Original Article

Analysis of mitochondrial ND1 gene in human colorectal cancer

Mansoureh Akouchekian\(^a\), Massoud Houshmand\(^b^\), Mohammad Hassan Hosseini Akbari\(^c\), Behnam Kamalidehghan\(^d\), Masoumeh Dehghan\(^e\)

Abstract

BACKGROUND: Colorectal cancer as a mortal disease affected both sexes of all ethnic and racial human groups. Former studies have indicated some mutations in the mitochondrial DNA (mtDNA) in different human cancers. Complex I NADH has the most subunits encoded by mtDNA. For a better understanding of the mtDNA abnormality in colorectal cancer some genes of this complex is screened for existence of mutations.

METHODS: One of the main regions of the mtDNA encoding protein was screened by PCR-RFLP followed by DNA sequencing. The obtained sequences were aligned with the revised Cambridge Reference Sequence (rCRS). Each alteration recorded as single nucleotide polymorphisms (SNPs), deletions or insertions.

RESULTS: Eight mutations were found in 15 samples out of 30 studied populations and no mutation detected in other 15 samples. Among these 15 mutated samples, 7 different mutations were found in 7 patients, that means one mutation per patient and the 8th mutation (T4216C) was common in the rest of 8 samples; in other words T4216C mutation in 27% of tested samples was identified (8 patients out of 30 patients). The existence of T4216C mutation was found to be significantly different \(p \leq 0.05\) between tumoral patient’s tissue and adjacent normal tissue.

CONCLUSIONS: Results showed that a high frequency of somatic alterations of mtDNA occurs during the carcinogenesis and/or the progression of colorectal cancer. Based on the mtDNA mutation pattern observed in this study and other previously studies it is believed that looking for somatic mutations in mtDNA would be one of the diagnostic values in early detection of cancer.

KEYWORDS: DNA, Mitochondrial, Colorectal Neoplasms, Electron Transport Complex I, MT-ND1 Protein, Human, Oxidative Phosphorylation, Reactive Oxygen Species.

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Colorectal cancer is a common mortal disease and second leading cause of cancer death in both sexes; namely it caused 10 to 11% of cancer deaths overall.\(^1\) Former studies have indicated some mutations in the mtDNA in different human cancers.\(^2\,^\text{a}\,^\text{b}\,^\text{c}\) Mitochondrial genome in the human cells is a 16.6 kb in size and it is a closed-circular, double-stranded DNA molecule. It is a genetic locus independent of the nuclear genome. The only 13 polypeptides encoded by human mtDNA involved in oxidative phosphorylation (OXPHOS) and 2 rRNAs, and a set of 22 tRNAs that are essential for protein synthesis in mitochondria are encoded by mtDNA.\(^5\) All of the mitochondrial protein coding genes encode subunits of the OXPHOS enzymes that are responsible for the energy generation pathway.

Reduced nicotinamide-adenine dinucleotide dehydrogenase or complex I NADH has the most subunits encoded by mtDNA; therefore it

\(^a\) Department of Genetic and Molecular Biology, Iran University of Medical Sciences, Tehran, Iran.
\(^b\) Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology (NGIEB), Tehran, Iran.
\(^c\) Department of Pathology, Baghyatallah Hospital, Tehran, Iran.
\(^d\) Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.
\(^e\) Department of Genetic, Special Medical Center, Tehran, Iran.
\(^\text{Corresponding Author}\
E-mail: massoudh@nigeb.ac.ir
is exceptionally susceptible to defects if mtDNA mutations are happened.

One of the major cellular generators of reactive oxygen species (ROS) is the electron transport chain of mitochondrial, which include the hydroxyl free radical (OH•) hydrogen peroxide (H2O2), and superoxide. Mutations in mtDNA has been suggested to have the ability for actuating an increase in ROS level, followed by deterioration of oxidative phosphorylation and mitochondrial respiration which leads to oxidative stress that can modify the DNA. Higher amounts of superoxide production in vivo have also been demonstrated in the cells with complex I deficiency.6,7

In particular, the production of ROS probably occurs when complex I activity is deprived; as scientists have reported not only it happened in the cells carrying the T9957C COX III mutation, but also cells carrying the G3460A ND1 mutation.8 A3243G in Leu tRNA, A8344G in Lys tRNA 9 and T8993G mutations.10 Additionally, the existence of particular mutation patterns has been indicated in the mtDNA of various cancers by researchers.11,12 Abnormal metabolic and apoptotic procedures in neoplastic cells are considered as consequence of these mutations in some cases.13 Certainly, mutational hot spots in the NADH dehydrogenase subunits 3, 4 and 5 from complex I, were determined in renal and colorectal tumors.12,14 To investigate mtDNA mutation of colorectal cancer, presence of mutations in one of the important mtDNA genes was checked, namely MTND1 gene, in cancerous tissues with comparison to non-cancerous adjacent tissues from the CRC patients.

Methods

Samples Preparation and DNA Extraction

Thirty tissue blocks of colorectal cancer patients were collected from the Baghiyatallah Hospital in Tehran from 2005 to 2007. The tissues were fixed in formalin and embedded in paraffin. The patients consisted of 13 and 17 women and men, respectively. The age range of the cases was 34-78; their mean ± SD of their age was 54.6 ± 13.1. In this study existence of mutations in tumoral tissues is compared to its existence in non-tumoral adjacent tissues of the same samples. Five μm sections of samples were prepared on slides then were deparaffinized and stained with hematoxylin and eosin (H & E) using standard operating procedure for pathological diagnosis.

All tumoral tissues and adjacent non-tumoral tissue specimens were confirmed by pathological diagnosis. Based on the patients file the patients in this study have not received chemotherapy or radiation therapy before surgery. DNA was extracted from Formalin-fixed, paraffin embedded samples according to standard methods.15 The patients were informed about the aims of the study and then gave their informed consents for the genetic analysis.

Sequence Analysis

The mitochondrial ND1 gene from each sample was amplified by PCR (mt3187F primer 5'-CTCAACTTAGTATTACCC-3', located at 3187-3206 bp; and mt4650R primer 5'-GGAAATACTTGATGCCAGCT-3' located at 4650-4631 bp). The amplified sequence with this primer is a 1463 bp in size. The nucleotides of this amplified sequence were directly determined by automated sequencing using ABI 3700 machines.

A multiple sequence alignment was made between the obtained mtDNA sequences interface, CLUSTAL X, with comparison to rCRS (http://www.gen.emory.edu/mitomap/mitoseq.html).

PCR-RFLP

A 384 bp PCR fragment was amplified from mitochondrial ND1 gene for using in RFLP analysis. The following primer pair was used for amplifying the requested PCR fragment mt4491F located at 4491-4510 (5'-GTCATCTACTCTACCATCTTT-3') and mt4144R located at 4144-4126 (5'-TGCGGGGATGCTGTCGTT-3'). The 384 bp amplified sequence was digested with NlaIII (Fermentas, Germany). The T→C variant at nt 4216 are used for RFLP method, because this variation created a recognition site
for the restriction endonuclease NlaIII. The recognition site of NlaIII is 5'-CATG^X3', PCR product was digested overnight at 37°C; mutant samples were cut into two 291 bp and 93 bp fragments but there was not any cut points in normal samples.

Statistical Analysis
Fisher's exact probability test was used in order to compare qualitative variables. A p value ≤ 0.05 has been considered statistically significant correlation.

Results
Mitochondrial genome, as former studies suggested, is particularly susceptible to mutations. Insomuch, the generation of high level reactive oxygen species (ROS) in this organelle, associated with a low level of mismatch repair gene in the mitochondrial genome.

The presented result revealed a T4216C point mutation in 8 samples out of 30 CRC samples, which cause an amino acid change of Tyrosin with the amino acid Histidin (Y→H) in MTND1 gene. Furthermore the T3290C, T3456C, A3480G, C3622T, C3741T, T3777C and T3847C point mutations were found in 7 of the 30 patients. Each of mentioned point mutations was found and detected in one sample (Table 1). None of the mutations were detected in adjacent normal tissues as compared with tumoral tissue samples of the patients.

The T4216C mutation was reported in LHON and insulin resistance diseases.

The T4216C mutation was determined in 8 patients out of 30 CRC patients; therefore the prevalence of this mutation affirming homoplasry is determined to be 27% in these examined patients (Figure 1). The existence of T4216C mutation was found to be significantly different (p ≤ 0.05) between patient's tumoral tissue and their adjusted normal tissue (Table 1).

Figure 2 and 3 shows the mutation at nucleotide position T4216C of mtDNA in patients who carry the mutation and those who are devoid of mutation, respectively.

Discussion
Based on the present results, seven out of eight mutations were found in the coding region. All these mutations were T/C transitions while only one A/G transversion was detected. Five of them did not result in amino acid modifications. One of them was seen in non-coding region. The C3622T consequences Leu(2) to Leu(1) amino acid and the T4216C mutation that was detected in 27% (8 patients out of 30 patients) of tested samples, resulted in the substitution of Tyrosin with the amino acid Histidin. This T4216C variation has been observed and was reported in LHON and insulin resistance diseases.
MT-ND1 gene in human colorectal cancer

Figure 2. Detection of somatic mtDNA mutations in colorectal cancer patient

Table 1. The identified mutations of MTND1 gene in colorectal cancer patient

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>Locus</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Number of patients</th>
<th>Number of non-tumoral tissues (without mutation as controls)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 3290</td>
<td>MT-TL1</td>
<td>T→C</td>
<td>non-coding</td>
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<td>30</td>
<td>0.99</td>
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<tr>
<td>2 3456</td>
<td>MT-ND1</td>
<td>T→C</td>
<td>syn</td>
<td>1</td>
<td>30</td>
<td>0.99</td>
</tr>
<tr>
<td>3 3480</td>
<td>MT-ND1</td>
<td>A→G</td>
<td>syn</td>
<td>1</td>
<td>30</td>
<td>0.99</td>
</tr>
<tr>
<td>4 3622</td>
<td>MT-ND1</td>
<td>C→T</td>
<td>Leu (2) → Leu (1)</td>
<td>1</td>
<td>30</td>
<td>0.99</td>
</tr>
<tr>
<td>5 3741</td>
<td>MT-ND1</td>
<td>C→T</td>
<td>syn</td>
<td>1</td>
<td>30</td>
<td>0.99</td>
</tr>
<tr>
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<td>T→C</td>
<td>syn</td>
<td>1</td>
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<td>0.99</td>
</tr>
<tr>
<td>8 4216</td>
<td>MT-ND1</td>
<td>T→C</td>
<td>Y→H</td>
<td>8</td>
<td>30</td>
<td>0.0045</td>
</tr>
</tbody>
</table>

Figure 3. Sequences of non-tumoral adjacent tissues from the same CRC patient devoid of T4216C mutations
It was suggested that tumoral cells with indistinguishable nuclei accompanying mitochondrial mutations grow faster than cells destitute of mitochondrial mutations.\textsuperscript{25}

The contribution of mtDNA mutations to the malignant phenotype of tumor cells in most cases still remains unknown and need more investigations. But it is to speculate that, existence of mtDNA mutations in cancer cells is consequent of mitochondrial DNA intrinsic susceptibility to damage because of ROS generation in mitochondria. On the other hand, it is demonstrated that tumors with mutation also significantly generate more ROS (confirmed by dihydroethidium staining).\textsuperscript{23} There is also another report that speculate probable role of other mitochondrial mutations with known OXPHOS defects in tumorgenesis.\textsuperscript{26}

Mitochondrial DNA abnormal variations appear to be a common shape of malignant cells. Abnormal expression of mtDNA-encoded proteins caused by mutation, deletion or insertion in mtDNA has been reported in various solid tumors.\textsuperscript{27-31} Detection of cancer specific mtDNA abnormalities has increased within recent years.

It is difficult to speculate about the role of this mutation in CRC. LHON mutations were shown to affect complex I in mitochondria and other investigators found other mutations in different parts of this gene. To show pathogenicity of this mutation or find its role in cancer, more investigations particularly in mtDNA of colorectal cancer patients are needed.

**Conclusions**

Based on the presented results it is suggested that a high frequency of somatic alterations of mtDNA may occur during the carcinogenesis and/or the progression of colorectal cancer. The molecular mechanisms by which mtDNA alterations or mitochondrial OXPHOS defects influence tumor formation and cancer progression is unclear and need further investigation. Based on the mtDNA mutation pattern observed in this study and other previously studies, it is believed that looking for somatic mutations in mtDNA, would be one of the diagnostic values in early detection of cancer.

**Conflict of Interests**

Authors have no conflict of interests.

**Authors’ Contributions**

MA designed and performed the experiments, analysis of data, writing the conclusion and preparation of the manuscript. MH was the scientific consultant and supported the project. MHHA did the pathological diagnosis of tumoral and normal adjustment tissues of patient's sample. BK did the DNA extraction of the samples. MD Helped in performing the laboratory experiments. All authors have read and approved the content of the manuscript.

**References**