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Markers of Mitochondrial Metabolism in Tumor Hypoxia, Systemic Inflammation, and Adverse Outcome of Rectal Cancer[1,2]

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Abstract

Tumor hypoxia contributes to therapy resistance and metastatic progression of locally advanced rectal cancer (LARC). We postulated that the tumor mitochondrial metabolism, manifested by reactive oxygen species (ROS) and mitochondrial DNA (mtDNA) damage, reflects how hypoxic conditions connect to cancer-induced systemic inflammation and poor outcome. Levels of ROS and mtDNA damage were analyzed in three colorectal cancer (CRC) cell lines cultured for 24 hours under normoxia (21% O_2) or hypoxia (0.2% O_2) and serum sampled at the time of diagnosis from 35 LARC patients participating in a prospective therapy study. Compared with normoxia, ROS were significantly repressed and mtDNA damage was significantly enhanced in the hypoxic CRC cell lines; hence, a low ratio of ROS to mtDNA damage was an indicator of hypoxic conditions. In the LARC patients, low serum ROS were associated with elevated levels of circulating carcinoembryonic antigen and tumor choline concentration, both indicative of unfavorable biology, as well as adverse progression-free and overall survival. A low ratio of ROS to mtDNA damage in serum was associated with poor local tumor response to the neoadjuvant treatment and, of note, elevated systemic inflammation factors (C-reactive protein, the interleukin-1 receptor antagonist, and factors involved in tumor necrosis factor signaling), indicating that deficient treatment response locally and detrimental inflammation systemically link to a hypoxic mitochondrial metabolism. In conclusion, serum ROS and damaged mtDNA may be markers of the mitochondrial metabolism driven by the state of oxygenation of the primary tumor and possibly implicated in systemic inflammation and adverse outcome of LARC.

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Introduction

Colorectal cancer (CRC) is a heterogeneous disease of high molecular complexity [1] and, accordingly, therapeutic response disparities that necessitate individualized treatment [2]. In this context, the impact of tumor hypoxia must be taken into consideration since it constitutes one of the main mechanisms of tumor resistance to cytotoxic therapy (radiation and chemotherapy) [3] and is significantly correlated to metastatic disease progression [4]. Of particular note, a hypoxic tumor microenvironment supports protumor inflammatory responses and enhances the immune tolerance [5]. At the molecular level, hypoxia drives the tumor metabolism through alteration of oxygen-sensitive regulatory mechanisms, ultimately leading to increased glycolysis [6,7]. Additionally, the malignant phenotype promotes aerobic glycolysis because of diminished mitochondrial oxidative phosphorylation, the phenomenon known as the Warburg effect [8]. Collectively, the mitochondrial function is essential in balancing enhanced energy needs with substrate generation for biogenesis in rapidly growing tumor cells within a hypoxic microenvironment.

The partial reduction in tissue molecular oxygen produces reactive oxygen species (ROS) [9], of which about 90% can be traced back to the mitochondrial respiratory chain [10]. ROS levels are higher in malignant than in normal cells, which are a reflection of the hypermetabolic state of the former but also the selection of cells with augmented mitochondrial ROS (as a result of the lower requirement for molecular oxygen) during tumorigenesis [11]. ROS are central mediators in mitotic, angiogenic, and T-lymphocyte signaling in cancer [5,12]. However, malignant cells with stemness-like properties are vulnerable to excessive ROS [13]; thus, certain tumor cell populations may die from the same ROS context in which other populations thrive.

The human mitochondrial DNA (mtDNA) is a ~16.6-kilobase circular molecule encoding subunits of the enzyme complexes that drive oxidative phosphorylation. Due to its close proximity to the high energy-converting reactions, mtDNA is predisposed to oxidative damage [14]. Yet, considering the maintenance of mtDNA integrity, recent research has indicated a diverse repertoire of oxidative mtDNA insults and repair mechanisms [15]. Interestingly, mtDNA homeostasis is not restricted to the organelle, as mtDNA has been identified extramitochondrially as well as extracellularly. As shown in experimental models, the relocalization into cytosol rises under hypoxia [16] and is capable of inducing a cytokine response [17,18]. While this particular process is ROS dependent [17], extracellular mtDNA release may occur independently of ROS [19]. In the circulation, cell-free mtDNA following cellular injury contributes to elicit the systemic inflammatory response [20–22].

The natural disease course of locally advanced rectal cancer (LARC), frequently growing as bulky tumors with predominant hypoxic regions within the pelvic cavity, makes this entity an expedient model for investigating attributes of tumor hypoxia that may be implicated in the systemic inflammation of therapy resistance and metastasis. Following local treatment, commonly consisting of pelvic chemoradiotherapy before surgical resection of the residual tumor, the histological tumor response is disparate. Moreover, metastatic progression beyond the pelvic cavity is a dominant cause of treatment failure, typically reported for 30%-40% of patients in recent clinical trials [23,24].

We hypothesized that components of the tumor mitochondrial metabolism, specifically in terms of ROS and mtDNA damage, might be retrieved in the circulation of LARC patients as indicators of tumor hypoxia and its potential implication in cancer-induced systemic inflammation and adverse outcome. Prior to analysis of patient samples, the methods were established in a pertinent experimental setting using CRC cell cultures kept under normoxic and hypoxic conditions. Of particular note, we expanded the application of an in-house–developed high-resolution method that measures the proportion of damaged mtDNA-mediated inhibition of restriction enzyme cleavage of total mtDNA [25,26] into the first-time use in serum specimens, collected from LARC patients at the time of diagnosis. The patients had long-term follow-up after treatment of the primary tumor with curative intent but with metastatic failure as a progression event in a number of cases.

Materials and Methods

Cell Cultures

The human CRC cell lines HCT-116, HT-29, and LoVo were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Gibco by Life Technologies, Grand Island, NY) and 2 mM L-glutamine (GE Healthcare–PAA Laboratories, Pashing, Austria). The cell lines were routinely tested and found free of mycoplasma infection, and identity was validated by short tandem repeat analysis. Prior to experiments, 1.0-1.5 × 10^6 cells (depending on the cell line) were seeded in T25 flasks and allowed to adhere for 24 hours in a humidified incubator containing 5% CO_2 before incubation for 24 hours under normoxic (21% O_2) or hypoxic (0.2% O_2) conditions, the latter obtained using the Invivo$_2$ 300 hypoxic chamber (Ruskinn Technologies, Leeds, UK). For ROS analysis, cell medium was collected, and cells were lysed by sonication in 500 µl PBS before the samples were stored at −80°C until analysis. For mtDNA damage analysis, cells were lysed in PBS containing proteinase K (diluted 1:10) before DNA isolation was undertaken as described below.

Ethics Approval and Consent to Participate

The two rectal cancer studies were approved by the Institutional Review Board and Regional Committee for Medical and Health Research Ethics of South-East Norway (reference numbers REK S-05059 and REK 2013/152) and were in accordance with the Helsinki Declaration. Written informed consent was required for participation.

The LARC-RRP Study

The patient population within the current report, which is from a prospective therapy study in rectal cancer (ClinicalTrials.gov NCT00278694), was enrolled from the 5 October 2005 to the 12 December 2009. Patient eligibility criteria, evaluation procedures, and review procedures of follow-up have been detailed previously [27]. Of particular note in the context of the mitochondrial metabolism, patients with diabetes mellitus were ineligible for study participation because of the significant risk of enhanced diabetes-induced sensory neuropathy by oxaliplatin [28], one of the study medications. Among the pleiotropic molecular mechanisms of the antidiabetic biguanide metformin, commonly prescribed for type 2 diabetes, is mitochondrial complex 1 inhibition [29]. For study-specific serum preparation at patient enrolment, blood was drawn in plain serum tubes with no additives for centrifugation to separate serum, which was left on ice for no more than 1 hour before storage at −80°C. Before the analyses for the current report, serum samples were centrifuged one more time (2000×*g* for 15 minutes) following

thawing. Patients were given neoadjuvant treatment consisting of short-course induction chemotherapy and sequential long-course chemoradiotherapy, both modalities containing oxaliplatin, before surgery 5-13 weeks after its completion [27]. The resected tumor specimens were histologically evaluated for the immediate local treatment response according to standard ypTN staging. In this patient population of mainly T3-4 stage disease, ypT0-2 outcome was considered as good response and ypT3-4 results as poor tumor shrinkage. Moreover, histological response was graded within one of five tumor regression grade (TRG) categories [30], where TRG1-2 was regarded as good histological regression and TRG3-5 as poor response. Regarding long-term progression-free survival (PFS; all PFS events were metastatic progression) and overall survival (OS), data were censored on the 8 August 2013, at which time 83% of study cases had been followed 5 years after enrolment.

The OxyTarget Study

Participants of this prospective biomarker study in rectal cancer (ClinicalTrials.gov NCT01816607), at present with immature outcome data, donated both whole blood on PAXgene Blood DNA Tubes (Qiagen Norge, Oslo, Norway), stored at −80°C, and serum at study enrolment. Serum was collected and processed in the same manner as described above.

ROS Assessment

Levels of ROS were determined applying the 96-well OxiSelect *In Vitro* ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA), according to the manufacturer's manual, essentially by the ability to oxidize dihydrochlorofluoroscein into a fluorescent derivative. Duplicate samples of cell lysate and medium (50 μl) and patient serum (25 μl), diluted 1:4 in PBS, were analyzed. For each sample, the mean value (obtained in nanomolar concentrations for the serum specimens) was used for downstream analyses.

Assessment of Total Antioxidant Capacity (TAC)

Serum TAC was determined applying the OxiSelect Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc.), according to the manufacturer's manual, by the ability of copper (I), as reduced from copper (II) by antioxidants, to react with a chromogenic reagent. Duplicate serum samples (20 μl), diluted 1:4 in PBS, were analyzed to provide concentrations in terms of copper-reducing equivalents, being proportional to the total reductive capacity.

Quantification of mtDNA Damage

This assay relies on the ability of a modification on the template DNA to inhibit restriction enzyme cleavage, as detailed previously [26]. Total DNA was isolated from cultured CRC cell lines, patients' whole blood (50 μl), or serum samples (50 μl) using the DNeasy Blood & Tissue Kit (Qiagen Norge) according to the manufacturer's instructions. Because this kit purifies total DNA, the amount of isolated nuclear DNA versus mtDNA was estimated by quantitative PCR analysis using primers for a nuclear gene (NDUFA9) and the mtDNA gene of interest, revealing 4- to 11-fold higher amounts of the latter in serum samples (not shown). A sequence flanking a TaqI restriction enzyme site in the 12S ribosomal RNA gene (MT-RNR1) was amplified using the forward (5′-AAACTGCTCGCCAGAACACT-3′) and reverse (5′-CATGGGCTACACCTTGACCT-3′) primers in the absence and presence of the enzyme. For quantitative PCR analysis, applied both for cell lines and patient samples, the resulting ΔC_t was calculated as $C_t^{TaqI} - C_t^{nd}$, with C_t^{TaqI} and C_t^{nd} corresponding to values of TaqI-digested and nondigested mtDNA, respectively. The frequency of mtDNA damage (i.e., resistant TaqI sites) was given by $2^{\Delta Ct}$. For the droplet digital PCR (ddPCR), applied in analysis of 29 LARC-RRP serum specimens, samples were partitioned by the QX200 Droplet Generator (Bio-Rad Laboratories, Oslo) and analyzed with the QX200 Droplet Reader (Bio-Rad Laboratories). Data were given as the percentage of nondigested mtDNA [($mtDNA^{TaqI}$ copies per μl − $mtDNA^{nd}$ copies per μl) × 100]. For these specimens, measures by quantitative PCR and ddPCR were correlated (r = 0.59; P = .001; by Pearson product correlation). The latter data set, representing the first-time application of ddPCR in combination with the particular method to measure mtDNA damage, was used in statistical analyses.

Data Normalization

Measured ROS levels showed significant interexperimental variations in the cell line studies. Thus, for ROS fluorescence and hence the ratios of ROS to mtDNA damage, the value of each data point was normalized with respect to the global mean of the respective setup, i.e., within each setup calculated as (ROS / global mean) × 100 and (ROS to mtDNA damage / global mean) × 100, respectively. Similarly, for the serum measures of ROS to mtDNA damage ratios, normalized data were used in downstream analyses.

Analysis of Other Circulating Factors

Routine blood tests were done within the standard patient workup. Exploration of selected inflammation factors in the study-specific serum samples was undertaken with a customized Luminex Multiplex Assay (R&D Systems, Minneapolis, MN).

Analysis of Tumor Metabolites

Tumor tissue was sampled at study enrolment as minuscule superficial biopsies endoluminally (i.e., by endoscopy), away from hypoxic tumor components invading beyond the bowel wall. The biopsies were analyzed by high-resolution magic angle spinning magnetic resonance spectroscopy, as detailed previously [31], to determine tissue concentration of 10 metabolites.

Statistical Analysis

Analyses were performed using IBM SPSS Statistics for Mac version 25.0 or GraphPad Prism version 7.0d. Groups were compared by Student's t test. Correlations were determined by Pearson product correlation analysis after logarithmic transformation. PFS was calculated from the time of study enrolment to the date of distant metastasis, death of any cause, or end of follow-up, whichever occurred first. OS was measured from the date of enrolment to death of any cause or final censoring. Associations between variables related to ROS and mtDNA damage and either PFS or OS were analyzed with univariate Cox proportional hazards models, and results were presented as hazard ratio (HR) with 95% confidence interval (CI). All tests were two-sided. P values of less than .05 were considered statistically significant. The Benjamini-Hochberg method was applied, as appropriate, to account for the false discovery rate from multiple testing.

Results

ROS in CRC Cell Cultures

The HCT-116, HT-29, and LoVo cell lines were incubated under normoxia or hypoxia for 24 hours, and ROS were assessed.

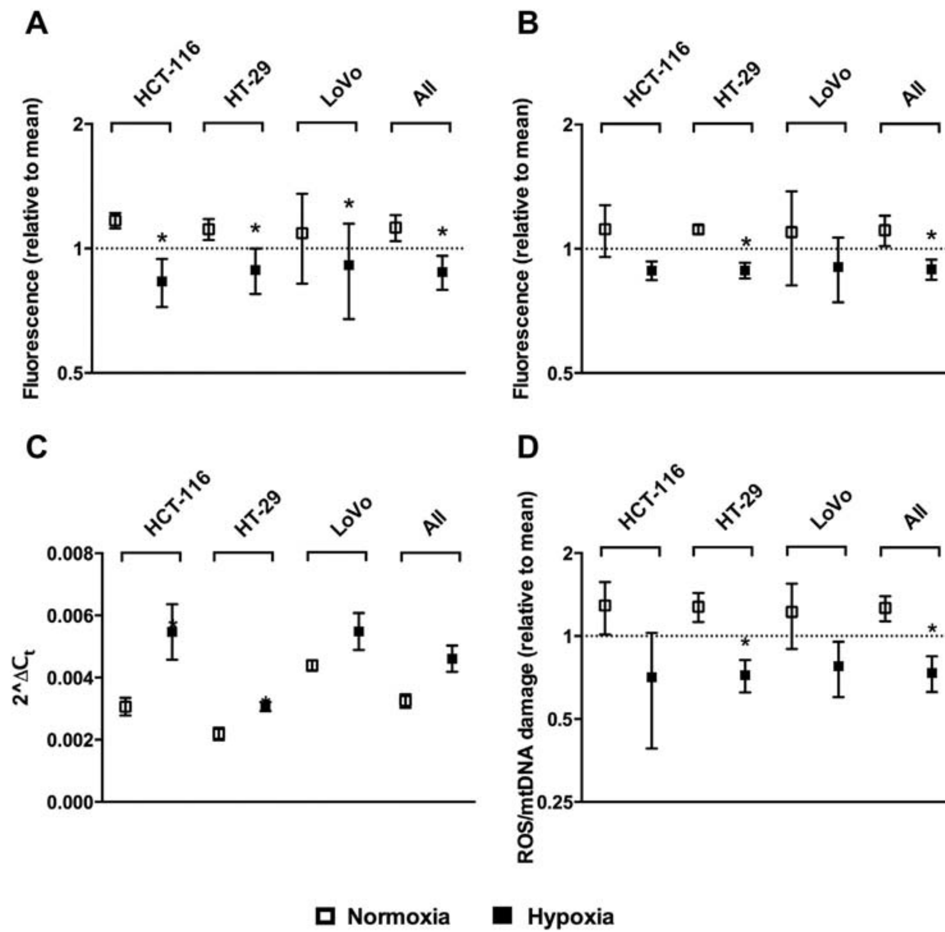

Figure 1. ROS and damaged mtDNA in normoxic and hypoxic colorectal cancer cell lines. Three cell lines were incubated for 24 hours and analyzed. ROS concentrations were measured in (A) cell lysate and (B) medium. (C) Levels of mtDNA damage were measured in cell lysate. (D) Ratios of ROS to mtDNA damage were calculated, applying panels A-C values. Normalized values are shown as mean ± standard error of the mean from three independent experiments, each performed in triplicates, for all conditions. All values for normoxic and hypoxic cells, respectively, from each set of experiments pooled together are also shown. $^*P < .05$ on comparison of hypoxic with corresponding normoxic conditions.

Compared with the normoxic condition, the ROS level was significantly reduced in lysate from all of the three cell lines under hypoxia (Figure 1*A*). Medium from hypoxic HT-29 cells, but not from the two other cell lines, was also significantly lower in ROS content (Figure 1*B*). In order to obtain robust results that were not dependent of a particular cell line or the interexperimental variations, all normalized data from all of the cell lines were pooled. Hypoxic cells and their media showed significantly less ROS than the normoxic counterparts (Figure 1, *A* and *B*).

Serum ROS at LARC Presentation

Having established that extracellular ROS mirrored the intracellular level in the CRC models, we next analyzed serum ROS in a balanced sample selection of 35 patients (17 females and 18 males, 18 T2-3 and 17 T4 cases) from the entire LARC-RRP study population [27]. The patients had no evidence of disease disseminated outside the pelvic cavity at diagnosis. Median age was 62 (range, 31-73) years. As shown in Table 1, the ROS level [median 180 (range, 20.0-666) nM] was weakly correlated with body mass index [median 24 (range, 17-34) kg/m^2] but not age. Moreover, higher ROS were seen with higher hemoglobin [median 13.3 (range, 9.6-15.2) g/dl], while inverse correlations were found with thrombocytes [median 329 (range, 177-990) × 10^9/l], leukocytes [median 7.3 (range, 4.5-16.2) × 10^9/l], neutrophils [median 4.8 (range, 2.5-12.8) × 10^9/l], and the CRC tumor marker carcinoembryonic antigen [median 4.0 (range, 0-156) μg/l]. In a subset of 16 patients with available data from high-resolution magic angle spinning magnetic resonance spectroscopy, a strong negative correlation was noted between serum ROS and tumor choline [median 0.37 (range, 0.25-1.6) μmol/g of tissue], which is a precursor for phosphocholine in cellular membranes [32], indicating that ROS generation and release may be lower in rapidly proliferating tumors with an active membrane phospholipid metabolism [33]. However, ROS levels were not significantly different in T4 versus T2-3 cases, or in females and males (Table 2).

Damaged mtDNA in CRC Cell Cultures

Next, mtDNA damage was quantified in the three cell lines following exposure to normoxia or hypoxia for 24 hours (Figure 1*C*). Compared with the respective normoxic condition, the frequency of mtDNA damage was significantly higher in hypoxic HCT-116 and HT-29 cells but not in the LoVo cell line. Again, when all data from all of the cell lines were pooled, hypoxic cells showed significantly

Table 1. Circulating ROS and Correlations with Patient, Circulating, and Tumor Metabolic Factors

	n	*r*	*P*	*P* Adjusted *
Age	35	0.13	.47	NA
BMI †	35	0.35	.040	NA
Hemoglobin	35	0.37	.030	NA
Thrombocytes	35	−0.49	.003	NA
Leukocytes	35	−0.38	.023	NA
Neutrophils	35	−0.39	.020	NA
Lymphocytes	35	−0.11	.53	NA
Monocytes	35	−0.22	.21	NA
CEA	35	−0.34	.046	NA
Choline	16	−0.68	.004	.040
Glycerophosphocholine	16	−0.56	.022	.17
Glucose	15	−0.40	.14	.17
Myoinositol	14	−0.33	.26	.17
Phosphocholine	16	−0.23	.40	.17
Taurine	15	−0.14	.62	.17
Glycine	15	0.070	.81	.17
Lactate	16	0.020	.94	.17
Creatine	16	−0.20	.94	.17
Alanine	16	−0.010	.98	.17

BMI, body mass index; *CEA,* carcinoembryonic antigen; *NA,* not applicable; *ROS.* reactive oxygen species.

* According to the Benjamini-Hochberg method with a false discovery rate set at 0.05.

† Calculated as body weight in kilograms divided by the height in meters square.

more mtDNA damage than the normoxic counterparts. As depicted in Figure 1*D*, acknowledging the indicated dependence of ROS (Figure 1, *A* and *B*) and mtDNA damage (Figure 1*C*) to hypoxia, a low ratio of ROS to mtDNA damage might be an indicator of hypoxic CRC.

Damaged mtDNA in Serum Versus Whole Blood

The arithmetic ratio of ROS to mtDNA damage might also provide information of tumor hypoxia in CRC patients if feasible to measure in the circulation. The commercial OxiSelect *In Vitro* ROS/RNS Assay Kit used in the current analyses of cell line and serum samples might in principle have been applied as a diagnostic assay. In contrast, our assay for quantification of mtDNA damage was developed to analyze cells or tissues [26]. In order to investigate if this assay might also allow measurement of mtDNA damage in the noncellular compartment of blood, we took advantage of the parallel sampling of PAXgene whole blood and serum in the OxyTarget study for rectal cancer. As shown in Figure 2, analysis of specimens from six randomly selected patients (5 males and 1 female, age 56-76 years, disease stage T2-4N0-2M0) eligible for neoadjuvant oncologic therapy demonstrated detectable damaged mtDNA which in serum,

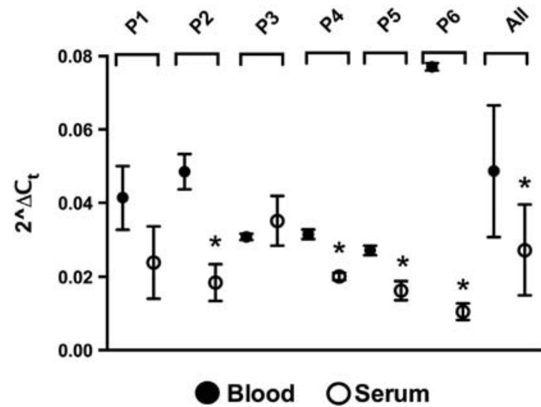

Figure 2. mtDNA damage in rectal cancer patients' whole blood and serum. Values are shown as mean ± standard deviation of triplicate measurements of specimens from six randomly selected patients (P1-P6), as well as all respective values pooled from the OxyTarget biomarker study. **P* < .05 on comparison of values from whole blood and serum.

Table 2. Circulating ROS and Damaged mtDNA and Correlations with Tumor Factors and Survival End Points

		ROS Level [nM]			Ratio of ROS to mtDNA Damage *		
		n	Mean (SD)	*P*	*n* †	Mean (SD)	*P*
Sex	Female	17	190 (149)		13	83.6 (79.1)	
	Male	18	183 (104)	.98	16	64.4 (38.5)	.58
T stage	2-3	18	197 (69.7)		13	72.4 (26.9)	
	4	17	175 (168)	.081	16	73.6 (78.0)	.22
N stage	0-1	7	228 (95.1)		5	86.4 (50.2)	
	2	28	176 (132)	.20	24	70.3 (62.1)	.45
ypT stage ‡	0-2	16	205 (67.7)		11	103 (76.6)	
	3-4	18	177 (163)	.074	17	57.1 (39.2)	.023
ypN stage ‡	0	23	217 (131)		17	84.6 (69.6)	
	1-2	11	134 (99.1)	.069	11	60.1 (39.5)	.19
TRG score ‡	1-2	22	195 (69.4)		18	88.7 (67.8)	
	3-5	12	180 (196)	.15	10	50.2 (32.1)	.044
		n	HR (95% CI) §	*P*	*n*	HR (95% CI) §	*P*
PFS ‡		34	0.52 (0.29-0.93)	.028	28	0.62 (0.29-1.3)	.23
OS		35	0.49 (0.26-0.95)	.034	29	0.71 (0.33-1.5)	0.38

CI, confidence interval; *HR,* hazard ratio; *mtDNA,* mitochondrial DNA; *OS,* overall survival; *PFS,* progression-free survival; *ROS,* reactive oxygen species; *SD,* standard deviation; *TN,* tumor-node; *TRG,* tumor regression grade; *yp,* histological response to neoadjuvant therapy.

* Because the ROS levels were several orders of magnitude higher than the percental values of damaged to total mtDNA (between 0.60% and 7.2%), the calculated value of each data point (ratio of ROS to mtDNA damage) was normalized with regard to the global mean (set as 100).

† Analysis of serum mtDNA damage was technically successful in 29 of 35 cases.

‡ One patient had disease progression in the pelvic cavity during the neoadjuvant treatment and therefore proceeded to palliative surgery; as a consequence, histological tumor response was missing, and the single case was omitted from analysis of treatment outcome.

§ Low HR will indicate favorable PFS and OS with high value of the measured factor.

Table 3. Circulating ROS to Damaged mtDNA Ratio and Correlations with Circulating Inflammatory Factors

	n	*r*	*P*	*P* Adjusted *
CRP †	27	−0.60	.001	NA
CD40	16	−0.44	.086	.18
CX3CL1	16	0.17	.54	.65
CXCL1	16	0.18	.50	.65
CXCL2	16	−0.51	.044	.11
CXCL5	16	−0.16	.56	.11
IFNG (IFNγ)	16	−0.52	.038	.11
IL1RN (IL1ra)	16	−0.80	.000	.000
IL4	16	0.064	.81	.81
IL10	16	−0.35	.19	.32
IL12B	16	−0.28	.30	.45
S100A8	16	−0.41	.12	.23
TNF (TNFα)	16	−0.63	.009	.045
TNFRSF1A (TNFR1)	16	−0.61	.013	.049
TNFRSF10B (TRAILR2)	16	−0.64	.007	.045
TNFRSF10C (TRAILR3)	16	−0.090	.74	.81

CRP, C-reactive protein; *IFNγ,* interferon-γ; *IL1ra,* interleukin-1 receptor antagonist; *mtDNA,* mitochondrial DNA; *NA,* not applicable; *ROS,* reactive oxygen species; *TNFα,* tumor necrosis factor-α; *TNFR1,* TNF receptor 1; *TRAILR2,* TNF-related apoptosis-inducing ligand receptor 2; *TRAILR3,* TNF-related apoptosis-inducing ligand receptor 3.

Each protein is listed by the gene name with the commonly used protein name in brackets if different from the former.

* According to the Benjamini-Hochberg method with a false discovery rate set at 0.05.

† Measurement was lacking for two cases.

likely as cell-free molecules, was in significantly lower quantity than in whole blood for the majority of patients.

Serum ROS Versus TAC

However, the serum ratio of ROS to mtDNA damage might not be informative in the actual clinical context if the ROS effects are counterbalanced by antioxidant defense mechanisms. We therefore used the OxyTarget serum biobank to directly compare ROS and TAC levels in 12 randomly selected patients (9 males and 3 females, age 48-67 years, disease stage T2-4N0-2M0-1). The individual ROS levels [median 100 (range, 18.5-130) nM] did not correlate with the corresponding TAC values [median 1.21 (range, 0.94-1.45) mM] (r = 0.45; P = .14; by Pearson product correlation). Moreover, since the extent of the variance from the median value was wider for the ROS measures than for TAC despite the former being several orders of magnitude lower, the individual patient's serum ROS level was probably independent of the overall reductive capacity.

Serum ROS and Damaged mtDNA in LARC Outcome

ROS and the ratio of ROS to mtDNA damage at the time of diagnosis were measured in stored serum biobank samples (collected at patient enrolment, 2005-2009) from the selected LARC-RRP study patients, who following diagnosis had been given an intensified neoadjuvant treatment regimen before the pelvic surgery [27]. Analysis of mtDNA damage was technically successful in 29 of the 35 cases, and the proportion of damaged mtDNA varied from 0.60% to 7.2% (not shown). As shown in Table 2, lower serum ratio of ROS to mtDNA damage was associated with poor histological tumor response to the neoadjuvant therapy (ypT3-4 stage and TRG3-5 scores; P = .023 and P = .044, respectively), but ROS as isolated measure was not. The local treatment response is regarded an indicative surrogate marker of long-term outcome in LARC [34], but in the case of PFS and OS, it was higher ROS level that was associated with favorable outcome (PFS: HR 0.52, 95% CI 0.29-0.93, P = .028; OS: HR 0.49, 95% CI 0.26-0.95, P = .034). While 5-year PFS was 54%, 71% of patients were alive after 5 years. For cases with recorded PFS events or death at censoring, median time to development of metastatic disease was 17 (range, 4-52) months and 27 (range, 1-57) months to death. When censored, median follow-up time was 65 (range, 45-65) months for patients still alive.

Serum ROS and Damaged mtDNA in LARC Inflammation

Finally, we found strong inverse correlations between the ratio of ROS to damaged mtDNA and serological markers of systemic inflammation in the LARC-RRP patients (Table 3), i.e., the levels of C-reactive protein [CRP; median 10 (range, 1-248) mg/l] and, in a subset of 16 patients with available data, interleukin-1 receptor antagonist [IL1ra; median 825 (range, 400-6204) ng/l] and factors involved in tumor necrosis factor (TNF) signaling: TNFα [median 30.8 (range, 28.8-42.6) ng/l], TNF receptor-1 [TNFR1; median 4.46 (range, 2.62-173) μg/l] and TNF-related apoptosis-inducing ligand receptor-2 [TRAILR2; median 70.7 (range, 58.8-138) ng/l].

Discussion

Presuming that the mitochondrial metabolism connects tumor hypoxia to systemic inflammation and adverse outcome of LARC, we assessed ROS and mtDNA damage in three CRC cell lines as template for the succeeding examination of these factors in patients' circulation. The cell line experiments unambiguously demonstrated the combination of repressed ROS and enhanced mtDNA damage under hypoxia. In LARC patients, low serum ROS at the time of diagnosis were associated with an elevated level of circulating carcinoembryonic antigen and tumor choline concentration, both indicative of unfavorable biology [33,35], as well as adverse long-term outcome (PFS and OS). Likewise, a low ratio of ROS to mtDNA damage in serum was associated with poor local tumor response to the neoadjuvant treatment and, of note, elevated systemic inflammation factors. Our strategy identified circulating ROS and cell-free damaged mtDNA as potential markers of the mitochondrial metabolism driven by the state of oxygenation of the primary tumor and possibly implicated in systemic inflammation and adverse outcome of LARC.

Malignant cells are regarded to be higher in mitochondrial ROS production than normal cells, and this ROS may stabilize the molecular effects of hypoxia [11,12]. In this context, the reduced ROS associated with hypoxic treatment of the CRC cell lines might reflect one or more of multiple respiratory chain adaptations [36], if not an incipient selection over the 24-hour treatment period toward stemness-like cell populations thriving in hypoxic conditions [13]. Contrary to the classic view that mtDNA base lesions are induced by ROS [14], hypoxic treatment with repressed ROS in the three CRC cell lines explicitly enhanced mtDNA damage. Importantly, ROS as such are not reflecting the submitochondrial level of peroxide, which has higher propensity for damaging mtDNA [37].

For the LARC patients, serum ROS were independent of age and sex. It was not surprising that subjects with higher hemoglobin, i.e., better tissue oxygenation capacity, had higher ROS. The inverse correlation with tumor choline probably reflected insufficient oxygenation of rapidly proliferating tumors, which may further have contributed in the observed correlation between high ROS and favorable long-term outcome, as hypoxia is a main mechanism of tumor resistance to cytotoxic therapy [3] and metastatic progression [4]. However, the isolated ROS measure was not predictive of local treatment outcome of the neoadjuvant therapy; instead, patients with a low serum ratio of ROS to mtDNA damage were at higher risk of poor histological tumor response. Collectively, this set of data indicates that low circulating ROS alone or in combination with heightened mtDNA release, provided both reflected the generation by the patients' intrapelvine tumor manifestations, were associated with tumor and systemic factors indicative of detrimental disease biology. In this context, it would have been valuable to have insight into the hypoxic tumor fraction; however, the available endoscopic tumor samples did not represent relevant tumor components for this purpose.

Perhaps most intriguingly, though data were available for only a subset of patients, the inverse connection between ROS and mtDNA damage in serum showed strong correlations with CRP, measured in clinical practice as a general marker of inflammation, as well as with IL1ra, TNFα, TNFR1, and TRAILR2—higher levels of these factors were found in patients with lower ROS and higher mtDNA damage. The inverse correlations between ROS and counts of thrombocytes, leukocytes, and neutrophils, commonly elevated in the inflammatory response, were also in line with these findings. Reported investigations of the role of circulating cell-free mtDNA in systemic inflammation comprise total mtDNA—not damaged mtDNA—primarily in nonmalignant conditions [20–22]. Interestingly, we detected significantly lower mtDNA damage in serum than in whole blood for four of six examined patients, implying a preference for extracellular release of intact over damaged mtDNA molecules. Although unraveling the biological impact of circulating mtDNA in

cancer is still in its infancy, it was recently shown that extracellular vesicles provide metabolically functional mtDNA that supports metastatic progression [38]. The specific mtDNA damage analyzed in the current report appeared at rather low occurrence. Furthermore, we cannot rule out tissues of origin other than the rectal primary tumor. Nevertheless, the strong correlation of a low ROS to mtDNA damage ratio with a deficient treatment response locally along with detrimental systemic inflammation, the latter likely cancer-induced in the study subjects [39], implies causality. IL1ra has not been much investigated in oncology. High circulating levels of this factor as well as of TNFα correlated with *ex vivo* cytotoxic activity of neutrophils in breast cancer patients, but the clinical implications of these findings are not known [40]. In patients with coronary artery disease, however, high circulating IL1ra correlated with elevated CRP and were independently predictive for adverse outcome [41]. While the TNFα-TNFR1 signaling pathway has been thoroughly studied in inflammatory bowel disease and CRC development, its precise function in established CRC and treatment response has not yet been determined [42]. Experimental studies have shown that TRAILR2, in addition to its cell death-inducing activity, is involved in activation of proinflammatory gene transcription programs, cell proliferation, and cell migration [43].

There are evident limitations of this hypothesis-generating exploration. First, the cohort is small, and results must be interpreted cautiously. On the other hand, the selected LARC-RRP study population was balanced in terms of sex and T2-3 versus T4 stage disease and representative for age groups in which rectal cancer prevails [44]. These factors did not confound the subjects' circulating components of the tumor mitochondrial metabolism. Only body mass index was higher in individuals with higher ROS, but the correlation was weak. Next, the analyses reported here had not been planned at the time of study conduct. Hence, the observed inverse correlation between the individual study patient's serum ROS and mtDNA damage and correlations between these factors and patients' local and systemic disease manifestations might be chance findings. Nevertheless, the association between low serum ROS (reflecting tissue hypoxia) and rapidly proliferating tumors (in terms of tumor choline) was strong even in the low number of patients with available data. This was also the case for ROS (molecules diffusing freely) and cell-free damaged mtDNA (likely tumor-released) with regard to inflammation factors in the circulation. A prospective therapy study incorporating explorative investigations that cover such aspects could provide further mechanistic insights.

In conclusion, our study provided data suggesting that low ROS and high cell-free mtDNA damage in the circulation reflect tumor hypoxia and contribute to cancer-induced systemic inflammation and thereby an adverse outcome of LARC. It might be that these findings have revealed a previously unheeded mechanism of the host response to aggressive cancer.

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