

Expression of Uncoupling Protein-2 in Human Colon Cancer

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ABSTRACT

Purpose: Cancer cell survival depends on adaptive mechanisms that include modulation of oxidative stress. One such mechanism may be via up-regulation of uncoupling protein-2 (UCP2), a mitochondrial inner membrane anion carrier recently found to provide cytoprotection in nontumor cells by acting as a sensor and negative regulator of reactive oxygen species production. We hypothesized that UCP2 expression may be increased in colon cancer as part of tumor adaptation.

Experimental Design: UCP2 expression was characterized by real-time polymerase chain reaction and Western blotting using paired human colon adenocarcinoma and peritumoral specimens. Oxidant production was characterized by tissue malondialdehyde levels. Tissue microarrays constructed of 107 colon adenocarcinomas as well as representative specimens of hyperplastic polyps and tubular adenomas were used for UCP2 immunohistochemistry.

Results: UCP2 mRNA and protein levels were 3- to 4-fold higher in adenocarcinomas, and UCP2 mRNA levels showed significant correlation with increased tumor tissue malondialdehyde contents. Immunohistochemistry on tissue microarrays showed positive staining for UCP2 in most adenocarcinomas (86.0%); positive staining for UCP2 was seen less often in tubular adenomas (58.8%) and rarely seen in hyperplastic polyps (11.1%).

Conclusions: UCP2 expression is increased in most human colon cancers, and the level of expression appears to correlate with the degree of neoplastic changes. These findings may foster the idea that UCP2 is part of a novel adaptive response by which oxidative stress is modulated in colon cancer.

INTRODUCTION

Tumor growth in general results from an imbalance between cell proliferation and cell attrition (1). Adaptive mechanisms in cancer cells include resistance to growth inhibition and evasion of apoptosis, cellular events appreciably affected by oxidative stress (2, 3). Reactive oxygen species (ROS) primarily originate in mitochondria during oxidative phosphorylation (4). Uncoupling protein-2 (UCP2) is a recently identified mitochondrial inner membrane anion carrier, which is emerging as a negative regulator of ROS production (5). In various cell types, UCP2 acts as a sensor of mitochondrial oxidative stress and may be activated by superoxide (6, 7) or by subsequently formed lipid peroxidation products (8). Loss of UCP2 function may result in increased generation of ROS (5, 9), whereas UCP2 overexpression conveys cytoprotection to various tissues by limiting oxidative injury (10, 11). Consequently, UCP2 appears to be an essential component of a local feedback mechanism controlling the production of mitochondrial ROS. We hypothesized that increased expression of UCP2 is one of the adaptive mechanisms that result from oxidative stress in cancer cells. In this study, we provide the initial characterization of UCP2 expression in normal and neoplastic human colon tissue including various stages of the adenoma–carcinoma sequence of colorectal carcinogenesis.

MATERIALS AND METHODS

Patients and Tissue Specimens. Tissue materials for this study originated from two different cohorts: (a) After sufficient tissue was taken for diagnostic purposes, mucosa from 10 colon tissue specimens obtained from surgical interventions on patients diagnosed with colon adenocarcinoma at Rhode Island Hospital (RIH) was macroscopically divided into tumor-containing portions and adjacent normal-appearing portions, snap-frozen for additional studies, and stored in an anonymous tissue bank. No further clinicopathological data were available in these cases. (b) Immunohistochemical evidence of UCP2 expression was also determined on 9 representative archival cases of hyperplastic polyps, 17 cases of tubular adenoma, and on tissue microarray slides consisting of colon cancer tissue from 107 consecutive tumor-node-metastasis (TNM) stage II patients retrieved nonselectively from the archives of the RIH Department of Pathology between the years of 1983 and 1994. The mean age of these latter patients at initial surgery was 72 years (range, 31–96 years), and 50 males and 57 females were included in this study. No rectal or rectosigmoid cases were included. The degree of tumor differentiation as defined by the American Joint Committee on Cancer (12) was as follows: 18 high-grade cases and 89 low-grade cases; and 21 mucinous cases and 86 nonmucinous cases. Evidence of lymphatic or vascular invasion was present in 10 cases. The mean duration of follow-up was 96 months (range, 1–216 months). Nineteen patients had recurrent disease (6 had local disease; 13 had distal metastases). Seventy-two patients died during the follow-up period; 18 died of colon cancer, and 45 died of unrelated causes,

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whereas the cause of death for 10 patients was unclear. This study was approved by the RIH Institutional Review Board.

Tissue Microarray Construction. Paraffin blocks containing areas consisting of pure invasive carcinoma were identified on corresponding hematoxylin and eosin-stained sections. Areas of interest that represented the “invasive front” of the tumor, rich in nonnecrotic tumor glands, were identified and marked on the source block. The source block was cored, and a 1-mm core was transferred to the recipient “master block” using Beecher Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). Three to six cores were arrayed per specimen. Representative sections of normal colon were taken from approximately 30% of the cases. At least three cores were scored per case. The analysis of three cores per case has been shown to be comparable with the analysis of the whole section in a recent study (12). The vast majority of cases exhibited a uniform degree of staining between all cores, and in those that did not, an average score was determined. All sections were scored independently by J. R. and M. B. R. without knowledge of the clinicopathological features or clinical outcome. There was a high level of correlation between the two scorers, and in the few discrepant cases a consensus was reached after joint review.

Immunohistochemistry. Tissue slides were stained with goat polyclonal anti-UCP2 antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using peroxidase (Vector Laboratories, Burlingame, CA). Negative control slides were treated in an identical manner with goat IgG (Vector Laboratories) instead of primary antibodies. The intensity of UCP2 staining was scored as negative (0), weak (+1), moderate (+2), or strong (+3).

Real-Time Polymerase Chain Reaction Amplification.

After extraction of total RNA from snap-frozen colonic surgical specimens with TRIzol reagent (Invitrogen, Carlsbad, CA) and removal of contaminating genomic DNA with DNase I, RNase-free (Roche Diagnostics Corp., Indianapolis, IN) reverse transcription was performed using first-strand cDNA synthesis kit (Roche Diagnostics Corp.). PCR reactions were performed using an iCycler iQ Multi-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The relative kinetic method was applied using separate standard curves for UCP2 and 18S rRNA genes. The pBSK(-) plasmid containing a full-length human UCP2 cDNA was a gift of Drs. Chen-Yu Zhang and Brad Lowell (Harvard Medical School, Boston, MA). The reference pCR2.1 plasmid containing a 495-bp sequence of human 18S rRNA was a gift of Dr. Phillippe Merle (Brown Medical School, Providence, RI). Sample cDNAs (equivalent of 5 ng of total RNA) and serial dilutions of the UCP2 and 18S rRNA plasmids were used as template with the following primers: UCP2, 5'-GGTGGTCGGAGATACCAAAG (sense) and 5'-CTCGGGCAATGGTCTTGTAG (antisense); and 18S rRNA, 5'-GGACACGGACAGGATTGACA (sense) and 5'-ACCCACGGAATCGAGAAAGA (antisense). Thermal cycling conditions included an initial step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Length of PCR products for UCP2 and 18S rRNA was 51 and 50 bp, respectively. Each sample was normalized using its 18S rRNA content as endogenous reference, and data are given as relative abundance of UCP2 mRNA over 18S rRNA.

Immunoblotting. Sample preparation for immunoblotting was performed essentially as described previously (13). Equal amounts of protein were size-fractionated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Perkin-Elmer Life Sciences, Boston, MA), and immunoblots were developed using anti-UCP2 antibody (C-20; Santa Cruz Biotechnology). Whole tissue lysates obtained from spleens of wild-type and *ucp2*^{-/-} mice (14) were used as positive and negative control, respectively. Equal loading was confirmed using β -actin antibody (Sigma, St. Louis, MO). The membranes were incubated with secondary goat antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology), and an enhanced chemiluminescence kit (Perkin-Elmer Life Sciences) was used for visualization.

Assessment of Oxidative Stress. The colonic tissue content of malondialdehyde (MDA), a terminal product of lipid peroxidation, was measured by the thiobarbituric acid reduction method using a commercially available kit (Oxis Research, Portland, OR).

Statistical Analysis. Data are expressed as mean \pm SE. Associations between categorical groups were evaluated using the χ^2 or Fisher's exact test, when appropriate. The influence of prognostic factors on tumor-related recurrence as well as survival was assessed by Kaplan-Meier estimates, and subgroups were compared by the log-rank test for univariate analysis. The multivariate Cox's proportional hazard model was applied using a stepwise forward method to detect independent predictors of recurrence/survival. When appropriate, linear regression and Pearson *r* correlation were calculated. Two-tailed *P* values of ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We assessed the expression of UCP2 mRNA in colonic mucosa from paired surgical specimens of patients undergoing resection for colon adenocarcinoma by quantitative real-time PCR. As shown in Table 1, UCP2 mRNA levels were several-fold higher in the tumor mucosa when compared with the grossly normal peritumoral colonic mucosal specimens used as control (3.55 ± 0.85 ; $n = 10$). Because UCP2 is under considerable translational regulation (15), we also assessed the amounts of UCP2 protein (Fig. 1) and found similar tumor to peritumoral tissue (T/P) ratios when quantifying the Western blots by densitometry (3.94 ± 0.91 ; $n = 8$). There was a strong linear correlation between the T/P ratio of UCP2 mRNA and protein expression ($r = 0.91$; $P = 0.0015$), suggesting that increased UCP2 expression in colon adenocarcinoma is largely determined at the transcriptional level.

Because expression of UCP2 has been associated with ROS levels in various tissues (10, 11), we decided to analyze the relationship between increased UCP2 expression and oxidative stress in the colonic cancer tissue. We assessed the amount of oxidants in mucosal specimens from colon adenocarcinoma and peritumoral regions by measuring tissue levels of MDA, a marker of lipid peroxidation. Table 2 indicates that we found increased MDA levels in colon adenocarcinomas compared with peritumoral regions with a T/P ratio of 2.13 ± 0.40 ($n = 10$). A statistically significant, positive correlation was found between MDA levels and expression of UCP2 mRNA in these colon

Table 1 UCP2 mRNA expression by quantitative real-time PCR in paired surgical specimens of human colon adenocarcinoma and peritumoral tissue

Patient no.	Tissue source	UCP2 mRNA (relative abundance, $\times 10^{-7}$)	T/P ratio
1	T	7.28	1.38
	P	5.26	
2	T	13.56	4.43
	P	3.06	
3	T	22.12	2.40
	P	9.20	
4	T	53.14	3.47
	P	15.30	
5	T	25.41	1.83
	P	13.89	
6	T	181.72	9.73
	P	18.68	
7	T	62.04	3.57
	P	17.30	
8	T	63.10	0.74
	P	85.58	
9	T	54.10	6.05
	P	8.94	
10	T	3.75	1.85
	P	2.02	
Average T/P ratio			3.55

NOTE. UCP2 mRNA values were normalized to 18S RNA. Abbreviations: T, tumor; P, peritumoral.



Fig. 1 UCP2 protein expression in human colon cancer. A, representative immunoblots of UCP2 expression detected in whole tissue lysates from paired surgical specimens of colon adenocarcinoma (T) and peritumoral tissue (P). +, positive control; -, negative control (whole tissue lysates were obtained from spleens of wild-type and *ucp2*^{-/-} mice, respectively). Western blot analysis was performed using specific antibody against UCP2 (C-20; Santa Cruz Biotechnology). β -Actin was used as loading control.

tumor specimens ($r = 0.65$; $P = 0.04$), but the association between MDA levels and UCP2 protein expression was not significant ($r = 0.60$; $P = 0.11$; Fig. 2). Although these data link UCP2 expression to ROS production in tumor cells, the cause and effect relationship remains unclear. Keeping in mind the relatively small number of cases, lack of a correlation between MDA and UCP2 protein levels may also result from efficient control of ROS production at higher UCP2 levels. Additional studies in which UCP2 expression is experimentally altered will be needed to definitively answer this question.

To further characterize UCP2 protein expression in the human colon, we applied immunohistochemistry to colon tissue sections including nonneoplastic hyperplastic polyps, neoplastic tubular adenomas, and a tissue microarray set of colonic adenocarcinomas. The epithelium of normal colon and hyperplastic polyps was negative for UCP2 expression (Fig. 3), although occasional weak epithelial immunopositivity was seen in the

superficial mucosal layers. Scattered UCP2-positive cells were seen in the stroma without a particular distribution pattern. Some of the UCP2-positive stromal cells were morphologically consistent with macrophages, as illustrated by the positive staining of tingible body macrophages seen in Fig. 3D. In contrast, strongly positive staining for UCP2 with a diffuse distribution pattern was identified throughout the mucosa in most tubular adenomas and adenocarcinomas (Fig. 3A and B). These findings indicate that colonic epithelium is the primary source of increased UCP2 expression in colon cancer. As shown in Table 3, whereas colonic UCP2 expression was rare in hyperplastic polyps, about half of the tubular adenomas and the vast majority

Table 2 MDA content in paired surgical specimens of human colon adenocarcinoma and peritumoral tissue

Patient no.	Tissue source	MDA (nmol/mg tissue)	T/P ratio
1	T	3.301 \pm 0.018	2.46
	P	1.341 \pm 0.018	
2	T	1.808 \pm 0.009	2.12
	P	0.852 \pm 0.014	
3	T	2.871 \pm 0.008	3.92
	P	0.732 \pm 0.010	
4	T	1.114 \pm 0.010	1.26
	P	0.887 \pm 0.010	
5	T	2.238 \pm 0.013	0.75
	P	2.967 \pm 0.018	
6	T	3.708 \pm 0.214	3.96
	P	0.935 \pm 0.014	
7	T	1.939 \pm 0.016	1.63
	P	1.186 \pm 0.003	
8	T	0.529 \pm 0.006	0.39
	P	1.341 \pm 0.013	
9	T	3.421 \pm 0.066	3.24
	P	1.055 \pm 0.009	
10	T	1.091 \pm 0.000	1.42
	P	0.768 \pm 0.005	
Average T/P ratio			2.13

NOTE. Data \pm SE from three separate measurements. Abbreviations: T, tumor; P, peritumoral.

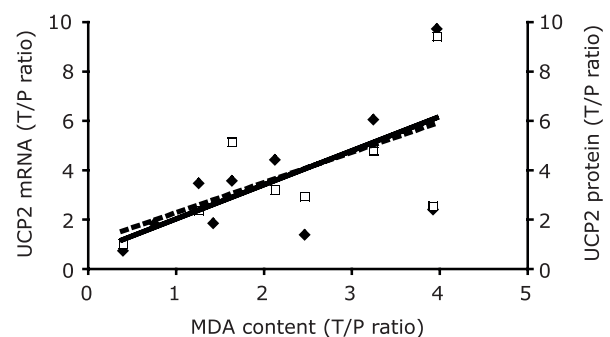


Fig. 2 Association between UCP2 expression and oxidative stress levels in human colon cancer. Tissue MDA levels plotted against expression levels of UCP2 mRNA (\blacklozenge) and protein (\square) as measured in paired surgical specimens of colon adenocarcinoma and peritumoral tissue and expressed as T/P ratios. Linear regression and Pearson r correlation were performed. Solid line, UCP2 mRNA versus MDA, $r = 0.65$, $P = 0.04$. Dotted line, UCP2 protein versus MDA, $r = 0.60$, $P = 0.11$.

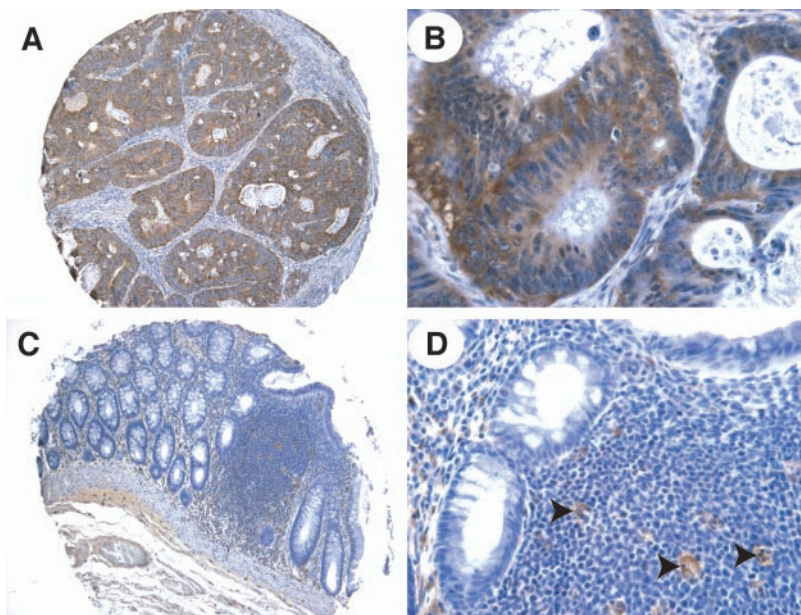


Fig. 3 Immunohistochemical assessment of UCP2 expression in human colon cancer. Representative photomicrographs of tissue microarrays featuring colon adenocarcinoma (A and B) and normal colonic mucosa (C and D) stained for UCP2 (C-20; Santa Cruz Biotechnology). Sections were counterstained with hematoxylin. Several macrophages that stained positive for UCP2 in normal colonic mucosa are marked with *arrowheads*. Magnification: $\times 100$ (A and C) and $\times 400$ (B and D).

of adenocarcinomas stained positive for UCP2, suggesting that UCP2 expression may intensify along the adenoma–carcinoma sequence. However, analysis of patient survival data and histologic grades of colon cancer in conjunction with the intensity of UCP2 staining showed no statistically significant correlation (data not shown).

Very few studies have addressed the potential role of UCP2 in carcinogenesis and the experimental data are somewhat controversial. Protein microarray profiling of LoVo human colon carcinoma cells treated with ionized radiation indicated moderately increased levels of UCP2 compared with untreated controls (16). Similarly, DNA microarrays after radiation of an apoptosis-sensitive lymphoma cell line revealed increased amounts of UCP2 transcripts when compared with an apoptosis-resistant subline (17). The authors of these reports proposed that the decrease in mitochondrial membrane potential mediated by UCP2 action may contribute to activation of cell death pathways. A recent multilaboratory effort on characterizing the energy metabolism and survival strategy of tumor cells offers a different conclusion. Drug-resistant sublines of various tumor cell lines were found to have increased expression of UCP2 with lower mitochondrial membrane potential and diminished susceptibility to oxidative stress (18). According to these observations, tumor cells may use UCP2 in their metabolic adaptation to avoid ROS-mediated apoptosis. Evidence that UCP2 may serve a cytoprotective role is certainly mounting in nontumor cells.

Overexpression of UCP2 was shown to reduce neuronal cell death in transgenic mice and in cell culture on exposure to hypoxia and glucose deprivation, coinciding with a decrease in net mitochondrial ROS formation and absence of extensive mitochondrial depolarization (10). Similarly, overexpression of UCP2 in cultured cardiomyocytes limited mitochondrial ROS production and suppressed the loss of mitochondrial membrane potential elicited by H_2O_2 treatment (11). These findings suggest that UCP2 overexpression may protect a wide array of cell types from apoptosis and that the cytoprotective effect of UCP2 is likely based on reduction of mitochondrial ROS generation.

It has been known for over 70 years that glycolysis is the preferred energy-producing pathway in rapidly growing cancer cells, while their mitochondrial respiration is diminished (19). Changes in cancer cell bioenergetics are often associated with more aggressive tumor growth and drug resistance, resulting in worse prognosis (20, 21). It is possible that the primary goal of this metabolic switch in cancer cells is to steer away reducing equivalents from the mitochondria to limit ROS generation in that organelle. UCP2 acts in nontumor cells to their benefit (10, 11), and it may well serve a similar purpose in cancer. Negative control of ROS production may therefore take precedence over efficiency of oxidative phosphorylation.

In summary, we have demonstrated that UCP2 expression is increased in human colon cancer and correlates with the degree of neoplastic changes. Our data leave the link between

Table 3 Immunohistochemistry scores of UCP2 expression in human colon tissue exhibiting various stages of neoplastic changes

Histology	n	Negative	Weak (1+)	Moderate (2+)	Strong (3+)	% Positive
Hyperplastic polyps	9	8	1	0	0	11.1
Adenomas	17	7	2	2	6	58.8
Adenocarcinomas	107	15	32	36	24	86.0

UCP2 expression and oxidative stress in colonic tumor tissue ambiguous. However, in light of the bidirectional relationship recently evidenced between UCP2 and mitochondrial ROS production, it is tempting to speculate that this mitochondrial carrier molecule is more than another marker of ROS production in cancer cells and may serve as an adaptive tool to reduce oxidative stress.

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