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Pitfall of Real-Time PCR Method to Find Delta mtDNA in Colon Cancer

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Abstract

In this study, we investigated the presence of a high incidence of common mitochondrial deletion in mtDNA of colon cancer tissues and in blood of both patients and control samples. For this purpose, one of the common regions of the mitochondrial genome was screened using different techniques including PCR amplification, Southern blot, and real-time PCR followed by DNA sequence analysis. Isolated DNA was applied for amplification of hypervariable regions ATPase8/6, COXIII, ND3, ND4, and ND5 of delta mtDNA4977. In 25 colon cancer patients, delta mtDNA4977 was identified using quantitative RT-PCR in 6 cases (4.5%) of the tumoral tissues and 18 cases (4.47%) of the non-tumoral tissues that were adjacent to the tumors. Delta mtDNA4977 was detected more in non-tumoral tissues than in adjacent tumoral tissues, but less common deletion was observed (0.017%). Additionally, no 4,977-bp common mitochondrial deletion was observed in blood of patients and 100 control samples. Unknown genetic aspects, ambiguous environmental factors, and reactive oxygen species (ROS) are events that cause the delta mtDNA4977 mutation rate to be elevated more in non-tumoral tissues as compared to adjacent tumoral tissues of colon cancer. The results of our study propose that delta mtDNA4977 percentage in tumoral tissue is less prevalent and is untolerable, perhaps due to high metabolism and ROS production.

Keywords: Common mitochondrial deletion, human colon cancer, 4,977-bp deletion

Introduction

Cancer is a broad group of diseases involving uncontrolled cell growth. Colorectal cancer (CRC) is one of the most common malignancies worldwide with high incidence in Iran. Because of the importance of early prevention of CRC in reduction of hospital costs and mortality/morbidity of patients, regular screening of old populations is strongly recommended by American Cancer Society and U.S. Preventive Services Task Force[1,2].

Many factors are recognized to increase the cancer risk including Genetic alterations smoking, dietary factors, some virus infections, exposure to radiation, low physical activity, overweight, and environmental pollutants[3]. One of the causes of cancer is virus. For example, hepatitis B and C is the major reason of liver cancer. WHO reported that there were 350 and 170 million hepatitis B and C virus respectively infected people in the worldwide in 2010[4].

Many recommendations have proposed to decline cancer risk. For instance, Internal and external antioxidants cause protective effect against cancer by neutralizing free radicals[5]. Sirappuselvi S, et al. showed that Cassia tora has an antioxidant property. Aweng E.R, et al. also, reported that *Vitex Trifolia Var and Simplicifolia* and Jamaican ball moss have anticancer properties[6,7].

Sequencing entire mitochondrial genome or human mitochondrial DNA (mtDNA) is a common clinical screening tool for detection of CRC and finding malignant genome mutations in human colorectal tumour[8].

Human mtDNA genome, a circle of double-stranded DNA of 16,569 bp, encodes various respiratory enzyme subunits in the mitochondrial electron transport chain[9]. This 16,569-bp genome encodes 13 polypeptides containing different respiratory chain complex subunits, 22 tRNAs, and two rRNAs used in synthesis of mitochondrial protein. Harmful effects of reactive oxygen species (ROS) - produced by oxidative phosphorylation -along with a defective mtDNA repair systems, leads to increases mtDNA mutation rate 10 times higher than Ndna[10].

The study of mitochondria biology proposes that mitochondrial genome can be considered as a fascinating target for mutations driving tumorigenesis. Mitochondria as the source of ROS production are highly mitogenic molecules at slightly elevated concentrations, and also mutagenic to the nuclear and mitochondrial genomes[11]. They are also known to play a pivotal role in regulating or affecting apoptosis[12].

The occurrence of mtDNA alterations could be observed in benign and malignant tissues. The so-called 'common deletion', a 4,977-bp mtDNA deletion and also a frequent mtDNA alteration, is detected in a variety of human tumors but at lower levels than in the surrounding normal tissue[13].

One study showed that mtDNA is 10-20 times more sensitive to ROS-induced DNA damage due to its relatively unprotected genome structure and less efficient DNA repair mechanism[14]. Accumulation of mtDNA deletions has been demonstrated to be an important contributing factor in human aging and degenerative diseases[14-16].

More than 50 different types of mtDNA deletions have been identified in human diseases. Some of which are found only in a certain type of tissue, whereas others may occur in many organs or tissues[17-19]. The 4,977-bp deletion is the most prevalent and abundant type of mtDNA deletion in human[14].

To investigate qualitative alterations in mtDNA of human cancer, we examined the presence of mtDNA deletions (probebly from mtDNA6000 to mtDNA16000) in tumoral tissues and compared them with non-tumoral adjacent tissues from Iranian patients with colon cancer. Moreover, we investigated the role of colon cancer in size and percentage of the mtDNA deletions in both patients and control samples.

Material and Methods

Patient population: Twenty-five colon cancer patients (28-77 years with median age of 53.66 years) were contributed in this study. The patients were referred to the National Cancer Institute (NCI) at Imam Khomeini Hospital Complex, Tehran, Iran from Nov. 2007 to Nov. 2009. Tumor tissue and adjacent non-tumor tissue samples were obtained from Iranian National Tumor Bank (INTB) at NCI. Each specimen was immediately frozen following resection and stored at -80°C until DNA extraction. The pathologic changes in tumor samples were confirmed by two expert pathologists as adenocarcinoma according to the American Joint Committee on Cancer. The colon tumoral tissue, adjacent non-tumoral tissue, blood of patients and blood of 100 control samples were randomly selected and obtained for the following analysis. All patients were notified on the aim of the study and signed an informed consent for genetic analysis, which was approved by INTB Ethical Committee.

PCR amplification: DNA isolation was performed using the Qiagen Kit (Qiagen, Hilden, Germany) based on the standard protocols. The purity of DNA was quantified by evaluating optical density at A 260 and A 280 [20]. General PCR analysis was performed in a Techne Flexigene thermal cycler (Techne, UK). PCR was carried out using three sets of primers: ONP89/ONP86 (pair A), ONP74/ONP25 (pair B) and ONP12/ONP13 (pair C). Pair A showed wild-type and deleted mtDNA, pair B showed only common 4,977-bp mitochondrial deletion and pair C showed wild type. To amplify a fragment of 279 bp in each specimen in a rarely deleted region as an internal control, we used two ets of sprimers: ONP89 (5'-GGCG GGAGAAGTAGATTGAA-3') and ONP86 (5'-CCCTTACCACGCTACTCCTA-3'), which are located at 5740-5721 and 5461-5480 bp of the mtDNA, respectively. In addition, ONP74 (5'-GGTTGACCTGTTAGGGTGAG-3') and ONP25 (5'-CTACGGTCAATGCTCTGAAA- 3') primers, located at 13640-13621 and 8161-8180 bp of the mtDNA, respectively, were used to amplify a 502-bp region created by the ΔmtDNA 4,977 deletion. Furthermore, ONP12 (5'-TCGTAGTAACAGCCATTCTC-3') and ONP13 (5'-GAGGTTAGCGAGGC TTGCTA-3') primers, located at 11646-11665 and 11860-11841 bp of the mtDNA, respectively, were used to amplify a 214-bp fragment at a frequently deleted region (table-1 and figure-1).

The three mtDNA fragments were amplified using PCR for 35 cycles, and PCR was carried out in a 25-µl reaction mixture, containing 100-200 ng total DNA, 10 pmol each primer, 2.5 mM $MgCl_2$, 200 mM each dNTP and 0.5 µL DNA Taq polymerase (Takara, Japan). PCR amplification was also conducted based on the following conditions: denaturation at 95ºC for 5 min and 35 cycles of denaturation at 94ºC for 45 s, hybridization at 56ºC for 30 s, extension at 72ºC for 30 s and a final extension at 72ºC for 5 min. PCR products were separated by electrophoresis in a 1.5% agarose gel at 110 V for 40 min, stained with ethidium bromide, and visualized using gel documentation system under UV transillumination.

Real-time PCR analysis: qRT-PCR was performed in a 15 µl reaction mixture, containing 1 µl 5 ng DNA, 7.5 µl SYBR Premix EX Taq (Takara Bio INC, Japan, code RR820A) and 10 pM primers for each reaction. Reactions were run at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 58°C for 20 s and 72°C for 15 s using *Rotor-Gene*™ 6000 (*Corbett Life Science*, Sydney, Australia). All PCR reactions were performed in triplicate, and the specificity of the reaction was detected by melting curve analysis at the dissociation stage. A standard curve method was used to obtain each genes DNA levels. The STD 10 dilution was used as the calibrator to correct the variation of PCR efficiency among different batches. Sample loading variations and tube-to-tube efficiency of PCR for each unknown sample were improved by using replicate analysis.

Table-1
Primer pair sequences for finding deletions

Primer	Sequence	Location	Size (bp)	TM (ºC)
Pair A	ONP89: 5'-GGCG GGAGAAGTAGATT GAA-3' ONP86: 5'- CCCTTACCACGCTACTCCTA-3'	5740-5721 5461-5480	279	56
Pair B	ONP74:5'-GGTTGACCTGTTAGGGTGAG-3' ONP25: 5'-CTACGGTCAATGCT CTGAAA- 3'	13640-13621 8161-8180	502	56
Pair C	ONP12: 5'-TCGTAGTAACAGCCATTCTC-3' ONP13: 5'-GAGGTTAGCGAGGCTTGCTA-3'	11646–11665 11860-11841	214	56

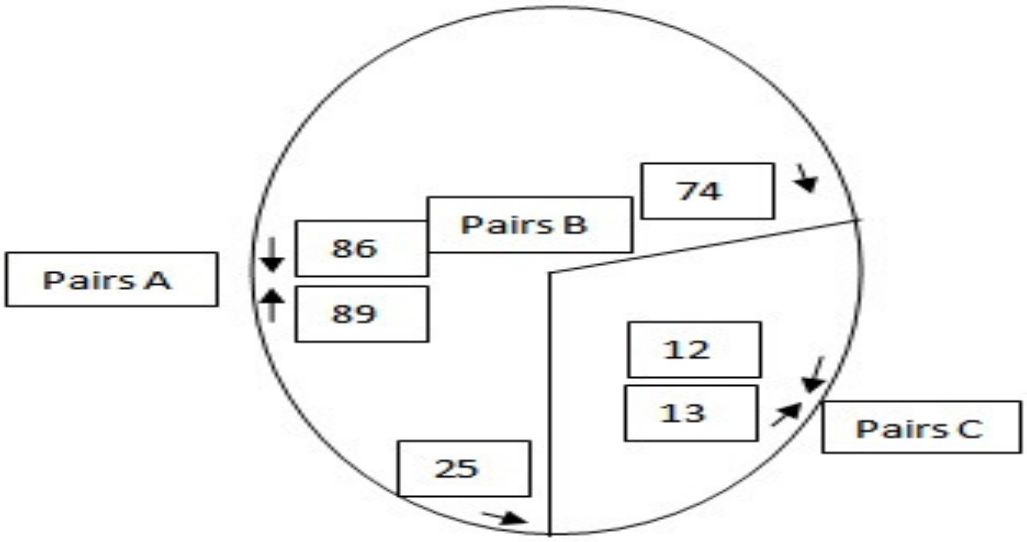

Figure-1
Schematic picture of mitochondrial DNA and positions of primers

Expand long PCR: Multiplex *PCR and long*-range *PCR* were *performed* with Phusion Flash High-Fidelity PCR Master Mix kit (Thermo Scientific, USA) according to manufacturer's instructions. Also to amplify a 10689-bp fragment, PCR was carried out with two sets of primers: ONP86 (5'-CCCTTACCACGCTACTCCTA-3') and ONP91 (5'-GTGGTCAAGTATTTATGGTA -3'), located at 5461-5480 and 1631-16150 bp of the mtDNA, respectively.

Statistical analysis: Fisher's exact probability test (using SPSS, version 13) was used to examine the association between the two groups. p value < 0.05 was regarded as statistically significant.

Results and Discussion

The analysis of 4,977-bp-deleted mtDNA and total mtDNA: The comparison between pairs A and B (figure 1) showed that there are no deletion differences between tumoral cells and adjacent non-tumoral tissues. In four cases, mitochondrial deletion was found in both cancer cells and adjacent normal cell when comparing primer pairs A and B. However, these deletions were not confirmed by Southern blot analysis, multiple PCR and expand long PCR and primer pairs C. Moreover, changing the primer pairs B with D and comparing with primers A showed no deletion in both tissues.

The specificity and the sensitivity of the primer and also the designed PCR condition was established using Melting curve analysis, which was performed in all RT-qPCR and presented a single PCR product for both pairs of primer. All primer sets were found to show good linearity in the target detection range. The coefficient of variation of replicates in each sample and in the standard curve was lower than 3% in all experiments. This fact indicates the high reproducibility of the analysis. The mean value of the calculated initial DNA concentration for each test sample was used to determine the proportion of 4,977-bp-deleted mtDNA/total mtDNA.

Molecular and clinicopathological characteristics: Our study showed that there was no significant correlation among mtDNA deletions and also among clinicopathological characteristics (p > 0.05), suggesting that mtDNA deletion variants are not probably responsible for clinicopathological features.

To the best of *our knowledge*, this is the first study analyzing the mtDNA common deletion in colon cancer and adjacent normal tissue with Real Time PCR.

Tan and his colleagues[21] revealed that the ratio of 4,977 bp-deleted mtDNA between non-tumor tissues cancer tissues, regardless of the history of chewing areca quid, was significantly lower. Moreover, the ratio of large-scale mtDNA deletion in non-tumor tissue of the chewers to non-chewers was significantly higher[21].

By comparison with the limited detection rate in the previous studies, all 25 paired samples in the current study could be quantitatively analyzed for their deleted mtDNA with high reproducibility. The detected proportion of 4,977-bp-deleted mtDNA in our patients was a broad range of 0.42% up to 50%.

Previous studies on aging-associated mtDNA mutations have suggested that oxidative stress could shift the rate of accumulation of mtDNA mutations from linear to exponential[22].

Turner and his colleagues' studies[23] showed that the deleted DNA fragment may escape from the mitochondria and

translocate into the nucleus, because insertion of mtDNA sequences into the nuclear DNA was observed.

In 2013, Warowicka et al[24] found that about 70% of the mtDNA copy number has a 4997-bp deletion in low grade squamous intraepithelial lesion. They also observed that ROS generation increased during cervical cancer development[24]. On the other hand, Zheng et al[25] results suggested that mtDNA 4,977-bp deletion is a common phenomenon in hair, and it is increased with age. Chen and his collogues[26] results indicated that the mtDNA 4,977-bp deletion may have a role in the early stage of colon cancer and also in alteration of mtDNA content in cancer cells. However, in our experience, the common deletion has no specific primary role either in the early or advanced stage of cancers, but it has possible secondary effects reflected in the carcinogenesis process[27].

Conclusion

Our results indicate that: i. the amount of the deletions is not different between colon cancer tissues and the adjacent normal tissue. It may depend on changes in molecular level but not in pathological one and ii. choosing the right primer is very important to investigate mtDNA common deletion in colon cancer. Therefore, we suggest that using two different primer pairs for finding the mitochondrial deletion in colon cancer if the real-time PCR applies as a detection method.

The RT-qPCR system not only allows the analysis of mtDNA mutation or deletion to be performed more accurately but also offers a quick and high-throughput profiling of the mitochondrial genomic alterations, which may happen during both the initiation and progression of carcinogenesis. The pitfall of this method is choosing the right primes and avoiding any contamination in primers, which can affect the results. Investigation of mitochondrial function and its role in cancer and normal cells can help us to prevent cancer through substitute of energy therapy and regulation of intrinsic pathway of apoptosis.

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