

RESEARCH ARTICLE

Proteins that underlie neoplastic progression of ulcerative colitis

Teresa A. Brentnall¹, Sheng Pan², Mary P. Bronner³, David A. Crispin¹, Hamid Mirzaei⁴, Kelly Cooke⁴, Yasuko Tamura¹, Tatiana Nikolskaya^{5,6}, Lellean JeBailey⁶, David R. Goodlett⁷, Martin McIntosh⁸, Ruedi Aebersold^{4,9}, Peter S. Rabinovitch² and Ru Chen¹

¹ Department of Medicine, University of Washington, Seattle, WA, USA

² Department of Pathology, University of Washington, Seattle, WA, USA

³ Department of Anatomic Pathology, Cleveland Clinic Foundation, Cleveland, OH, USA

⁴ Institute for Systems Biology, Seattle, WA, USA

⁵ Vavilov Institute for General Genetics, Russian Academy of Sciences, Moscow, Russia

⁶ GeneGo Inc., St Joseph, MI, USA

⁷ Department of Medicinal Chemistry, University of Washington, Seattle, WA, USA

⁸ Fred Hutchinson Cancer Research Center, Molecular Diagnostics Program, Seattle, WA, USA

⁹ Institute of Molecular Systems Biology, ETH-Zurich and Faculty of Science, University of Zurich, Switzerland

Patients with ulcerative colitis (UC) have an increased risk for developing colorectal cancer. Because UC tumorigenesis is associated with genomic field defects that can extend throughout the entire colon, including the non-dysplastic mucosa, we hypothesized that the same field defects will include abnormally expressed proteins. Here, we applied proteomics to study the protein expression of UC neoplastic progression. The protein profiles of colonic epithelium were compared with (i) UC patients without dysplasia (non-progressors), (ii) non-dysplastic colonic tissue from UC patient with high-grade dysplasia or cancer (progressors), (iii) high-grade dysplastic tissue from UC progressors, and (iv) normal colon. We identified differential protein expression associated with UC neoplastic progression. Proteins relating to mitochondria, oxidative activity, and calcium-binding proteins were some of the interesting classes of these proteins. Network analysis discovered that Sp1 and c-myc proteins may play roles in UC early and late stages of neoplastic progression, respectively. Two over-expressed proteins in the non-dysplastic tissue of UC progressors, carbamoyl-phosphate synthase 1 and S100P, were further confirmed by immunohistochemistry analysis. Our study provides insight into the molecular events associated with UC neoplastic progression, which could be exploited for the development of protein biomarkers in fields of non-dysplastic mucosa that identify a patient's risk for UC dysplasia.

Received: April 6, 2009
Revised: June 26, 2009
Accepted: August 8, 2009

**Keywords:**

Biomarker / Cancer / Dysplasia / Neoplastic progression / Ulcerative colitis

Correspondence: Dr. Ru Chen, Department of Medicine, Division of Gastroenterology, University of Washington, Seattle, WA 98195, USA

E-mail: ruc@medicine.washington.edu

Fax: +1-206-685-9478

Abbreviations: CPS1, carbamoyl-phosphate synthase 1; HGD, high-grade dysplasia; IBD, inflammatory bowel disease; IHC, immunohistochemistry; NEG, negative for dysplasia; TPP, Trans-Proteomic Pipeline; TRF5, interferon regulatory factor 5; UC, ulcerative colitis

1 Introduction

Ulcerative colitis (UC) patients have an elevated rate of colon cancer and the colonoscopic surveillance of these patients is expensive, time consuming, and invasive. Moreover, evaluation of UC neoplasia is a subjective interpretation; even the highly regarded, experienced gastrointestinal pathologists who developed the histologic standards for UC neoplasia could only agree on the diagnosis of indefinite for dysplasia half the time [1]. Obviously, a biomarker of

dysplasia would aid the clinical management of cancer risk in UC patients. Of even greater utility would be biomarkers that are present in large fields surrounding UC dysplasia, allowing their detection without the need for the very large numbers of biopsies now needed to safely identify focal dysplasia [2].

Neoplastic progression in UC occurs in a stepwise manner of histological changes from negative → indefinite for dysplasia → low-grade dysplasia → high-grade dysplasia (HGD) [1]. UC cancer is preceded by and co-exists with dysplastic precursors; thus it is possible to analyze the cancer precursors to investigate biomarkers that could be exploited to improve cancer surveillance. Previous investigations have identified several markers of dysplasia including sialosyl-Tn, sphingomyelin, p53 abnormalities, changes in cyclin D1, abnormalities in Bcl-2 expression, and aneuploidy; unfortunately many of these markers are not sufficiently wide spread and/or not sensitive enough for regular use as an ideal biomarker [3–5]. Our laboratories and others have previously showed that genetic factors (chromosomal instability, genomic instability, and microsatellite instability) and epigenetic factors (methylation) play a role in UC neoplastic progression [6–9]. These genetic biomarkers are still under evaluation for their potential as diagnostic biomarker for UC dysplasia.

Although it is clear that the histologically normal-appearing mucosa of UC patients with cancer/pre-cancer is abnormal at the genome/DNA level, little exploration has taken place to examine whether protein expression is also abnormal. The study of various grades of UC dysplasia at the protein level may discern previously unrevealed events in UC tumorigenesis, and this knowledge could, in turn, be exploited for the development of biomarkers that predict UC dysplasia. The emerging field of proteomics provides systematic investigation of proteome expression within a complex biological system. Although a large number of proteomic investigations have studied a variety of diseases and developed biomarkers for early cancer diagnosis, only a few have focused on inflammatory bowel disease (IBD) [10]. UC is one of the two major forms of IBD. Two reports using 2-DE and MS were able to identify proteins associated with IBD [11, 12], but a global quantitative proteomic study of UC neoplastic progression has not been explored.

To date, one of the most effective and versatile quantitative proteomics approaches is the combination of high-resolution MS/MS and stable isotope labeling [13]. The introduction of the stable isotope labeling technique allows for proteome identification and the direct comparison of two or more samples in a single measurement. The iTRAQ labeling technique [14] that allows simultaneous detection of up to eight samples for quantitative proteomic comparison at a global scale; thus, it is ideal for comparing multiple UC biopsies of varying degrees of dysplasia. Since both the identification and the quantification of peptides is achieved at the MS/MS level, the iTRAQ approach provides the ability for a multiplex comparison without complicating the MS

spectrum, thereby minimizing ion suppression and, ultimately, enhancing the peptide identification and quantification.

UC tumorigenesis involves genomic abnormalities throughout the whole colon, including the non-dysplastic mucosa. This is referred to as molecular field defect or genomic field defect [6–9]. Because of this genomic field defect in the colonic mucosa, we hypothesized that the same field defect would be associated with abnormally expressed proteins. In this report, we carried out an exploratory/discovery study to test this hypothesis. We used quantitative proteomics with iTRAQ labeling to compare the proteomes of both dysplastic and non-dysplastic mucosa from UC progressors (UC patients with HGD or cancer) to the colonic mucosa of UC non-progressors (UC patients who are dysplasia-free) and normal non-UC colon. We identified differentially expressed proteins that occur during the process of UC tumorigenesis, as the non-dysplastic mucosa turns into HGD. These differentially expressed proteins were further investigated to reveal the associated biological pathways, which might underlie tumorigenesis. Finally, immunohistochemistry (IHC) was used to confirm two over-expressed proteins that are present at the earliest stages of neoplastic progression and that may serve as future candidate biomarkers to identify UC progressors.

2 Materials and methods

2.1 Patients and tissue specimen

Tissue specimens from patients with UC and normal non-UC colons were collected in accordance with approved human subject's guidelines at the University of Washington and the Cleveland Clinic *via* institutional internal tissue banks. Once procured, all specimens were assigned with study IDs and specimen IDs. The specimens obtained at the time colonoscopy or from surgical resections were placed in frozen media containing minimal essential medium with 10% DMSO and kept frozen at -70°C until use. For proteomic analysis, colonic epithelial samples from five subjects (patients) from each of the following categories were used: (i) normal non-UC colon, (ii) UC non-progressors, (iii) non-dysplastic biopsies from UC progressors (UC progressors negative for dysplasia (NEG)) (these specimens were from UC patients who had HGD ($n = 3$) or colon cancer ($n = 2$), but the particular specimens tested were histologically NEG), (iv) HGD biopsies from UC progressors (UC progressors HGD). The histological grades for each specimen were made by co-author (M.P.B.) who has extensive experience in evaluating the pathology of IBD samples. The rationale for selecting five subjects from each disease category was based on the notion that over 80% of the markers present in only 30% of patients can be identified when five cases are pooled for discovery study.

The clinical characteristics of these patients are listed in Supporting Information Table 1. Efforts were made to match the progressor and non-progressor specimens for patient age, duration of disease, and inflammatory status of the samples.

2.2 Isolation of epithelial cells and protein lysis

Colonic epithelial cells were isolated from specimens by EDTA shake-off, which provides over 90% purity of epithelial cells as previously described [9]. Protein lysates were obtained by lysing the epithelial cells in T-Per (Pierce, Rockford, IL, USA) or CHAPS (Millipore, Billerica, MA, USA) with 1 × Protease Inhibitor Cocktail (Pierce).

2.3 iTRAQ labeling and MS

Four sample categories were compared using iTRAQ method, including normal non-UC colon, UC non-progressors, UC progressors NEG, and UC progressors HGD. For each sample category, a pooled sample of five was generated using 50 µg of protein lysates from each sample. The final 250 µg of protein lysate from each sample category was resuspended in iTRAQ (Applied Biosystems, Foster City, CA, USA) dissolution buffer (0.5 M triethylammonium bicarbonate). The cysteine groups of the proteins were reduced with 50 mM tris-(2 carboxyethyl)phosphine and blocked with 200 mM methyl methanethiosulfonate. The proteins were then digested with trypsin (trypsin to protein ratio of 1/50) overnight (16–18 h) and labeled with one channel of iTRAQ reagents *per* category according to the manufacturer's instructions. The samples were then combined after labeling. The combined samples were separated with strong cation exchange into 12 fractions for LC MS/MS analysis. The samples were analyzed in triplicate.

The reverse phase chromatographic separation was performed on a 100 mm × 75 µm nanoAcquity 1.7 µm BEH C18 analytical column (Waters, Milford, MA, USA) using a nanoAcquity Ultra Performance Liquid Chromatography system (Waters). The column temperature was maintained at 35°C and eluted with a gradient of 2–25% B over 50 min and 25–35% B over 10 min, where A refers to deionized H₂O with 0.1% formic acid and B refers to ACN with 0.1% formic acid. The flow rate used was 300 nL/min and 2 µL of each sample was injected. Samples were analyzed on a Waters Q-TOF Premier mass spectrometer (Micromass, Manchester, UK) by data-dependent acquisition using a standard Waters nano-ESI source operating in the positive mode. The capillary and cone voltages were 3.5 kV and 36 V, respectively. The source temperature was set to 90°C with a nanoflow gas pressure of 40 000 pascals.

2.4 Proteomics data processing

The MS/MS data were exported in .RAW format from Waters Q-TOF instrument using MassLynx software (v4.1.) and processed using Trans-Proteomic Pipeline (TPP, <http://tools.proteomecenter.org/software.php>). The MS/MS data was first converted into mzXML format using massWolf software provided with TPP and then searched against IPI human protein database (version 3.38, 70 856 protein entries, European Bioinformatics Institute) using the SEQUEST algorithm (version 27) [15]. The searches were performed using the following parameters: a mass tolerance of 3.0 Da, full trypsin specificity, static modification of iTRAQ labeling on N-terminus and lysine (+144.10), and blocking on cysteine using methyl methanethiosulfonate (+45.99). All the peptide identifications were validated using PeptideProphet [16]. PeptideProphet uses various SEQUEST scores and a number of other parameters to estimate the accuracy of peptide assignments to MS/MS spectra made by database search algorithms. A cutoff probability score of 0.9 was used for this study. A target/decoy search was performed and it revealed a false positive rate of less than 1% based on a PeptideProphet probability score cutoff at 0.9 [17]. The false positive rate is defined as the percentage of the number of false positive identifications in the forward database to the total number of peptides identified with a probability score of 0.9 or higher.

The iTRAQ quantification was achieved using LIBRA program [18]. The threshold for the peptides used for quantification was a probability of 0.9. Protein quantitation is derived from the group of peptides associated with the protein. The integrated intensity of each peptide was normalized by the sum of its channel intensities. All of the normalized peptides of a protein were then averaged and the standard deviation of the mean was determined for each protein. Channel 114 was designated as a reference normalization channel, and the protein quantitation of other channels (115, 116, and 117) was normalized with respect to this channel. Furthermore, the channel 115, 116, and 117 ratios were normalized by summing all iTRAQ ratios across the entire data set, calculating an overall ratio (0.86 for 115, 1.00 for 116, and 1.02 for 117) and dividing the ratios for each protein by these values. This effectively accounts for experimental and potential errors introduced by isotopic overlap between the iTRAQ reagent fragments [19]. More information about TPP, PeptideProphet, LIBRA, and other programs can be obtained from the Seattle Proteome Center (<http://tools.proteomecenter.org>).

2.5 Networks analysis using MetaCore

MetaCore (GeneGo, St. Joseph, MI, USA) was used to map the differentially expressed proteins into biological networks. MetaCore is an integrated software suite for functional analysis of experimental data. It is based on a

Table 1. Proteins over-expressed in UC progressor by at least twofold (proteins with at least two peptides quantification and $p < 0.1$ were included here)

Database ID ^{a)}	Protein description	Gene symbol	NP/NL (115:114)	SEM	NEG/NL (116:114)	SEM	HGD/NL (117:114)	SEM	Peptides for quantification
IP100385149	Putative uncharacterized protein	PTMA	1.2	0.2	1.0	0.1	2.2	0.4	9
IP100258804	ATPase inhibitory factor 1 isoform 3 precursor	ATP1F1	1.3	0.5	1.1	0.5	2.1	1.2	10
IP100004962	Golgi integral membrane protein 4	GOLIM4	2.7	4.0	1.1	1.0	2.6	3.3	5
IP100003734	Putative S100 calcium-binding protein H_NH0456N16.1	LOC347701	1.0	0.2	1.2	0.3	2.0	0.6	14
IP100027412	Carcinoembryonic antigen-related cell adhesion molecule 6	CEACAM6	1.4	0.1	1.3	0.2	2.4	0.3	5
IP100019872	Estradiol 17- β -dehydrogenase 2	HSD17B2	1.3	0.3	1.3	0.1	2.6	0.5	12
IP100793199	Annexin IV	ANXA4	1.2	0.1	1.4	0.3	2.1	0.5	78
IP100001539	3-Ketoacyl-CoA thiolase, mitochondrial	ACAA2	1.6	0.3	1.4	0.3	2.0	0.5	118
IP100026328	Thioredoxin domain-containing protein 12 precursor	TXNDC12	1.4	0.2	1.5	0.3	2.1	0.4	11
IP100030122	Isoform 1 of urotensin-2 precursor	UTS2	1.7	0.0	1.5	0.0	2.1	0.1	2
IP100020599	Calreticulin precursor	CALR	1.5	0.2	1.6	0.3	2.3	0.6	139
IP100386755	ERO1-like protein α precursor	ERO1L	1.7	0.3	1.6	0.5	4.0	1.4	16
IP100009904	Protein disulfide-isomerase A4 precursor	PDI4A	1.5	0.3	1.6	0.4	2.3	0.7	152
IP100171421	Uncharacterized protein C8orf55 precursor	C8orf55	1.5	0.1	1.7	0.1	2.5	0.4	2
IP100013895	Protein S100-A11	S100A11	1.1	0.1	1.7	0.3	2.9	0.5	22
IP100024993	Enoyl-CoA hydratase, mitochondrial precursor	ECHS1	1.7	0.4	1.8	0.5	2.0	0.6	99
IP100008934	Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor	HMGCS2	1.7	0.4	1.8	0.5	2.0	0.7	180
IP100025252	Protein disulfide-isomerase A3 precursor	PDI3	1.6	0.3	1.8	0.4	2.4	0.6	282
IP100010491	Ras-related protein Rab-27B	RAB27B	0.9	0.0	1.8	0.4	2.0	0.1	2
IP100007427	AGR2	AGR2	1.2	0.5	1.8	0.6	2.3	1.0	117
IP100010796	Protein disulfide-isomerase precursor	P4HB	1.9	0.4	1.8	0.4	2.7	0.7	292
IP100021828	Cystatin-B	CSTB	1.5	0.6	1.8	0.9	2.0	1.0	3
IP100025062	Isoform 1 of cathepsin E precursor	CTSE	0.7	0.1	1.8	0.3	3.7	0.7	5
IP100220362	10kDa heat shock protein, mitochondrial	HSPE1	1.6	0.2	1.8	0.3	2.1	0.4	110
IP100008840	POU domain, class 3, transcription factor 1	POU3F1	1.1	0.1	1.9	0.1	2.3	0.2	14
IP100010290	FABP1 protein (fragment)	FABP1	2.4	1.0	1.9	0.9	2.0	0.9	157
IP100216071	Eosinophil lysophospholipase	CLC	1.6	0.0	2.0	0.0	1.5	0.0	2
IP100008367	Isoform 1 of inactive carboxylesterase 4 precursor	CES4	1.5	0.2	2.0	0.2	1.4	0.2	4
IP100005668	Aldo-keto reductase family 1 member C2	AKR1C2	1.4	0.3	2.0	0.4	1.2	0.2	4
IP100006663	Aldehyde dehydrogenase, mitochondrial precursor	ALDH2	2.0	0.4	2.0	0.5	2.0	0.5	234
IP100027028	Paraneoplastic antigen Ma2	PNMA2	2.1	0.0	2.0	0.0	1.7	0.0	2
IP100167072	Isoform 5 of abhydrolase domain-containing protein 11	ABHD11	1.5	0.3	2.0	1.0	1.8	0.9	7
IP100011603	26S proteasome non-ATPase regulatory subunit 3	PSMD3	3.1	3.0	2.0	1.9	2.6	2.5	2
IP100027463	Protein S100-A6	S100A6	1.1	0.1	2.1	0.5	2.9	0.8	45
IP100103436	Intelectin-2 precursor	ITLN2	0.7	0.1	2.1	0.4	1.5	0.2	8
IP100216932	Isoform 1 of acetyl-coenzyme A synthetase 2-like, mitochondrial precursor	ACSS1	1.5	0.4	2.1	0.7	1.7	0.4	5
IP100027701	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADS	1.7	0.5	2.2	0.8	1.7	0.4	62
IP100291737	Intelectin-1 precursor	ITLN1	0.9	0.2	2.3	0.5	1.7	0.4	28

Table 1. Continued

Database ID ^{a)}	Protein description	Gene symbol	NP/NL (115:114)	SEM	NEG/NL (116:114)	SEM	HGD/NL (117:114)	SEM	Peptides for quantification
IP100018909	Trefoil factor 3 precursor	TFF3	1.4	0.4	2.4	0.9	1.7	0.7	17
IP100242956	IgGf-c-binding protein precursor	FCGBP	1.4	0.3	2.5	0.8	2.0	0.6	258
IP100008339	78 kDa protein		2.1	0.5	2.5	0.3	2.6	1.3	2
IP100791006	Mucin 4	MUC4	4.6	2.4	3.3	1.9	2.8	1.8	3
IP10020716	Isoform 2 of cathepsin E precursor	CTSE	1.3	0.2	5.0	0.5	11.9	1.2	2
IP100011062	Isoform 1 of carbamoyl-phosphate synthase, mitochondrial	CPS1	1.1	0.3	5.3	4.1	3.6	2.5	43
IP100017526	Protein S100-P	S100P	1.3	0.3	6.4	3.8	6.5	3.9	18

NP, non-progressor; NL, normal.

a) When there are multiple matches in database, only the first IPI ID is listed here.

proprietary, manually curated database of human protein–protein, protein–DNA, and protein–compound interactions; metabolic and signaling pathways; and effects of bioactive molecules. The differentially expressed proteins were converted into Entrez protein identifiers and uploaded into the MetaCore platform for analysis. The biological process enrichment was analyzed based on gene ontology processes. The Analyze Network algorithm was used to map protein pathways within two-step interactions. This algorithm generates sub-networks of a large network, where interactions are expanded from each gene (from our list) until the sub-networks intersect. Enrichment analysis was performed using MetaCore, in addition to the Database for Annotation, Visualization and Integrated Discovery (DAVID) [20] tool from the National Institute of Allergy and Infectious Diseases (NIAID), NIH.

2.6 IHC analysis

Paraffin-embedded, lightly paraformaldehyde-fixed slides were processed using a modification of previous protocols [21]. The deparaffinized sections were processed for antigen retrieval using heat induced epitope retrieval techniques in citrate buffer (0.1 M, pH 6.0), followed by cooling to room temperature and then primary antibody incubation. Dilution of the primary antibody carbamoyl-phosphate synthase 1 (CPS1) (Abcam, Cambridge, MA, USA) and S100P (R&D Systems, Minneapolis, MN, USA) were 1:250 and 1:500, respectively. Dilution of the secondary antibodies for CPS1 and S100P were 1:500 and 1:1000, respectively. IHC staining was graded as 0–4+, from negative to intensely positive.

3 Results

3.1 Identification of differentially expressed proteins in UC and associated dysplasia

The overall workflow for the quantitative proteomics analysis of UC colonic epithelium is presented in Fig. 1. UC colons have various degrees of leukocytes (inflammatory cells) in the epithelium depending on the degree of inflammation. However, these inflammatory cells usually stay in the lamina propria and sub-mucosa; thus after epithelial cells isolation procedure, they are no longer in the epithelial cell fraction. The tryptic digestion of the samples was labeled with the appropriate iTRAQ reagents: normal non-UC colon with iTRAQ 114, UC non-progressors with iTRAQ 115, UC progressors NEG with iTRAQ 116, and UC progressors HGD with iTRAQ 117. The MS/MS spectra were searched against the IPI human protein database using the SEQUEST algorithm and peptide identifications were validated using PeptideProphet with a cutoff probability score of 0.9. At this cutoff, a false positive rate of

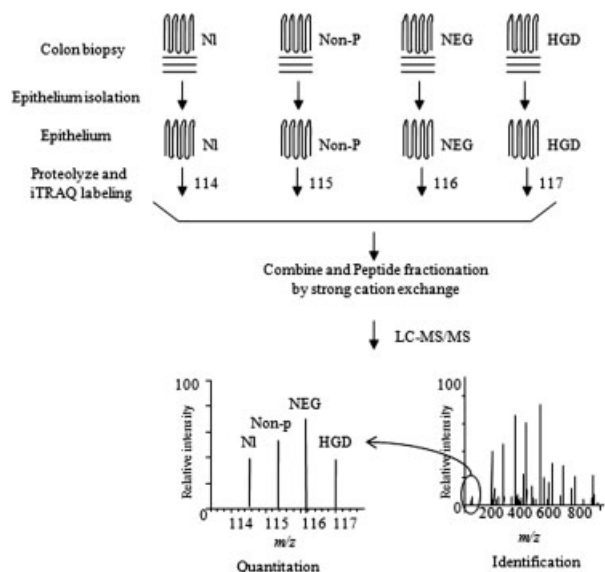


Figure 1. Workflow for quantitative proteomics analysis of colon epithelium. Colonic epithelium was first isolated from colon specimens. The protein lysates from colon epithelium were proteolyzed and iTRAQ labeled with different iTRAQ channel reagents. The labeled samples were then combined, fractionated, purified, and analyzed by tandem mass spectrometer. The proteins were identified by database search of the tandem mass spectrum for matching peptides. The relative abundance was determined by the ratio of signal intensities of the signature peaks of the target sample compared with normal colon (NI). Non-p refers to non-progressors.

0.65% was estimated based on a target/decoy database search. The final, filtered data set contains 4035 non-redundant peptides leading to the identification of 1107 proteins/protein groups including 269 single-peptide hits.

The iTRAQ quantification was achieved using the LIBRA program. We performed technical replicates for the experiment. There is a strong correlation in the protein ratios between the replicates: $r^2 = 0.94$ ($p < 0.0001$). This level of variation (6%) is consistent with the results reported in the literature [22]. For each iTRAQ channel, the data were normalized to reduce the systematic error and a Z-score was calculated for each protein based on the normalized iTRAQ ratio. Using a 2.0-fold change in protein expression as cutoff value, there were 60 differentially expressed proteins in UC non-progressors compared with normal non-UC colon, comprising 45 down-regulated and 15 up-regulated proteins. The numbers of differentially expressed proteins in the UC progressor samples (compared with normal non-UC colon) were increased to 98 and 99 for UC progressors NEG and UC progressors HGD, respectively. Although the number of down-regulated proteins in UC progressors NEG ($n = 73$) was much higher than in UC non-progressors ($n = 45$), the number of under-expressed proteins in HGD ($n = 49$) was close to that of UC non-progressors. In contrast, the number of over-expressed proteins increased as the UC colon

was becoming more dysplastic: non-progressors ($n = 15$ proteins)NEG ($n = 25$ proteins)→HGD ($n = 50$ proteins) (Supporting Information Fig. 1A). A subset of over-expressed proteins in UC progressors with at least twofold expression change and at least two-peptide quantification is presented in Table 1. The protein identification information is provided in Supporting Information Table 2. As shown in Supporting Information Fig. 1B, 44 proteins were differentially expressed in all UC samples compared with normal non-UC colon. These included 8 up-regulated and 36 down-regulated proteins. There were 6, 35, and 37 differentially expressed proteins in UC non-progressors, UC progressors NEG, and UC progressors HGD, respectively.

We do not rule out the possibility that some of the differentially expressed proteins may be due to biological heterogeneity. As we will discuss in the following sections, many of the proteins in Table 1 are found to be functionally associated with neoplastic progression. It is also interesting to note that several keratins were identified as under-expressed in the UC diseased samples compared with the normal non-UC colon. Keratins can be possible contaminations in proteomics analysis. However, in this particular study, we used isolated colonic epithelial tissue, which is particularly rich in keratin content, for proteomics analysis. Thus, it is not surprising to observe the presence of keratins in our samples. In addition, it has been previously observed that defects in keratin result in a variety of tissue diseases that can be reflected by the changes in the keratin expression profile.

3.2 Functional analysis of differentially expressed proteins in UC non-progressors and progressors

To functionally annotate the differentially expressed proteins identified in this study, these proteins (≥ 2.0 -fold change) were entered into GeneGo's MetaCore and DAVID databases for enrichment and network analysis.

3.2.1 Common biological processes in UC

First, we examined the common biologic processes that were represented by the shared differentially expressed proteins present in all UC samples. This was accomplished using GeneGo's Compare Experiments Workflow from the MetaCore platform. The top five common network processes were: (i) interphase of mitotic cell cycle and cell cycle; (ii) mitotic spindle organization and biogenesis and microtubule-based movement; (iii) localization of cell, cell motility, and actin cytoskeleton organization and biogenesis; (iv) structure, organ, and system development; and (v) cell differentiation and cellular developmental process. These common processes reflect the underlining biological events in UC condition: active cellular turnover, cellular regeneration, and tissue repair.

3.2.2 Unique biological processes in UC neoplastic progression

We then analyzed the biologic processes for the differentially expressed proteins unique for each stage of UC neoplastic progression using the MetaCore platform mentioned earlier. The most relevant unique processes for the UC non-progressors (by degree of significance) were nuclear transport, recombination, spindle organization and biogenesis, and mRNA processing, indicating the very active cellular organelle activity in UC conditions. The most relevant unique processes for UC progressors NEG were triacylglycerol mobilization, cellular component organization and biogenesis, cell development, apoptosis, and programmed cell death. Finally, in UC progressors HGD, the unique processes included immune response, multi-cellular organismal process, system development, and multi-cellular organismal development.

3.2.3 Mitochondrial proteins were frequently differentially expressed

Mitochondrial proteins were among the most differentially expressed proteins in UC non-progressors and even more in the UC progressors: 6, 8, and 12 mitochondrial proteins were differentially expressed by at least twofold in UC non-progressors, UC progressors NEG, and UC progressors HGD, respectively. The enrichments of mitochondrial proteins among the differentially expressed proteins were all statistically significant ($p = 0.049$, 0.05 , and 0.0005 for UC non-progressors, UC progressors NEG, and UC progressors HGD, respectively).

3.2.4 Oxidative activity

UC is associated with increased level of ROS and oxidative stress. The number of differentially expressed proteins in oxido-reductase activity increased with UC neoplastic progression: 5 in UC non-progressors, 7 in UC progressors NEG, and 13 in UC progressors HGD ($p = 0.072$, 0.059 , and 0.0017 for UC non-progressors, UC progressors NEG, and UC progressors HGD, respectively).

3.3 Unique differentially expressed networks associated with UC neoplastic progression

To analyze the broader interacting network among the differentially expressed proteins in UC progressors, we used GeneGo's network algorithms from the MetaCore platform using the Analyze Network option, with a 50-node limit. The top sub-network for UC progressors NEG brought together 12 differentially expressed proteins (≥ 2.0 -fold) with the most prominent network protein being transcriptional factor Sp1. Sp1 displays direct interactions with half of the targets in the UC network; 12 of the 25 were the differentially expressed proteins in UC progressors NEG. The top network for UC progressors HGD brought together eight differentially expressed proteins with ≥ 2.0 -fold changes. In contrast to the central role of Sp1 in UC progressors NEG, the transcriptional factor c-myc played a role in the HGD protein network. Among the eight differentially expressed proteins in the network, c-myc directly interacted with six of them and had two-step (*e.g.* indirect) interactions with the other two proteins.

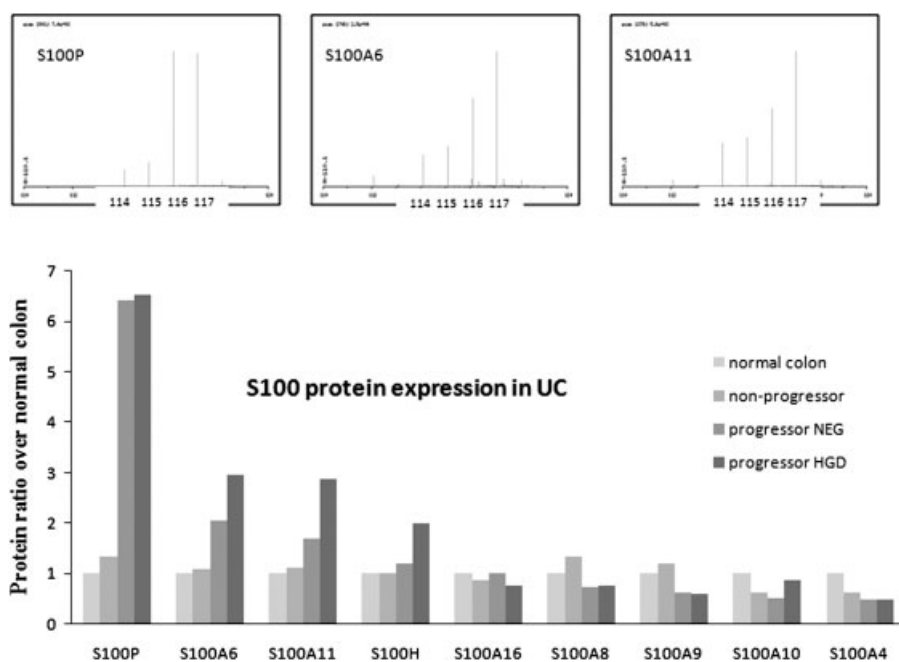


Figure 2. S100 protein expression in UC. The upper panel shows the ion chromatogram of the signature peaks for fragments 114, 115, 116, and 117, which represent normal colon, UC non-progressors, UC progressors NEG, and UC progressors HGD, respectively. The lower panel displays the ratio of S100 abundance in the four sample types.

3.4 Progressive S100 protein dysregulation associated with UC neoplastic progression

The S100 proteins are a family of calcium-binding proteins that are involved in a broad range of intra- and extra-cellular functions. Several members of this S100 family were differentially expressed in UC neoplastic progression. Four S100 proteins, including S100P, S100A6, S100A11, and S100H, displayed increased expression in the UC neoplastic progression, e.g. normal colon < UC non-progressors < UC progressors NEG < UC progressors HGD, with HGD samples having the highest expression (Fig. 2). Four other proteins, S100A8, S100A9, S100A10, and S100A4, displayed various degrees of decreased expression in UC progressors NEG and UC progressors HGD.

3.5 Over-expression of CPS1 and S100P in UC progressors: IHC confirmation

The two proteins with highest degree of over-expression in UC progressors, CPS1 and S100P, were selected for further IHC analysis.

3.5.1 CPS1

Proteomic analysis revealed that CPS1 was over-expressed in UC progressors (5.3-fold in NEG and 3.6-fold in HGD) and its expression was normal in UC non-progressors (Table 1). IHC staining was performed to confirm this result. As showed in Fig. 3B, colonic epithelium from UC progressors NEG exhibited strong staining, while the UC non-progressor mucosa stained mildly positive (Fig. 3A). The mean IHC-staining scores in UC progressors are significantly higher than those of UC non-progressors ($p = 0.0001$, Fig. 4). All but one of the 17 specimens from UC progressors displayed IHC score of 3+ or 4+. If the IHC score of 3+ or higher was used as a cutoff, CPS1 could achieve 80% specificity and 94% sensitivity in distinguishing UC progressors from UC non-progressors.

3.5.2 S100P

S100P was over-expressed in UC progressors NEG (6.4-fold) and UC progressors HGD (6.5-fold) by proteomic analysis. IHC staining revealed that majority of non-progressor samples had negative or mild (1+) staining of S100P (Fig. 3C). In contrast, the majority of UC progressors showed strong epithelial staining (Fig. 3D). The mean IHC staining scores in UC progressors are significantly higher than those of UC non-progressors ($p = 0.0001$, Fig. 4). All but three of the 17 specimens

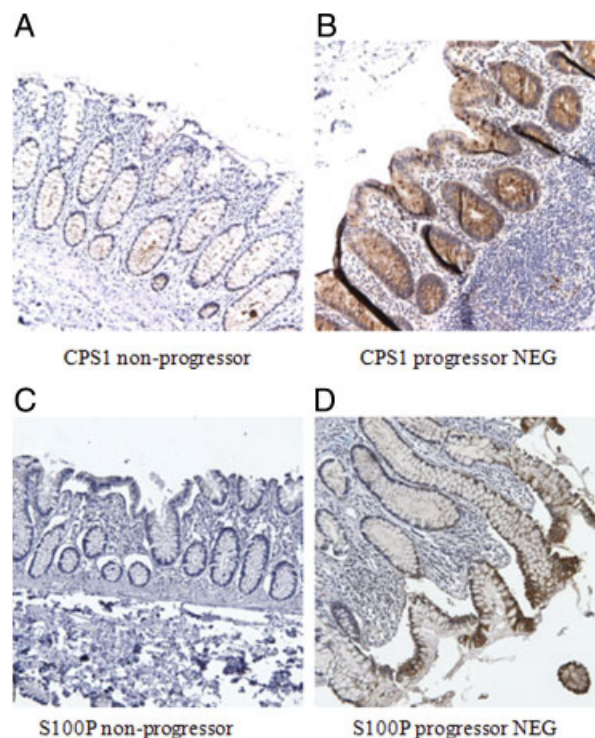


Figure 3. IHC analysis of CPS1 and S100P protein expression in UC non-progressors and UC progressors. (A and B) CPS1 is mildly positive in the epithelium of non-progressors (A); in contrast, over-expression of CPS1 was apparent in the epithelium of UC progressors NEG (B). (C and D) Note the over-expression of S100P in the epithelium of UC progressors NEG, while UC non-progressor colon has absence of staining.

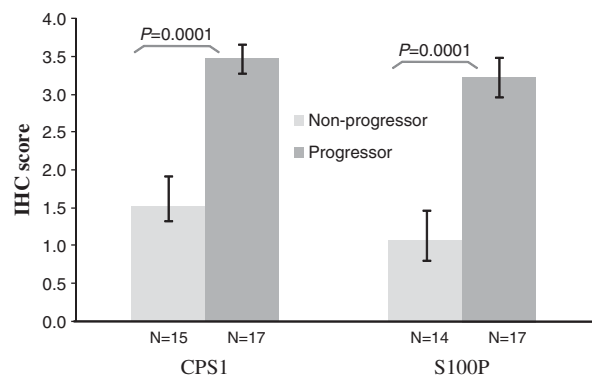


Figure 4. IHC scores of CPS1 and S100P. The mean IHC score for progressor is significantly higher than non-progressors for both CPS1 and S100P. IHC scores ranges from 0 to 4+, 0 being negative and 4+ being strongest. *N* refers to number of specimens tested.

from UC progressors displayed IHC score of 3+ or 4+. If the IHC score of 3+ or higher was used as a cutoff, S100P could achieve 79% specificity and 82% sensitivity in distinguishing UC progressors from UC non-progressors.

4 Discussion

In this study, we investigated the proteome that underlies UC neoplastic progression. The protein profiles of colonic epithelium from UC non-progressors *versus* UC progressors (both the non-dysplastic and dysplastic mucosa) were compared with normal colonic epithelium from non-UC colons. Not surprisingly, the colonic mucosa of UC progressors displayed more differentially expressed proteins than the UC non-progressors. What was surprising was the discovery that the proteome of the *non-dysplastic* mucosa from the progressors was also abnormal – that it was more akin to the proteome of HGD than it was to the non-dysplastic mucosa of non-progressors. These findings suggest that there are changes in protein expression early in the neoplastic progression, before the histologic changes become evident in the epithelial cells. IHC studies provided confirmation of the over-expression of two proteins in UC progressors (in both the dysplastic tissue and non-dysplastic tissue) compared with absent or minimal expression in UC non-progressors and normal colon. The data from this proteomic analysis may help shed light on the process of UC tumorigenesis and provide candidates for future biomarkers. In considering the changes in protein expression between UC non-progressor epithelium and the UC progressors, it is theoretically possible that some of the changes could be due to the inflammatory cells retained in the isolated epithelial cell fractions used for proteomics analysis. However, we used the following strategies to minimize the chance of identifying biomarkers from inflammation: our epithelial cell isolation protocol usually obtains over 90% purity of epithelial cell, the degree of inflammation varies between patients and biopsies, and we use inflammation matching for each group of pooled specimens used in this study including some matched UC specimens that had minimal to no inflammation.

4.1 Differentially expressed mitochondrial proteins

One of the enriched classes of differentially expressed proteins in both UC non-progressors and UC progressors was the mitochondrial proteins (8 and 12% of all differentially expressed proteins, respectively). As the main source of energy and endogenous oxidative stress in the cell, mitochondria require continuous turnover and regeneration. Inability to maintain mitochondrial function and integrity is associated with degenerative diseases, cancer, and aging. We have previously identified UC as a disease of premature aging of the colon, with the colon of a young person with 8 years of disease sharing the same colonic telomere lengths and DNA damage markers as those of a person who is 60 years old [21]. In UC, the colon epithelium undergoes repeated cycles of inflammation and tissue repair, resulting in oxidative stress and accumulation of ROS. Mitochondrial DNA is more susceptible than nuclear

DNA to damage by ROS because there are no adequate repair mechanisms for mtDNA. Mutations in mitochondrial DNA have been found in UC colonic epithelium, and animal models of colitis suggest that these mutations may be preventable with the use of selenium [23]. It is well known that mitochondrial dysfunction and mitochondrial mutations are associated with and accumulate in cancer cells, although the precise role of impaired mitochondria in tumorigenesis is still unclear. One mechanism in which the mitochondria could influence tumorigenesis stems from observations that impaired mitochondria can lead to premature senescence [24]. Senescent cells have recently been recognized as having a potential role in promoting neoplastic progression, *e.g.* by promoting a pro-inflammatory micro-environment that promotes tumor evolution [25]. Thus, increasingly impaired mitochondria may help drive the process of UC tumorigenesis. In one study, the transfer of mitochondrial DNA from cells that have a high metastatic potential into cells that have a low metastatic potential leads a change in the functional behavior of the cells. The cells take on the phenotype that is associated with the mitochondria – in this case the low metastatic cells became highly metastatic. Interestingly, the process was reversed by the introduction of anti-oxidants [26]. The premature aging in UC colons, and the increased cancer risk, is not just due to injury of nuclear DNA but perhaps due to chronic injury to the mitochondria as well.

4.2 Sp1 in the earliest stages of UC progression

Sp1 is a ubiquitously expressed transcription factor, which can both positively and negatively regulate genes. In addition to the major targeted genes that encode proteins for intermediary metabolism, Sp1 target genes are key players in cell proliferation and oncogenesis [27] as well as the UC susceptibility gene of interferon regulatory factor 5 (TRF5) [28]. A polymorphism in the TRF5 gene provides additional binding sites for Sp1, increasing the transcription of the TRF5 and likely causing an increase in production of pro-inflammatory cytokines and the perpetuation of inflammation [28]. In our study, GeneGo network analysis revealed that Sp1 played a central role in the most significant network of the differentially expressed proteins in UC progressors NEG. This result suggests that Sp1 may play a role not only in UC susceptibility by increasing transcription of TRF5, but it also appears to be important in early UC neoplastic progression.

4.3 c-myc may regulate advanced UC dysplasia

The transcription factor c-myc is a key regulator of cell proliferation, cell growth, differentiation, and apoptosis. Our previous studies have identified c-myc as a common prominent regulatory protein in pancreatic cancer and

chronic pancreatitis [29]. In the current study, in UC progressors HGD, the most significant network of differentially expressed proteins merged through c-myc regulation. This finding is supported by previous studies showing enhanced c-myc expression, as measured by IHC and c-myc mRNA, in UC and particularly in UC dysplasia [30]. Together these findings suggest that c-myc plays an important role in late UC neoplastic progression (HGD). Recent *in vitro* studies in human colon cancer cell lines revealed that high doses of mesalamine, an anti-inflammatory agent used to treat UC patients, can significantly reduce expression of c-myc mRNA and protein and increase apoptosis in a dose-dependent manner [31]. Chemoprevention of UC dysplasia using mesalamine to reduce inflammation and c-myc over-expression would certainly be an area of future interest [32].

4.4 Dysregulation of S100 proteins

The S100 proteins are a multi-gene calcium-binding family comprising 20 known human members [33]. They have a broad range of intracellular and extracellular functions, including regulation of protein phosphorylation and enzyme activity, calcium homeostasis, regulation of cytoskeletal components, and regulation of transcriptional factors [33]. Some S100 proteins are located on the mitochondrial membrane and some play a role in apoptosis by inducing a rapid decrease in the mitochondrial membrane potential [34, 35]. Several members of S100 proteins were differentially expressed in the colonic epithelium of UC progressors compared with UC non-progressors in our study, suggesting their importance in UC tumorigenesis.

S100P is frequently over-expressed in several epithelial tumor types including pancreas [36] and colon [37]. A recent study suggests that S100P may play an important role in sporadic colon cancer by stimulating cell growth, migration, and signaling pathways that affect a range of pro-inflammatory molecules [37]. Up-regulation of S100P is an early molecular event in the development of pancreatic cancer and it is expressed at high levels in both precursor lesions and invasive cancer [38, 39]. In a similar vein, we detected over-expression of S100P in UC-associated HGD and colon cancer and in the non-dysplastic mucosa of UC progressors. Furthermore, IHC analysis verified that S100P protein expression was markedly higher in the non-dysplastic colonic tissue in UC progressors than in normal or UC non-progressor colonic mucosa. Further studies are warranted to investigate whether S100P could be a potential biomarker for predicting colon cancer development in the setting of UC.

In addition to dysregulation of S100P, three other S100 proteins displayed increased expression in UC progressors (S100A6, S100A11, and S100H (putative S100 calcium-binding protein H)) and two other S100 proteins displayed decreased expression. Given the substantial changes in the

expression of S100 proteins in UC progressors, it would be worthwhile to explore further the role of these calcium-binding proteins in UC tumorigenesis.

4.5 CPS1

CPS1 is a mitochondrial enzyme involved in the urea cycle. The protein, which is expressed mainly in intestinal epithelial and liver cells, detoxifies ammonia and, together with other enzymes of the urea cycle, is the *de novo* source of arginine. Variations in the supply of arginine, due to alterations in urea cycle function, affect the production of nitric oxide [40]. Nitric oxide, in turn, can cause DNA damage and laxity in DNA repair, and it is associated with inflammation-associated cancers [41]. The rate of CPS1 gene amplification is elevated 50- to 100-fold in human cell lines deficient in mismatch repair (due to MLH1 or MSH6) as compared with mismatch repair-proficient control cells. Previous studies have showed increased expression of CPS1 in gastric cancer [42] and down-regulation in human hepatocellular carcinoma [43]. Our study identifies over-expression of CPS1 in UC progressors, in both histologically normal and dysplastic tissues. CPS1 is an abundant protein that constitutes 20–30% of mitochondrial matrix protein. There is usually an excess amount of CPS1 that is actually required for its function. Further study is needed to investigate if increased CPS1 protein expression level reflects an increase in its functional activity.

5 Concluding remarks

In this study, we applied quantitative proteomics to investigate differentially expressed proteins associated with UC neoplastic progression. Proteins relating to mitochondrion, oxidative activity, and calcium ion binding were some of the interesting classes of these differentially expressed proteins. Further network analysis discovered that Sp1 and c-myc may play roles in the early and late events of UC tumorigenesis, respectively. Finally, two up-regulated proteins in both the dysplastic and non-dysplastic mucosa of UC progressors, CPS1 and S100P, were confirmed by IHC analysis. The differentially expressed proteins identified in this study, especially CPS1 and S100P, could be exploited for the future development of biomarkers that predict UC dysplasia.

The authors thank Rosa Ana Risques, Josephine Maurer, Allyn Stevens, Jimmy Eng, and Ashley Eastham for technical support and study coordination. This work was supported by grants from Crohn's & Colitis Foundation (RC), NIH NCI R01CA068124 (TB), and National Heart, Lung and Blood Institute NIH, under contract # NOI-HV-28179 (RA).

The authors have declared no conflict of interest.

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