Gastroenterology 2014;146:1313–1324

Biomarkers of Coordinate Metabolic Reprogramming in Colorectal Tumors in Mice and Humans

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BACKGROUND & AIMS: There are no robust noninvasive methods for colorectal cancer screening and diagnosis. Metabolomic and gene expression analyses of urine and tissue samples from mice and humans were used to identify markers of colorectal carcinogenesis. **METHODS:** Mass spectrometry–based metabolomic analysis of urine and tissues from wild-type C57BL/6J and *Apc*$^{Min/+}$ mice, as well as from mice with azoxymethane-induced tumors, was employed in tandem with gene expression analysis. Metabolic profiling was also performed on colon tumor and adjacent nontumor tissues from 39 patients. The effects of β-catenin activity on metabolic profiles were assessed in mice with colon-specific disruption of *Apc*. **RESULTS:** Thirteen markers were found in urine associated with development of colorectal tumors in *Apc*$^{Min/+}$ mice. Metabolites related to polyamine metabolism, nucleic acid metabolism, and methylation, identified tumor-bearing mice with 100% accuracy, and also accurately identified mice with polyps. Changes in gene expression in tumor samples from mice revealed that derangement of metabolites were a reflection of coordinate metabolic reprogramming in tumor tissue. Similar changes in urinary metabolites were observed in mice with azoxymethane-induced tumors and in mice with colon-specific activation of β-catenin. The metabolic alterations indicated by markers in urine, therefore, appear to occur during early stages of tumorigenesis, when cancer cells are proliferating. In tissues from patients, tumors had stage-dependent increases in 17 metabolites associated with the same metabolic pathways identified in mice. Ten metabolites that were increased in tumor tissues, compared with nontumor tissues (proline, threonine, glutamic acid, arginine, N1-acetylspermidine, xanthine, uracil, betaine, *symmetric* dimethylarginine, and *asymmetric*-dimethylarginine), were also increased in urine from tumor-bearing mice. **CONCLUSIONS:** Gene expression and metabolomic profiles of urine and tissue samples from mice with colorectal tumors and of colorectal tumor samples from patients revealed pathways associated with derangement of specific metabolic pathways that are indicative of early-stage tumor development. These urine and tissue markers might be used in early detection of colorectal cancer.

Keywords: Azoxymethane; *Apc*$^{Min/+}$; Colorectal Cancer; Metabolomics; Cancer Biomarker; Metabolic Reprogramming.

Colorectal cancer is a leading cause of cancer mortality worldwide.[1,2] Although the disease has good therapeutic response at early stages, advanced stages are associated with poor prognosis. Therefore, early diagnosis is pivotal to therapeutic success. Recent studies have shown that regular screening could reduce mortality by almost 50%.[3] Large-scale screening and diagnosis would be facilitated with high-throughput noninvasive screening methods. Although fecal occult blood test is clinically used and fecal genetic tests are promising,[4,5] endoscopy and biopsy remain the most definitive methods for diagnosis. However, these are low-throughput, costly, and invasive procedures. The lack of high-throughput noninvasive markers continues to contribute to avoidable health care burden and mortality.

Metabolomics is a promising approach for the identification of changes in biochemical signatures associated with pathogenesis that could be used for diagnosis. A number of metabolomic studies[6] have reported that metabolite compositions of tissue[7] as well as biofluids[8,9] from colorectal cancer patients differ from those of healthy controls. However, invasiveness of tissue sampling and sensitivity of the biofluid metabolome to factors, such as genetic composition, food, and environment, warrants exploration of the mechanisic link between biofluid biomarkers and molecular signatures of tumor tissue to identify robust biomarkers. There has been a lack of comprehensive studies simultaneously investigating changes in tumor tissue and biofluids to establish such mechanistic links. This is the first instance where an unbiased high-throughput approach was adopted to identify progressive changes in the urine metabolome

Abbreviations used in this paper: **AOM, azoxymethane; *Apc*$^{Min/+}$, mouse bearing heterozygous mutation in *adenomatous polyposis coli* gene that develops multiple intestinal neoplasia; CoA, coenzyme A; PRMT, protein arginine methyl transferase; ROC, receiver operating characteristics.**

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2014.01.017

and link it to changes in the biochemical landscape of colorectal tumors as shown in Supplementary Scheme 1 (Supplementary Material). Mutations in the *Apc* gene and consequent activation of β-catenin signaling is frequently an early event in the development of colorectal cancer.[10] This study coupled metabolomic and gene expression analysis using mice harboring germline and colon-specific disruption of the *Apc* gene to identify noninvasive biomarkers mechanistically associated with metabolic derangements in colorectal carcinogenesis. The robustness of this association was also established using azoxymethane (AOM)-induced sporadic colorectal carcinogenesis model. Finally, analysis of human tumors and adjacent nontumor tissue showed that similar metabolic reprogramming also occurs in human colorectal tumors.

Materials and Methods

Human Samples

Tissue samples (demographic summary in Supplementary Table 1) were provided by the Cooperative Human Tissue Network, a National Cancer Institute supported resource (Southern, Eastern and Midwestern Divisions). Colon tumor and adjacent nontumor tissues used in this study were obtained during 2004−2012. Tumor staging was performed by a pathologist in accordance with the seventh edition of the cancer staging manual of the American Joint Committee on Cancer.[11] This study was approved by the Institutional Review Boards of the involved institutions.

Animal Studies

Six age-matched wild-type and 6 $Apc^{Min/+}$ (mice bearing heterozygous mutation in *adenomatous polyposis coli* gene that develops multiple intestinal neoplasia) littermates (C57BL/6J background) were cohabited in cages containing equal numbers of wild-type and $Apc^{Min/+}$ and used as the discovery cohort for longitudinal metabolomic studies. Mice were fed normal chow and water *ad libitum*. Urine samples were collected monthly starting from 2 months up to 6 months of age by placing mice in metabolic cages for 24 hours and samples were stored at −80°C. Urine samples were also collected from an independent set of age-matched (6 wild-type and 6 $Apc^{Min/+}$) nonlittermate mice at 6 months of age. These samples were used to test and validate biomarkers identified in the discovery cohort. Serum samples were collected by retro-orbital bleeding. Animals were killed by CO_2 asphyxiation at the end of the study, and intestines were flushed with isotonic saline, longitudinally opened, tumors counted under light microscope, tissue samples harvested, and all samples stored at −80°C. For colon-specific disruption of *Apc,* the $Apc^{CDXERT2}$ mice[12] or littermate control $Apc^{F/F}$ were treated with 100 mg/kg of tamoxifen and killed 24 hours later, and colon mucosa scraped and stored at −80°C. For the sporadic colorectal carcinogenesis study, 6 age-matched wild-type 129P3/J mice were weekly injected with AOM (10 mg/kg body weight; intraperitoneal) for 6 weeks and 6 control mice received saline.[13] Urine samples were collected using metabowls 5 months after the last AOM injection. Subsequently, mice were killed and colons examined to confirm the presence of tumors under light microscopy.

Biochemistry

Serum alanine aminotransferase and aspartate aminotransferase levels were measured using VetSpec kits (Catachem Inc., Bridgeport, CT) following the manufacturer's instructions.

Proliferation Assay

Increase in proliferation due to acute colon-specific disruption of the *Apc* gene was examined by bromodeoxyuridine staining (see Supplementary Material).

Metabolomics

Deproteinated urine samples and tissue extracts were analyzed by chromatography on a Xevo G2 ESI-QTOFMS coupled with Acquity UPLC BEH C_{18} or amide column (Waters Corp., Milford, MA) for reverse-phase or hydrophilic interaction liquid chromatography analysis, respectively. The data analysis was performed as described earlier.[14] Metabolites were quantitated on a Xevo triple-quadruple platform coupled with an Acquity UPLC BEH amide column through multiple reaction monitoring. See Supplementary Material for detail.

Gene Expression

Gene expression was analyzed by quantitative polymerase chain reaction using SYBR GreenER Reagent System (Invitrogen, Carlsbad, CA) in a 7900 HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). See Supplementary Tables 4–8 for primers.

Protein Interaction Network Analysis

The Search Tool for the Retrieval of Interacting Genes/Proteins[15] was used to examine the functional association of genes involved in metabolic pathways. For mice, enzymes corresponding to genes that showed significant changes in expression by quantitative polymerase chain reaction (Supplementary Tables 4–8) were used to construct the network. All proteins with a Search Tool for the Retrieval of Interacting Genes/Proteins score <0.7 were excluded.

Statistics

Statistical significance of changes in metabolite abundance and gene expression were calculated by 2-tailed Mann-Whitney test with 95% confidence interval using Graphpad Prism (San Diego, CA) unless otherwise mentioned. The creatinine-normalized urinary excretion of metabolites was used to test the predictive power of individual metabolites or metabolite panels by receiver operating characteristic (ROC) analysis using STATA software (StataCorp, College Station, TX). The statistical significance of the change in metabolite abundances in matched human samples were calculated using 2-tailed paired *t*-test with 95% confidence interval. *P* value $<.05$ was considered statistically significant.

Results

Apc$^{Min/+}$ Mice Develop Distinct Metabolic Traits

Age-matched littermate male wild-type and $Apc^{Min/+}$ mice showed no difference in body weight or liver enzyme