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Helicase-like transcription factor-deletion from the tumor microenvironment in a cell line-derived xenograft model of colorectal cancer reprogrammed the human transcriptome-S-nitroso-proteome to promote inflammation and redirect metastasis

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ABSTRACT

Methylation of the *HLTF* gene in colorectal cancer (CRC) cells occurs more frequently in men than women. Progressive epigenetic silencing of *HLTF* in tumor cells is accompanied by negligible expression in the tumor microenvironment (TME). Cell line-derived xenografts (CDX) were established in control ($Hltf^{+/+}$) and *Hltf*-deleted male $Rag2^{-/-}IL2rg^{-/-}$ mice by direct orthotopic cell microinjection (OCMI) of $HLTF^{+/+}$HCT116 Red-FLuc cells into the submucosa of the cecum. Combinatorial induction of *IL6* and *S100A8/A9* in the *Hltf*-deleted TME with *ICAM-1* and *IL8* in the primary tumor activated a positive feedback loop. The proinflammatory niche produced a major shift in CDX metastasis to peritoneal dissemination compared to controls. Inducible nitric oxide (*iNOS*) gene expression and transactivation of the *iNOS-S100A8/A9* signaling complex in *Hltf*-deleted TME reprogrammed the human S-nitroso-proteome. POTEE, TRIM52 and UN45B were S-nitrosylated on the conserved I/L-X-C-X_2-D/E motif indicative of iNOS-S100A8/A9-mediated S-nitrosylation. 2D-DIGE and protein identification by MALDI-TOF/TOF mass spectrometry authenticated S-nitrosylation of 53 individual cysteines in half-site motifs (I/L-X-C or C-X-X-D/E) in CDX tumors. POTEE in CDX tumors is both a general S-nitrosylation target and an iNOS-S100A8/A9 site-specific (Cys^{638}) target in the *Hltf*-deleted TME. *REL* is an example of convergence of transcriptomic-S-nitroso-proteomic signaling. The gene is transcriptionally activated in CDXtumors with an *Hltf*-deleted TME, and REL-SNO (Cys^{143}) was found in primary CDX tumors and all metastatic sites. Primary CDX tumors from *Hltf*-deleted TME shared 60% of their S-nitroso-proteome with all metastatic sites. Forty percent of SNO-proteins from primary CDX tumors were variably expressed at metastatic sites. Global S-nitrosylation of proteins in pathways related to cytoskeleton and motility was strongly implicated in the metastatic dissemination of CDX tumors. *Hltf*-deletion from the TME played a major role in the pathogenesis of inflammation and linked protein S-nitrosylation in primary CDX tumors with spatiotemporal continuity in metastatic progression when the tumor cells expressed HLTF.

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Materials and Methods

1 Reagents and kits

Abcam (Cambridge, MA) was the source of the following antibodies: mouse monoclonal (ab22506) to S100A9 + Calprotectin (S100A8/A9 complex), mouse monoclonal (ab18672) to IL8,rabbit polyclonal anti-iNOS (ab15323), rabbit monoclonal anti-ICAM-1 (ab109361), goat anti-rabbit IgG H&L (HRP; ab97051), goat anti-mouse (HRP; ab205719); and mouse on mouse polymer IHC kit (ab 127055). For immunoprecipitation and Western blotting, Abcam was the source of rabbit polyclonal anti-HLTF (ab17984) to human HLTF aa 600-700 that reacts with mouse and human. For immunohistochemistry, Sigma (St Louis, MO) was the source of rabbit polyclonal anti-HLTF (HPA015284) to human HLTF aa 164-300 that reacts with mouse and human.Biotinylated goat-anti-mouse (BA-9200) and goat anti-rabbit (BA-1000) IgG antibodies and the ABC-enzyme complex were purchased from Vector Laboratories, Inc. Harris Modified Hematoxylin (HHS16) were purchased from MilliporeSigma (St. Louis, MO). XenoLight D-luciferin potassium salt (122799) was purchased from PerkinElmer (Waltham, MA). ThermoScientific (Waltham, MA) was the source of the following Pierce™ reagents: S-nitrosylation Western blot kit (90105), HENS buffer (90106), mouse anti-TMTantibody (90075), immobilized anti-TMT resin (90076), TMT elution buffer (90104) and, iodoTMTsixplex™ label reagent set (90101). BioRad (Hercules, CA) was the source of 7.5% Mini-PROTEAN TGX precast protein gels (4561024), Clarity Western ECL-substrate (170-5060), Precision Protein StrepTactin-HRP conjugate (1610381) and Kaleidoscope SDS-PAGE Standards (1610324). PerkinElmer (Waltham, MA) was the source of Bioware® Brite Cell Line HCT116 Red-FLuc (BW124318).

2 Cell Culture

Bioware® Brite Cell Line HCT116 Red-FLuc is a cell line derived from the parental cell line (ATCC, CCL-247) from adult male colorectal carcinoma by stable transduction with red-shifted lentivirus containing firefly luciferase from Luciola Italica (Red-FLuc) under the control of human ubiquitin C promoter. HCT116 Red-FLuc cells were confirmed

to be pathogen free by the IMPACT Profile I (PCR) at the University of Missouri Research Animal Diagnostic and Investigative Laboratory. HCT116 Red-FLuc cells have a mutation in codon 13 of the ras proto-oncogene, and express transforming growth factor beta 1 (TGFβ1) and tumor protein 53 (TP53), HCT116 Red-FLuc cells grown in McCoy's 5a Modified Medium (ATCC, 30-2007) supplemented with 10% fetal bovine serum and puromycin (2µg/mL) have an average doubling time of 16 hours. For each experiment, cell stocks in liquid nitrogen at passage 2 were thawed using T25 flasks, expanded in T150 flasks for 2 days, passaged overnight and harvested at 70-75% confluency. This protocol provided a unified framework for comparative gene expression analysis.

3 *Hltf*-deleted and control mice

Global *Hltf*-deleted mice were developed in collaboration with genOway (Lyon, France) and bred to be fully congenic (N11) on the C57BL/6J genomic background. Global *Hltf*-deleted mice present a neonatal lethal phenotype. However, when the*Hltf*-deletion was bred (IACUC# 02007) into the recombinase activating gene 2 (Rag2)/common gamma (IL2rg) double knockout background, i.e. mice lacking lymphocytes (NK-, T- B-; alymphoid), the perinatal lethal phenotype was eliminated—clearly showing the perinatal lethal phenotype requires an immune component.

Immune-deficient mice were housed with a 12:12 light/dark cycle with access to food and water *ad libitum* and bedding was changed 2-3 times/week. Routine testing of sentinel mice ensured the colony was disease free. All studies and the anticipated mortality were conducted in accord with the NIH Guidelines for the Care and Use of Laboratory Animals, as reviewed and approved by the Animal Care and Use Committee at Texas Tech University Health Sciences Center (NIH Assurance of Compliance A3056-01; USDA Certification 74-R-0050, Customer 1481, S1 Checklist). TTUHSC's IACUC (# 02009) specifically approved this study.

The orthotopic *HLTF*$^{+/+}$HCT116 xenograft model was established as follows: randomly selected six- to eight-week old *Hlt-deleted* (n=24) and *HLTF*$^{+/+}$ (n=14) male *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice received direct orthotopic cell microinjections (OCMI) of *HLTF*$^{+/+}$HCT116 Red-Fluc cells (2x10^{6} cells/10 µl) between the mucosa and the muscularis layers of the cecal wall. Hereafter the mice were designated *Hltf*-deleted and control. All surgery was performed with isoflurane (Isothesia) and the SomnoSuite® Low-Flow anesthesia system (Kent Scientific) with far infrared warming pads during surgery and recovery. Additional efforts to minimize suffering included an IP injection of Buprenorphine (Buprenex, 0.1 mg/kg) prior to surgery to manage incisional pain followed by a second dose 4-8 hours later. The cecum was exteriorized via a small midline laparotomy on the vertical linea alba to eliminate bleeding. Non-invasive bioluminescence imaging (BLI) with an IVIS Spectrum *In Vivo* Imaging System was used to validate the quality and accuracy of the injection, and to track and quantify tumor growth and metastasis. Histopathology at necropsy confirmed placement of the inoculum. Mouse behavior and well-being were monitored daily. Tumor growth/metastasis was monitored weekly with BLI.

4 Postmortem analysis

Necropsy was performed at 35 days post CDX establishment. Mice under continuous Isothesia were imaged and killed immediately (< 15 seconds) by cervical dislocation. Primary tumor xenografts and metastatic tumors were quickly removed, rinsed in physiological saline and either flash frozen for biochemical evaluation (RNA-seq, Western blotting, MALDI-TOF/TOF MS, nanoLC-MS/MS) or fixed in formalin and processed for either routine histopathology or immunohistochemistry.

5 Immunohistochemistry

Tissue blocks were serially sectioned (3-4 µm). Two sections were placed on each slide and deparaffinized prior to staining. Beginning with the first slide, sections on every fifth slide were stained with hematoxylin and eosin (H&E) for evaluation by light microscopy. Sections on alternate slides were processed for immunohistochemistry with heat-induced epitope retrieval. Two tissue sections per slide facilitated the use of one section for positive immunostaining, and the serial section for negative (minus primary antibody) control staining. All primary antibodies (1:50) were paired with an appropriate HRP-conjugated secondary antibody (1:200) depending upon the species in which the primary antibody was generated. Nuclei were counterstained (blue) with hematoxylin.

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6 Immunoprecipitation and Western blotting

Immunoprecipitation and Western analysis were performed as previously reported [33, 35]. Briefly, whole cell lysates from two T150 flasks of HCT116 Red-FLuc cells (80% confluent) were immunoprecipitated with rabbit polyclonal anti-HLTF (ab17984) to human HLTF aa 600-700 at a concentration of 5 ug/ml. Western blotting was achieved with the same primary antibody (1:5000) followed by HRP-conjugated mouse anti-rabbit (1:5000). Signal was detected by chemiluminescence with the Clarity Western ECL Substrate Kit.

7 Tumor transcriptome analysis (RNA-seq)

Primary tumor xenografts (1 per individual mouse x 3 biological replicates for *Hltf*-deleted and control male mice = 6 total samples) were flash frozen and sent to Otogenetics Corp. (Norcross, GA) for RNA-seq assays. Briefly, total RNA was isolated, and evaluated for its integrity and purity with an Agilent Bioanalyzer. RNA samples were rRNA-depleted prior to random-primed cDNA preparation/QC, Illumina library preparation/QC; PE100-125 and HiSeq2500 sequencing at a minimum of 40 million reads. Paired-end 106 nucleotide reads were subjected to species-specific mapping against reference genomes for mouse (mm10) and human (hg38). Differential expression (DE) analysis and alternative splicing analysis for each species was done with cufflinks.cuffdiff (2.2.1). Data were imported into iPathwayGuide (Advaita Corporation, Plymouth, MI) and evaluated using the **q-value of 0.05 for statistical significance** and a log-fold change (logFC) of expression with an absolute value $\leq$ 0.6. Cuffdiff generated q-values are adjusted p-values that consider the false discovery rate (FDR). The q-value is an essential statistic when measuring thousands of gene expression levels from a relatively small sample set because it has a greater ability (power) to identify significant changes in gene expression. All RNA-seq data in this publication are accessible through NCBI's Gene Expression Omnibus (GEO) Series accession number GSE161961 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE161961).

With iPathwayGuide, data were analyzed in the context of pathways obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 90.0+/05-29, May 19), gene ontologies from the Gene Ontology Consortium database (2019-Apr26), miRNAs from the miRBase (Release 22.1, October 2018) and TARGETSCAN (Targetscan version: Mouse:7.2, Human:7.2) databases,network of regulatory relations from BioGRID: Biological General Repository for Interaction Datasets v3.5.171. March 25th, 2019, and diseases from the KEGG database (Release 90.0+/05-29, May 19).

8 Tumor S-nitroso-proteome analysis

Step-wise tumor S-nitroso-proteomic analysis leading to iNOS-S100A8/A9 site-specific analysis was performed with iodoTMT-switch labeling, affinity enrichment and high-resolution LC-MS/MS analysis by Applied Biomics, Inc (Hayward, CA). Briefly: primary tumor xenografts from *Hltf*-deleted (n=2 mice) and control (n=2 mice) male mice were homogenized/sonicated (Polytron) in 4 volumes HENS buffer (100mM HEPES, pH 7.8, 1 mM EDTA, 0.1 mM Neocuproine, 1% SDS). Protein concentrations were determined (OD_{280}) with NanoDrop One (ThermoFisher), and adjusted to a final concentration of 2 µg/µl with HENS buffer. Two hundred microgram samples were incubated for 30 minutes at room temperature with 20 mM methyl methanethiosulfonate (MMTS) in dimethylformamide (DMF) to block free cysteine thiols. Proteins were precipitated with 6 volumes pre-chilled (-20° C) acetone for a minimum of 60 minutes to remove MMTS, pelleted by centrifugation (10,000 x g) for 10 minutes at 4° C, and dried for 10 minutes. Following sample resuspension in HENS buffer (100 µl), S-nitrosylated cysteines were selectively reduced with ascorbate (protected from light) and irreversibly labeled with iodoTMTzero reagent for 2 hours at room temperature. Proteins were precipitated with 6 volumes pre-chilled (-20° C) acetone for a minimum of 60 minutes, pelleted by centrifugation (10,000 x g) for 10 minutes at 4° C, and dried for 10 minutes.Samples were resuspended in HENS buffer (100 µl), added to anti-TMT resin and incubated overnight with end-over-end mixing at 4° C. Resin was washed 3X with 1XTBS, 3X with water, and eluted with 4 volumes TMT elution buffer. Eluates were frozen and lyophilized to dryness. In experiment 1, global S-nitrosylation of CDX tumors from TMT-tagged *Hltf*-deleted and control samples was analyzed by NanoLC-MS/MS with a data base search for human genes in SwissProt using MASCOT. **E**xponentially **M**odified**P**rotein **A**bundance **I**ndex (emPAI defined as 10^{PAI}-1) values, where PAI (Protein Abundance Index) is the ratio of observed to observable peptides, is approximately proportional to the logarithm of protein concentration and indicates absolute protein abundance.

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In experiment 2, *Hltf*-deleted and control TMT-tagged samples were resolved by two-dimensional gel electrophoresis (2DE)with mouse anti-TMT as the primary antibody (1 µg/ml final concentration) and Cy5-labeled donkey anti-mouse IgG (1:2000) as the second antibody. In experiment 3, TMT-labeled proteins were co-labeled with different color fluorescent dyes, i.e. Cy3 for *Hltf*-deleted and Cy2 for control for two-dimensional difference gel electrophoresis (2D-DIGE), which detected as little as 1.0 fmol of protein in each sample. Spots were excised and proteins identified by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF/TOF). Database search was for human proteins in SWISSProt using MASCOT.

In experiment 4, unmodified cysteines in proteins (1 mg/ml) from primary tumor xenografts (n=4 mice) and metastatic tumors (n=2 mice) from *Hltf*-deleted mice were blocked with MMTS, selectively reduced with ascorbate (protected from light), and individually tagged with iodoTMTsixplex isobaric reagent such that a unique mass reporter (126-131 Da) in the low-mass region of the MS/MS spectrum was generated for samples from six locations. Equal amounts of six different samples (1 mg each) were combined into a single sample for enrichment with anti-TMT resin and NanoLC-MS/MS analysis. Relative expression for each protein fragment, i.e. the average ratio(s) for the protein, together with the number of peptide ratios that contributed (N) and the geometric standard deviation (SDgeo) were calculated.

In all experiments, peptides labeled with iodoTMT were quantified. Off-target (non-cysteine) labeling was <5%, and authentic cysteine nitrosylation as well as authentic iNOS/S100A8/A9 consensus sequences [29] were confirmed by peptide sequence. Gene symbols for differentially expressed proteins were input to the **P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships (PANTHER) database for functional classification and pathway identification. Protein-protein networks (cluster analysis) constructed with STRING with EMPAI values identified direct (physical) and indirect (functional) interactions.

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