial DNA and ROS generation as a site of production makes it more sensitive to oxidative damage and may lead to cause mutations in mitochondrial genome and subsequently related to cancer incidence (Chatterjee et al., 2006).

Various copy of mitochondrial genome had found in each cell of human and animal bodies; therefore, some changes in mitochondrial DNA were known as heteroplasmy and can distinguish from other normal cell type or wild type. The percentage of heteroplasmy in mitochondrial genome within each may differ by the time. This type of mutation, which changes the mitochondrial DNA, would lead polymorphism between individuals within same family regardless of carrying the same pathogenic mtDNA mutation (Wallace, 1992; Carew et al., 2003).

Both homoplasmic and heteroplasmic mtDNA mutations have been observed in cancer cells. In our investigation, all mutations in the d-loop region of mtDNA in subjects with ALL were identified as single nucleotide polymorphisms with a homoplasmic distribution, which is the most common pattern of mtDNA mutations (Chatterjee et al., 2006). The mechanisms by which such homoplasmy arises from heteroplasmic mutations in cancer cells still remain to be defined. Coller et al. (2001) used extensive computer modeling to suggest that if an mtDNA mutation occurs in a tumor progenitor cell, homoplasmy can be achieved entirely by chance through unbiased mtDNA replication and sorting during cell division, without selection for physiological advantage.

Several studies demonstrated that mtDNA mutation is common in cancer (Lu et al., 2009; Cook and Higuchi, 2011). Mitochondria play vital functions in ATP metabolism, free radical generation, and regulation of apoptosis, subsequently changes in mitochondrial DNA would affect cellular energy capacities, increase oxidative stress, trigger ROS-mediated damage to DNA, and alter the cellular response to apoptosis induction by anticancer agents (Carew et al., 2003).

Yacoub et al. (2014) concluded that a thirteen mutant alleles in the d-loop region of mtDNA occurred with a high frequency and novel alleles and locations were also identified in that region as follows: 89 G insertions (40%), 95 G insertions (13%), 182 C/T substitutions (5%), 308 C insertions (19%), and 311 C insertions (80%).

Many mutations in mitochondrial DNA have been characterized in different types of human cancer. Mutations in mitochondrial DNA have been appeared

in different regions within a certain genome and most of these mutations were reported as homoplastic in nature (Chatterjee et al., 2006). In addition, it illustrates how mtDNA alterations activate mitochondria-to-nucleus retrograde signaling to modulate the expression of relevant nuclear genes or induce epigenetic changes and promote malignant phenotypes in cancer cells.

In this investigation, we were used direct DNA sequencing method for ATP synthase subunit 6 gene of mtDNA which has advantage when compared with alternative techniques for identification such as PCR-RFLP, PCR using specific primers offers the advantages of being less expensive and more useful for routine analysis of large numbers of samples.

In conclusion, in our study, thirty-one mutant sites were exploited at 14 location in ATP synthase subunit 6 of mtDNA in ALL subjects and these kind of mutations have not been conducted before in any other studies. Novel haplotypes were identified in ATP synthase subunit 6 gene of mtDNA in this investigation. Haplotype (G) was recorded 34% in diagnosed patients and it was not shown in control ones. The second haplotype was (C) with frequency of 13% in ALL subjects and it was not observed in control samples as well. These haplotypes were identified at the first time in acute lymphoblastic leukemia patients and have not been conducted before in any other studies. Five mutations were be able to change amino acids synthesis at ATP synthase subunit 6 and are associated with acute lymphoblastic leukemia patients. This investigation could be used to provide an overview of incidence frequency of acute lyphoblastic leukemia (ALL) in Saudi patients based on molecular level.

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