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The Mitochondrial DNA 4977-bp Deletion in Patients with Colorectal Cancer: A Case-control Study in Iran

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Abstract

Background: Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide, and its occurrence can be ascribed to genetic susceptibility. Mitochondrial DNA 4977-bp (mtDNA 4977), as the most described mtDNA deletion, has been long proposed to be involved in various types of cancers. However, a few studies on mtDNA 4977-bp deletion in Iranian patients with CRC have been reported. The current study aimed to determine mtDNA 4977 frequency in CRC and its association with cancer susceptibility.

Patients and Methods: We conducted a case-control study in which a total of 26 patients with CRC, 26 tumor tissues, adjacent normal tissues, peripheral blood samples, and peripheral blood samples from 50 healthy subjects were included. mtDNA 4977 was detected using multiplex PCR technique and direct DNA sequencing. Real-time PCR was also used to determine deletion levels.

Results: mtDNA 4977 was observed in six patients (23.07%), four (15.3%) in both tumor and matched surrounding normal tissues, and two (7.69%) in adjacent normal tissues, but not detected in both patients and control samples in peripheral samples. A significant difference was found between mtDNA 4977 deletion in tumoral and adjacent normal tissues (P=0.001). No relation was observed between mtDNA 4977 and categorical variables, including age and gender, and tumor stage.

Discussion: Our study indicates no association between the mtDNA 4977-bp deletion and susceptibility to colorectal cancer in Iranian patients. However, more extensive studies are required to confirm or reject these findings.

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1. Introduction

Despite improving treatment and prognosis in cancer issues, colorectal cancer (CRC) is now the third leading cause of cancer-related death [1]. The global incidence of CRC mortality rate reflects factors, including the absence of obvious symptoms in the early stages and the absence of rational programs for cancer prevention [2]. Genetic factors, accompanied by traditional risk factors such as age, family history, lifestyle, and ecological factors, have influential roles in pathogenesis of CRC [3]. Based on the studies, CRC occurrence can be partially (12–35%) attributed to genetic susceptibility [4, 5]. In this regard, based on the alteration in molecular biomarkers, including protein, DNA, and RNA-based biomarkers found in blood, tissue, or other body fluids, molecular genetic

tests seem to be as sensitive and specific methods in CRC diagnosis. Recent studies have proposed novel diagnostic biomarkers for tumor tissue, blood, and stool samples in CRC diagnosis, although the identification of ideal biomarkers for clinical use remains a challenge [6]. Detection of blood-based biomarkers seems to be a screening tool for CRC diagnosis [7], while stool specimens might be more acceptable for early detection of cancer compared to blood samples [8].

Mitochondrial DNA (mtDNA) alterations could broadly affect mitochondrial function and contribute to increased cancer susceptibility, aging, and diverse forms of human diseases [9, 10]. mtDNA is a double-stranded circular molecule of ~16.5 kbp that contains 37 genes encoding 13 essential polypeptides of the oxidative phosphorylation system (OXPHOS), two rRNAs, and 22 tRNAs. Proposedly, the plenty of mtDNA mutations could be mainly due to the proximity of the mtDNA to the OXPHOS, and the reactive oxygen species (ROS) formed through ATP production [11]. In recent years clinical value of mtDNA has been frequently defined in CRC and since has attracted the attention of many researchers. Some studies suggest that mtDNA can be the potential CRC biomarker to assess the risk and prognosis of disease [12]. Among the mtDNA alterations, large-scale deletions in mtDNA are one of the critical mtDNA mutations related to human diseases. The 4,977-bp deletion, as a marker of mtDNA damages, is located between nucleotides 8,469 and 13.447, which includes several essential oxidative phosphorylation genes encoding ATPase6/8, cytochrome oxidase III, NADH dehydrogenase subunit 3 (ND3), ND4, and ND5. This mtDNA common deletion has been reported in several types of cancers, such as gastric cancer [13] and lung cancer [14], as well as the early stage of CRC [13, 15]. Consequently, it can potentially be a biomarker for cancer occurrence in the tissues [16].

Given the multifactorial causation of CRC, identifying genetic and environmental factors involved in its pathogenesis would help to improve our understanding of better disease management programs [17]. Unlike most cancers, CRC is often preventable (through avoiding known risk factors, adopting a healthy lifestyle, and regular screening) and treatable in early diagnosis [18]. However, one of the main problems in CRC is late diagnosis; therefore, there is a growing need for molecular techniques and studies that recognize biological markers for early diagnosis [19].

Hence, to investigate the frequency of mtDNA 4977-bp deletion in colorectal cancer and whether the deletion is a risk factor for susceptibility to cancer, we evaluated the tumoral and matched surrounding normal tissues and peripheral blood samples from Iranian patients with colorectal cancer.

2. Patients and Methods

2.1. Patients

This case-control study included 26 colon cancer patients referred to the National Cancer Institute (NCI) at Imam Khomeini Hospital Complex, Tehran, Iran, from May 2021 to July 2022. Patients who had received chemotherapy or radiation therapy before sampling were excluded from the study. The tumor tissue and adjacent non-tumor tissue samples were taken from patients undergoing colonoscopy following a positive pathologic diagnosis of CRC. Their peripheral blood samples were taken as study cases, and the peripheral blood samples from 50 healthy subjects were used as the control in this study. The clinicopathological information, such as age, gender, and tumor stage, was obtained from patients. According to Tumor-node-metastasis (TNM) Staging System [20], the tumors were categorized from I-IV. All subjects signed an informed consent approved by the Iranian National Tumor Bank (INTB) Ethical Committee.

2.2. DNA extraction

Genomic DNA was extracted from tissues (tumor tissues and their matched surrounding normal tissues) and patients' peripheral blood samples and controls' peripheral blood samples using QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The extracted DNA's quantity and quality were measured using Thermo Scientific NanoDrop 1000 and agarose gel electrophoresis (1%). All extracted qualified DNA samples were frozen and stored at −80°C until later analysis.

2.3. Detection of mtDNA with 4977-bp Deletion

For detecting mtDNA 4977-bp deletion, a multiplex polymerase chain reaction (PCR) was performed using two sets of primers; the ONP 86/ONP 89 and ONP 25/ ONP 74 (**Figure 1**). The first pair amplified a 279 bp fragment of normal mtDNA (279 bp) that served as a control for the PCR analysis (**Table 1**). A 497-bp band using the second primer pair on a 1.5% agarose gel indicated an expression of the 4977-bp deletion, which was verified by sequencing analysis. On the other hand,

normal mtDNA would not represent this fragment on the agarose gel. The specificity of primers was checked using Primer-BLAST from NCBI (http://www.ncbi.nlm.nih.gov/). PCR was performed with the following protocol: 95ºC for 5 minutes followed by 35 cycles at 94°C for 45 seconds, annealing time at 57.5°C for 30 seconds, and extension at 72°C for 40 seconds with a final extension time of 5 minutes at 72°C.

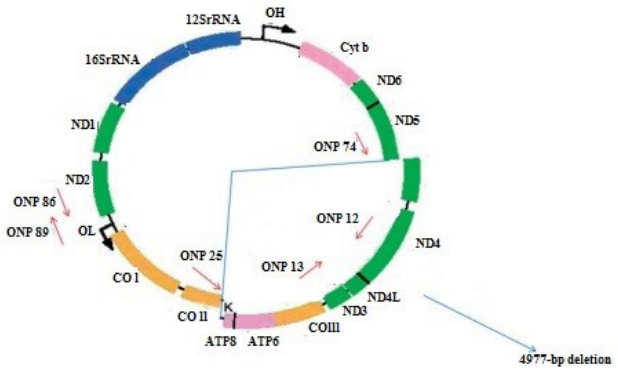

Figure 1. Detection of the mtDNA 4,977-bp deletion. Human mitochondrial genome with o the 4,977-bp deletion and the positions of primers used in multiplex and qPCR.

2.4. Determination of 4,977 bp deletion levels

The mtDNA content was measured in patients carrying this mutation by a real-time PCR and normalized by β-actin (**Table 1**) as a housekeeping gene. QPCR was performed in a 20 µl reaction containing SYBR master mix (Real QPCR 2x Mix, Ampliqon) and primer pairs (ONP 86/ONP 89 and ONP 12/ONP 13). The ONP 12/ ONP 13 primers were; ONP 12 (5'-TCGTAGTAACAGCCATTCTC-3') and ONP 13 (5'-GAGGTTAGCGAGGCTTGCTA-3'). The target region of this primer pair (214-bp) was located within the mitochondrial common deletion and amplified a specific product only in wild-type mtDNA. The PCR conditions were 95°C for 15 minutes, followed by 35 cycles at 95°C for 30 seconds and 58°C for 60 seconds. All samples were run in triplicate. The Ct method (ΔCt) was used to determine relative levels of mtDNA 4977 in tissues that carried the deletion. The relative amounts of deleted mtDNA were measured as the ratio of the deleted region to total mtDNA.

2.5. Statistical analysis

All statistical analyses were conducted using IBM SPSS statistics ver.23 (IBM. Armonk, NY, USA). Mean (standard deviation) was calculated in continuous data and the number (percent) for categorical data. Mann-Whitney U test was used to determine whether there is a relationship between mtDNA 4977 deletion and clinicopathological parameters. Furthermore, categorical variables were analyzed by the Fisher exact test. The P-value was considered statistically significant at a level of <0.05.

3. Results

Of the 26 patients with colorectal cancer recruited in this study, 11 were male, 15 were females, and aged from 35 to 74 years (mean: 58.9 ± 9.98). According to cancer stage classification, four patients were at stage I, 11 at stage II, nine at stage III, and two at stage IV. There was no significant correlation between categorical variables, including age and gender, with tumor stage (P>0.05) **(Table 2).** Based on our results, six patients (23.07%) showed 4,977-bp deletion, four (15.3%) in both tumor and matched surrounding normal tissues, and two (7.69%) only in adjacent normal tissues **(Figure 2).** A significant difference was found between mtDNA 4977 deletion in tumoral and adjacent normal tissues (4 (11.11%) vs. 6 (16.66%); P=0.001) **(Figure 3).** This deletion was not detected in both patients or control samples in peripheral samples. Of the patients who carried mtDNA 4977, three patients (50%) with deletions in tumor tissues and one with a deletion in adjacent normal tissue were in early-stage cancer (I, II). Based on the results, we found no relation between mtDNA 4977-bp deletion in tumor and adjacent normal tissues with tumor stage (P=0.61 and P=1, respectively). Furthermore, there was no significant difference between age (<65 and >65) and mtDNA 4977-bp deletion in tumoral (P=0.62) and adjacent normal tissues (P=016). We also analyzed mtDNA 4977 levels, and our results showed that deletion levels in patients carrying this deletion varied from 0.42% to 5.7% of the total mtDNA.

4. Discussion

The mitochondria serve as a metabolic modulator of cellular function. mtDNA variations, such as mutations, single nucleotide polymorphisms (SNPs), and dysregulated mitochondrial genes, are associated with the survival of cancer patients. Various mtDNA alterations, such as deletions, are believed to increase with advancing age in human tissues, so it has been identified in the aged human brain, skeletal muscles, heart, and colon tissues [21]. Among the mtDNA alterations, large-scale deletions are one of the most in

Table 1: Primers used for mtDNA deletion analysis.

Primer	*Sequence*	Location	Size
ONP 86/89	ONP86 (F): 5'-CCCTTACCACGCTACTCCTA-3' ONP89 (R): 5'-GGCG GGAGAAGTAGATT GAA-3'	5461-5480 5740-5721	279-bp
ONP 25/74	ONP 25 (F): 5'-CTACGGTCAATGCT CTGAAA- 3' ONP 74 (R): 5'-GGTTGACCTGTTAGGGTGAG-3'	8161-8180 13640-13621	497-bp
B-actin	F: 5'- AGACGCAGGATGGCATGGG-3' R: 5'- GAGACCTTCAACACCCCAGCC-3'	603-621 461-481	161-bp

Table 2: Demographic and Clinical Characteristics of the CRC Patients.

Patients' parameters	*Total number (n=26)*	mtDNA 4,977-bp (Tumor tissue)	P Value	mtDNA 4,977-bp (Non-tumor tissue)	P Value
Gender					
Male	11	3 (27.2%)	0.15	4 (36.3%)	0.16
Female	25	1(4%)		2(8%)	
Age, y					
>65	10	2 (20%)	0.70	4(40%)	0.21
<65	16	2 (12.5%)		2(12.5%)	
Stage					
I, II	15	3 (20%)	0.61	4(26.6%)	1
III, IV	11	1 (9.09%)		2(18.1%)	

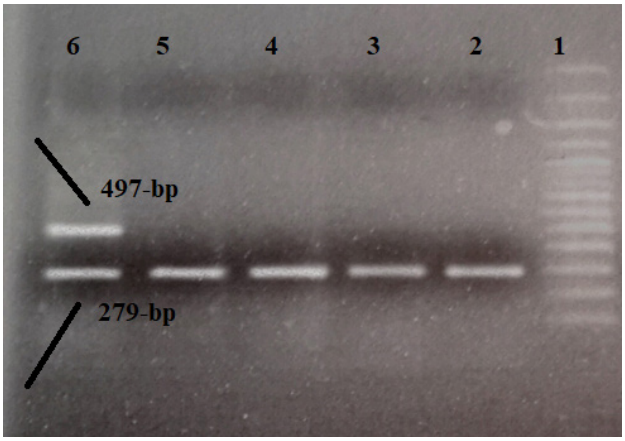

Figure 2. Electrophoretogram of Multiplex-PCR product amplified from mtDNA. Lane 1 indicate the PCR products amplified from mtDNA with the specific 4977-bp deletion, lanes 2, 3, 4, 5, show the internal control (297-bp), and lane 6 is a 100-bp DNA size marker.

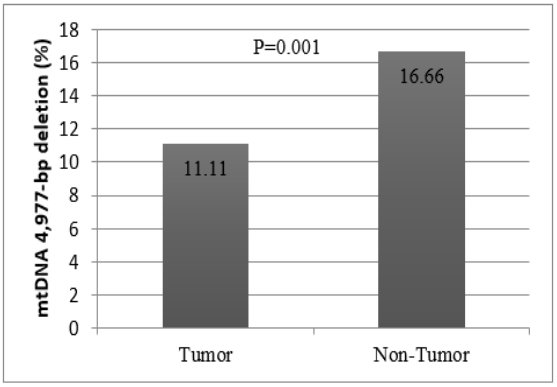

Figure 3. Comparison of mtDNA 4,977-bp deletion between tumor and non-tumor tissues. 4 and 6 patients carrying the deletion in tumor and non-tumor tissues, respectively.

human diseases [22]. The 4977-bp deletion is one of the best-described large-scale mtDNA deletions found in numerous disorders, including mitochondrial diseases and many different types of cancer [23]. The hypoxic in cancer cells microenvironment activates ROS generation in the mitochondria that can provoke damaging oxidative events in cells and lead to genomic instability in the mitochondria and the nucleus [24]. Indeed, mtDNA, due to proximity to the respiratory chain, is permanently exposed to ROS [25]. It has been postulated that mtDNA deletions might occur either via spontaneous errors of mtDNA replication or abnormal repair of DNA double-strand breaks [26]. The primary effect of mtDNA 4977 can cause deficiencies in components of the OXPHOS system and remove all tRNA and some coding region genes crucial for supporting normal mitochondrial function [27]. There is considerable evidence from the literature supporting this idea.

A high prevalence of mtDNA 4977 and its relation with clinicopathological features in brain tumors [28], breast [29], colorectal [13], and endometrial [30] cancers have been reported in published articles in recent years. Despite the presence of this deletion in tissue samples, it was absent in the peripheral blood sample of our patients and control samples. Based on the results of the current study, a significant difference was found between mtDNA 4977 deletion in tumoral and adjacent normal tissues. Of the patients with colorectal cancer, 23.07% showed mtDNA 4977, about 15% in both tumor and matched surrounding normal tissues, and about 8% in their matched surrounding normal tissues. In other words, this deletion was more frequent in non-tumor tissue compared to tumoral tissue. Recent researches suggest that the mtDNA 4977 bp is frequently detected in cancerous tissues and, therefore, can be considered a biomarker [16]. Mohamed Yusoff, in a study from a Malaysian group, reported the absence of this deletion in the peripheral blood of patients as well as subjects with normal brain tissue [28]. In another study by Chen, the mtDNA 4977 was present only in colorectal cancer tumor tissues, not in their matched normal tissues [15].

Furthermore, evidence suggests that mtDNA 4977 may serve as a biomarker in the early stage of tumor development. Also, there is a correlation between the cancer stage and the 4977-bp in patients with the common deletion [15, 16]. Based on the results obtained, four patients carrying mtDNA 4977 were diagnosed with the early stage of colorectal cancer. However, no statistically significant relationship was found between mtDNA 4977 and cancer stage in our patients. One possible reason for these contradictory results may be due to the small sample size, as the major limitation of the current study.

The mtDNA 4977 bp deletion has been extensively studied as a pathogenic mutation for two decades, even though mtDNA 4977 can occur as a common phenomenon with increasing age in normal tissues [31, 32]. Our findings showed no significant difference between age (<65 and >65) and mtDNA 4977-bp deletion in tumoral and non-normal tissues. In a colorectal cancer study, Dimberg reported no statistically significant association between mtDNA 4977 and clinical parameters, such as gender, age, and tumor localization [33]. In contrast, Chen's results showed that the deletion was more present in tumor tissues of patients < 65 [15].

Our results showed that mtDNA content was higher in tumor tissues than in adjacent normal tissues. Indeed, the exact cause of the changes in the ratio of mtDNA deletion levels in different tissues is not fully understood. Although, evidence indicated that oxidative stress inside mitochondria could be attributable to mtDNA large deletions [34]. In a study, NG Ericson et al. found that the frequency of mtDNA mutations is reduced in tumor tissues compared to normal tissues [35]. Our results agree with studies that reported lower deletion levels in tumor tissues than adjacent normal tissues [13, 15]. In addition, several studies have indicated that the levels of mtDNA deletion in tumor tissues reduce with advancing cancer.

Since research study limitations are a natural occurrence, we represented limitations of our study that may influence the outcomes and conclusions of the research. The small sample size was one of the significant limitations that may prevent to detection of the statistically valid association of mDNA 4977 with colorectal cancer risk. To investigate the mtDNA deletion association with CRC, analyses by ethnicity seem necessary, although it was impossible because of the small sample size.

4. Conclusion

In conclusion, our analysis confirmed no association between the mtDNA 4977-bp deletion and susceptibility to colorectal cancer in Iranian patients.

However, more extensive studies are required to confirm or reject these findings.

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Authors' Contribution

Study concept and design, Sh. K; Analysis and interpretation of data, Sh. K., and A. AM; Drafting of the manuscript, Sh. k, and F. A; Critical revision of the manuscript for important intellectual content, Sh. K., F. A., and V. Z.

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Conflict of Interests:

The authors declare that they have no conflict of interest.

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