Aug 29, 2019

Helicase-like transcription factor (Hltf) gene-deletion promotes oxidative phosphorylation (OXPHOS) in colorectal tumors of AOM/DSS-treated mice

PLOS One

Rebecca Ann Helmer[1], Gurvinder Kaur[2], Lisa Ann Smith[3], Beverly S. Chilton[4]

[1]becky@helmeronline.com, [2]gurvinder.kaur@ttuhsc.edu, [3]lisa.ann.smith@ttuhsc.edu, [4]beverly.chilton@ttuhsc.edu

1 Works for me dx.doi.org/10.17504/protocols.io.4argsd6

Beverly Chilton

ABSTRACT

The *helicase-like transcription factor (HLTF)* gene – a tumor suppressor in human colorectal cancer (CRC) - is regulated by alternative splicing and promoter hypermethylation. The detection of hypermethylated HLTF DNA in fecal occult blood tests is an indicator of disease recurrence and poor survival. *Hltf*-deficiency in the $Apc^{Min/+}$ mouse strain increased the formation of intestinal adenocarcinoma with a high incidence of gross chromosomal instabilities. To investigate *Hltf*-deletion effects in CRC without cross-breeding into a tumorigenic strain, *Hltf*-deletion was studied in mice treated with the carcinogen azoxymethane (AOM) and the proinflammatory agent dextran sodium sulfate (DSS). *Hltf*-deletion resulted in weight loss beginning at treatment week 6, and poor survival (Kaplan-Meier survival plot). *Hltf*-deletion increased tumor multiplicity compared to controls, and dramatically shifted the topographic distribution of lesions into the rectum. Differential isoform expression analysis of lesions from control mice revealed both the truncated isoform that lacks a DNA-repair domain and the full length isoform capable of DNA damage repair are present (3:1.8 ratio) during adenocarcinoma formation. iPathwayGuide identified 51 dynamically regulated genes of 10,967 total genes with measured expression. Oxidative Phosphorylation (Kegg: 00190), the top biological pathway perturbed by *Hltf*-deletion, resulted from increased transcription of *Atp5e, Cox7c, Uqcr11, Ndufa4* and *Ndufb6* genes, concomitant with increased endogenous levels of ATP (p=0.0176). Upregulation of gene expression, as validated with qRT-PCR, was accompanied by a stable mtDNA/nDNA ratio. This is the first study to show *Hltf*-deletion in an inflammation-associated CRC model elevates mitochondrial bioenergetics. The distal shift in tumorigenesis indicates the detection of hypermethylated HLTF DNA in stool samples might be a prognostic biomarker for distal (left-sided) CRC in patients with inflammatory bowel disease.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0221751

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Helicase-like transcription factor (Hltf) gene-deletion promotes oxidative phosphorylation (OXPHOS) in colorectal tumors of AOM/DSS-treated mice - submitted to PLOS

1

Materials and methods

Reagents and kits

Abcam (Cambridge, MA) was the source of the luminescent ATP detection assay kit (ab113849). DNeasy® Blood & Tissue Kit (69506) was purchased from Qiagen (Valencia, CA) for isolation of genomic DNA from tail biopsies and tumor samples. Applied Biosystems TaqMan®MGB (minor groove binder) probes with a 5´ reporter (FAM or VIC) and a 3´ nonfluorescent quencher (MGB-NFQ), Invitrogen™TRIzol™ Reagent (15596026), Invitrogen SuperScript™Vilo™cDNA Synthesis Kit (11754050), and SequalPrep™ Long PCR Kit with dNTPs (A10498) were purchased from ThermoFisher Scientific (Waltham, MA). PCR primers were synthesized by Midland Certified Reagent Company (Midland, TX). OmniPur agarose (2120) was purchased from Calbiochem division of EMD4Biosciences (San Diego, CA). Promega (Madison, WI) was the source of the Lambda DNA/EcoRI + HindIII agarose gel markers (G173A). Azoxymethane (A2853) was purchased from Sigma-Aldrich (St. Louis, MO) and dextran sulfate sodium salt colitis grade (160110) was purchased from MP Biomedicals (Santa Ana, CA). Mouse Mitochondrial DNA copy number assay kit (MCN 3) was purchased from Detroit R&D, Inc. (Detroit, MI). Alcian-blue staining solution was purchased from EMD Millipore Corp (Burlington, MA).

2 ### *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. PCR screening reactions were used to authenticate the *Hltf*-deleted and wild type control genotypes. Mice were housed with a 12:12 light/dark cycle with access to food and water *ad libitum*. Bedding was changed 2-3 times/week. Routine testing of sentinel mice ensured the colony was disease free. All studies 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, Checklist S1). TTUHSC's IACUC specifically approved this study. Mice were monitored at least twice daily by BSC and veterinary staff. All mice experienced increased stooling with stool consistency softer but solid. There was no incidence of diarrhea. Minimal frank blood associated with rectal prolapse was evident in cage litter toward the end of the study. All efforts were made to minimize pain and suffering, i.e. if mice became lethargic with evidence of piloerection they were removed from the study and euthanized (CO_2 followed by cervical dislocation).

To recreate the aberrant crypt foci – adenoma – carcinoma sequence in human CRC, randomly selected six- to eight-week old *Hltf*-deleted and control male mice were given a single intraperitoneal (IP) injection of 10 mg/kg AOM, a potent carcinogen used to induce colon cancer in rodents. All experimental protocols began at 8 AM in the LARC housing facility. After 3-days of recovery, mice were given the first of four cycles of 3% DSS *ad libitum*. Each cycle lasted five days followed by a 16-day recovery period except for the last cycle in which mice were sacrificed 26-days after the last DSS treatment. Mice were weighed every seven days throughout the treatment protocol.

3 Techniques

The entire colon (from the anus to the cecum) for each mouse was excised, flushed in a physiologically accurate direction (proximal to distal) with ice-cold phosphate buffered saline, and cut open longitudinally along the mesenteric side. For some colons, alcian blue dye (1%) was used to highlight the texture of the colon and demarcate the borders of individual tumors. Each colon was subdivided into three roughly equal regions, i.e. proximal colon (with rugae), mid or central colon, and distal (colon/rectum) colon. Tumors were counted and measured (BSC) with a dissecting microscope fitted with an eyepiece reticle. Measurements were confirmed (LAS) in histological preparations.

Some colons were rolled with the mucosa outwards, or tumors were snared with forceps and carefully separated from the muscularis mucosae. After histological processing (fixed in 4% paraformaldehyde, paraffin embedded, sectioned @ 3-4 mm, and processed for staining), all lesions were evaluated (double-blind) by LAS and BSC, and scored for degree of dysplasia, lymphocytic response, and invasive colorectal carcinoma (LAS). Human criteria were used for tumor staging with stage 0 the earliest stage followed by a range from I through IV. For a diagnosis of stage 1, the cancer has either grown through the muscularis mucosa into the submucosa (pT1), or the muscularis propria (pT2), but not invaded local lymph nodes or distant sites.

OneTouch Ultra Mini and OneTouch Ultra Mini Blue test strips were purchased from LifeScan (Malpitas, CA), a Johnson & Johnson Company, for blood glucose monitoring. Serum glucose from non-fasting males was tested with blood from a tail-snip prior to euthanasia. Luminescent ATP detection assay (96-well plate) was performed according to the manufacturer's instructions with tumor samples from *Hltf*-deleted (n=8) and control (n=8) mice. DNA was quantified with a PicoGreen dsDNA assay. A Kaplan-Meier survival plot (log-rank Mantel-Cox test, $p<0.05$) and hazard ratio (Manel-Haenszel) were calculated for *Hltf*-deleted (n=109) and control (n=47) mice. With the exception of RNA-seq data analyses, all statistical procedures were performed with GraphPad Prism v8.1.1 (significance, $p<0.05$).

4 Tumor transcriptome analysis (RNA-seq)

Individual samples (tumors from an individual mouse/sample 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 Illumina library preparation and sequencing (HiSeq2500). Paired-end 106 nucleotide reads were mapped against the reference genome mm10 with star2.4.0j. Comparison of expression level (fpkm_tracking) for differential expression (DE) analysis as well as alternative splicing (isoform) analysis was conducted with cufflinks.cuffdiff (2.2.1). All RNA-seq data in this publication are accessible through NCBI's Gene Expression Omnibus (GEO) Series accession number GSE132814 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132814). DE data were imported to iPathwayGuide (Advaita Corporation, Plymouth, MI) to utilize a systems biology analysis that considers the role, type, function, position and interactions of each gene in various pathways to identify significantly impacted genes in a specific condition. Standard enrichment parameters (0.6, $p<0.05$) were used for bioinformatics analyses.

iPathwayGuide scored pathways with the Impact Analysis method that uses two types of evidence: the over-representation of DE genes in a given pathway and the perturbation of that pathway computed by propagating the measured expression changes across the pathway topology. These aspects are captured by two independent probability values that are combined in a unique pathway-specific p-value. The underlying pathway topologies, comprised of genes and their directional interactions, were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 84.0+/10-26, Oct 17), gene ontologies from the Gene Ontology Consortium database (2017-Nov6) miRNAs from the miRBase (Release 21) and TARGETSCAN (Targetscan version: Mouse:7.1, Human:7.1) databases, network of regulatory relations from BioGRID: Biological General Repository for Interaction Datasets v3.4.154. October 25th, 2017, and diseases from the KEGG database (Release 84.0+/10-26, Oct 17).

5 ***RNA isolation and qRT-PCR***

Tumors (n=14 control mice, 23 *Hltf*-deleted mice) were dissolved in 1ml of Trizol reagent and RNA was extracted. The RNA was DNAse-treated and cDNA was synthesized from 100ng of RNA using the Superscript VILO^{TM} kit. Real time PCR with target-specific primers (ATP5e, Assay ID-Mm01239887_m1; Uqcr10, Assay ID-Mm01186961_m1; Cox7c, Assay ID-Mm01340476_m1; Nufa4, Assay ID-Mm04208480_g1; Nufb6, Assay ID-Mm01208591_g1; and Rn18s, Assay ID-Mm04277571_s1) was performed using TaqMan Gene expression assays. Real time PCR was conducted in triplicate for the 37 biological samples. Non-template controls contained water instead of cDNA. The expression level of the gene of interest was evaluated using the comparative Ct method (ΔΔCt method). Threshold values (Ct) for the gene of interest and the reference gene Rn18s were determined using QuantStudio^{TM} 12K Flex software (Applied Biosystems Technology). Ct values for the gene of interest were normalized to Ct values for Rn18s in each sample and then the fold change for the gene of interest was calculated relative to the level in the control sample.

6 **Mitochondrial DNA copy number (qPCR)**

Tumors (n=6 control mice, 16 *Hltf*-deleted mice) were flash frozen prior to total DNA isolation with the DNeasy® Blood & Tissue Kit. Concentrations of dsDNA were determined with a NanoDrop™ One^{C} microvolume UV-Vis spectrophotometer (Thermo Scientific). Mitochondrial (mt) copy number was achieved with the Mouse Mitochondrial DNA Copy Number Assay Kit. Real time PCR was conducted in duplicate for each biological sample. Non-template controls contained water instead of cDNA. The kit provided a positive reference control, i.e. total DNA isolated from liver of B6 mouse, SYBR™ green master mix, and primers for amplification of either the nuclear (n) gene beta actin (Actb) or the mitochondrial (mt) gene NADH dehydrogenase 4 (Nd4). Threshold values (Ct) for nuclear and mitochondrial genes for each sample were determined using QuantStudio^{TM} 12K Flex software (Applied Biosystems Technology). The relative mtDNA content was quantified by normalizing the mitochondrial gene to the nuclear gene using the comparative Ct method (ΔΔCt method). A total of 20ng genomic DNA was used for mitochondrial and nuclear DNA markers