Frequencies of mtDNA mutations in primary tissue of colorectal adenopolyps

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1. ABSTRACT

To investigate the potential role of mtDNA alterations during the onset of colorectal cancer, the occurrence of mtDNA variants in colorectal adenomatous (Tubular, Tubulovillous, and Villous) polyps, were studied. High resolution endonucleases and PCR-based sequence were applied to examine mtDNA variants in the *ND* and *ATPase* genes of 64 primary tissues of colorectal adeno-polyps and their matched normal controls. Forty-two variants were observed and 57% (24/42) were not previously reported in the MITODAT reference. Fifty-eight percent of these variants were germline and homoplasmic transitions. The distribution of observed mtDNA variants includes: 31% (13/42) tubular, 52% (22/42) tubulovillous, 45% (19/42) villous, and 45% (19/42) cancer (including FAP and JVP). Notably, an unreported germline variant in the *ATPase 8* gene at nucleotide position (np) G8573A was observed in tubulovillous adenomas tissues. The results suggest that some specific mtDNA variants may serve as a potential biomarker for colorectal adenomatous polyps.

2. INTRODUCTION

Mitochondria are necessary intracellular organelles that, as the nucleus, posses a separate genome and all the enzymatic machinery translating genetic information into proteins (1-3). Human mitochondrial gene mutations are increasingly being associated with unclear pathophysiological significances for various cancers (4-9). MtDNA is a circular molecule of 16.6 kb containing 37 genes including 2 ribosomal RNAs, 22tRNAs, required for translation of mtDNA, and 13 polypeptides that encode subunits of the respiratory chain (10). Mitochondria are mainly responsible for the production of energy in the form of ATP and are an important source of excited oxygen species (11). Mitochondria have been implicated in cancer given their role in oxidative phosphorylation of Reactive Oxygen Species (ROS) and apoptosis (6). Apoptosis serves an essential role in cellular response to anticancer agents and development of cancer. Mitochondria contribution to tumorigenesis is necessary to understand the biology of cancer development. Past studies indicated that

Table 1. Demographic characteristics of tumor and patients

Characteristics	n (%)
Age (mean± years)	57.1±7
Gender	
Male	16 (47)
Female	17 (50)
Unidentified	1 (3)
Race	
African American	3 (9)
Caucasian	23 (68)
Unidentified	8 (24)
Localization	
Right colon	10 (29)
Colon and Rectum	14 (41)
Sigmoid	4 (12)
N/A	6 (18)
Depth of invasion	
T0	2 (6)
T1	14 (41)
T2	5 (15)
T3	6 (18)
T4	2 (6)
N/A	5 (15)
Lymph node metastasis	
N0	19 (56)
N1	7 (21)
N2	1 (3)
N3	0 (0)
N/A	7 (21)
Adenomatous Type (64)	
Tubular and NST	18 (28)
Tubulovillous and NST	20 (31)
Villous and NST	18 (28)
FAP and NST	3 (5)
JVP and NST	2 (3)
Cancer and NST	3 (5)

Characterization of 33 patients and 64 collected samples of these patients

mtDNA content can regulate cancer progression, and accumulation in mtDNA mutations have been associated with the impairment of mitochondrial function which is involved in the etiology of aging and several degenerative pathologies (12-15). Additionally, studies have suggested that mitochondrial function decreases with aging and is associated with the process of tumorigenesis (16; 17; 18). Moreover, mtDNA abnormalities in the hypervariable regions and mtDNA-encoded Complexes I, III- IV, have been found in cancer tissues (8).

Given that colorectal cancer (CRC) is an age related disease, we examined the correlation of mtDNA variants with early colorectal adenopolyps. In this communication we report the finding of a high incidence of mtDNA alterations in the *ATPase* 8 gene in the tissues of tubulovillous of colorectal adeno-polyps. Based on a relative distribution of mtDNA mutations observed in this study, a 2.2kb of mtDNA encompassing the ND and ATPase 8 regions may be useful in providing a framework for further differentiation studies of colorectal adenopolyps tissues.

3. MATERIALS AND METHODS

3.1. Subjects

We collected sixty-four frozen colorectal adenomatous polyp tissues including their precancerous normal surrounding tissues (NST) of the three polyps and cancer (Tubular: 9 Tumor, 9 NST; Tubulovillous: 10 Tumor, 10 NST; Villous: 9 Tumor, 9 NST; Familial Adenomatous Polyps (FAP): 1 Tumor, 2 NST; Juvenile Polyps (JVP): 1 Tumor, 1 NST; Cancer: 2 Tumor, 1 NST) (Table 1). The tissue samples used were from the Tissue Procurement Network at the University of Alabama at Birmingham, Cooperative Human Tissue Network (CHTN) and the Department of Surgery of Morehouse School of Medicine/Grady Hospital. For reference purposes, samples of JVP, FAP and cancer (n=9) mtDNA were amplified and sequenced along with the colorectal adenomatous polyps. The mean age of patients that tissues were obtained was 57.1±7y. Demographic characteristic of the samples were grouped based on the clinical diagnosis. Diagnosis of the colorectal adenomatous polyps, JVP, FAP and cancer were confirmed by histological examinations of biopsied specimens for all patients and their pathological tumor staging were based on American Joint Committee on Cancer (AJCC) (Table 1). All studies were implemented under protocols approved by Institutional Review Boards of Morehouse School of Medicine and University of Alabama-Birmingham.

3.2. High resolution restriction endonucleases and PCR-based analysis of mtDNA variants

To increase the quality of target DNA extracted from small samples, laser-capture micro-dissection was performed on the selected tissue samples with the use of an Arctus PixCell II microscope (Arcturus Engineering) for replication of the distance between the polyps and matched surrounding precancerous normal-appearing cells.

Extracted genomic DNA from each tissue sample was Polymerase Chain Reaction (PCR) amplified with mtDNA specific primers according to Aikhionbare *et al.* 2007. The primer sets (*for-5'-CCCCTCTAGAGCCCACTGTAAAGC-3'*, position 8282-to-8305 and *rev-5'- GTAGTAAGGCTAGGAGGGTG-3', position 10107-to-10088*); (*for-5'-CCCCCTGAAGCTT CACCGG-3', position* 11673-to-11691 and *rev*-5'-GGGGATTGTGCGGTGTGTG-3', position 13950-to-13932) flanking MT-NC7, MT-TK, MT-ND3, ATPase 6, ATPase 8, COX III, COX III, ND3, ND4L, ND4 and ND5 regions respectively. Restriction Fragment Length Polymorphisms (RFLP) was performed with HaeIII, HaeII, and HinfI according to the manufacturer's protocols (New England Biolab). PCR fragments were directly sequenced with same PCR primers to identify the exact nature of new length variants detected by restriction analysis and assess presence of any other mtDNA mutations that exist among and within samples. Sequences of both sense and anti-sense strands were derived with ABI 3130xl Gene Analyzer.

3.3. Analysis of the mtDNA Sequences

Sequence alignments were performed using ABI Sequence Scanner Version 1.0. The results of the mtDNA sequence analysis were compared with MITOMAP database to verify if the changes detected had previously been reported (Table 2). Sequence variants present in both tumor and NST were scored as germ-line variants. Any mtDNA sequence variant that was different between the tumor and the NST were scored as somatic mtDNA mutations. Sequence variants within a tissue type of tumor or normal were define as heteroplasmy.

Table 2. Mitochondrial mutations

MTDNA VARIANTS	MT GENES/ REGION	% Frequency Adenomatous Polyps/ Cancer				NOTES
		Tubular	Tubulovillous	Villous	Cancer	
G8573A	ATPase 8		100			NR*
A8860G	ATPase 6	100	100	100	100	R*
C11867G	MTND4			57		NR*
C11868T/A	MTND4		67	14	38	NR*
C11870T	MTND4	44		57		NR*
A11873G	MTND4	67	78	71	38	NR*
C11874G	MTND4	67	67	71	38	NR*
T11875G	MTND4	56	67	71	38	NR*
G11891A	MTND4			29		NR**
A11905C/G	MTND4		11	29		NR**
G11906T	MTND4		11			NR**
T11907A	MTND4			14		NR**
A11912C	MTND4			14		NR**
12413insC	MTND5	11				NR**
12439insT/A	MTND5	11				NR**
G12476T	MTND5	11				NR**
C12553A	MTND5				0.125	NR**
A12554C	MTND5				0.25	NR**

MtDNA mutations of 33 samples used in regions mtDNA4 and mtDNA5 reported and non-reported in MITOMAP database. * Germ-lines mutations/polymorphisms found in some of our studied tissue samples. Thirty-three total samples including precancerous-normal surrounding tissue (NST) (15 tumors and 18 matched surrounding normal tissues: Tubular-4T, 5NST; Tubulo-villous-4T, 5NST; Villous-3T, 4NST; Cancer-1T, 2NST; JVP-1T, 1NST, FAP-2T, 1NST). ** Somatic mutations found in matched surrounding precancerous normal tissues. R Mutations/polymorphisms are previously reported. NR Mutations/polymorphisms are unreported.

4. RESULTS AND DISCUSSION

Two regions of mtDNA were analyzed from 64 samples of colorectal adenomatous polyps and their matched surrounding precancerous normal tissues (32 tumor and 32 NST) with primer spanning 8282 to 10107 and 11673 to 13950 bp, including *MT-NC7, MT-TK, MT-ND3, ATPase 6, ATPase 8, COX III, ND3, ND4L, ND4 and ND5* genes. Among the 64 colorectal adenomatous polyp samples, a total of f 42 variants were observed over a span of 4.0 kb fragments. The identified variants included: 31% (13/42) Tubular, 52% (22/42) Tubulovillous, 45% (19/42) Villous, 45% (19/42) Cancer, (including FAP and JVP) (Table 2). The observed cumulative frequency of mtDNA variants in tubular and tubulovillous samples is consistent with our previous studies except the observed variants were lower in cancer samples, which could be an indication of the small cancer samples size used in this study (6). Eighteen of the 42 (43%) mutations were not previously reported based on the MITOMAP reference (http://www.mitomap.org). Variant C11867G was unreported and found in ~60% of the selected samples of villous adenopolyps and NST. Notably, a heteroplasmic variants C11868T/A was found in tubulovillous adenopolyps (~70%), also in villous (14%) and cancer samples (38%), but not tubular adenopolyps. The relatively high incidence of the heteroplasmic mtDNA variants observed in tubulovillous samples may be an indication of colorectal tumor cells transformation because tubulovillous is the intermediate between the tubular and villous architecture. A variant, A8860G, was found in 100% of all colorectal adenomatous polyp samples which is consistent with others showing nucleotide variations at this position (6; 18). Unreported germline variant G8573A (Figure 1A and 1B) in *ATPase 8* gene was found 100% in the tubulovillous and the normal surrounding tissues of adenopolyps only. Several studies have suggested mtDNA variants in the ATPase 8 region with Leigh Syndrome patients (19-27). In the ND5 region, we observed a somatic variant of A12553C, and a germline variant at np C12554A in our reference samples, cancer subtype tissues, n=3 (not JVP or FAP). Diseases, such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; Leber Hereditary optic neuropathy; Leigh syndrome and, Mitochondrial complex I deficiency have also been associated with mtDNA variants in this region (7; 28-33). Furthermore, the ATPase 8 and the ND5 of the NADH subunits, are essential for protein synthesis of the mitochondria, any dysfunctions in these genes affect the oxidative phosphorylation (OXPHOS). Therefore, determination of the functionality of these observed variants in association with transformation of colorectal adenopolyps in a large population will be interesting.

In conclusion, this study demonstrates a high frequency of mtDNA variants in colorectal tubulovillous and villous adenopolyp tissue samples possessed more morphology of colorectal carcinoma based on the observed level of frequency of 45% (19/42) mtDNA variants in villous and cancer tissues used in this study. Given that villous carcinoma are associated with the highest morbidity and mortality rates than of all polyps, it could be harboring carcinoma *in situ* or invasive carcinoma more frequently than the other adenomas (7). Whether these observed mtDNA variants are directly associated with the development and progression of colorectal cancer, is still unclear and was not the purpose of our current study but it will be an interesting area that warrants further investigation.
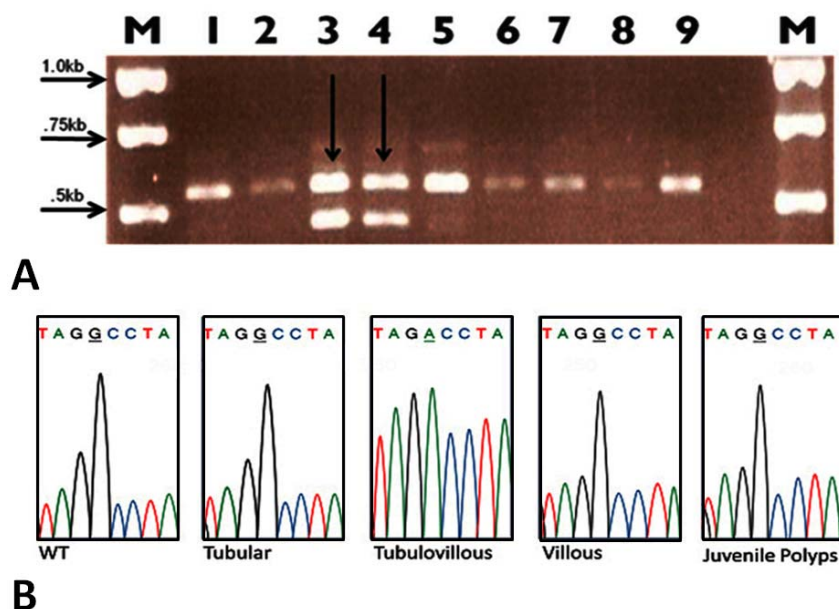
Figure 1. The HaeIII restriction enzyme digestion gel profile of PCR-product obtained with primer that flanked the MT-NC7, MT-TK, MT-ND3, ATPase 6, ATPase 8, COX III genes of mtDNA in adenomas and their normal surrounding tissues. Lanes: M-Marker 1-T; 2-NST T; 3-TV; 4-NST TV; 5-V; 6-NST V; 7-JVP; 8-NST JVP; 9- control. B. Electropherogram representative of restricted amplicon showed in fig1 depicting a mtDNA variant at position G8573A of tubulovillous samples at ATPase 8 gene.

of the manuscript. OII participated in the confirmation of some of the results. WG provided the clinical samples and participated in the review of the manuscript. XY participated in the review of the manuscript. All authors read and approved the final version. FOA conceived, designed and coordinated the study and participated in data analysis and drafted the manuscript. This study was funded in part as a result of the grants from NIH-NCI CA150317, CA150039 and NIH-NIGMS GM099663 awarded to Dr. Felix O. Aikhionbare.

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Abbreviations: Colorectal Cancer (CRC); Tubular (T); Tubulovillous (TV); Villous (V); Juvenile Polyps (JVP); Familial Adenomatous Polyps (FAP); mitochondrial DNA (mtDNA); reactive oxygen species (ROS); American Joint Committee on Cancer (AJCC); UAB Cooperative Human Tissue Network (CHTN); precancerous normal surrounding tissues (NST); Mitochondria databank (MITOMAP).

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