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Mitochondrial DNA copy number in colorectal cancer: between tissue comparisons, clinicopathological characteristics and survival

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Abstract

Low mitochondrial DNA (mtDNA) copy number in tumors has been associated with worse prognosis in colorectal cancer (CRC). This study further deciphers the role of mtDNA copy number in CRC by comparing mtDNA copy number between healthy, adenoma and carcinoma tissue, by investigating its association according to several clinicopathological characteristics in CRC, and by relating it to CRC-specific survival in CRC patients. A hospital-based series of samples including cancer, adenoma and adjacent histologically normal tissue from primary CRC patients (n = 56) and recurrent CRC (n = 16) was studied as well as colon mucosa samples from healthy subjects (n = 76). Furthermore, mtDNA copy number was assessed in carcinomas of 693 CRC cases identified from the population-based Netherlands Cohort Study (NLCS). MtDNA copy number was significantly lower in carcinoma tissue (P = 0.011) and adjacent tissue (P < 0.001) compared to earlier resected adenoma tissue and in primary CRC tissue compared to recurrent CRC tissue (P = 0.011). Within both study populations, mtDNA copy number was significantly lower in mutated *BRAF* (P = 0.027 and P = 0.006) and in microsatellite unstable (MSI) tumors (P = 0.033 and P < 0.001) and higher in *KRAS* mutated tumors (P = 0.004). Furthermore, the association between mtDNA and survival seemed to follow an inverse U-shape with the highest HR observed in the second quintile of mtDNA copy number (HR = 1.70, 95% CI = 1.18, 2.44) compared to the first quintile. These results might reflect an association of mtDNA copy number with various malignant processes in cancer cells and warrants further research on tumor energy metabolism in CRC prognosis.

Introduction

An often observed phenomenon in cancer progression is the alteration of energy metabolism (1). More specifically, an increased glucose uptake, a higher lactate production and an increased glycolytic activity in the presence of sufficient oxygen are observed, especially in larger tumors with high metastatic potential (2,3). Since mitochondria are the main aerobic producers of ATP in the cell and major regulators of apoptosis (4), studies on mitochondrial alterations in relation to cancer etiology, progression and therapy response provide an interesting avenue in cancer research to explore further.

Mitochondrial DNA (mtDNA) copy number, the amount of copies of the complete mtDNA, is an important aspect of mitochondrial genetics. MtDNA copy number plays a part in mitochondrial biogenesis (5) and regulating mitochondrial function (6). MtDNA copy number itself is regulated mainly by mitochondrial transcription factor A (TFAM), which is encoded by the nuclear DNA (5). Nevertheless, other factors are also involved in mtDNA copy number regulation, especially availability of nucleotide pools and functioning of the replication machinery,

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Abbreviations

FFPE	formalin-fixed paraffin embedded
mtDNA	mitochondrial DNA
MSI	microsatellite instability
MSS	microsatellite stable

involving amongst others mtDNA polymerase γ (POLG) (7,8). MtDNA copy number in human cells generally varies from 0 to 10^5 copies per cell depending on the energy demand of the cell or tissue (9).

MtDNA copy number has been reported to be lower in advanced cancer stages in colorectal tumors in several studies (10–14). In one of these studies, the lowest mtDNA copy number category was associated with shorter disease-free survival compared to the middle or highest mtDNA copy number categories in 194 tumor samples; this association was on the borderline of being statistically significant in a multivariable analysis after adjusting for TNM stage, mutations in p53 and lymphovascular invasion (12). It has also been suggested that mtDNA depletion and thus low mtDNA copy number predicts worse response to chemotherapy (15,16). Relatively low mtDNA copy number is associated with shorter survival and other unfavorable characteristics like increased tumor size and poor differentiation of tumors (12,13,17–21). Therefore, it has been hypothesized that relatively low mtDNA copy number is associated with worse prognosis and could reflect several malignant alterations such as increased tumor invasiveness and glycolytic deficiencies in cancer cells.

This study aims to contribute to understanding the role of mtDNA copy number in colorectal cancer (CRC) prognosis and etiology by investigating three research goals. First, differences in mtDNA copy number between carcinoma—adjacent normal—and adenoma tissues within CRC patients and normal colon mucosa from individuals without CRC were investigated. Secondly, the relation between mtDNA copy number and clinicopathological characteristics such as disease stage and important molecular markers of prognosis was explored. Three established and common molecular markers of prognosis in CRC were investigated in this study: *BRAF* V600E mutation status, which is associated with unfavorable prognosis, microsatellite instability (MSI) status which is associated with favorable prognosis and different response to chemotherapeutics, and activating *KRAS* mutation status which is common in CRC (30–50% of tumors) and associated with poorer survival and response to chemotherapeutics (22–25). Finally, the association of mtDNA copy number with CRC-specific survival was assessed. Two series of samples were used; one hospital-based series of CRC patients and individuals without CRC and one large sample of CRC patients prospectively identified within the Netherlands Cohort Study (NLCS).

Materials and methods

Study populations

Hospital-based series

Tissue samples obtained at diagnosis from 74 CRC patients were retrospectively collected from the archive of the Department of Pathology at Maastricht University Medical Centre+ (MUMC+) as described earlier (26). All patients in this series did not receive any therapy prior to surgery. This hospital-based series of samples consisted of formalin-fixed, paraffin embedded (FFPE) tumor- and adjacent normal tissues from patients (n = 74) who were older than 50 years at the time of CRC diagnosis during 1995–2003. Of these 74 cases, 16 had a history of CRC, 1 case had an unknown history status and for 1 case mtDNA copy number could not be determined so these 18 cases were excluded for further analysis. The mtDNA copy number data from cases with a positive history of cancer were only used to compare mtDNA copy number in carcinoma tissue between primary and recurrent CRC cases, since it is hypothesized that recurrent CRC tissue is different with regard to mtDNA copy number. When available in the tissue archive, adenoma tissue from these CRC patients was also collected. Of the 25 available adenoma's, eight were resected before cancer development and the remaining 17 simultaneously with the cancer resection. In addition, histologically normal colon mucosa from individuals who did not develop CRC within 10 years of sample collection within the same age range who underwent endoscopy during 1987–2004 for non-specific abdominal complaints (n = 76) was obtained. Normal colon tissues were excluded if the individual without CRC had been diagnosed with CRC in the past or during follow-up (until 2004). CRC patients who had been diagnosed with cancers other than CRC (excluding non-melanoma skin cancer) were also excluded. This means patients with earlier colorectal tumors were included as patients with a history of CRC. Relevant patient information such as patient demographics was collected from medical files. Disease stage was originally coded using Dukes stage, the most widely used staging system for CRC at the time that these samples were collected (27), but was transformed to TNM stage in the dataset (28). This study population and available tissues are summarized in panel A of Figure 1.

NLCS series

An additional series of FFPE tumor samples obtained at diagnosis were available from CRC patients prospectively identified during 7.3 years of follow-up in the NLCS (excluding the first 2.3 years). The NLCS is a nationwide prospective cohort study, initiated in 1986 among 120 852 participants aged 55–69 years and is described in more detail elsewhere (29). FFPE tumor samples have been obtained for ~90% of all individuals who had developed CRC between 1989 and 1993 and who could be linked to the national pathology registry (PALGA) (30,31). Earlier studies within the NLCS have utilized DNA from these samples to investigate several molecular markers in relation to CRC (31–33). Therefore, for this study not all 733 collected samples could be used due to DNA depletion of some samples and insufficient quality of FFPE DNA material. Finally, 693 tissue samples were available for assessment of mtDNA copy number. This sample collection is summarized in panel B of Figure 1. Within this sample, there were three cases who had undergone radiotherapy before surgery, however since their mtDNA copy number scores were within the second and third quintile of scores it was decided to include these cases because there were no reasonable suspicions of these cases being outliers. Furthermore, CRC-specific survival has been determined by assessing vital status through linkage with the Dutch municipal registries and by obtaining the cause of death through linkage with the Dutch Central Bureau for Statistics (CBS). Information on date of incidence, localization and stage according to TNM classification was retrieved through the Netherlands Cancer registry. In cases for which the cause of death was unclear, e.g. when liver metastases were coded as the cause of death, the involvement of other forms of cancer was checked. If no other primary cancers had been registered, these patients were labeled as CRC-related deaths.

DNA isolation

A 5-µm section, cut from each FFPE tissue block, was stained with haematoxylin and eosin (HE) for histopathological examination by a pathologist. Five 20 µm sections of tumor tissue were cut for DNA isolation. Tumor tissue was macrodissected from normal colon epithelium using the HE section as a reference. Genomic DNA was extracted from the macrodissected tumor tissue using proteinase K (Qiagen, St Louis, MO) and the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) (31). Purity of the DNA samples was assessed through Nanodrop technology before mtDNA copy number assessment and samples without sufficient DNA yield were not included for mtDNA copy number analysis.

MtDNA copy number assessment

MtDNA copy number was determined by amplification of a nuclear DNA (beta-2-microglobulin) and a mtDNA (D-loop) fragment in a real-time PCR (ABI Prism 7900HT Fast Real-time PCR System, Taqman®) for all available samples. The DNA input was 10 ng and all samples were measured independently in duplo. The mean value between duplo's was used as

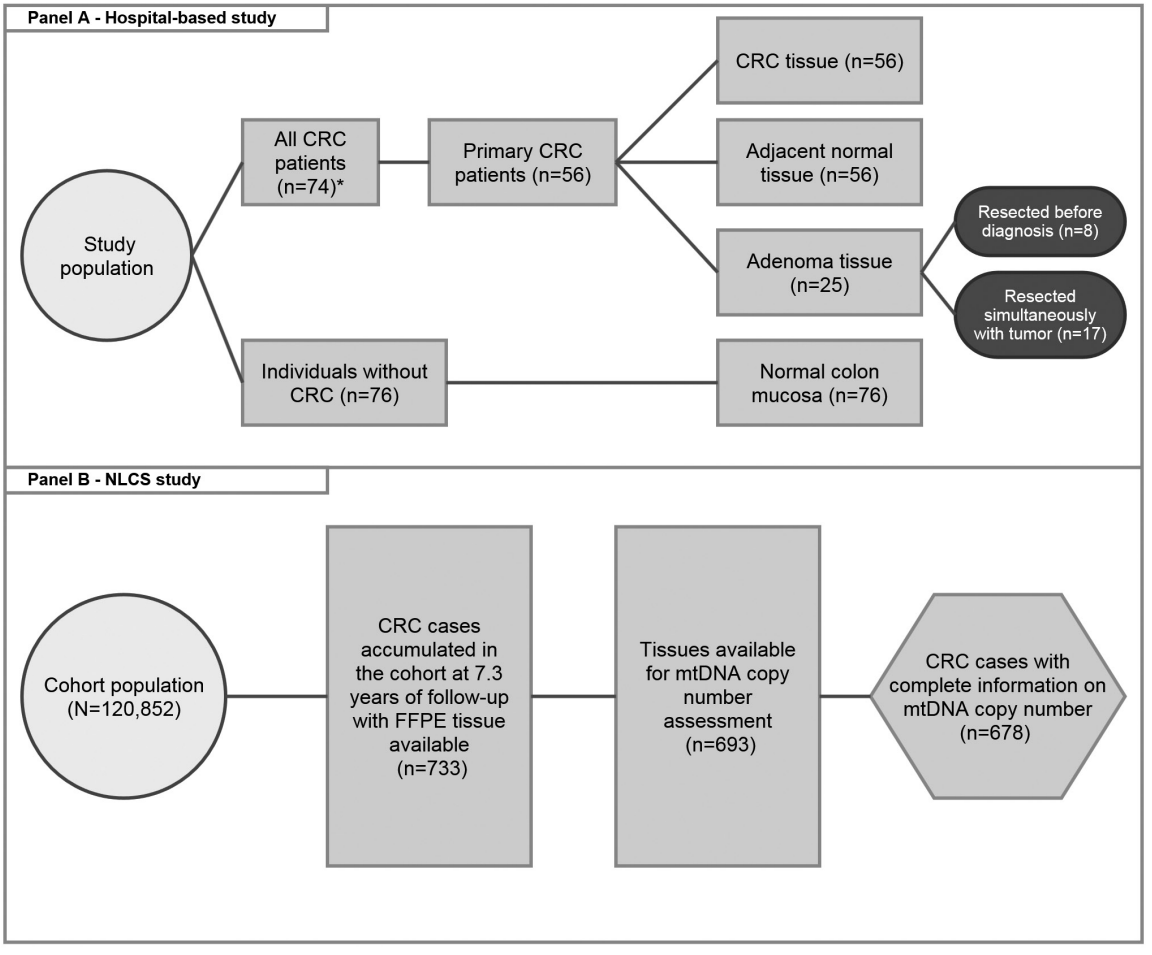

Figure 1. Flowchart of sample collection in the hospital-based study (**A**) and NLCS study (**B**).

mtDNA copy number score for each individual. Primers for beta-2-microglobulin were 'TGCTGTCTCCATGTTTGATGTATCT' as forward primer and 'TCTCTGCTCCCCACCTCTAAGT' as reverse primer. For quantification of the D-loop region 'CATCTGGTTCCTACTTCAGGG' was used as forward primer and 'TGAGTGGTTAATAGGGTGATAGA' as reverse primer. The PCR reaction was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Finally, mtDNA copy number was calculated as the ratio of Ct values for the β-2-microglobulin and D-loop fragments. MtDNA copy number was successfully determined in 73 out of 74 CRC patients, in all 76 individuals without CRC from the hospital-based series (intraclass correlation coefficient = 0.49) and in 678 out of 693 tumor samples (intraclass correlation coefficient = 0.84) available from the NLCS at 7.3 years of follow-up.

Molecular analyses

BRAF mutation status has been assessed by a seminested PCR and subsequent sequencing of the PCR product. The common BRAF V600E mutation in exon 15 was analyzed by restriction fragment length polymorphisms analyses (32). MSI status was determined by pentaplex PCR using mononucleotide repeats BAT-26, BAT-25, NR-21, NR-22 and NR-24. If allelic size varied in more than three repeats, it was considered a marker for MSI whereas other tumors were marked as microsatellite stable (MSS) (34). In the NLCS study, KRAS mutation analysis (codon 12 and 13) was performed by a nested PCR, directly followed by sequencing of purified fragments. This method has been described in more detail elsewhere (31). In the hospital-based series, the PCR has only been performed for mononucleotide repeat BAT-26 and not for the other repeats, which has been shown to be sufficient to detect MSI in a majority of tumors from Caucasian populations (34,35).

Statistical analyses

Since mtDNA copy number is expressed as a ratio, by dividing the mtDNA copy number by the amount of nuclear DNA copies within the same tumor tissue (36), a large standard deviation of mtDNA copy number was observed in all tissues within this study and mtDNA copy number did not seem to be normally distributed (Shapiro–Wilk's $W = 0.494$, $P < 0.001$) but skewed towards the right. Therefore, non-parametric statistical tests were used to investigate differences in mtDNA copy number between tissues and clinicopathological characteristics. Fortunately, by performing non-parametric tests the impact of mtDNA copy number variability (control DNA samples which were analyzed on multiple PCR plates showed 6–30% variation in mtDNA copy number scores) on reproducibility of results was minimized because mtDNA copy numbers scores are ranked using this approach. Differences were assessed by Mann-Whitney's *U* test for comparisons of tissue types within patients and Wilcoxon's signed rank tests for comparisons between patients. The association of mtDNA copy number with clinicopathological characteristics (personal history of CRC, BAT-26 stability, TNM stage, dysplasia, differentiation grade and tumor sublocation) as well as the associations with the *BRAF* V600E mutation were investigated by the Mann–Whitney's U and Kruskal–Wallis tests, where applicable. Tumors with two or more affected regional lymph nodes were combined in the analyses as N2 + N3 because of the small number of available N3 tumors. In a sensitivity analysis, a logarithmic transformation of mtDNA copy number scores was used, which normalized the distribution of mtDNA copy number. No differences "no differences between results" results were observed.

The mtDNA copy number data from the NLCS series ($n = 678$) were also skewed to the right. Therefore, analyses were performed using non-parametric tests. In addition, analyses were performed in separate strata

for MSI status and TNM stage, since both are strongly associated with CRC prognosis. Differences between mutated and non-mutated *BRAF* variants were analyzed using Mann–Whitney's *U* test, in the same way as in the hospital-based series. Within the NLCS series survival analyses were also performed. Cases with unknown or missing mortality status, who were diagnosed at autopsy or who deceased after 1 month of diagnosis ($n = 38$) were excluded, resulting in 655 CRC patients at risk of CRC-related death and a total of 258 CRC related deaths during 5 years of follow-up. Survival analyses were truncated at 5 years of follow-up, since most deaths that occur after 5 years of follow-up are not CRC-related. Three hierarchical survival models were applied; an unadjusted model, a model adjusting for age at diagnosis and sex, and a third model additionally adjusting for tumor sublocation and differentiation grade as well as TNM stage and MSI. There was no adjustment for BRAF mutation, because of the risk of collinearity between BRAF and MSI. The proportional hazards assumption was checked in all Cox regression models and was not violated in any of the models. To investigate the association of mtDNA copy number with CRC specific survival, Cox regression analysis was performed comparing quintiles of mtDNA copy number, modelled as dummies with the lowest quintile as reference category. Additionally, mtDNA copy number quintile distribution across TNM stages was tested using Pearson's chi-square. *P* values lower than or equal to 0.05 were considered statistically significant using two-sided tests. Data analysis was performed using SPSS (version 21; SPSS Inc., Chicago, IL) and Stata (version 13; Stata Corp., College Station, TX).

Results

Available baseline characteristics included age, sex, TNM stage, differentiation grade, sublocation, (familial) history and MSI and *BRAF V600E* mutation status; these data are listed for both the hospital-based series and the NLCS in Table 1. The CRC patients from both populations were largely comparable except for that the hospital-based population was older (71 versus 63) and were more often stage III cancer patients than the NLCS based group.

MtDNA copy number in different colon tissues from CRC patients and non-CRC subjects

In the hospital-based series of CRC patients, normal tissue from individuals without CRC (444) was not significantly different from histologically normal tissue from CRC patients (377) or cancer tissue from CRC patients (351) (Table 2). However, significantly higher mtDNA copy number was observed in recurrent CRC patients (590) compared to primary CRC patients (351, $P = 0.011$). In the 25 cases with previous adenomas, mtDNA copy number was significantly higher in adenomas (590) compared to adjacent normal tissue (382, $P < 0.001$) and cancer tissue (461, $P = 0.009$) from the same patients. Additionally, significantly higher mtDNA copy number in adenomas was also observed when only including the eight adenomas which were resected before cancer development (628) ($P = 0.025$ compared to carcinomas and $P = 0.012$ compared to adjacent normal tissue from the same patients). The mtDNA copy number of carcinomas from the NLCS series (393) did not differ from the mtDNA copy number in carcinomas from the hospital-based series (351).

MtDNA copy number and clinicopathological characteristics

No statistically significant differences in mtDNA copy number between differentiation grades were observed within both series of samples (Table 3). In spite of this, when only considering MSI tumors within the NLCS series ($n = 77$), mtDNA copy number was significantly lower (mean difference=334, $P = 0.043$) in 23 poorly differentiated MSI tumors compared to 33 moderately differentiated tumors. No difference in mtDNA copy number was observed between CRC patients with familial history of CRC compared to those without a familial history of CRC. In the hospital-based and NLCS series, respectively, mtDNA copy number was lower in tumors with a *BRAF* V600E mutation ($P = 0.027$ and $P = 0.006$) and in tumors with unstable *BAT-26* and MSI ($P = 0.033$ and $P < 0.001$) compared to tumors without these aberrations (Table 3). *BRAF* mutated tumors and MSI tumors showed a large overlap in both sets of samples (e.g. five out of eight *BRAF* mutated tumors also exhibited MSI in the hospital-based series). Furthermore, mtDNA copy number was significantly higher in KRAS-mutated tumors (482) compared to non-activated KRAS tumors (368, $P = 0.004$) within the NLCS. Finally, mtDNA copy number was similar across TNM stage in both series of samples ($P = 0.480$ in the hospital-based series and $P = 0.185$ in the NLCS series), although median mtDNA copy number seemed lower in every subsequent disease stage in the NLCS series. A similar decreasing trend in mtDNA copy number was observed in CRC patients from the NLCS with increasing size of the primary tumor, although not statistically significant ($P = 0.067$).

Table 1. Baseline characteristics of primary CRC patients within both study populations

	Hospital-based series	NLCS
	CRC patient ($n = 56$)[a]	CRC patient ($n = 678$)[a]
Age (mean, standard deviation)	70 (9)	63 (4)
Sex (*n*, %)		
Male	31 (55)	372 (55)
Female	25 (45)	306 (45)
TNM stage (*n*, %)		
1	10 (18)	174 (27)
2	16 (29)	221 (34)
3	22 (39)	165 (26)
4	8 (14)	85 (13)
Differentiation grade (*n*, %)		
Poor	5 (9)	98 (17)
Moderate	45 (80)	424 (72)
Well	6 (11)	66 (11)
Sublocation (*n*, %)		
Total colon	30 (54)	—
Proximal colon	—	222 (33)
Distal colon	—	211 (32)
Sigmoid	10 (18)	0 (0)[b]
Rectosigmoid	9 (16)	76 (11)
Rectum	6 (11)	160 (24)
Familial history of CRC (*n*, %)		
Yes	—	70 (10)
No	—	605 (90)
BAT-26 unstable/MSI (*n*, %)[a]		
Yes	7 (13)	77 (12)
No	48 (87)	560 (78)
BRAF mutation (*n*, %)[a]		
Yes	8 (15)	105 (16)
No	47 (85)	555 (74)
KRAS mutation (*n*, %)[a]		
Yes	—	237 (35)
No	—	441 (65)

[a]Number of patients in columns do not always add up to the total number of patients within both studies because of missing values.
[b]The analyzed subset of samples from the NLCS cohort did not contain tumors of the sigmoid a. In the hospital-based series, there was one missing value for both BAT-26 instability and BRAF mutation.

Table 2. Median mtDNA copy number and 25th to 75th percentile range in all tissues within both study populations

		MtDNA copy number	
	Tissue type	Median	25–75th percentile range
Hospital-based series	Normal colon mucosa from healthy subjects (*n* = 76)	444	301, 634
	Normal from primary CRC patient (*n* = 56)	377	282, 496
	Carcinoma from primary CRC patient (*n* = 56)	351	237, 617
	Carcinoma from recurrent CRC patient (*n* = 16)	590[b,c]	426, 811
	Adenoma from CRC patient (*n* = 25)	590	517, 850
	Normal from CRC patient with previous adenoma (*n* = 25)[a]	382[d,e]	341, 503
	Carcinoma from CRC patient with previous adenoma (*n* = 25)[a]	461[d,e]	300, 534
NLCS	Carcinoma from CRC patient (*n* = 678)	393	215, 778

[a]These 25 patients were all patients of the total of 56 patients with primary CRC who also had adenoma tissue available.
[b]MtDNA copy number in carcinoma tissue was compared between primary and recurrent CRC patients.
[c]Statistically significant median difference at $\alpha = 0.05$ level.
[d]Statistically significant median difference at $\alpha = 0.01$ level.
[e]MtDNA copy number in adenoma tissue from CRC patients was compared to mtDNA copy number in carcinoma- and adjacent normal tissues from the same CRC patients.

No statistically significant differences were observed between tumors with and without distant metastases or between numbers of regional lymph nodes involved.

MtDNA copy number and CRC-specific survival

Within the 655 CRC cases in the NLCS at risk of dying, 258 CRC-related deaths were recorded during 5 years of follow-up culminating in a total of 5152 person-years at risk (Table 4). Kaplan–Meier curves (Figure 2) showed that the survival rate significantly differed between mtDNA copy number quintiles ($P < 0.001$). The Kaplan–Meier curves show that the second quintile had the worst CRC specific 5-year survival (53%) and the fifth and highest quintile of mtDNA copy number the most favorable 5-year survival (72%). This is also reflected in the HRs per quintile in Table 4 where the second quintile has the highest and only statistically significant HR (1.70, 95% CI (1.18, 2.44)) compared to the lowest quintile of mtDNA copy number. This association remained statistically significant after adjusting for age and sex (HR = 1.69, 95% CI (1.17, 2.44)), however not when adjusting for other prognostic factors including TNM stage (HR = 1.33, 95% CI (0.89, 1.99)). When inspecting all HRs across quintile categories the relation between mtDNA copy number and CRC-specific survival appears to be inversely U-shaped. There seems to be a linearly decreasing trend in HRs from the second quintile towards the highest quintile. The observed distribution of mtDNA copy number quintile scores across TNM stages differed significantly (Pearson's chi-square= 26,837; $P = 0.043$) from expected values. TNM stage 4 tumors were relatively more common and TNM stage 1 tumors were relatively less common in quintile 2 compared to the other quintiles (Supplementary Table 1, available at *Carcinogenesis* Online). Additionally, since normality could not be assumed, the log-transformed mtDNA copy number was investigated as a continuous variable in relation to CRC mortality. The observed unadjusted HR of 0.72 (95% CI (0.54, 0.97)) indicates that with 1 unit increase in log(mtDNA copy number) the hazard of dying due to CRC significantly decreases by 28%. The multivariable-adjusted estimate (HR = 0.78, 95% CI (0.58, 1.06)) was comparable though not statistically significant.

Discussion

This study is one of the first investigating mtDNA copy number in several CRC tissues resected at different time points within the same patient and, to our knowledge, also the study with the largest sample size investigating mtDNA copy number in CRC patient tissues to date. Significantly lower mtDNA copy number was observed in carcinomas and also adjacent normal tissue compared to adenomas in CRC patients. Also, mtDNA copy number was significantly lower in carcinoma tissue of primary CRC patients compared to recurrent CRC patients. Regarding clinicopathological characteristics in carcinomas, the *BRAF* V600E mutation was associated with lower mtDNA copy number compared to wild-type *BRAF* V600E, and MSI was associated with lower mtDNA copy number compared to MSS carcinomas while *KRAS* mutation was associated with higher mtDNA copy number. Moreover, mtDNA copy number was associated with CRC-specific survival in a non-linear way, which seemed to follow an inverse U-shaped curve of HRs with increasing mtDNA copy number.

Colorectal adenoma tissue was significantly higher in mtDNA copy number than cancer- and adjacent normal tissue, which were resected later in life than the adenoma samples. This comparison was not made in any comparable studies, to our knowledge. A possible explanation for the observed high mtDNA copy number in adenomas is that adenomas are proliferating tissues with good oxygenation (37) whereas later carcinomas are mostly in a hypoxic state; downregulating aerobic glycolysis (38), which might affect mtDNA copy number and suggests that alterations in mtDNA copy number in carcinomas might be a consequence of tumor progression. MtDNA copy number was also significantly higher in carcinoma tissue of recurrent CRC patients compared to carcinoma tissue from primary CRC patients. Potentially primary tumor patients with higher mtDNA copy number more often survive long enough to develop a recurrent tumor. However, this is highly speculative since it is based on only 16 recurrent CRC patients for whom we do not have mtDNA copy number in the primary tumor tissue. The association with survival and progression is suggested because lower mitochondrial activity leads to lower oxidative stress levels which is an advantage for cancer progression since the tumor is protected from oxidative damage (39). In other studies, a decrease in activity of respiratory chain enzyme complexes in the mitochondria has been associated with a decrease in mtDNA copy number in tumor tissue, which may point to a role for anaerobic glycolysis in carcinoma tissue as discussed in more detail later (40,41).

Low mtDNA copy number in carcinomas has been associated with several clinicopathological characteristics such as increased tumor size and histological grade of tumors (17–21),

Table 3. Median mtDNA copy number within CRC tissue for several clinicopathological characteristics

	Hospital-based series			NLCS		
	n[a]	Median mtDNA copy number[b]	*P* value*	*n*[a]	Median mtDNA copy number[b]	*P* value*
Differentiation grade						
Poor	5	179 (115, 530)		98	344 (192, 655)	
Moderate	45	342 (217, 536)		424	409 (225, 839)	
Well	6	538 (178, 805)	0.357	66	396 (195, 767)	0.091
Sublocation						
Total colon	30	336 (203, 538)		—	—	
Proximal colon	—	—		222	331 (187, 688)	
Distal colon	—	—		211	441 (232, 801)	
Sigmoid	10	394 (130, 628)		—	—	
Rectosigmoid	9	285 (194, 498)		76	388 (197, 820)	
Rectum	6	505 (289, 762)	0.535	160	452 (257, 841)	0.024[c]
Familial history of CRC						
Yes	—	—		70	407 (210, 835)	
No	—	—	—	605	393 (217, 774)	0.889
BAT-26 unstable/MSI						
Yes	7	179 (106, 311)		77	250 (127, 484)	
No	47	383 (221, 534)	0.033	560	437 (231, 826)	<0.001
BRAF mutation						
Yes	8	251 (166, 302)		105	318 (163, 632)	
No	46	394 (205, 538)	0.027	555	425 (236, 830)	0.006
KRAS mutation						
Yes	—	—		237	482 (259, 844)	
No	—	—	—	441	368 (193, 756)	0.004
TNM stage						
1	9	360 (185, 517)		174	476 (248, 830)	
2	16	352 (186, 649)		221	384 (214, 752)	
3	22	329 (184, 473)		165	372 (190, 765)	
4	8	538 (218, 745)	0.480	85	332 (221, 652)	0.185
Extent of primary tumor						
T1	1	221 (-)		59	558 (319, 1059)	
T2	9	390 (201, 527)		139	434 (214, 825)	
T3	39	329 (194, 534)		394	374 (210, 751)	
T4	6	503 (182, 760)	0.759	46	345 (214, 686)	0.067
Regional lymph nodes						
N0	27	321 (179, 534)		384	419 (224, 798)	
N1	17	289 (167, 421)		160	368 (197, 723)	
N2+N3	11	507 (403, 729)	0.050	59	369 (203, 784)	0.838
Distant metastases						
M0	47	329 (189, 527)		349	419 (220, 756)	
M1	8	538 (218, 745)	0.215	85	332 (221, 652)	0.147

[a]Numbers do not always add up to total number of cases because of missing values.
[b]25th to 75th percentile range of mtDNA copy number is presented between parentheses.
[c]MtDNA copy number is significantly higher in rectal tumors compared to proximal colon tumors ($P = 0.010$).
*P values presented are for non-parametric comparative tests (Kruskal–Wallis, Mann–Whitney *U* test and Wilcoxon signed rank tests).

including studies in CRC (12,13), suggesting a role for mtDNA copy number in cancer progression. Studies investigating mtDNA copy number in CRC also observed a decrease of mtDNA copy number as disease stage progressed, although sample size was relatively small in these studies, ranging from 44 to 194 CRC cases (11–13). However, within this study no statistically significant decrease of mtDNA copy number by TNM stage or histological grade was found, although median mtDNA copy number seemed to decrease with increasing TNM stage and tumor extent within the NLCS sample. Although the numbers were small, mtDNA copy number in MSI tumors was significantly lower in poorly differentiated tumors compared to moderately differentiated tumors. Poorly differentiated tumors tend to grow and spread at a higher rate than averagely differentiated or well-differentiated tumors (42), emphasizing the possible involvement of mitochondrial alterations in malignant transformation. However, caution should be taken when interpreting the results from the hospital-based series since the numbers were small, the study was not set up *a priori* to investigate these associations and the study population possibly did not represent the general population of CRC patients because patients with stage II and III CRC appeared to be oversampled (Table 1).

MtDNA copy number was consistently lower in tumors with a *BRAF* V600E mutation or MSI and higher in *KRAS* mutated tumors. The prognostic role of *BRAF* mutation does not seem to be differential for MSI status in CRC (43) possibly explaining why mtDNA copy number was observed to be lower in both *BRAF* mutated tumors and MSI tumors. A *BRAF* V600E mutation is a predictor of worse prognosis (23), so it is in line with this study's hypothesis that mtDNA copy number was

Table 4. Crude and adjusted hazard ratios (HR) and 95% confidence intervals (CI) for colorectal cancer-related deaths according to quintiles of mtDNA copy number at 5 years of follow-up for three different Cox regression models from the NLCS study population

		MtDNA copy number quintile				
		1	2	3	4	5
	N at risk	133	128	130	133	131
	N fatal events	49	70	55	47	37
	Time at risk (person-years)	1,076	849	992	1103	1131
Unadjusted estimates	HR	1.00	1.70[a]	1.17	0.94	0.74
	(95% CI)	Reference	(1.18, 2.44)	(0.80, 1.72)	(0.63, 1.40)	(0.48, 1.12)
Age and sex adjusted estimates	HR	1.00	1.69[a]	1.17	0.94	0.74
	(95% CI)	Reference	(1.17, 2.44)	(0.79, 1.72)	(0.63, 1.40)	(0.48, 1.13)
Multivariable adjusted estimates	HR	1.00	1.33	1.12	1.03	0.63
Adjusted for age, sex, sublocation, differentiation grade, TNM stage and MSI	(95% CI)	Reference	(0.89, 1.99)	(0.74, 1.70)	(0.66, 1.62)	(0.40, 1.01)

[a]Statistically significant HR at $\alpha = 0.05$ level.

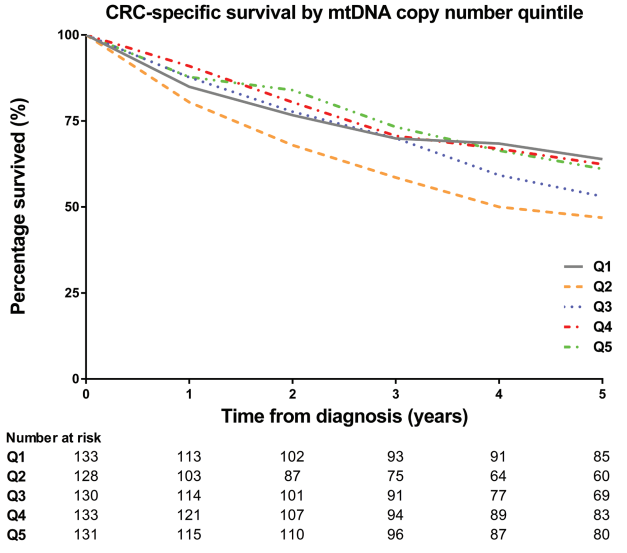

Figure 2. Kaplan–Meier curves showing 5-year CRC-specific survival per mtDNA copy number quintile within the NLCS series until 7.3 years of follow-up.

significantly lower in *BRAF* mutated tumors. Because *BRAF* mutation has been linked with several malignant responses such as cell proliferation and invasion (44,45) and since mitochondrial dysfunction is thought to be involved in cell migration in gastric cancer (46) it is most likely that mutated *BRAF* and mtDNA copy number are linked because they are both partially involved in altering the invasive properties of tumors. When considering MSI status, findings were not in accordance with our hypothesis; mtDNA copy number was lower in the MSI tumors compared to the MSS tumors even though MSI is known to be an indicator of favorable prognosis (47), although this might also be explained by MSI/*BRAF* combinations since there was a large overlap between *BRAF* mutated and MSI-high tumors In stratified analyses the difference in mtDNA copy number between MSI and MSS tumors was most profound in TNM stage 2 (data not shown). *KRAS* mutations are an established marker for epidermal growth factor (EGFR)-specific antibody therapy resistance but their application as an overall prognostic marker remains ambiguous (48). In addition to its association with *EGFR*, which is able to modulate mitochondrial function through COX-II (49), *KRAS* has been shown to be a possible regulator of mitochondrial metabolism through inducing hypoxia-inducible factors in colon cancer cell lines (50). The difference in association with mtDNA copy number as observed between *BRAF* and *KRAS* mutated tumors might be explained by the fact that mutated *BRAF* and *KRAS* genes are markers of distinct oncogenic pathways which is supported by the lack of co-occurrence of mutations in these genes (51,52). This was also shown in our sample where only 12 of the 105 *BRAF* mutated tumors showed a *KRAS* mutation.

Moreover, within the NLCS sample mtDNA copy number seemed to decrease with increasing tumor size, however not statistically significant, indicating that there might be an association between mtDNA copy number and tumor size. Since TNM stage 2 also marks the stage in which cancers invade the muscularis propria (28), alterations in mtDNA copy number might reflect processes which cause this malignant transformation. In addition to this, a 2012 publication by The Cancer Genome Atlas initiative concluded that MSI alone might not always be the best indicator of prognosis (53), thereby indicating that other factors (e.g. total mutation rate) also play an important role in prognosis and malignant transformation, underlining the importance of additional information in clinical settings. Our results indicate that more research on tumor metabolism might also add to this spectrum of prognostic markers in CRC.

MtDNA copy number was significantly associated with CRC-specific survival, but not linearly. Although a statistically significant HR of 0.72 was observed for a log-transformed mtDNA copy number, the association is more likely to follow an inverse U-shape. CRC patients with very high mtDNA copy number (in the highest quintile) were at the lowest risk of dying due to CRC. Patients in the second quintile of mtDNA copy number were markedly at the highest risk of dying due to CRC compared to the first quintile. This relatively large difference at the lower end of the mtDNA copy number distribution might point to different disease types for those with severely depleted mtDNA (quintile 1) versus those with low mtDNA (quintile 2). Indeed, patients in quintile 2 less frequently had TNM stage 1 tumors and more frequently had TNM stage 4 tumors than those in other quintiles. This implies that mtDNA copy number might be involved in determining the malignant potential of tumors. This association with disease-specific survival could be explained by the metabolic wave model which describes the interplay between energy metabolism, oncogenes and tumor progression as proposed by Jose *et al.* (54). This model defines four waves in which alterations in energy metabolism occur in cancer progression through alteration of several (onco)genes, such as tumor protein p53, proto-oncogenic transcription factor *Myc* and peroxisome proliferator-activated receptor gamma coactivator 1-alpha

(*PGC1α*). Alteration of these factors influences tumor growth and other malignant properties of tumors have also been related to mtDNA copy number and thus possibly explain the different distribution of TNM stages across mtDNA copy number quintiles. The significant associations between mtDNA copy number and CRC-specific survival are in line with findings in other studies investigating mtDNA copy number in relation to CRC survival (10,12,14).

Both studies have different strengths and limitations. In the hospital-based series the control subjects without CRC were included only if they had a colonoscopy at the hospital, possibly introducing selection bias for the comparison between normal colon tissue between cases and controls. Bias might have also been introduced in the hospital-based study by the overrepresentation of TNM stage 2 and 3 patients which probably occurred because of hospital-based sampling (Table 1), however because the variation of mtDNA copy number was large in all groups this might not have been too influential in the analyses regarding TNM stage. Also, due to this large variation in relatively small number of samples, the specificity of the mtDNA copy number assay seemed low compared to the NLCS sample. Although there were some limitations, most data on all subjects was gathered from a detailed and reliable hospital database. Within the population-based NLCS study, cases were significantly more equally distributed across TNM stages since cases were not selected but accumulated over time within the cohort. Furthermore, most other studies investigating mtDNA copy number compare the tumor mtDNA copy number to paired histologically normal samples to normalize the distribution of mtDNA copy number (10–14). This comparison was impossible to make with the available sets of samples, since extra biopsies would have to be done to obtain paired normal samples further away from primary tumors. This might also explain why several studies consistently observed statistically significant differences in mtDNA copy number between TNM stages (10–14) in contrast to our findings. However, it has also been observed that mtDNA copy number is lower in later TNM stages only if a mtDNA 4977-bp deletion is present in CRC, possibly because the impairment of mitochondrial function associated with this deletion (11). Even though the associations in this study between TNM stage and mtDNA copy number were weak, significant results were observed regarding CRC-specific survival and mtDNA copy number.

In conclusion, our results suggest that mtDNA copy number alterations might reflect different malignant processes, possibly involving tumor growth and invasiveness of the tumor cell at several disease stages. Although mtDNA copy number is associated with several clinicopathological properties and CRC-specific survival, its usefulness as a biomarker in patient care is limited due to the large interpersonal range in mtDNA copy number scores. However, when investigated in combination with relevant oncogenes and energy metabolism proteins, it can reflect metabolic pathways in cancer cells involved in prognosis and possibly etiology. Although the role of mtDNA copy number as either a driver or consequence of carcinogenesis needs to be specified further, mtDNA copy number should further be investigated in relation to metabolic pathways which can be targeted in cancer therapy such as the targetable mammalian target of rapamycin (mTOR) pathway and hypoxia-inducible factor-1-α (HIF1α) (55,56) because of their apparent association with several malignant properties of CRC. Identifying a metabolic profile of CRC tumors associated with a low mtDNA copy number could provide interesting new insights in the metabolic processes involved in CRC prognosis.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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