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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