TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Increased Levels of Oxidative Damage in Liver Metastases Compared with Corresponding Primary Colorectal Tumors

Association with Molecular Subtype and Prior Treatment

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High levels of oxidative stress in disseminated colorectal cancer tumor cells may form a therapeutically exploitable vulnerability. However, it is unclear whether oxidative stress and damage persist in metastases. Therefore, we analyzed markers of oxidative damage in primary colorectal tumors and their corresponding liver metastases. Markers of generic and oxidative DNA damage [phosphorylated histone H2AX (γ H2AX) and 8-hydroxy-2'-deoxyguanosine (8-OHdG)] were significantly higher in liver metastases compared with their corresponding primary tumors. Chemotherapy and/or radiotherapy before tumor resection was associated with increased persistent oxidative DNA damage, and this effect was more pronounced in metastases. Immunohistochemistry-based molecular classification into epithelial- and mesenchymal-like molecular subtypes revealed that untreated mesenchymal-like tumors contained lower levels of oxidative DNA damage compared with epithelial-like tumors. Treated mesenchymal-like tumors, but not epithelial-like tumors, showed significantly higher levels of γ H2AX and 8-OHdG. Mesenchymal-like tumors expressed significantly lower levels of phosphorylated nuclear factor erythroid 2-related factor 2, a master regulator of the antioxidant response, and nuclear factor erythroid 2-related factor 2-controlled genes. Of interest, a positive 8-OHdG status identified a subgroup of mesenchymal-like metastases with a poor overall survival. An increased capacity to tolerate therapy-induced oxidative damage in mesenchymal-like colorectal cancer may explain, at least in part, the poor responsiveness of these tumors to chemotherapy, which could contribute to the poor survival of this patient subgroup. (*Am J Pathol* 2018; 188: 2369–2377; <https://doi.org/10.1016/j.ajpath.2018.06.008>)

Colorectal cancer (CRC) is frequently a lethal disease.^{1,2} Approximately 20% of patients with CRC present with metastases at diagnosis.² Despite multiple improvements in cancer care, such as more effective systemic therapies and improved surgical techniques, the median overall survival (OS) of metastatic CRC (mCRC) after chemotherapy combined with targeted agents is still poor, up to approximately 30 months.^{3,4} Clearly, more effective treatment approaches are needed. Characterization of the dependencies and

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vulnerabilities of metastasized tumor cells may help in designing such therapies.

Oxidative stress is characterized by an imbalance between the production of reactive oxygen species (ROS) and their neutralization by antioxidants. Oxidative stress is known to be a common characteristic of cancer cells that can be exploited therapeutically.^{5,6} Moderately increased levels of ROS stimulate tumorigenesis by acting as second messengers in tumor-promoting signaling pathways.⁷ Antioxidants are important for maintaining the reduction-oxidation (redox) balance in tumor cells, thereby preventing cell death and senescence.⁶ Of interest, metastasizing tumor cells experience high levels of oxidative stress, which is an important barrier to successful establishment of distant metastases.^{8–10} However, not much is known about oxidative stress in established metastases, which are the main targets of systemic therapy in mCRC. Chemotherapy regimens for mCRC are based on fluoropyrimidines (5-fluorouracil or capecitabine) in combination with

oxaliplatin and/or irinotecan.⁴ These chemotherapeutic drugs work, at least in part, by enhancing ROS levels and causing oxidative damage.^{11,12} Moreover, a signature reflecting increased oxidative signaling has been associated with the aggressive mesenchymal (MES) CRC subtype,⁹ now commonly referred to as consensus molecular subtype 4 (CMS4).¹³

Increased oxidative stress during metastatic outgrowth, after chemotherapy or as a consequence of aggressive tumor biology, could generate a therapeutically exploitable vulnerability.¹⁴ To gain insight into patient subgroup(s) that might benefit from such a therapeutic approach, we assessed the levels of oxidative damage and expression of an antioxidant transcription factor in a large series of primary CRC and corresponding colorectal liver metastases (CRLMs) in relation to molecular subtypes. During data analysis, prior chemotherapy and/or radiotherapy and molecular subtype were considered as potential factors influencing oxidative stress status.

Figure 1 Increased oxidative DNA damage in colorectal liver metastases. **A:** Representative images of immunohistochemical expression of phosphorylated histone H2AX (γ H2AX) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in primary colorectal cancer (CRC) samples and corresponding colorectal liver metastases (CRLMs). Images of 10 $\times$ microscope objective whole-tissue microarray tissue cores and 40 $\times$ microscope objective (**insets**) are shown. **B** and **C:** Bar graphs showing quantification of γ H2AX expression (**B**) and 8-OHdG accumulation (**C**) in CRC tumors versus CRLMs. **B** and **C:** A paired *t*-test was applied to the log-transformed data to compare γ H2AX expression and 8-OHdG accumulation as continuous variable in CRC versus CRLMs. Error bars represent the geometric means and corresponding 95% CIs (**B** and **C**). **P* $\leq$ 0.05, ***P* $\leq$ 0.01. Scale bars: 100 μ m (**A**, main images); 50 μ m (**A**, insets).

Materials and Methods

Patients and Tissues

The patient cohort was described before.¹⁵ CRC patients who underwent a resection for CRLMs at the University Medical Center Utrecht (Utrecht, the Netherlands) between 2003 and 2014 were included in a prospective database. Their primary tumors were resected in the University Medical Center Utrecht, Diaconessenhuis Utrecht, St. Antonius Hospital Nieuwegein, or Rijnstate Hospital at Arnhem. Patients with either synchronous or metachronous CRLMs were included. Eventually, 158 mCRC patients were included. Clinicopathological data were available from 129 patients (86 males and 43 females). Median follow-up time was 76 months. Mean age was 62 years (range, 37 to 83 years) at the time of surgery for liver metastases. The use of anonymous or coded leftover material for scientific purposes is exempt from informed consent, according to Dutch law. Standard treatment agreements include an opt-out for such use.^{16,17}

TMA Construction

A 0.6-mm (triple-core) tissue microarray (TMA) of primary CRC tumors and corresponding CRLMs was constructed,¹⁵ from which multiple sections (4 μ m thick) were cut for immunohistochemical staining.

Treatment Status

Primary tumors were considered treated when neoadjuvant chemotherapy and/or radiotherapy was administered. Tissue from liver metastases was considered treated when patients received neoadjuvant chemotherapy for metachronous CRLMs. Tissue from synchronous metastases was considered treated when neoadjuvant chemotherapy for primary tumors and/or liver metastases was administered. An overview of treatment status is given in [Supplemental Table S1](#).

Mesenchymal versus Epithelial Subtypes

All tumors included within the TMA were previously classified as MES-like or epithelial (EPI)-like,¹⁵ using a validated immunohistochemistry-based classifier.¹⁸ In short, this assay identifies EPI- and MES-like CRC tumors using expression of homeobox protein CDX-2 (CDX2), FERM domain-containing protein 6 (FRMD6), 5-hydroxytryptamine receptor 2B (HTR2B), zinc finger E-box-binding homeobox 1 (ZEB1), and pan-cytokeratin. High expression of the differentiation marker CDX2 and low immunohistochemical expression of the epithelial-mesenchymal marker ZEB1 identify EPI-like cancers and increased levels of the vasoactive neurotransmitter HTR2B and goblet cell marker FRMD6 MES-like cancers. In addition, pan-cytokeratin was used to normalize the other markers for epithelial content.

On the basis of the immunohistochemistry staining per marker, a prediction probability for the MES-like subtype was calculated (MES probability score). Each individual TMA core was classified as EPI- or MES-like using an MES probability cutoff of 0.6 (EPI < 0.6; MES $\geq$ 0.6), and subtype classification was obtained by majority consensus scoring. This individual per core MES probability score was used to look for putative correlations between per core marker expression and the MES probability scoring.

Immunohistochemistry

Three immunohistochemical stainings were performed on serial sections of the TMA: phosphorylated histone H2AX (γ H2AX), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and phosphorylated nuclear factor erythroid 2-related factor 2 (NRF2; alias NFE2L2). [Supplemental Table S2](#) gives an overview of antibodies and dilutions, incubation conditions, and antigen-retrieval methods for each marker. For all stainings, sections were deparaffinized with xylene and rehydrated with serial dilutions of ethanol and water. Next, endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide diluted in phosphate-buffered saline for

Figure 2 Prior treatment is associated with DNA damage and 8-OHdG accumulation. **A** and **B**: Bar graphs showing quantification of phosphorylated histone H2AX (γ H2AX) expression (**A**) or 8-hydroxy-2'-deoxyguanosine (8-OHdG) accumulation (**B**) in primary colorectal cancer (CRC) and colorectal liver metastases (CRLMs), treated versus untreated tumors. **A**: Therapy-exposed CRC and CRLMs express higher levels of the DNA damage marker γ H2AX. **B**: Therapy-exposed CRC and CRLMs accumulate 8-OHdG. **A** and **B**: An unpaired *t*-test was applied to the log-transformed data to compare groups. Error bars represent the geometric means and corresponding 95% CIs (**A** and **B**). **P* $\leq$ 0.05, ***P* $\leq$ 0.01.

30 minutes. Then, antigen retrieval was performed by boiling samples for 20 minutes in citrate buffer (pH 6.0) for γ H2AX and 8-OHdG or EDTA buffer (pH 9.0) for phosphorylated NRF2. Protein Block (Novolink Polymer Detection Systems, Leica Biosystems, Newcastle upon Tyne, UK) was applied, according to the manufacturer's instructions, to block nonspecific binding of the anti-8-OHdG antibody. Primary antibody incubation was performed as follows: anti- γ H2AX (Ser139; rabbit; Cell Signaling, Danvers, MA; 2577; 1:50; overnight at 4°C), anti-8-OHdG (N45.1; mouse monoclonal; Abcam, Cambridge, UK; ab48508; 1:500; 1 hour at room temperature), and anti-NRF2 (phosphorylated S40; rabbit monoclonal; Abcam; ab76026; 1:500; 1 hour at room temperature). Subsequently, the secondary antibody BrightVision poly-horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (ImmunoLogic, Duiven, the Netherlands) was incubated for 30 minutes at room temperature. All sections were developed with diaminobenzidine for 10 minutes, followed by a hematoxylin counterstaining. Air-dried slides were mounted in a ClearVue Coverslip (Thermo Fisher Scientific, Waltham, MA). To check for nonspecific binding of the secondary antibody, slides were incubated without primary antibodies in the presence of the secondary antibody. Tissues previously validated in the literature as positive controls were used throughout.

Evaluation of Staining

Scoring was performed by consensus of two investigators (L.M.W. and K.V.) blinded to clinicopathological data and

EPI- versus MES-like subtype, after training by a pathologist (P.J.D.). Damaged and empty TMA cores and those not containing cancer cells were excluded. For 8-OHdG and phosphorylated NRF2, nuclear staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) (Supplemental Figure S1), ignoring cytoplasmic staining or staining within clearly necrotic regions. For 8-OHdG and phosphorylated NRF2, the percentage of positive nuclei was scored per intensity, calculating H-scores¹⁹ as follows:

$[1 \times (\% \text{ cells with intensity } 1) + 2 \times (\% \text{ cells with intensity } 2) + 3 \times (\% \text{ cells with intensity } 3)]$.

Because of the highly heterogeneous intensity of the nuclear γ H2AX staining per cell, only the percentage of positive tumor cells was scored. The mean H-scores (8-OHdG and phosphorylated NRF2) or percentage positivity (γ H2AX) of three cores for each primary tumor specimen or metastatic specimen per patient were calculated. TMA raw data are provided in Supplemental Table S3.

Bioinformatic Analysis

All bioinformatic analyses were performed using the R2 Genomics Analysis and Visualization Platform (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>, registration required). Expression data were obtained from a large composite primary CRC cohort data set and a CRLM cohort data set.^{13,20}

Statistical Analysis

All analyses were performed using IBM SPSS statistics version 23 (IBM Corp., Armonk, NY) and Excel version

Figure 3 Oxidative DNA damage is increased in treatment-exposed mesenchymal (MES)-like tumors characterized by low basal levels of DNA damage. **A:** Bar graphs showing expression of phosphorylated histone H2AX (γ H2AX) in primary colorectal cancer and colorectal liver metastases stratified according to treatment status and subtype. **B:** Bar graphs showing levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in primary colorectal cancer and colorectal liver metastases stratified according to treatment status and subtype. **A** and **B:** A one-way analysis of variance and Fisher's least significant difference post hoc test was applied to the log-transformed data to compare groups. Error bars represent the geometric means and corresponding 95% CIs (**A** and **B**). * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. EPI, epithelial.

Figure 4 Low basal levels of phosphorylated nuclear factor erythroid 2-related factor 2 (NRF2) and its target genes in mesenchymal (MES)-like tumors. **A:** Bar graph showing quantification of phosphorylated NRF2 expression and representative images of immunohistochemical nuclear expression of phosphorylated NRF2 in primary colorectal cancer (CRC) and corresponding colorectal liver metastases (CRLMs). Images of 10× microscope objective whole-tissue microarray tissue cores and 40× microscope objective (insets) are shown. A paired *t*-test was applied to the log-transformed data to compare phosphorylated NRF2 expression as a continuous variable in CRC versus CRLMs. **B:** Low basal levels of phosphorylated NRF2 in mesenchymal-like tumors. A one-way analysis of variance and Fisher's least significant difference post hoc test were applied to the log-transformed data to compare groups. **C:** Expression of NRF2 target genes within 3232 colorectal cancer tumors classified according to their molecular subtype.¹³ The following NRF2 target genes were included in the analyses: *ABCC4*, *ALDH3A1*, *AOX1*, *CAT*, *CHAC1*, *ENTPD5*, *FAM3B*, *GCLC*, *GCLM*, *GSR*, *HMOX1*, *HTATIP2*, *IDH1*, *LRP8*, *MAGOHB*, *MGST1*, *MOCOS*, *NQO1*, *PRR13*, *RNF183*, *SLC19A2*, *SLC22A23*, *SLC7A11*, *TRIB3*, *VASN*, and *ZFP36*. Analysis of variance *P* values were determined. The box represents the median and middle 50% of the scores. The upper and lower whiskers represent scores outside the middle 50%. **D:** Kaplan-Meier curves showing the differences in overall survival between NRF2 high and low subgroups in a cohort consisting of patients undergoing resection for their colorectal liver metastases.²⁰ Levels of NRF2 mRNA in liver metastases were used to generate high and low subgroups, using the average as a cutoff point. NRF2 mRNA levels in both groups are shown in the inset. Significance was tested using the log-rank test. Error bars represent the geometric means and corresponding 95% CIs (**A** and **B**). ***P* ≤ 0.01, ****P* ≤ 0.001. Scale bars: 100 μm (**A**, main images); 50 μm (**A**, insets). CMS, consensus molecular subtype; EPI, epithelial.

2013 (Microsoft Corp., Redmond, WA). GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, CA) was used for graphical presentation of the data. *P* < 0.05 (two tailed) was considered statistically significant. All clinicopathological characteristics of the

patients were assessed using a χ^2 test and an independent-sample *t*-test for the continuous variable age. Correlations between expression of the different immunohistochemical markers within primary CRC tissue or CRLM tissue cores and MES probability scoring

Table 1 Correlations between Expression of the Different Immunohistochemical Markers within Primary CRC Tissue or CRLM Tissue Cores and Mesenchymal Probability Scoring

Marker	CRC				CRLM tissue core			
	Kendall's τ^*	95% CI	<i>P</i> value [†]	<i>n</i> [‡]	Kendall's τ^*	95% CI	<i>P</i> value [†]	<i>n</i> [‡]
γ H2AX	−0.130	−0.263 to 0.002	0.0540	220	−0.057	−0.190 to 0.075	0.398	201
8-OHdG	−0.046	−0.206 to 0.114	0.573	233	−0.052	−0.203 to 0.098	0.496	215
Phosphorylated NRF2	−0.206	−0.312 to −0.099	0.0002	220	−0.142	−0.272 to −0.012	0.0318	214

*Kendall's rank correlation coefficient (Kendall's τ).
†Two-sided *P* values are given.
‡*n* = number of XY pairs.
8-OHdG, 8-hydroxy-2'-deoxyguanosine; CRC, colorectal cancer; CRLM, colorectal liver metastasis; γ H2AX, phosphorylated histone H2AX; NRF2, nuclear factor erythroid 2-related factor 2.

were determined using the Kendall's τ test. Hydrogen peroxide metagene expression within CRC molecular subtypes was compared using a one-way analysis of variance. Because the immunohistochemistry expression data were right skewed, the data were log transformed before analyzing and comparing mean differences in expression. The results are reported on the original scale by back transformation, yielding geometric means and corresponding 95% CIs. To account for values of zero using this approach, 0.5 was added to all data points before log transformation, and this value was subtracted again from the back-transformed results. A paired *t*-test was applied to the log-transformed data to compare γ H2AX expression, 8-OHdG accumulation, and phosphorylated NRF2 expression in primary CRC samples and corresponding CRLMs; an independent-sample *t*-test was used to compare expression in treated and untreated tumor samples. All other outcomes were compared using a one-way analysis of variance, followed by a Fisher's least significant difference test, to compare individual groups. For survival analysis, OS was measured from time of liver surgery until death or end of follow-up. Kaplan-Meier survival analyses were performed, and survival curves were compared between the different groups by log-rank tests.

Results

Increased Oxidative DNA Damage in Liver Metastases Compared with Primary Colorectal Tumors

To study DNA damage accumulation in primary CRC tumors and corresponding liver metastases, phosphorylation of histone H2AX was analyzed as a marker of the formation of DNA double-strand breaks. γ H2AX initiates repair of double-strand breaks and is a useful marker for their quantification.²¹ γ H2AX levels were significantly increased ($P \leq 0.01$) in liver metastases compared with corresponding primary tumors (Figure 1, A and B), indicating an increased load of DNA double-strand breaks or repair in mCRC. ROS may cause oxidative damage to the nucleotide precursor pool, which—on incorporation into DNA—can lead to DNA damage. DNA bases, predominantly guanine, are also susceptible to direct oxidation by ROS, and the oxidized base 8-OHdG is, therefore, a frequently used marker of oxidative DNA damage.²² A significantly increased accumulation of 8-OHdG ($P \leq 0.05$) was observed in metastases compared with the primary tumors from which they were derived (Figure 1, A and C).

Figure 5 8-hydroxy-2'-deoxyguanosine (8-OHdG) positivity identifies a group of poor-prognostic mesenchymal (MES)—like metastases. **A:** Kaplan-Meier curves showing the differences in overall survival between 8-OHdG—positive and 8-OHdG—negative MES-like metastases. Significance was tested using the log-rank test. **B:** Kaplan-Meier curves showing the differences in overall survival between 8-OHdG—positive and 8-OHdG—negative epithelial (EPI)—like metastases. Significance was tested using the log-rank test.

Table 2 Patient and Liver Metastasis Characteristics of the Classified Patients

Characteristics	Epithelial-like metastases (<i>n</i> = 69)							Mesenchymal-like metastases (<i>n</i> = 60)						
	8-OHdG UNK (<i>n</i> = 11)		8-OHdG negative (<i>n</i> = 27)		8-OHdG positive (<i>n</i> = 31)		<i>p</i> value*	8-OHdG UNK (<i>n</i> = 14)		8-OHdG negative (<i>n</i> = 22)		8-OHdG positive (<i>n</i> = 24)		<i>p</i> value*
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	
Age, mean (range), years	-	-	63	(37–82)	61	(42–77)	0.415	-	-	63	(46–83)	59	(43–78)	0.110
Sex														
Male	-	-	20	74	22	71	0.792	-	-	14	64	14	58	0.713
Female	-	-	7	26	9	29		-	-	8	36	10	42	
Onset														
Synchronous	-	-	14	52	22	71	0.134	-	-	12	55	10	42	0.382
Metachronous	-	-	13	48	9	29		-	-	10	46	14	58	
Treatment status														
Treated	-	-	6	22	9	29	0.555	-	-	0	0	9	38	0.001
Untreated	-	-	21	78	22	71		-	-	22	100	15	63	

*All χ^2 test (categorical data), except for the continuous variable age at surgery (independent-sample *t*-test).

8-OHdG, 8-hydroxy-2'-deoxyguanosine; UNK, unknown.

Accumulation of Oxidative Damage in Therapy-Exposed Tumors

Chemotherapy and radiotherapy generate oxidative damage.^{11,12,23–25} Therefore, we hypothesized that the increased accumulation of oxidative DNA damage in CRC liver metastases could be a consequence of differences in treatment status between primary CRC tumors and their corresponding liver metastases. To test this hypothesis, tissues were stratified according to their treatment status, and expression of the different markers was reanalyzed. Treatment before tumor resection was associated with a significant increase of γ H2AX levels, both in primary tumors and metastases ($P \leq 0.05$) (Figure 2A). In addition, γ H2AX levels were higher in previously untreated metastases compared with untreated primary tumors (Figure 2A).

Levels of 8-OHdG were increased in therapy-exposed metastases ($P \leq 0.01$) (Figure 2B), and a similar trend was observed in primary tumors (Figure 2B). Similar to γ H2AX, 8-OHdG levels were also increased in previously untreated liver metastases when compared with untreated primary tumors (Figure 2B).

Accumulation of Oxidative Damage in Therapy-Exposed Mesenchymal-Like Tumors

The recent classification of colorectal tumors into molecular subtypes¹³ offers a new paradigm for developing subtype-targeted therapy. One of the subtypes, CMS4, is characterized by a mesenchymal gene expression profile and is associated with a poor prognosis¹³ and high expression of hydrogen peroxide-regulated genes (Supplemental Figure S2).⁹ To assess whether high expression of hydrogen peroxide-regulated genes in mesenchymal-like tumors is associated with increased oxidative damage, immunohistochemistry-based molecular classification was

used to discriminate between EPI- and MES-like tumors.¹⁸ Previously untreated primary tumors and metastases of the MES-like subtype showed significantly less accumulation of γ H2AX, and there was a trend toward less accumulation of 8-OHdG ($P = 0.054$ for 8-OHdG primary; $P = 0.057$ for 8-OHdG metastasis) (Figure 3, A and B). Of interest, levels of γ H2AX and 8-OHdG lesions were significantly higher in MES-like tumors (primary and metastases) that received prior treatment, but not in EPI-like tumors (Figure 3, A and B).

Low Levels of Phosphorylated NRF2 and Its Target Genes in Mesenchymal-Like Tumors

NRF2 is a transcription factor that induces expression of many antioxidant genes in normal and cancer cells and is considered to be (one of) the most important orchestrators of the cellular antioxidant response.¹⁴ Therefore, it was tested whether the levels of active (phosphorylated S40) NRF2 were different between primary tumors and metastases, and between MES- and EPI-like tumors. Primary tumors and liver metastases contained similar levels of phosphorylated NRF2 (Figure 4A). Furthermore, a highly significant negative correlation was found between phosphorylated NRF2 levels and an MES-like tumor phenotype (Figure 4B and Table 1). Analysis of NRF2 target genes (ie, genes containing NRF2-binding sites, as demonstrated by chromatin immunoprecipitation sequencing²⁶) in a large composite CRC cohort showed that MES-like tumors (CMS4) express NRF2 target genes—as a proxy for active NRF2—at significantly lower levels than epithelial subtypes (CMS2 and CMS3) (Figure 4C and Supplemental Figure S3). In addition, low *NRF2* mRNA levels in liver metastases were also associated with poor OS, and this effect was most prominent in MES-like tumors (Figure 4D).

8-OHdG Staining in Mesenchymal-Like Metastases Is Associated with Poor Survival

The abundance of oxidative damage in resected tumors may not only serve as an indicator of prior treatment but could also be related to protumorigenic oxidative stress signaling. Therefore, OS of patients with EPI- and MES-like metastases with positive (H-score > 0) and negative (H-score = 0) 8-OHdG staining was analyzed. A positive 8-OHdG status ($n = 23$) in MES-like metastases showed a trend toward significance ($P = 0.119$) as a marker for poor OS (Figure 5A). In contrast, a positive 8-OHdG status in EPI-like metastases was not associated with survival (Figure 5B). In addition, a positive treatment status was associated with a positive 8-OHdG status in MES-like metastases but not in EPI-like metastases (Table 2).

Discussion

CRC patients with metastatic disease have a poor prognosis,^{3,4} and effective therapeutic approaches that have an impact on long-term survival are urgently needed. Targeting the increased redox status of tumor cells could be such an approach. These data show that CRC liver metastases are characterized by high levels of oxidative DNA damage. This is in line with a recent report showing that metastatic melanoma nodules express significantly higher levels of ROS in a human melanoma xenograft model.¹⁰ Of interest, these data point to higher oxidative stress levels in treatment-naïve CRC metastases compared with primary tumors. This indicates that factors distinct from treatment status, such as microenvironmental cues and/or intrinsic differences between metastasis-competent and metastasis-incompetent tumor cell clones, could be responsible for the different levels of oxidative damage in primary tumors and metastases.

Expression of the DNA damage markers γ H2AX and 8-OHdG in CRC liver metastases was significantly higher in patients who had received treatment. This may be because of a sustained chemotherapy- and radiotherapy-induced elevation of ROS^{12,23} and 8-OHdG lesions.²⁴ Additional experiments will be necessary to assess the causal relationship between treatment and redox status. Such studies will require the prospective collection of tumor biopsy specimens before and after treatment.

Effective therapies for poor-prognosis MES-like CRC tumors^{13,15,18} are urgently needed. These tumors were found to be characterized by low expression of γ H2AX, 8-OHdG, and phosphorylated NRF2. Although phosphorylated NRF2 might not adequately reflect NRF2 activation status,²⁷ MES-like tumors also expressed low levels of NRF2 target genes. Moreover, low mRNA levels of *NFE2L2* (the gene encoding NRF2) in primary tumors were associated with poor survival in the colorectal adenocarcinoma cohort, as shown

by the Human Protein Atlas (<https://www.proteinatlas.org/ENSG00000116044-NFE2L2/pathology/tissue/colorectal+cancer>, last accessed October 31, 2017). Finally, low levels of NRF2 mRNA in CRC metastases identified a subgroup of MES-like tumors with a poor OS.

The correlation of low phosphorylated NRF2 protein levels, low *NFE2L2* mRNA, and low NRF2 target gene expression with an aggressive tumor phenotype is somewhat surprising, considering that sustained and constitutive activation of NRF2 is known to promote tumor growth²⁸ and chemotherapy resistance.²⁹ Possibly, mRNA or phosphorylated NRF2 levels may not adequately reflect total NRF2 activity. Alternatively, NRF2 regulation of cancer cell viability may be context dependent. Finally, the levels of NRF2 expressed at the time of tumor resection may not reflect the ability of these tumors to mount an NRF2 response and increase their antioxidant capacity in response to ROS insults. Indeed, phosphorylated NRF2 protein levels were significantly higher in MES-like tumors that had been exposed to prior treatment. Although markers of oxidative damage were also higher in these tumors, they clearly survived treatment. This suggests that MES-like tumors may have a higher tolerance for therapy-induced oxidative damage than EPI-like tumors, which may (partly) explain their relative resistance to chemotherapy.³⁰ Of importance, a definitive assessment of the role of NRF2, its phosphorylation, and activation of its target genes in colorectal cancer progression requires further studies in model systems and human tissues. In particular, it will be interesting to investigate whether a higher tolerance for oxidative stress, possibly relying on NRF2 activity, contributes to the higher metastatic potential of aggressive MES-like tumors.

Given that moderately increased levels of oxidative stress may have tumor-promoting effects,⁷ it was explored whether a positive 8-OHdG status identifies aggressive CRC metastases. Of interest, these data suggest that patients with 8-OHdG-positive MES-like metastases have a shorter OS, whereas 8-OHdG status did not seem to be associated with OS in patients with EPI-like tumors. These results, therefore, suggest that a further subclassification (based on redox status) may improve the prognostic value of colon cancer subtyping, at least in the metastatic setting. Finally, this may help selecting patients for specific types of targeted therapies, particularly those targeting the redox status of tumor cells. The value of such an approach should now be evaluated in preclinical studies using models of MES- and EPI-like CRC.

In conclusion, therapeutics that interfere with redox status might be particularly effective in targeting metastases, therapy-surviving tumor cells, and MES-like tumors and might be administered in combination with conventional oxidative stress-inducing radiotherapeutics and chemotherapeutics. Further studies will be needed to clarify whether markers of redox status could have prognostic and/or predictive value.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.06.008>.

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