Novel and Differential Accumulation of Mitochondrial DNA Deletions in Swedish and Vietnamese Patients with Colorectal Cancer

JAN DIMBERG[1*], THAI TRINH HONG[2*], MARITA SKARSTEDT[3], STURE LÖFGREN[3], NIKLAS ZAR[4] and ANDREAS MATUSSEK[5]

[1]*Department of Natural Science and Biomedicine, University College of Health Sciences, Jönköping Sweden;*
[2]*Key Laboratory of Enzyme and Protein Technology, College of Science, Vietnam National University, Hanoi, Vietnam;*
Departments of [3]*Clinical Microbiology,* [4]*Surgery and*
[5]*Laboratory Services, Ryhov County Hospital, Jönköping, Sweden*

Abstract. *Background: Mitochondrial DNA (mtDNA) has been proposed to be involved in carcinogenesis and aging. The mtDNA 4977 bp deletion is one of the most frequently observed mtDNA mutations in human tissues and may play a role in colorectal cancer (CRC). In the present study, we aimed to evaluate the frequency of mtDNA 4977 bp deletion in CRC tissues and its association with clinical factors. Patients and Methods: We determined the presence of the 4977 bp common deletion in cancer and normal paired tissue samples from 105 Swedish and 88 Vietnamese patients with CRC using polymerase chain reaction (PCR) assays. Results: The mtDNA 4977 bp deletion was shown to be significantly more frequent in normal tissues in comparison with paired cancer tissues in both Swedish and Vietnamese patients. The 4977 bp common deletion was significantly more frequent in cancer tissues of the Vietnamese patients compared to the Swedish patients, and in Vietnamese cancer tissues, the 4977 bp deletion was significantly over represented in those with localized disease compared to those with disseminated disease. Moreover, we detected nine novel mtDNA deletions and found a significantly higher rate of these in CRC tissues in Swedish in comparison to Vietnamese patients. Conclusion: The mtDNA 4977 bp deletion seems to have an impact on the clinical outcome of CRC in Vietnamese patients, that the Swedish patients accumulate more of the detected novel deletions in CRC tissue compared to Vietnamese patients probably indicates divergent mechanisms in colorectal carcinogenesis.*

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*These Authors contributed equally to this study.

Correspondence to: Dr. Andreas Matussek, Department of Laboratory Services, Ryhov County Hospital, SE-551 85, Jönköping, Sweden. E-mail: Andreas.Matussek@lj.se

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Colorectal cancer (CRC) is the third most common cancer in women and the fourth most common in men worldwide (1, 2). The incidence of CRC is rapidly rising in Asian countries and is beginning to reach the same rate as in Western countries (1, 3). Mutations in nuclear genes such as tumour-suppressor genes and oncogenes are fundamental to the process of malignant transformation in the colon and rectum (4, 5).

Alongside the nuclear genome, the human cell contains hundreds to several thousand copies of the 16,569 base pair closed circular mitochondrial DNA (mtDNA) including 37 genes (6, 7). Within cells the mtDNA has the capacity to form a mixture of both wild-type and mutant mtDNA genotypes in a state called heteroplasmy (6, 7). mtDNA has been proposed to be involved in carcinogenesis and ageing (6, 7), and somatic mtDNA mutations have been reported in various types of cancer, including CRC (6, 8-10).

The main reason for its involvement in carcinogenesis is probably that mtDNA has a high susceptibility to undergo mutations due to its lack of histones and limited repair mechanisms (6, 7). The mitochondrial 4977 bp deletion, also known as the common deletion, is one of the most frequently observed mtDNA mutations. The deletion occurs between nucleotides 8,470 and 13,447 and spans five tRNA genes and seven genes encoding subunits of cytochrome *c* oxidase, ATPases and complex I (11, 12). It has been associated with esophageal squamous cell carcinoma and CRC (10, 13). On the contrary, this 4977 bp deletion has been found to be less abundant in epithelial tumours as compared with non-tumour adjacent tissue (11, 12). The area which undergoes this deletion is proposed to protect against tumour-promoting effects of other somatic mutations

Table I. *Primer sequences and product sizes for mtDNA 4977 bp deletion analysis.*

Primer	Primer sequence	Position	Product	Note
mtDNA-forward	5'-GACGCCATAAAACTCTTCAC-3'	3457-3476	433 bp	Represents ND1 region
mtDNA-reverse	5'-GGTTGGTCTCTGCTAGTGTG-3'	3889-3870		
4977-1forward	5'-TCAATGCTCTGAAATCTGTGG-3'	8167-8187	496 bp	Represents first PCR
4977-1reverse	5'-GTTGACCTGTTAGGGTGAGAAG-3'	13639-13618		
4977-2forward	5'-ACAGTTTCATGCCCATCGTC-3'	8196-8215	381 bp	Represents second PCR
4977-2reverse	5'-GCGTTTGTGTATGATATGTTTGC-3'	13553-13531		
10398-forward	5'-CCTGCCACTAATAGTTATGTC-3'	10307-10327	246 bp	Represents ND3 region
10398-reverse	5'-GATATGAGGTGTGAGCGATA-3'	10552-10533		

thereby preventing neoplastic growth (12). In this study, we determined the frequency of the 4977 bp deletion in cancer and normal paired tissue samples from 105 Swedish and 88 Vietnamese patients with CRC using polymerase chain reaction (PCR).

Patients and Methods

Patients and controls. This study comprised of consecutive patients with CRC from southeastern Sweden and from northern Vietnam. Tissue samples were collected when the patients underwent surgical resections for primary colorectal adenocarcinomas at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden and the National Cancer Hospital, Tamhiep, Hanoi, Vietnam. Clinicopathological characteristics from the patients were received from surgical and pathological records. Tumour tissue and adjacent normal tissue (about 5 cm from the tumor) from each patient were excised and immediately frozen a –80˚C until analysis.

The Swedish patient group consisted of 60 males and 45 females, with a mean age of 69 years (range 29-90 years). The tumors were localized in the colon (n=59) and rectum (n=46) and were classified according to Dukes' classification system: stage A (n=22), stage B (n=31), stage C (n=34) and stage D (n=18). The Vietnamese patients comprised 45 males and 43 females with a mean age of 57 years (range 26-95). The tumours were localized in the colon (n=40) and rectum (n=48) and were classified as stage A in 30, stage B in 24, stage C in 31 and stage D in three. Informed consent was obtained from each patient and the study was approved by the Ethics Committee at the Faculty of Health Sciences Linköping, Sweden (Dnr. 98113) and by local Ethics Committee in Vietnam (2422/QD-KHCN).

Polymerase chain reaction (PCR) assay. DNA was isolated from all CRC and paired normal tissues using QIAamp DNA Mini kit (Qiagen, Hilden, Germany). To screen for the mitochondrial 4977 deletion, a nested PCR method was developed to detect low levels of the deletion. Two pairs of PCR primers were designed for the first amplicon of 496 bp and the a second amplicon of 381 bp (Table I). For the first amplicon, the primers were designed to be distant enough to detect only mtDNAs containing deletions. To assess the presence of mtDNA and to detect heteroplasmy/homoplasmy regarding 4977 deletion, PCR primers were designed in the region of the genes NADH dehydrogenase 1(*ND1*) and *ND3* resulting in products of 433 bp and 246 bp, respectively (Table I).

Except for the second PCR run for 4977 deletion, DNA was amplified in a total volume of 12.5 μl containing 0.2 μM of each primer (TIB Molbiol, Berlin, Germany), 1.8 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.04 units *Taq* DNA polymerase and reaction buffer [20 mM Tris-HCl (pH 8.3), 20 mM KCl, 5 mM $(NH_4)_2SO_4$] (Fermentas, Burlington, Canada). Amplification was performed using an initial denaturation at 95˚C for 4 min followed by 35 cycles at 92 ˚C for 30 s (denaturation), 54˚C for 30 s (annealing), 72˚C for 45 s (extension) and final elongation at 72˚C for 10 min. For the second PCR run regarding the 4977 deletion, the conditions were the same as above except that we changed the annealing temperature to 60˚C and the total number of cycles to 32. The amplified PCR products were visualized by UV-illumination on 2% agarose gel containing Gel Red (Biotium, Inc., Hayward, CA, USA). The band reflecting the 4977 common deletion and all the other bands that were obtained at different levels on the gel were purified using Gel Extraction kits (Qiagen, Santa Clarita, CA, USA), followed by commercial sequencing (GATC Biotech, Köln, Germany).

Statistical analysis. Differences in the rate of mtDNA deletions were analyzed using the Chi-square test. Statistical analyses were performed using SPSS for Windows computer package (IBM SPSS Statistics, 2012, version 19; SPSS Inc., Chicago, IL, USA). Results were considered significant at $p<0.05$.

Results

Prevalence of mtDNA 4977 bp deletion in patients with CRC. All samples showed clear bands with mtDNA and 10398 primers representing 433 bp and 246 bp respectively (Figure 1). In lane 4 Figure 1, one novel deletion is illustrated (624 bp) which was confirmed by sequencing. For the 4977 bp deletion, represented by bands 381 bp, we defined three types of signals by nested PCR: negative, no band; weak, weak and narrow band; positive, clear strong band (Table II). In the tissues from Swedish patients, the deletion was detected in 67.6% (71/105) of cancerous tissues and 92.4% (97/105) of normal tissues (Table II) ($p<0.001$). In the Vietnamese patients (Table II), these figures were 80.7% (71/88) for cancerous tissues and 92.0% (81/88) for normal tissues ($p=0.028$). Furthermore, we recorded a significantly ($p=0.040$) higher rate of the 4977 bp deletion in cancer tissue of the Vietnamese compared to Swedish patients.

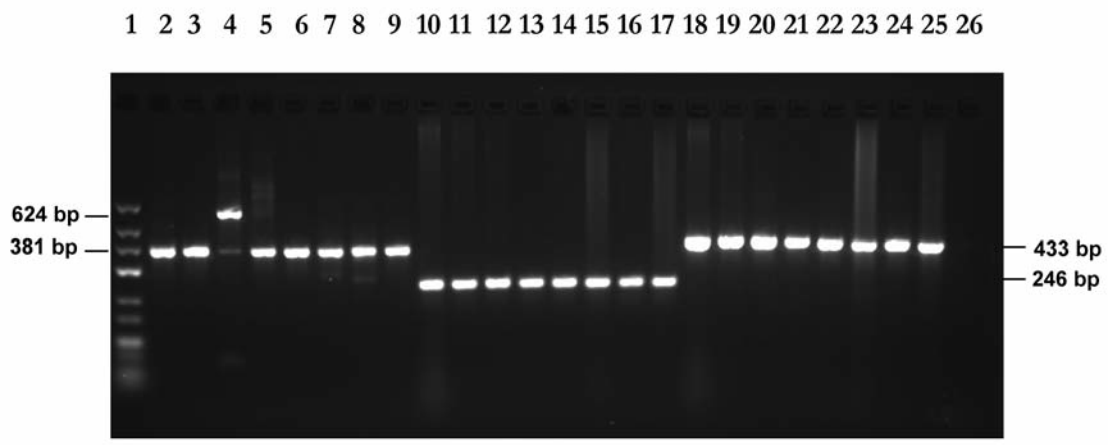

Figure 1. *Agarose gel showing polymerase chain reaction (PCR) products from four colorectal cancer tissue/normal paired tissue. Nested PCR (381 bp, lane 2/3, 4/5, 6/7, 8/9); 10398 (246 bp, lane 10/11, 12/13, 14/15, 16/17); mtDNA (433 bp, lane 18/19, 20/21, 22/23, 24/25) and discovered novel deletion (624 bp, lane 4). Lane 1, molecular marker and lane 26, negative control.*

With regard to disease stage, the patients were divided into two sub-groups of Duke A+B (localized disease) and Duke C+D (disseminated disease) (Table II). In Vietnamese cancer tissues, we found that the 4977 bp deletion was over-represented in those with localized disease at 87.0% (47/54) compared to those with disseminated disease at 67.6% (23/34) with an odds ratio (OR)=3.21 [95% confidence interval (CI)=1.10-9.37, p=0.028]. No corresponding significant difference was seen in the Swedish patients. There was no statistically significant association between the 4977 bp deletion and the clinical parameters gender, age or tumour localization in either ethnic group.

Detection of novel mtDNA deletions. After nested PCR, we detected nine different bands in addition to the 381 bp representing the 4977 bp deletion. Six of these bands were >381 bp and three were <381 bp, which are represented in Table III as numbers 1-6 and 7-9, respectively. Additionally, when the novel bands were detected, the 4977 bp deletion was either negative or weak. All detected bands were purified, sequenced and the corresponding deletions were analyzed using the program BLASTn (14). The deletions were checked against the MITOMAP database (15) and other possible reference sources, with the consequence that we characterized our findings as novel deletions. Table III summarizes the novel deletions with information about breakpoints, deletion size, repeat location and type.

We compared the prevalence of the novel deletions in the cancerous tissue of Swedish and Vietnamese patients and found a significantly (p=0.034) higher rate, 41.9% (44/105), in Swedish patients in comparison with 27.2% (24/88) in Vietnamese patients, with an OR=1.92 (95% CI=1.05-3.53, p=0.034). In normal tissues from the Swedish and Vietnamese patients, we found three and five novel deletions, respectively.

Table II. *Mitochondrial DNA 4977 bp deletion in Swedish and Vietnamese patients with colorectal cancer.*

		Prevalence of deletion (n)		
Parameters	No. of cases	Negative	Weak	Positive
Swedish patients				
Cancer tissues	105	34	18	53
Normal paired tissues	105	8	0	97
Dukes'				
A+B	53	15	11	27
C+D	52	19	7	26
Vietnamese patients				
Cancer tissues	88	17	11	60
Normal paired tissues	88	7	4	77
Dukes'				
A+B	54	7	7	40
C+D	34	11	3	20

There were no associations between the novel deletions and clinical characteristics such as Dukes' stage, age, location and gender (data not shown).

In one Swedish patient with deletion number 1 (sample 194) with breakpoints 8290:11279 and 11793:13117 (Table III), we found that this deletion contained an inverted and connected sequence of light (L) and heavy (H) strand (Figure 2). In this deletion, a DNA fragment of 515 bp is retained and inverted, and the fragments of 2988 and 1323 bp are deleted.

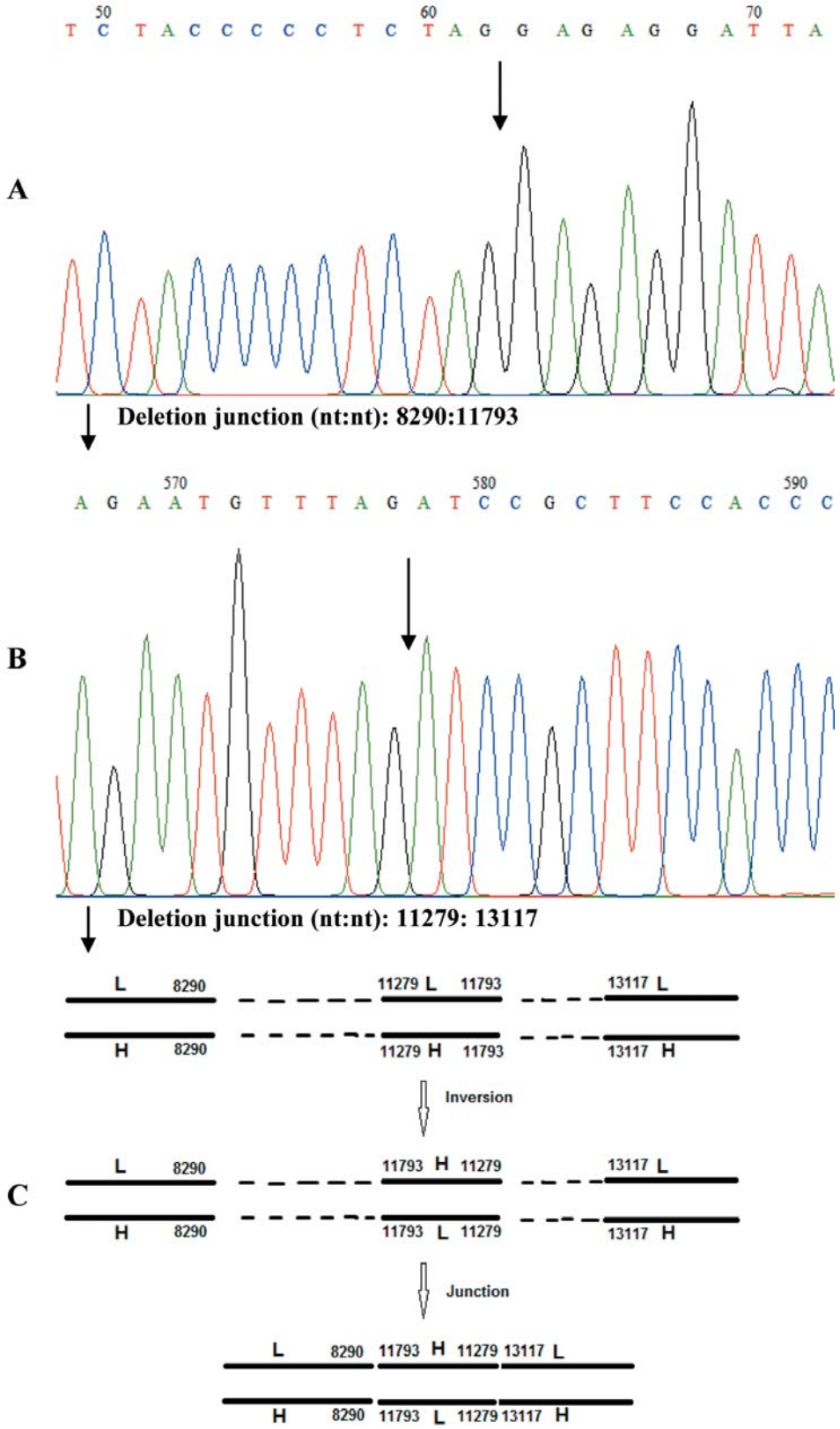

Figure 2. *The sequence of the deletion junctions from sample 194. A: 2988 bp deletion, deletion junction (nt:nt) is 8290:11793; B: 1323 bp deletion, deletion junction (nt:nt) is 11279: 13117; C: after deletion, inversion and junction of light strand (L) and heavy strand (H): nt 11279-11793.*

Table III. *Novel mtDNA deletion detected in colorectal cancer tissue.*

No.	Sample no.	Deletion junction (nt:nt)	Deletion size (bp)	Repeat location (nt)	Repeat type
1	194*	8290:11793	4311	8288-8290/11279-11281	D, 3/3
		11279:13117	(2988+1323)	11791-11793/13114-13116	D, 3/3
2	199	8563:13241	4677	8561-8563/13250-13252	D, 3/3
3	204	9097:13110	4012	9094-9097/13110-13113	D, 4/4
4	282	8569:13465	4895	8571-8577/13465-13470	D, 6/7
5	13B	8514:12761	4246	8515-8520/12765-12770	D, 6/6
6	44B	8243:12978	4734	8248-8253/12978-12982	D, 5/6
7	184	8367:13486	5118	8359-8365/13478-13483	D, 6/7
8	36B	8336:13481	5144		NR
9	58B	8380:13517 or 8382:13519	5136	8381-8382/83517-83518	D, 2/2

D, Direct repeat; NR, no repeat; nt, nucleotide; *inversion and junction of L-H, H-L:nt 11279-11793.

Discussion

The mitochondrial 4977 bp deletion has been found in several tumour types, in aging and even in apparently normal tissues (6, 7, 10, 13). Recently, reduced mitochondrial mutagenesis in CRC has been shown, as well as a higher frequency of mtDNA mutagenesis which may prevent CRC (16).

In the present study, the mtDNA 4977 bp deletion was found at a significantly higher frequency in normal tissues compared to paired cancer tissues in both Swedish and Vietnamese patients. We also observed a pervading heteroplasmy in the tissues. Our results are consistent with previous studies showing decreased proportions of the mtDNA 4977 bp deletion in various cancer types compared to adjacent normal tissues, such as breast (11), non-melanoma skin (9) and gastric cancer (17). One explanation of this phenomenon might be a dilution of the mtDNA 4977 bp deletion in tumour tissues caused by rapid cell proliferation, or that cells harbouring this deletion are eliminated by apoptosis (17). Moreover, the mtDNA 4977 bp deletion might confer a metabolic disadvantage to proliferating cells and thus is selected out in the highly proliferative tumour tissue (12). The selection pressure is presumably relaxed in normal tissue with a lower proliferative rate and therein the mtDNA 4977 bp deletion will be accumulated.

We found a significantly higher rate of the 4977 bp deletion in cancerous tissues of the Vietnamese patients compared to Swedish patients. Furthermore, in Vietnamese cancerous tissues we found that the 4977 bp deletion was significantly over-represented in those with localized disease compared to those with disseminated disease. No corresponding significant difference was seen in the Swedish patients. Our results might indicate an ethnic difference in the accumulation of the 4977 bp deletion in CRC tissue. Our findings that the frequency of 4977 bp deletion is decreased in the Vietnamese cancerous tissue with higher stage is in agreement with data from Chinese patients with CRC (10), representing another Asian population.

In addition to the 4977 bp deletion, we discovered nine novel deletions with over-representation in cancer tissue from Swedish and Vietnamese patients with CRC (Table III). These deletions were also significantly more frequent in Swedish patients in comparison to Vietnamese patients but were not associated with stage of cancer.

Mitochondrial retrograde signalling is a pathway of communication from mitochondria to the nucleus under normal and pathological conditions (18). It involves multiple factors that sense and transmit mitochondrial signals to bring about changes in nuclear gene expression and encompasses a wide assortment of cellular activities including growth control and ageing. It is possible that our novel deletions are involved in the mediation of tumour progression. However, our finding does not provide answers as to whether mtDNA alterations are contributing factors to carcinogenesis or whether they simply arise as part of secondary effects in cancer progression.

Among the nine novel deletions, one case showed a combination of deletion and inversion. To our knowledge, this is the first case of long inversion (515 bp), while the reported size of mtDNA pathogenic inversion is only 7 bp in mitochondrial myopathy (19). Whether our novel deletions have an impact on cancer development or not requires further investigation. Moreover, their possible impact on other diseases and aging needs to be evaluated.

In conclusion, our observations indicate that the mtDNA 4977 bp deletion seems to have an impact on the clinical course of CRC in Vietnamese patients and that Swedish patients accumulate more of the detected novel deletions in CRC tissue compared to Vietnamese patients, thereby possibly reflecting divergent mechanisms in colorectal carcinogenesis. Additional studies are required to define the importance of the mtDNA deletions found in prediction of clinical outcome, including recurrence and survival of patients with CRC.

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