Increasing Incidence of Colon Cancer in the Young: Assessing the Tumor Biology

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BACKGROUND: The overall incidence of colon cancer (CC) is decreasing, but with increasing early-onset colon cancer (EOCC < 50 years old). Our recent study revealed unique overexpression of cartilage oligomeric matrix protein (COMP) in EOCC and its association with aggressiveness. The aim of this study was to assess CC biology, especially in the young, by evaluating the role of COMP in CC carcinogenesis and cancer progression, detecting COMP in serum and its association with disease stage.

STUDY DESIGN: Cancer and matching noninvolved tissue blocks from 12 sporadic EOCC and late-onset colon cancer (LOCC) patients of 4 disease stages were obtained from pathology archives. Ribonucleic acid expression profiling of 770 cancer-related genes using nCounter platform was performed. The COMP levels from 16 EOCC and LOCC serum samples were measured by ELISA. Carcinoembryonic antigen levels from these 16 samples were taken at the time of diagnosis. Transwell assay was performed to elucidate the role of COMP in motility and metastases.

RESULTS: Expression profiling revealed increased COMP levels in higher disease stage. There was 7-fold higher COMP expression ($p \leq 0.05$) in stage III compare to stage I and its coexpression with GAS1, VEGFC, MAP3K8, SFRP1, and PRKACA. Higher COMP expression was seen in stage II compared with stage I ($p = 0.07$) and its coexpression withTLR2, IL8, RIN1, IRAK3, and CACNA2D2, and COMP was detectable in serum and showed significantly higher levels in EOCC compared with LOCC. Similar correlation was seen with CEA levels, but the difference was not significant. Transwell assay revealed significantly increased motility of HT-29 cells after treatment with recombinant COMP.

CONCLUSIONS: These findings suggest different tumor biology between EOCC and LOCC. Cartilage oligomeric matrix protein plays a significant role in CC carcinogenesis and has potential as biomarker for CC, especially aggressive EOCC. (J Am Coll Surg 2019;229:79–90.

Colon cancer (CC) is the third most commonly diagnosed cancer and second leading cause of cancer-related deaths in the United States. Globally, 1.2 million new cases and 0.6 million deaths are reported each year.[1-3] Approximately 97,380 of new CC cases were expected to be diagnosed in the US in 2018.[1]

Despite an overall decrease in incidence of CC over the past 30 years, the number of early-onset CC (EOCC < 50 years old) is rising alarmingly.[4,5] Most of these EOCC tumors (70% to 80%) are "sporadic," and not attributed to any hereditary cause. They tend to be more aggressive, with poorly differentiated mucinous histologic features and signet ring morphology.[3-8] Furthermore, these tumors are often diagnosed at a more advanced stage, and these patients usually have poorer survival.[9] The etiology and mechanism of EOCC and its enhanced aggressive features are still not well understood, but are likely multifactorial.

Due to a persisting increase in the incidence of EOCC, the American Cancer Society recently recommended

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Abbreviations and Acronyms

CC	= colon cancer
COAD-TCGA	= colon adenocarcinoma dataset - the cancer genome atlas
COMP	= cartilage oligomeric matrix protein
EOCC	= early onset colon cancer
FFPE	= formalin-fixed paraffin-embedded
LOCC	= late-onset colon cancer.

decreasing the age eligibility for first colonoscopy screening for average risk patients from 50 years of age to 45.[10] Model-recommendable strategies showed that starting screening at age 45 was more effective at identifying CC at earlier stages than starting at age 50.[11] Currently, CEA remains the only tumor marker with recognized efficacy for monitoring CC, although its elevated levels in blood samples can constitute a variety of other pathologic processes including gastric cancer, pancreatic cancer, or inflammatory conditions.[12] Moreover, CEA as a marker has shown only limited sensitivity and specificity.[13,14]

Although cancer-specific biomarkers are promising tools for cancer screening and diagnosis,[15] no reliable molecular markers for early detection of CC exist to date, especially in younger patients. Therefore, there is an urgent need to find molecular biomarkers for early detection of CC, as they can assess the risk of malignancy, its aggressiveness over time, and the probability that a patient will respond to a certain treatment, resulting in more personalized treatment decisions instituted by physicians.[16]

Our recently published genomic studies have shown that EOCC is a distinct disease from late-onset colon cancer (LOCC).[17] We demonstrated that sporadic EOCC expresses unique genes when compared with LOCC. Cartilage oligomeric matrix protein (COMP) was one of the highest uniquely up-regulated genes in EOCC.[17] This observation was confirmed by quantitative polymerase chain reaction (qPCR) and immunohistochemistry using anti-human cartilage oligomeric matrix protein (COMP) antibody. All EOCC tissues showed very strong staining for COMP compared with LOCC (unpublished data). Furthermore, we also demonstrated that COMP was found to be coexpressed with epithelial to mesenchymal transition (EMT) genes, suggesting its role in cancer progression and aggressiveness. Further survival analysis revealed poorer overall survival of CC patients with higher COMP expression levels.[18] Additionally, in vitro proliferation studies showed significantly increased cellular growth of HT-29 CC cells, expressing negligible levels of COMP at baseline, after they were treated with human recombinant COMP (unpublished data). These data suggest that COMP can potentially serve as a candidate molecular biomarker for more aggressive EOCC.

The aim of this study was to further assess the biology of CC, especially in the young, by investigating the role of COMP in carcinogenesis and disease progression. This aim was achieved by looking particularly at the correlation between COMP mRNA expression levels and stage of CC, in addition to evaluating the role of COMP in CC motility. The study will also detect the presence of COMP glycoprotein in serum and its association with CEA levels, which will confer the potential of COMP glycoprotein to be considered as a biomarker for noninvasive, early detection of more aggressive EOCC.

METHODS

Ethics statement

This study involved human subjects and was approved by the University of Arizona Institutional Review Board (Protocol # 1504771180A001).

Patient samples

Deidentified formalin-fixed paraffin-embedded (FFPE) CC tissues and matching noninvolved colon tissues were obtained from the University of Arizona Pathology archives (Table 1). The samples were propensity matched based on pathology. Blood samples from EOCC (n = 8) and LOCC (n = 8) (Table 2) were obtained from the University of Arizona CRC Biorepository. All samples were propensity matched based on stage of disease. Patients with Lynch syndrome, familial adenomatous polyposis, and inflammatory bowel disease were excluded from this study.

Tumor mismatch-repair protein expression

Standard immunostaining protocols were used to analyze expression of MLH1, MSH2, MSH6, and PMS2 mismatch-repair proteins using mouse anti-MLH1 (clone M1), anti-MSH2 (clone G219-1129), anti-MSH6 (clone 44), and rabbit anti-PMS2 (clone EPR3947) monoclonal antibodies (Cell Marque). Specimens were scored in a blinded fashion by a gastrointestinal pathologist. Signal intensity in the tissue sections was graded as (0), (1) weak, (2) moderate, or (3) strong. The proportion of positively stained cells was evaluated as a percentage. The score was calculated by multiplying the intensity and percentage of stained cells. A tumor was deemed negative for protein expression only in cases in which neoplastic epithelium lacked nuclear staining, while non-neoplastic epithelial or stromal cells retained normal expression of that protein.

Table 1. Clinical Characteristics of 12 Colon Cancer Samples Used for nCounter NanoString Gene Expression Analysis

Stage	TNM	Pathology	Sex	Age, y
I	pT2N0Mx	Moderately differentiated adenocarcinoma	M	77
I	pT2N0Mx	Invasive adenocarcinoma	F	46
I	pT1N0Mx	Invasive adenocarcinoma	M	76
II	pT4bN0Mx	Invasive adenocarcinoma	F	42
II	pT4N0M0	Moderately differentiated adenocarcinoma	M	39
II	pT3N0M1a	Moderately differentiated adenocarcinoma	M	63
II	pT4N0Mx	Moderately differentiated adenocarcinoma	F	78
III	pT3N2bMx	Invasive adenocarcinoma	F	52
III	pT4aN1aMx	Invasive adenocarcinoma	F	73
III	pT3N1bMx	Invasive adenocarcinoma	M	44
III	pT4aN2bMx	Mucinous adenocarcinoma	M	65
IV	pT3N2bM1	Invasive adenocarcinoma	M	40

Table 2. Clinical Characteristics of 16 Colon Cancer Serum Samples used for Detection of Cartilage Oligomeric Matrix Protein Glycoprotein Levels

Sex	Disease site	Stage	Stage by TNM	Surgery/treatment	CEA, ng/mL
M	Colon/sigmoid	IV	pT4a N2a M1	Laparoscopic colon resection of colonic adenocarcinoma/pre-treatment	5.6
F	Colon/sigmoid	II	pT4a N0 M0	Exploratory laparotomy, resection of rectosigmoid mass/pre-treatment	35.1
F	Colon	I	pT1 N0 M0	Laparoscopic extended right hemicolectomy/ pre-treatment	2.9
F	Rectosigmoid mass	IV	pT3 N2 M1	Exploratory laparotomy with resection of the rectosigmoid/pre-treatment	52
M	Colon/hepatic flexure	III	pT4 N2 M0	Right colectomy; loop ileostomy/pre-treatment	0.6
F	Colon/sigmoid	II	pT4a N0 M0	Laparoscopic low anterior resection/pre-treatment	1.6
M	Colon/rectosigmoid	III	pT4a N1a M0	Laparoscopic low anterior resection; laparoscopic mobilization of splenic flexure/ pre-treatment	13
F	Colon/sigmoid	III	pT3 N1 M0	Laparoscopic sigmoid colectomy/pre-treatment	1.8
M	Colon/rectosigmoid	II	pT3 N0 M0	Laparoscopic-assisted robotic low anterior resection/pre-treatment	1.9
M	Colon/rectosigmoid	III	pT4a pN1b M0	Laparoscopic-assisted robotic low anterior resection/pre-treatment	1.7
M	Colon/sigmoid	III	pT4 N1 M0	Low anterior resection with diverting loop ileostomy/pre-treatment	1.2
M	Colon/sigmoid	II	pT4b N0 M0	Low anterior resection; small bowel resection/ pre-treatment	4.3
M	Colon/cecum	IV	pT3 N0 M1	Laparoscopic right colon resection/pre-treatment	N/A
F	Colon/ascending	I	pT2 N0 M0	Laparoscopic right colectomy/pre-treatment	1.2
F	Colon/ascending	III	pT4a N1c M0	Laparoscopic extended right hemicolectomy/ pre-treatment	5.5
M	Colon/sigmoid	IV	pT4 N2 M1	Exploratory laparotomy with partial proctectomy = small bowel resection with primary anastamosis/recurrent	N/A

F, female; M, male; N/A, not applicable.

Table 3. List of Top 20 Differentially Expressed Genes Between Stage I and Stage II Patients

Gene	Log2 fold change	p Value	Gene set
HES1	1.48	.00271	Notch
RASA4	−1.65	.00278	Ras
FZD3	1.13	.00333	Wnt
IL1R2	−2.3	.00345	MAPK, transcriptional misregulation
GAS1	−2.01	.0041	Hedgehog
WNT4	3.09	.00909	Hedgehog, Wnt
COL24A1	−0.688	.0118	PI3K
IL1R1	−0.686	.0123	Cell cycle - apoptosis, MAPK
PPP3CA	0.614	.0132	Cell cycle - apoptosis, MAPK, Wnt
TET2	0.736	.0148	Driver Gene
COL1A2	−1.07	.0163	PI3K
CREB5	−1.45	.017	PI3K
NOS3	−1.13	.0186	PI3K
SFRP4	−1.59	.0201	Wnt
FGF13	−1.45	.0203	MAPK, PI3K, Ras
AKT3	−0.566	.0225	Cell Cycle - Apoptosis, JAK-STAT, MAPK, PI3K, Ras
HSPB1	−0.96	.0227	MAPK
IL3RA	−1.24	.0248	Cell Cycle - Apoptosis, JAK-STAT, PI3K
RUNX1T1	−0.701	.0254	Transcriptional misregulation
CSF3R	−1.91	.0262	JAK-STAT, PI3K

DNA isolation and tumor microsatellite instability analysis

Genomic DNA was extracted using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit (Promega) according to manufacturer's specifications. A panel of 6 mononucleotide markers (NR21, NR22, NR24, NR27, BAT25, and BAT26) was used for multiplexed PCR amplification, as described previously.[19] Polymerase chain reaction products were analyzed by capillary electrophoresis.[20] Tumors showing differences in marker size between normal and tumor DNA at 2 or more loci were classified as microsatellite instable[20] and excluded from gene expression studies.

Deparaffinization, macro-dissection, and total RNA isolation

Unstained tissue slides were incubated in series of 3 baths for 2 minutes each, with gentle agitation for the first 15 seconds in d-limonene (histology grade), and 100% ethanol. After complete drying, they were rehydrated in 3% molecular biology grade glycerol solution. Hematoxylin and eosin slides (taken continuously with the unstained sections) were used as a guide for removing surrounding nontumor tissue from unstained sections. Ribonucleic acid was isolated from remaining tumor tissue using the Roche HighPure FFPET RNA Isolation spin-column kit, according to manufacturer's instructions.

NanoString sample preparation and data analysis

One-hundred nanograms of the purified RNA was hybridized with the PanCancer Pathway Code Set (NanoString Technologies) at 65°C overnight. Further purification and binding of the hybridized probes to the optical cartridge was performed on an nCounter Prep Station, and finally, the cartridge was scanned on an nCounter Digital Analyzer. The nCounter data files (RCC files) from the NanoString Digital Analyzer were imported into nSolver 4.0 software (NanoString Technologies) and checked for data quality (quality control) using default settings. All samples passed quality control. Background subtraction

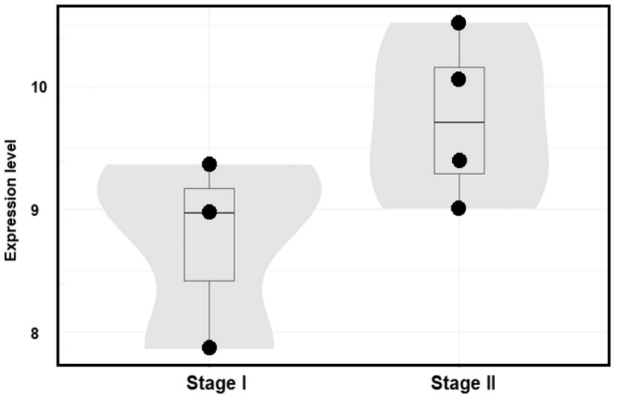

Figure 1. Cartilage oligomeric matrix protein mRNA expression levels between stage I and stage II patients with colon cancer.

was carried out by subtracting the mean value of the 8 negative control sequences from the raw counts of all endogenous genes. Samples were normalized using the geometric mean of the housekeeping genes and expression ratios, calculated by dividing the mean values of all samples in 1 experimental group (eg stage I tumors, etc) by the mean values of all samples in another experimental group (eg stage II tumors, etc). Values of p were calculated by Student *t*-test and all graphs were generated by nSolver 4.0 and PanCancer Pathways Advanced Analysis module.

Specifically, for the advanced analysis, we used the following arguments/parameters: "Type of data: raw; File type of plots: png; File type of plots: tiff; Low count threshold details: Remove Genes Below Specified Threshold: TRUE; Threshold count value: 20; Remove genes below the threshold at frequency greater than: 0.5; Sample annotation details: Unique sample identifier: Sample.Name; Covariate1: Prognosis (; Variable type: categorical; Reference level: normal; Normalization details: Perform normalization: TRUE; Auto-select number of housekeepers: TRUE;

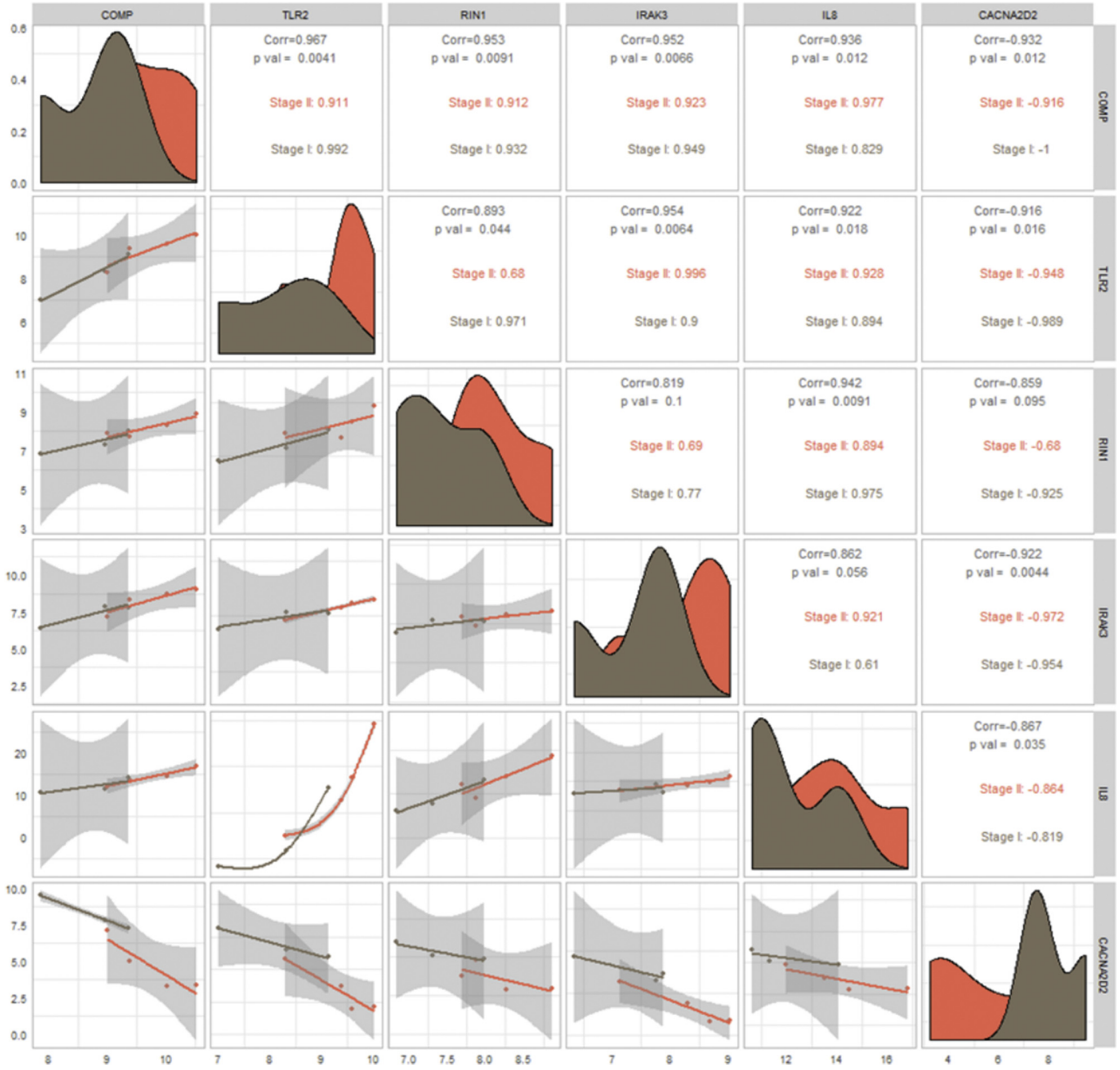

Figure 2. Pairwise expression association of cartilage oligomeric matrix protein and its top 5 correlated genes between stage I and stage II patients' colon cancer samples.

Table 4. List of Top 20 Differentially Expressed Genes Between Stage I and Stage III Patients

Gene	Log2 fold change	p Value	Gene set
PLA1A	−3.92	.000135	Ras
LIG4	−1.17	.000311	DNA damage - repair
HES1	1.73	.000377	Notch
BRCA2	−1.17	.000564	DNA damage - repair, driver gene
DDIT3	−0.633	.000579	MAPK, transcriptional misregulation
FZD3	1.7	.00105	Wnt
LEFTY2	−7.25	.00164	TGF-beta
MDM2	0.785	.00179	Cell cycle - apoptosis, PI3K, transcriptional misregulation
MAP2K4	0.714	.0018	MAPK
SFRP4	−2.4	.00336	Wnt
IDH2	0.921	.00418	Driver gene
XRCC4	−0.404	.00441	DNA damage - repair
SMARCB1	0.45	.00474	Driver gene
NCOR1	0.477	.00546	Driver gene, transcriptional misregulation
BMP2	2.08	.0066	Hedgehog, TGF-beta
DLL1	1.67	.0074	Notch
DDB2	1.08	.00764	DNA damage - repair
RFC3	−0.962	.00814	DNA damage - repair
PRKACA	−0.519	.0082	Cell cycle - apoptosis, Hedgehog, MAPK, Ras, Wnt
IDH1	0.794	.0085	Driver gene

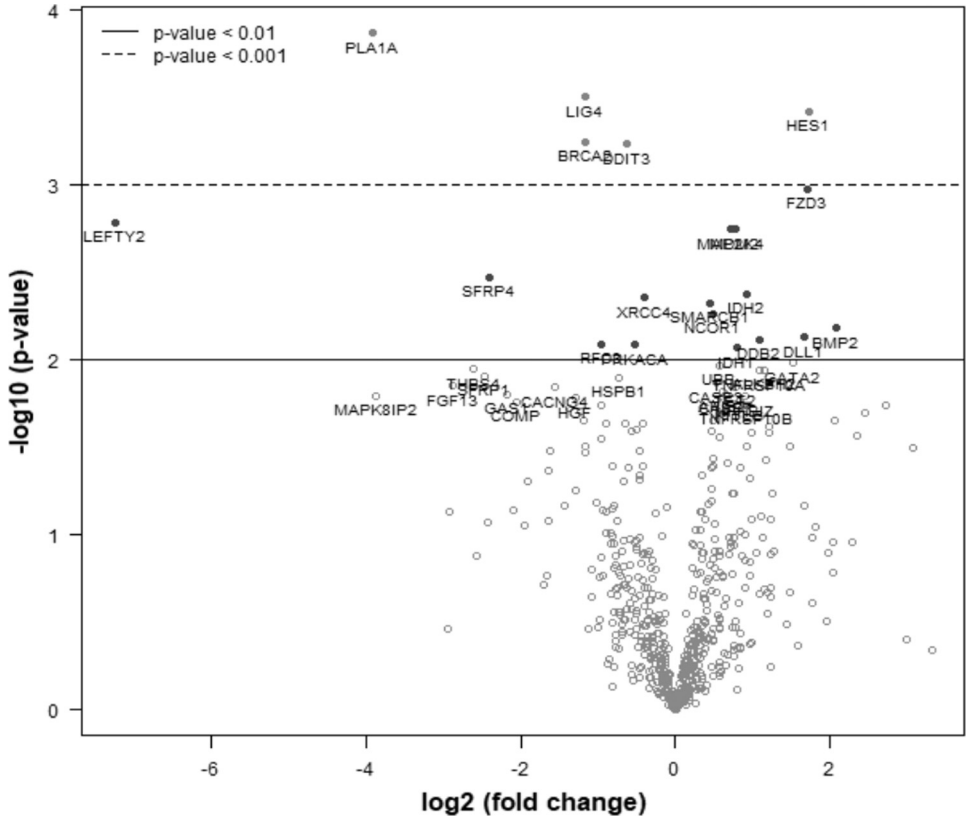

Figure 3. Volcano plot of differential expression between stage I and stage III patients with colon cancer.

Pathway scoring details: Perform pathway scoring: TRUE; Pathway scoring method: PC1; Pathway scoring baseline variable: Prognosis using normal; Plot pathway scores vs.: Prognosis; Adjust pathway scores for: Differential expression analysis details: Perform differential expression testing: TRUE; Predictors: Prognosis; Confounders: P-value adjustment: BY Run gene set analysis: TRUE; Pathview details: Display results using Pathview: TRUE; Color Pathview pathview plots by: Foldchange; P-value threshold: 0.05".

Human cartilage oligomeric matrix protein immunoassay (ELISA)

Fifty microliters of 100-fold diluted serum was used to measure COMP concentration using Quantikine ELISA human COMP immunoassay (R&D Systems, Inc), according to manufacturer's instructions. Optical density of each well was determined by a microplate reader set to 450 nm, with correction set up to 540 nm, to correct for optical imperfections in the plate. Total protein in each sample was measured by bicinchoninic acid method. Cartilage oligomeric matrix protein glycoprotein levels were normalized to a total protein.

Transwell migration assay

Eight-micrometer pore size translucent transwell migration chambers (BD Biosciences) in 24-well plates were used for migration analysis. Briefly, 600 µL of migration buffer (Dulbecco's Modified Eagle Medium [DMEM] containing 0.5% fetal bovine serum [FBS] and 0.1% bovine serum albumin [BSA]) was added to the bottom of each well, and a total of 2.5×10^4 HT-29 cells resuspended in 150 µL of migration buffer with or without 10 µg/mL Mito C or in the presence of recombinant human COMP protein (200 µg/mL) were seeded on the top of the membrane. After overnight incubation at 37°C, 5% CO_2, noninvading cells were removed by wiping the upper side of the membrane, and migrating cells were fixed with methanol and stained with crystal violet (Sigma Aldrich) for 1 minute. The number of cells migrating through the porous membrane was quantified by counting 10 random fields per filter at 400× magnification. At least 3 membrane filters were used for each condition within 1 experiment.

RESULTS

NanoString nCounter PanCancer Pathway code set[21] was used to analyze alterations in gene expression patterns between patients with sporadic CC at different stages of disease. Twelve CC patients (stage I: n = 3, stage II: n = 4, stage III: n = 4, and stage IV: n = 1, excluded from analysis due to only 1 sample being available) were used to assess the differences in gene expression profiles.

Cartilage oligomeric matrix protein mRNA expression levels are higher in stage II patients compared with stage I patients

Of 700 genes involved in cancer development and progression, expression of 37 genes was significantly different, with p values less than 0.05, between stage I and stage II CC patients. The top 20 statistically significant genes are shown in Table 3. Forty percent from these top 20 statistically significant deregulated genes are involved in PI3K signaling, suggesting its key role in CC progression. Two-fold higher expression of COMP, a member of a PI3K signaling pathway, was seen in stage II CC patients compared with patients with stage I disease (Fig. 1). Although the change was not statistically significant (p = 0.07), the trend toward increasing levels of COMP in patients with higher disease stage was observed. Pairwise expression association analysis revealed TLR2, RIN1, IRAK3, IL-8, and CACNA2D2 as the top 5 probes highly correlated with COMP expression (Fig. 2).

Cartilage oligomeric matrix protein expression levels are significantly increased in stage III patients compared with stage I patients

Changes in expression of 83 genes between stage I and stage III CC patients were statistically significant, with values of $p < 0.05$. The top 20 statistically significant genes are shown in Table 4. Volcano plot displaying each gene's -log10 (p value) and log2 fold change shows the 40 most statistically significant genes (Fig. 3). The COMP (p = 0.0174) gene was 1 of the top 40 most statistically significant genes with the most profound change (FC = 6.2), as documented in

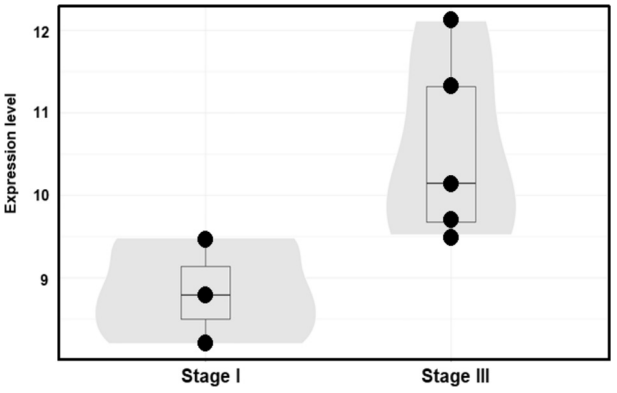

Figure 4. Cartilage oligomeric matrix protein mRNA expression levels between stage I and stage III patients with colon cancer.

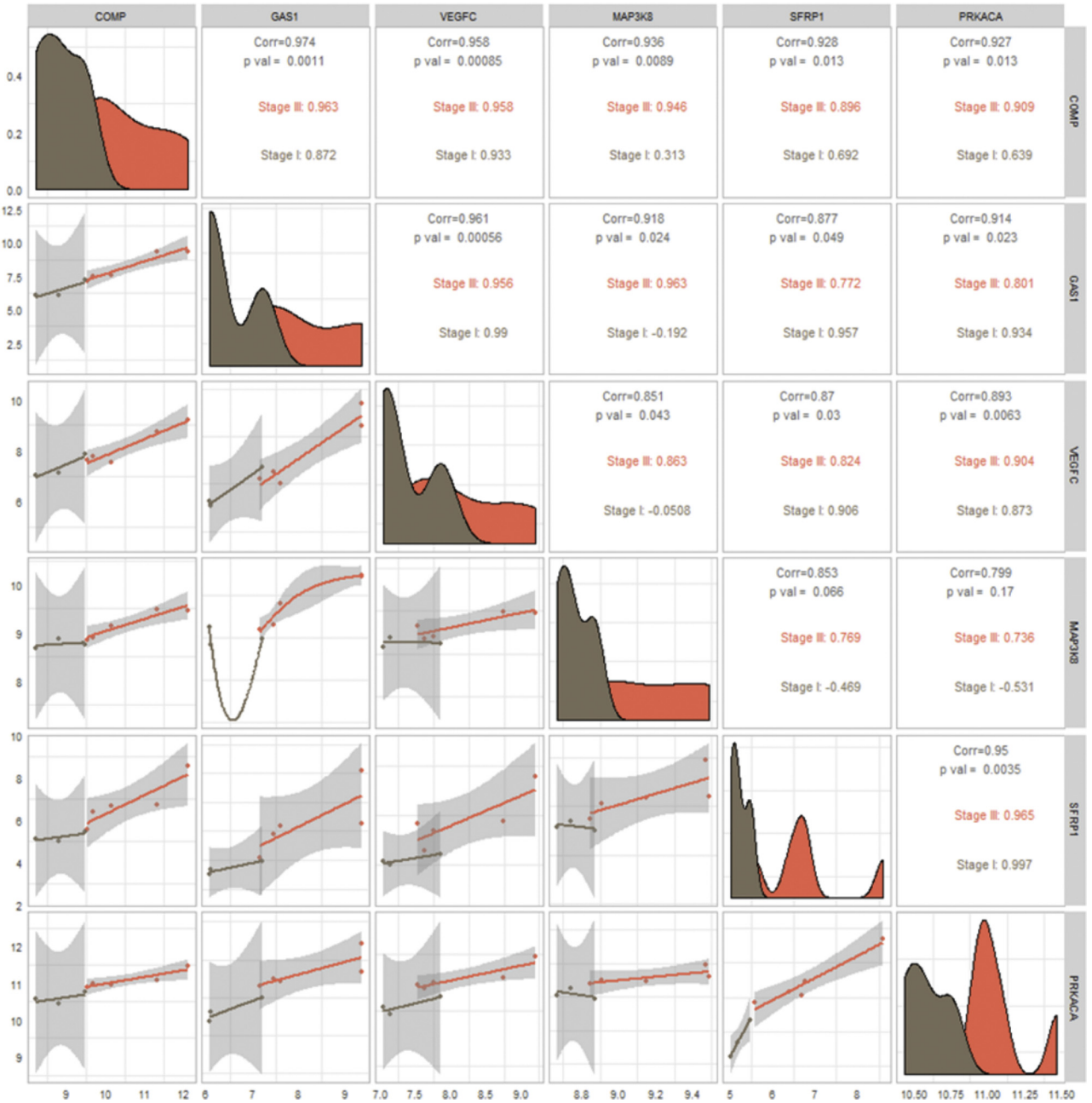

Figure 5. Pairwise expression association of cartilage oligomeric matrix protein and its top 5 correlated genes between stage I and stage III patients' colon cancer samples.

Figure 4. This observation confirms that increasing expression levels of COMP are associated with higher stage of CC. Expression of the top 5 highest correlated probes (GAS1, VEGFC, MAP3K8, SFRP1, and PRKACA) with COMP expression were defined by pairwise expression association analysis and are displayed in Figure 5.

Cartilage oligomeric matrix protein expression levels are higher in stage III patients compared with stage II patients

When stage II and stage III molecular profiles were compared, there were overall higher COMP expression levels in stage III CC patients compared with stage II

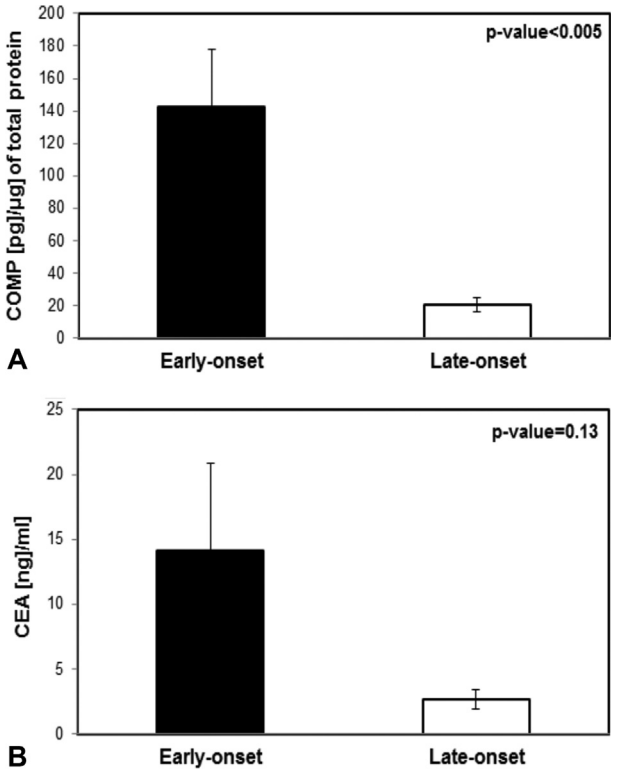

Figure 6. (A) Cartilage oligomeric matrix protein glycoprotein (COMP) levels and (B) carcinogenic embryonic antigen (CEA) levels in serum samples of colon cancer patients with early- and late-onset cancer.

patients; however, the change was not statistically significant ($p = 0.14$).

Cartilage oligomeric matrix protein glycoprotein levels are higher in serum samples of early onset colon cancer patients

Human COMP enzyme-linked immunosorbent assay (ELISA) revealed detectable levels of COMP protein in serum samples of CC patients. The COMP protein levels were normalized to total protein. As shown in Figure 6A, significantly higher COMP levels were found in EOCC patients when compared with LOCC patients (FC = 6; $p \leq 0.005$). The COMP glycoprotein levels were compared with CEA levels of assayed patient samples taken before the surgery. When averaged, CEA levels were higher in EOCC samples compared with LOCC samples, although this increase was not statistically significant (Fig. 6B). Elevation of CEA level correlated with increase in COMP level but was not statistically significant ($p = 0.13$).

Migratory abilities of colon cancer cells in vitro are significantly enhanced after a treatment with recombinant human cartilage oligomeric matrix protein

To test the role of COMP in CC motility, transwell migration experiments were performed using HT-29 colon cancer cells, which express only negligible levels of COMP at the baseline. After these cells were treated with recombinant human COMP protein, their migratory abilities increased significantly compared with untreated cells (Fig. 7).

DISCUSSION

Cartilage oligomeric matrix protein has a normal physiologic role in chondrogenesis and is abundant in ligaments, tendons, meniscal tissue, and articular cartilage.[22,23] It has been widely studied in various types of arthritis and demonstrated to be a biomarker for cartilage breakdown with the highest levels detected in synovial fluid. Although the implication of COMP glycoprotein in disease has been well documented in connective tissue disorders,[24,25] investigating the role of COMP in cancer development and cancer progression is only in its infancy.

Elevated COMP expression levels have been shown to confer breast and prostate tumor aggressiveness and poorer patient prognosis.[26-28] A recent study by Liu and colleagues[23] screened several colon cancer cell lines for expression levels of COMP and showed that COMP promotes colon cancer cell proliferation in vitro by activating the Akt signaling. This is in agreement with our unpublished data, which showed high levels of COMP expression in Caco-2 cells, but only negligible levels in HT-29 cells (unpublished data). We observed in that study that the proliferative ability of HT-29 cells, which typically express only negligible COMP levels at baseline, was significantly enhanced after 48- and 72-hour treatment with recombinant human COMP protein. We have further evaluated motility of these cells through the transwell membrane. The HT-29 cells treated with human recombinant COMP protein migrated significantly more than the same cells treated with vehicle (water) only (Fig. 7). These in vitro studies clearly demonstrate the crucial role COMP plays in colon cancer development and progression since cell proliferation and migration are important in carcinogenesis and metastasis.

A recent study analyzing a TCGA-COAD cohort of patients suggests COMP as a potential prognostic factor for CC.[23] Our genomic studies showed that COMP is uniquely overexpressed in EOCC with a 50-fold increase ($p \leq 0.05$) when compared with LOCC.[17,29] Moreover, our recently published analysis of RNAseq gene expression

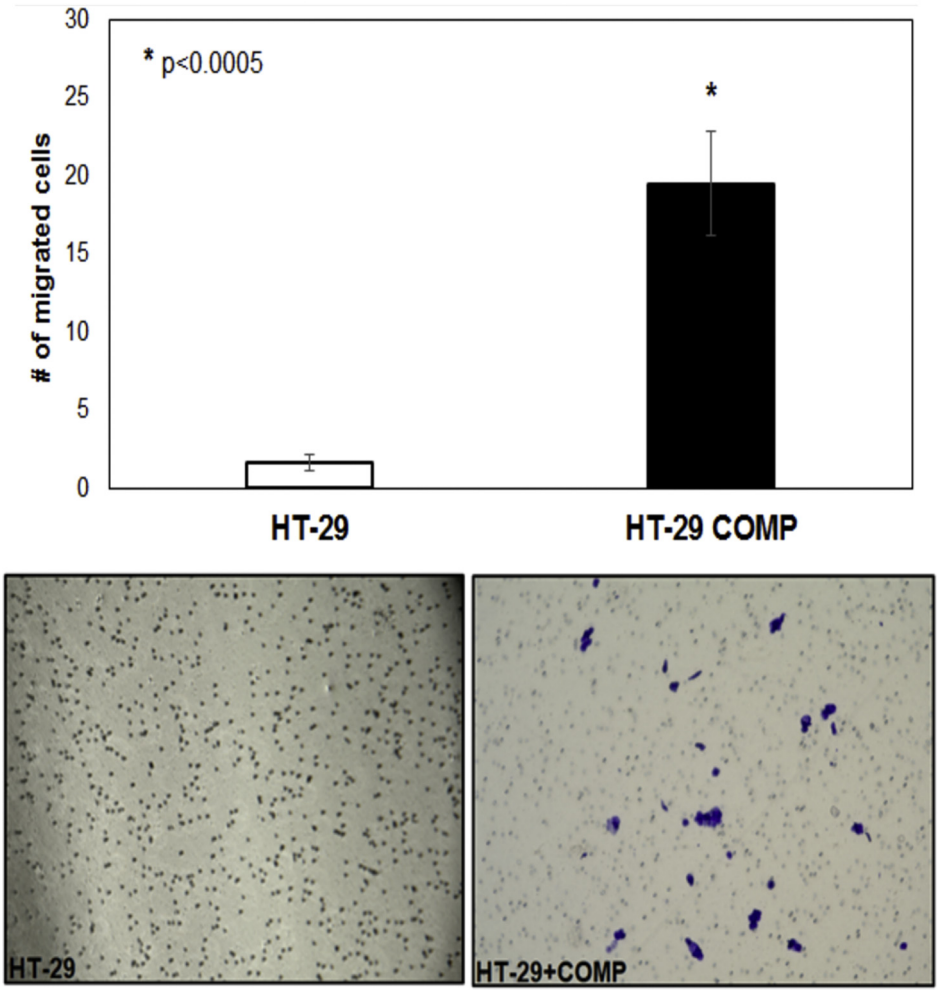

Figure 7. In vitro transwell migration assay using HT-29 cells after a treatment with human recombinant cartilage oligomeric matrix protein (COMP).

data (n = 286, CC primary tumors) from the COAD-TCGA dataset has shown coexpression of COMP with epithelial to mesenchymal transition-linked genes,[18] a mechanism that is implicated in cancer metastasis and invasion.[30,31] Further Kaplan Meier survival analysis of the same samples revealed significantly poorer overall survival of patients expressing higher COMP mRNA levels.[18]

Colon cancer in young patients is often diagnosed at advanced stages and presents with more aggressive tumors.[9] In our study, we sought to evaluate the association between COMP expression levels and stage of disease using NanoString nCounter gene expression platform. Our results showed increasing expression levels of COMP with more advanced stage disease (Figs. 1 and 4) and coexpression of COMP with other genes known to play key roles in cancer aggressiveness by way of cancer proliferation, metastases, and angiogenesis (Figs. 2 and 5). These findings suggest a significant role of COMP in CC carcinogenesis, especially in younger patients, as these tumors express high levels of COMP.

Currently, CEA is the main tumor marker for monitoring CC and its recurrence.[13,14,32] Unfortunately, CEA concentrations are rarely identified in stage I CC, and further CEA levels do not differentiate benign vs malignant polyps.[12] As a marker, CEA has been shown to have limited sensitivity and only 40% specificity, and it falls short of biomarker clinical qualification and therefore, transfer into routine clinical practice.[13] With the potentially increased number of CCs expected to be diagnosed with the lowered age recommended for colonoscopy screening, as well as the increasing incidence of EOCC, there is an increasing need to find novel CC

diagnostic and prognostic molecular biomarkers, especially in young patients.

We have shown in our studies that COMP has a potential to be a biomarker; it is a glycoprotein and therefore can be detectable in blood and/or urine. In our study, we analyzed serum samples from a group of EOCC and a group of LOCC patients to see not only if COMP will be detectable in serum, but also if there are differences in its levels between the 2 age groups of patients. Our results confirmed detectable levels of COMP glycoprotein in serum samples with significantly ($p \leq 0.005$) higher levels of COMP glycoprotein in EOCC samples compared with LOCC samples (Fig. 6A). Levels of CEA, when averaged for the same patients measured before the surgery, showed increased levels in EOCC compared with LOCC (Fig. 6B), although this increase was not statistically significant ($p = 0.13$). In addition, CEA levels tended to correlate with COMP levels, but it was not statistically significant.

Our study has shown that EOCCs have distinct tumor biology compared with LOCC. In addition, COMP plays a significant role in EOCC carcinogenesis and cancer aggressiveness. We also showed that COMP glycoprotein can be detected in serum and therefore should be considered as a potential prognostic biomarker for early detection and surveillance of aggressive EOCC, which is associated with poorer prognosis.

Our study has some limitations. First, we believe that using a larger sample size would have been more ideal. Because of the smaller sample size, we were not able to analyze the Stage IV CC patients to compare their COMP expression with other CC stages. However, despite this small sample size, we were able to find a statistically significant difference in the gene expression between stages I vs III, and differences that approached, but did not reach, statistical significance between stages I vs II and stages II vs III. Our second limitation is that we were not able to identically match the samples in the 2 groups in terms of demographics; however, serum samples were propensity matched based on stage of disease and tissue samples were matched based on pathology and sex.

Despite these limitations, our results strongly demonstrate a statistical difference in expression and regulation of a significant number of genes and pathways between different stages of CC. In terms of serum samples, we were able to see statistically significant changes in COMP glycoprotein levels between EOCC and LOCC. We consider this study a "proof-of-concept" of COMP as a biomarker that necessitates a bigger study with 2 larger cohorts of patients in discovery and validation arms.

Author Contributions

Study conception and design: Nfonsam, Jandova
Acquisition of data: Jandova
Analysis and interpretation of data: Jecius, Chen, Omesiete, Ewongwo
Drafting of manuscript: Nfonsam, Jecius, Jandova
Critical revision: Nfonsam, Elquza, Scott

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Discussion

DR JAMES SPITZ (Glenview, IL): From a basic science standpoint, for your transwell migration assay, how are you sure that your results are not due to excessive proliferation and not migration? Clinically, can you comment on the fact that there was no statistically significant difference in Cartilage Oligomeric Matrix Protein (COMP) levels between stage 2 and stage 3 disease? I did notice in reading your manuscript that your samples were all from Caucasians. Would you expect a COMP level change with different races?

DR VALENTINE NFONSAM (Tucson, AZ): To answer your first question, when we looked at proliferation studies, we saw significant proliferation when we used the HT-29 cells and Caco-2 cells. We saw there was significant change in proliferation at 72 hours. However, at 24 hours, you do not see a significant change. In our transwell migration study, we performed our experiment to last within 18 hours, so we stopped the experiment at 18 hours and actually saw there was proliferation. And we had done another study, looking at proliferation, where we used Mitomycin-C to slow down growth of the cells, and we saw similar results in proliferation, so we are pretty confident that our experiment measured migration of the cells and not proliferation.

Your second question was about why there was no significant difference in COMP levels between stage 2 and stage 3 patients. I actually went back and looked at that and tried to figure out why the COMP level did not significantly increase between the 2. What I noticed was that when we matched the samples, the stage 2 patients actually had T-4 lesions and not T-3, which makes a significant difference. Anyone who does oncology will know that T-4 patients fare even worse than a stage 3 disease patients. This might explain the reason why. My thought is that using a T-4 cancer patient, even though labeled as having stage 2 disease, might have been what made that difference. So, again, when we repeat our experiments, we are actually going to use a T-3 patient, and T-4, and compare to see if there is any difference.

As for the third question, yes, we used just Caucasians for our study, and that is happenstance. But, yes, African Americans have been shown to have significantly higher COMP levels, and patients with arthritis have also been shown to have significant COMP levels. As we try to look at COMP as a potential biomarker, those are the things we will consider. As we prepare our next grant, we are trying to have a bigger cohort of patients to not only to have a discovery cohort, but also to have a validation cohort, and all of those things will be factored in.