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

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Abstract

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 analyses 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. Metabolomics profiles were also determined on colon tumor and adjacent non-tumor 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

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Supplemental Info: Supplementary methods, tables, figures and texts are available online.

identified mice with polyps. Changes in gene expression in tumor samples from mice reflected the observed changes in metabolic products detected in urine; similar changes were observed in mice with azoxymethane-induced tumors and 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 12 metabolites associated with the same metabolic pathways identified in mice (including amino acid metabolism and polyamine metabolism). Ten metabolites that were increased in tumor tissues, compared with non-tumor tissues (proline, threonine, glutamic acid, arginine, N1-acetylspemidine, 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 metabolites associated with specific metabolic changes that are indicative of early-stage tumor development. These urine and tissue markers might be used in early detection of colorectal cancer.

Keywords

AOM; colon cancer; mouse model; mechanism

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 the mortality by almost fifty percent.³ Large-scale screening and diagnosis would be advanced with high-throughput noninvasive 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. Thus, the lack of high-throughput noninvasive markers continues to contribute to avoidable healthcare 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 studies⁶ have reported that metabolite compositions of tissue⁷ as well as biofluids^{8,9} from colorectal cancer patients differ from that of healthy controls. However, invasiveness of tissue sampling and sensitivity of biofluid metabolome to factors such as genetic composition, food, and environment, warrants exploration of the mechanistic 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 and link it to changes in the biochemical landscape of colorectal tumors as shown in Scheme S1 (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 *Apc* gene to identify noninvasive biomarkers mechanistically associated with metabolic derangements in colorectal carcinogenesis. The robustness of this association was established using AOM-

induced sporadic colorectal carcinogenesis model. Finally, analysis of human tumors and adjacent non-tumor tissue showed that similar metabolic reprogramming also occurs in human colorectal tumors.

Materials and methods

Human Samples

Tissue samples (demographic summary in Supplemental 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 non-tumor 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 (AJCC).¹¹ This study was approved by the Institutional Review Boards of the involved institutions.

Animal Studies

Six age-matched wild-type and six *Apc*^{Min/+} 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 two months up to six 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 (six wild-type and six *Apc*^{Min/+}) non-littermate mice at six 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 CO₂ asphyxiation at the end of the study, intestines 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} mice¹² or littermate control *Apc*^{F/F} were treated with 100mg/kg of tamoxifen and sacked 24 hours later, colon mucosa scraped and stored at –80 °C. For sporadic colorectal carcinogenesis study, six age-matched wild-type 129P3/J mice were weekly injected with AOM (10 mg/Kg body weight; i.p.) for six weeks while the six others received saline¹³. Urine samples were collected using metabowls five months after last AOM injection. Subsequently, mice were euthanized and colons were examined to confirm the presence of tumor under light microscope.

Biochemistry

Serum ALT and AST levels were measured by using VetSpec kits (Catachem Inc., Bridgeport, CT) following the manufacturer's instructions.

Proliferation Assay

Increase in proliferation due to acute colon-specific disruption of *Apc* gene was examined BrdU staining (see supplementary method).

Metabolomics

Deproteinized urine samples and tissue extracts were analyzed in Xevo G2 ESI-QTOFMS coupled with Acquity UPLC BEH C₁₈ or amide column (Waters Corp. Milford, MA) for reverse-phase or HILIC analysis, respectively. The data analysis was performed as described earlier.¹⁴ Metabolites were quantitated on a Xevo triple-quadrupole platform coupled with an Acquity UPLC BEH amide column through multiple reaction monitoring. See supplemental methods for detail.

Gene Expression

Gene expression was analyzed by qPCR 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

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING)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 qPCR (Supplementary Tables 4–8) were used to construct the network. All proteins with a STRING score < 0.7 were excluded.

Statistics

Statistical significance of changes in metabolite abundance and gene expression were calculated by two-tailed Mann-Whitney test with 95% confidence interval using Graphpad Prism (San Diego, California) unless mentioned otherwise. The creatinine-normalized urinary excretion of metabolites was used to test the predictive power of individual metabolites or metabolite panels by 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 two-tailed paired 't'-test with 95% confidence interval. P value < 0.05 was considered statistically significant.

Results

Apc^{Min/+} Mice Develop Distinct Metabolic Trait

Age-matched littermate male wild-type and *Apc*^{Min/+} mice showed no difference in body weight or liver enzyme levels (Supplementary Figure 1A and B). All *Apc*^{Min/+} mice were found to develop colorectal tumors along with multiple intestinal polyps, while wild-type mice were tumor-free at six months of age (Supplementary Figure 1C). Unsupervised principal components analysis (PCA) of the creatinine-normalized urinary metabolomics data acquired with HILIC (Figure 1A) or reverse-phase (Figure 1B) chromatography showed separation of wild-type and *Apc*^{Min/+} mice along the first principal component, although none of these mutants were visibly sick. These observations indicated that tumorigenesis in *Apc*^{Min/+} mice is associated with development of a distinct metabolic trait. In order to identify features contributing to segregation of metabolomes of naïve and tumor-bearing mice, supervised orthogonal projection to latent structure analysis was performed

(Supplementary Figure 2A and B). Ions significantly elevated or depleted in *Apc*^{Min/+} urine were selected using a cut-off value of $p(\text{corr}) [1] > 0.65$ or $p(\text{corr}) [1] < 0.65$, respectively from the S-plot (Supplementary Figure 2C). Identities of ions were confirmed with authentic standards, concentrations were measured and normalized with respect to creatinine to account for any changes in glomerular filtration rate to select final list of metabolites (Supplementary Table 2) that could serve as potential biomarkers for tumorigenesis.

Quantitation, Validation and Predictive Power of Biomarkers

Urinary excretion of amino acid metabolites such as glutamine, proline, N α -acetyllysine (Figure 1C–E) were progressively elevated whereas carnitine (Figure 1F) was depleted during tumorigenesis in *Apc*^{Min/+} mice. Carnitine is derived from permethylation of lysine. Two hypermethylated metabolites of arginine, namely, *symmetric*-dimethylarginine and *asymmetric*-dimethylarginine (Figure 2A and B) were also progressively elevated. In addition, metabolites related to the urea cycle and polyamine metabolism such as citrulline (Figure 2C), spermine (Figure 2D) were elevated whereas ornithine (Supplementary Figure 3A) was depleted in tumor-bearing mice. Acetylated polyamines, namely N1-acetylspermidine and N8-acetylspermidine (Figure 2E and 2F), were also progressively elevated during tumorigenesis. Nucleic acid metabolites such as xanthosine, inosine, xanthine, cytidine, deoxyuridine, and thymidine (Figure 3) were progressively elevated. In order to test the robustness of association of these metabolites with tumorigenesis, their urinary excretions were measured in an independent set of age-matched non-littermate wild-type and *Apc*^{Min/+} mice. Excretion of these metabolites was significantly deranged in the validation cohort except for cytidine, xanthine, cytidine, citrulline and ornithine (Supplementary Figure 3B). Although not statistically significant, deoxyuridine ($P = .065$) and thymidine ($P = .054$) showed the same trend of elevation (Supplementary Figure 3B) as observed in the discovery cohort. On the basis of their consistent and significant derangement in both cohorts (Supplementary Figure 4), thirteen metabolites were selected for evaluation of predictive power in the combined cohort of twelve wild-type and twelve tumor-bearing mice using ROC analysis. These included metabolites associated with amino acid metabolism (proline, glutamine and N-acetyllysine), polyamine metabolism (N1-acetylspermidine, N8-acetylspermidine and spermine), nucleic acid metabolism (xanthosine, inosine, deoxyuridine and thymidine) and methylation (carnitine, *symmetric*-dimethylarginine and *asymmetric*-dimethylarginine). Individual metabolites (see Supplementary Figure 5 and Supplementary Table 3 for details on range, AUCROC, sensitivity and specificity) showed moderate to high (75–96%) accuracy of prediction. However, combining them according to the aforementioned metabolic pathways of origin resulted in a significant improvement in predictive power (Figure 4B–D). In particular, metabolites related to polyamine metabolism, nucleic acid metabolism and methylation showed 100% accuracy in identifying tumor-bearing mice.

Urinary Biomarkers Reflect Dysregulation of Metabolic Networks in Tumors

In order to examine whether changes in these urinary biomarkers correlate with changes in the metabolic machinery in tumor, the abundance of these and related metabolites as well as expression of key genes involved in these pathways (Supplementary Table 4–8 contains detail of the corresponding reactions) were examined. A battery of genes involved in amino

acid metabolism, urea cycle and polyamine metabolism were found to be overexpressed in colon tumors (Supplementary Figure 7A and 7D) of *Apc*^{Min/+} mice. Consistent with this observation, abundances of metabolites in these pathways, including proline, glutamine and N1-acetylspermidine that were found to be elevated in urine, were also elevated in colon tumors (Figure 5A and 5B, Supplementary Figure 6). Genes involved in *de novo* synthesis as well as salvage of nucleic acid were overexpressed in colorectal tumor (Supplementary Figure 7B and C). Consequently, the abundance of deoxyuridine and thymidine that were elevated in urine were also found to be elevated in tumor tissue. ATP levels, which are related to energy and nucleic acid metabolism, were elevated in colon tumors (Supplementary Figure 6). Simultaneously, genes related to post-translational and epigenetic methylation machinery were also overexpressed (Supplementary Figure 7E). This resulted in elevation of hypermethylated metabolites such as dimethylarginine, carnitine, dimethylglycine and betaine in tumor tissue. Although not statistically significant, intermediates involved in methylation such as S-adenosylmethionine ($P = .052$) and S-adenosylhomocysteine ($P = .082$) also showed a trend of elevation in mouse tumor tissue (Supplementary Figure 6). These observations indicate that changes in urinary metabolic signature reflect extensive reprogramming of aforementioned metabolic pathways in tumor tissue.

Coordinate Reprogramming of Metabolic Pathways Results in Early Prediction of Tumorigenesis

In order to examine whether reprogramming of these metabolic pathways were concerted or unrelated, the functional cooperativity of genes that were significantly changed in *Apc*^{Min/+} mice were analyzed. The results showed significant interconnectivity between derangements of these metabolic pathways (Supplementary Figure 8), particularly, between urea cycle and polyamine metabolism, nucleic acid metabolism and methylation. This indicated a coordinate dysregulation of these metabolic pathways during tumorigenesis. Biomarkers representing these metabolic pathways were subsequently combined to evaluate their ability to identify animals undergoing tumorigenesis in earlier stages using ROC analysis (5 months, Supplementary Figure 9; 4 months, Supplementary Figure 10; 3 months, Supplementary Figure 11; 2 months, Supplementary Figure 12). The results showed that combination of biomarkers related to nucleic acid metabolism, polyamine metabolism and methylation predicted mutant mice at risk of developing colorectal tumors with 100% accuracy even at two months of age when they present only few polyps (data not shown).

Coordinate Metabolic Reprogramming is an Early Event in Proliferation

Since, combination of urinary biomarkers resulted in early identification of *Apc*^{Min/+} mice undergoing tumorigenesis, conditional knockout mice with colon-specific disruption of the *Apc* gene¹² were used to examine whether such coordinate metabolic reprogramming occurs in early stages of β -catenin signaling. Both Cre+ and Cre- mice were treated with tamoxifen. Only Cre+ mice showed colon-specific activation of β -catenin signaling as evidenced by overexpression of target genes such as *cMyc* and *Lgr5* (Supplementary Figure 13A and B) within twenty-four hours, due to inactivation of *Apc*. This resulted in increase in proliferation (Supplementary Figure 13C and D) with a concomitant elevation of metabolites related to the aforementioned pathways along with acetyl-CoA, which is involved in energy

production and central carbon metabolism, (Supplementary Figures 14–17) in the colon epithelium of Cre⁺ mice. This was also associated with dysregulation of gene expression in these pathways (Supplementary Figure 18). Notably, early activation of both type-I and type-II PRMTs and consequent elevation of dimethylarginines are novel observations in the context of colorectal cancer. These data show that coordinate metabolic reprogramming reflected by urinary biomarkers is an early event in tumorigenesis associated with proliferation.

AOM-induced Colorectal Carcinogenesis is Associated with Similar Changes in Urinary Metabolome

In order to investigate whether such changes in the metabolic signature in the *Apc*^{Min/+} mice is a model-independent feature associated with colorectal carcinogenesis, urinary metabolic profile of mice bearing AOM-induced colorectal tumors were analyzed. All AOM-treated mice developed colon tumors at five months after the last injection whereas no tumors were found in saline-treated mice. Similar to that observed in *Apc*^{Min/+} mice, AOM-treated mice showed elevation of excretion of glutamine, proline, N α -acetyllysine, N8-acetylspermidine, citrulline, deoxyuridine, thymidine, *symmetric*-dimethylarginine, and *asymmetric*-dimethylarginine (Supplementary Figure 19 and Table 9). Although not statistically significant, xanthine (P = .065), xanthosine (P = .132) and N1-acetylspermidine (P = .132) showed a trend of elevation. In addition, there was an elevation of threonine, arginine, deoxycytidine, uracil, cytosine, guanosine, betaine, and methionine (Supplementary Figure 19 and Table 9). However, unlike *Apc*^{Min/+} mice, AOM-treated mice showed an increase in the excretion of ornithine and carnitine. Nevertheless, the overall metabolic signature indicated a concerted dysregulation of the metabolic pathways similar to *Apc*^{Min/+} mice. This indicated that such metabolic reprogramming may be a common feature of colorectal carcinogenesis irrespective of etiological heterogeneity, as encountered in humans.

Human Colorectal Tumors Show Similar Derangement of Metabolic Pathways

In order to examine whether similar metabolic reprogramming is indeed associated with human colorectal carcinogenesis, targeted metabolic profiling of human colorectal tumors and matched adjacent non-tumor tissue was performed. The median age of patients (N = 39) were 69 years with almost equal representation of males (N = 19) and females (N = 20). The ethnicity was predominantly Caucasian (N = 32). Most tumors were well to moderately differentiated (N = 33) with all but one being adenocarcinoma. Since most of the colorectal tumors in the mouse models are found in the lower two-third of the large intestine, which is equivalent to the segment of human large intestine from transverse colon to rectum, all human tumor samples (N = 23) collected from this segment were grouped together for comparison with metabolic derangements observed mice. Results of the metabolic profiling showed that similar to that observed in mice, amino acid metabolites were elevated in human colorectal tumors (Figure 6, Supplementary Figure 18A). Metabolites connecting amino acid metabolism to urea cycle and polyamine metabolism, such as arginine and N1-acetylspermidine were also elevated (Figure 6). Nucleic acid metabolites such as uracil, hypoxanthine and xanthine (Figure 6) were elevated. Similar to *Apc*^{Min/+} mice, human tumors showed elevation of hypermethylated metabolites such as carnitine, *symmetric*-dimethylarginine, *asymmetric*-dimethylarginine, dimethylglycine and betaine along with S-

adenosylmethionine and S-adenosylhomocysteine (Figure 6). In the overall cohort, aspartic acid, glutamic acid, proline, threonine, lysine, arginine, uracil, xanthine, hypoxanthine, S-adenosylhomocysteine, S-adenosylmethionine, carnitine, *symmetric*-dimethylarginine, *asymmetric*-dimethylarginine, betaine and dimethylglycine were significantly elevated (Supplemental Figure 19). These samples were also subdivided in two groups according to TNM stage¹¹; stage I-II and stage III-IV. In stage I-II samples, proline, hypoxanthine, S-adenosylhomocysteine, S-adenosylmethionine, carnitine, *symmetric*-dimethylarginine and dimethylglycine were elevated (Supplementary Figure 20). In stage III-IV samples, proline, hypoxanthine, S-adenosylhomocysteine, carnitine, *symmetric*-dimethylarginine, dimethylglycine as well as lysine, aspartic acid, glutamic acid, threonine, uracil, xanthine, and betaine were significantly elevated (Supplementary Figure 21). Although not statistically significant, other metabolites, particularly, S-adenosylmethionine ($P = .07$) and *asymmetric*-dimethylarginine ($P = .06$) also showed trends of elevation, in stage III-IV samples (Supplementary Figure 21). It is interesting to note that most of the methylation-related metabolites were elevated in human tumors irrespective of the stage or location. These observations indicate that similar to that observed in mice, human colorectal tumorigenesis also involves progressive dysregulation of the same metabolic pathways found in mouse colon carcinogenesis.

Discussion

Activation of β -catenin signaling due to loss-of-function *Apc* mutation is frequent in colorectal cancer.¹⁰ This is the first report on the effect of colorectal tumorigenesis caused by loss-of-function *Apc* mutation on urine metabolome. This study showed a mechanistic connection between early events of proliferation and the coordinate reprogramming of metabolic network that was reflected by biomarkers for accurate identification of mice undergoing early stages of tumorigenesis. The robustness of derangements in amino acid metabolism, polyamine metabolism, nucleic acid metabolism and methylation during colorectal carcinogenesis was established using genetic as well as chemically-induced mouse models. This was further substantiated by the characterization of similar signatures of metabolic derangement in human colorectal tumors. A consistent and novel signature of aberrant methylation was found in colorectal tumors as reflected by hypermethylated urine biomarkers.

Uncontrolled proliferation that requires nutrients and energy is the hallmark of cancer cells. Cancer cells develop ability to increase nutrient uptake.^{16,17} The accumulation of essential amino acids (threonine and lysine) in human and mouse colon tumors indicates to development of such capability. Increased retention of carnitine along with elevation of acetylated metabolites in tumors as well as urine indicates activation fatty acid oxidation that produces acetyl-CoA. Acetyl-CoA was indeed elevated after activation of β -catenin signaling. Fatty acid oxidation produces ATP that was found to be elevated in tumor tissues. Cancer cells resort to a variety of metabolic reprogramming ranging from increased aerobic glycolysis (Warburg effect), Krebs cycle to fatty acid oxidation to harvest energy.^{18–21} Earlier studies showed that inhibition of fatty acid β -oxidation can contribute to inhibition of colorectal cancer growth.²² It was also shown that Wnt-signaling promotes mitochondrial biogenesis via c-MYC²³, which increases acetyl-CoA production.²⁴ This study reports

evidence that increased production of acetyl-CoA and ATP follows immediately after activation of β -catenin signaling, and remains high in colorectal tumors.

Glutamine and a number of metabolically related amino acids such as glutamic acid, proline, arginine and aspartic acid were elevated in mouse urine and/or tumors along with overexpression of genes involved in corresponding metabolic pathways. *c-Myc* activation in cancer cells was shown to cause glutamine addiction for *de novo* synthesis¹⁷ of amino acids as well as nucleic acids. Simultaneous accumulation of essential amino acids and increase in production of non-essential amino acids reflect metabolic rearrangements required to support protein synthesis for proliferation.

Genes involved in *de novo* synthesis of nucleic acids, including c-MYC targets such as *Cad* and *Impdh1*, were overexpressed in tumors. c-MYC was shown to increase flux through the pentose phosphate pathway⁴ required for *de novo* nucleic acid synthesis. Consequently, several nucleic acid and related metabolites were elevated in tumor tissue and urine. Interestingly, although elevated in urine, xanthine was depleted in mouse tumors. This may be due to reutilization of xanthine for nucleotide synthesis. This was evident from the overexpression of genes involved in the salvage pathway such as *Hprt1* and *Pnp*. These observations indicate increased nucleic acids synthesis via both *de novo* synthesis and salvage pathway during tumorigenesis.

Aspartic acid and glutamic acid that were elevated in tumor enter the urea cycle and contribute to polyamine biosynthesis. Polyamines were shown to promote proliferation.²⁵ Expression of genes including *Odc*, which is a c-MYC target, and metabolites involved in urea cycle were found to be elevated in tumor tissue. In addition, *Amd1*, a gene involved in production of S-adenosylmethionine, which is required for extension of polyamine chain, was overexpressed in tumor tissue. Thus, elevation of N1-acetylspermidine and N8-acetylspermidine in urine as well as tumor, which was reported earlier as well²⁶, is consistent with a simultaneous increase in polyamine biosynthesis and acetyl-CoA production in tumors.

As noted earlier, some of these metabolites were reported to be modulated in colorectal cancer tissue or urine.^{6-9,26} However, the untargeted approach enabled capture of noninvasive biomarkers of progressive dysregulation of all these pathways together in this study. In addition, these data revealed novel urinary signatures of aberrant methylation that was corroborated by metabolomic and gene expression analysis of mouse as well as human tumors. Acute colon-specific disruption of *Apc* in mice showed that such aberrant methylation is an early event colorectal carcinogenesis. Increase in metabolites involved in methylation machinery along with overexpression of genes involved in DNA methylation essentially indicate aberrant DNA methylation that has recently been recognized to contribute to the development and progression of colorectal cancer.^{27, 28} *Symmetric*- and *asymmetric*-dimethylarginines were elevated in tissue and urine reflecting aberrant post-translational protein methylation. Arginine methylation is a relatively less well-characterized phenomenon in the context of colorectal cancer. Arginine methylation was shown to influence a number of biological processes such as signal transduction, chromatin remodeling, DNA damage repair and proliferation.^{29,30} Type-I (*Prmt1*, *Prmt3*, *Carm1*, and

Prmt6) and type-II (*Prmt5* and *Prmt7*) protein arginine methyl transferases (PRMT) that produce *symmetric*- and *asymmetric*-dimethylarginines, respectively, were shown to act on different substrates and oppositely control gene expression.^{29,30} PRMT1 was shown to stabilize AXIN, a negative regulator of β -catenin.³¹ Thus the increase in PRMT1 activity may be an early adaptive response to loss of *Apc*. However, overexpression of both types of PRMTs upon activation of β -catenin signaling indicates a novel and wider role of arginine methylation in the initial events during colorectal carcinogenesis that warrants further investigation. Nevertheless, the elevation of *symmetric*- and *asymmetric*-dimethylarginine could act as noninvasive signature of the underlying dysregulation of molecular events and contribute to early diagnosis of colorectal carcinogenesis. Taken together, tandem metabolic profiling and gene expression analysis revealed a wide-spread and intricately interconnected reprogramming of metabolic network as shown in Figure 7A (see Supplementary text for elaboration on interconnectivity) that was reflected by urinary biomarkers identified by global metabolic profiling.

Each of these biomarkers may individually be affected by multiple factors and physiological conditions compromising diagnostic accuracy. For example, production of acetylspermidines was found to be elevated in IBD^{26,32} while *asymmetric*-dimethylarginine was elevated in urine of patients with muscular dystrophy.³³ Proliferation requires simultaneous activation of multiple pathways following oncogene activation. Amino acids, nucleic acids, polyamines and methylation are crucial for proliferation. This study showed activation of these metabolic pathways following acute disruption of *Apc* in colon epithelium. Therefore, the combined biomarker panel representing these pathways is more likely to capture neoplastic transformation than individual biomarkers or pathways. In fact, for the first time in this study it was shown that the functional and mechanistic interconnectivity among these metabolic pathways can be used to combine representative biomarkers, which resulted in identification of *Apc*^{Min/+} mice at early stages of tumorigenesis. Although, activation of β -catenin signaling is frequent, other pathways such as K-RAS, TGF β were also found to contribute to the pathogenesis of sporadic colorectal cancer. AOM-induced colorectal carcinogenesis in mice was shown to be associated with the activation of pathways such as K-RAS, TGF β and β -catenin signaling³⁴. The fact that these mice also showed similar metabolic derangements strongly suggests that coordinate metabolic reprogramming is a common feature in colorectal carcinogenesis. The fact that even the genetic background of *Apc*^{Min/+} and AOM-treated mice were different further indicates the robustness of the association. In fact, human colorectal tumors showed very similar derangement of metabolite levels including twelve metabolites (aspartic acid, glutamic acid, proline, threonine, lysine, arginine, N1-acetylspermidine, carnitine, *symmetric*-dimethylarginine, *asymmetric*-dimethylarginine, betaine and dimethylglycine) that were elevated in mouse tumors. Metabolites elevated in human tumors also included ten noninvasive biomarkers (glutamic acid, proline, threonine, arginine, N1-acetylspermidine, xanthine, uracil, betaine, *symmetric*-dimethylarginine, and *asymmetric*-dimethylarginine) observed in mice. These observations indicate that such combined biomarker panel representing coordinate metabolic reprogramming associated with proliferation may be useful in screening and early diagnosis of colorectal cancer as shown in Figure 7B.

Association of these pathways with proliferation also invites further investigation into their utility in prediction of therapeutic response and relapse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AOM	azoxymethane
BrdU	Bromo deoxyuridine
ALT	alanine aminotransferase
AST	aspartate aminotransferase
UPLC-ESI-QTOFMS	ultraperformance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry
HILIC	hydrophilic interaction liquid chromatography
<i>Apc</i>^{Min/+}	mouse bearing heterozygous mutation in <i>Adenomatous Polyposis Coli</i> gene that develops multiple intestinal neoplasia
ROC	receiver operating characteristics

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Figure 1.

Effect of tumorigenesis on global metabolic signatures and amino acid metabolism. Scores scatter plot for principal components analysis (PCA) of creatinine-normalized data obtained from (A) HILIC-ESI-MS and (B) RP-ESI-MS analysis of urine samples at five months in negative ionization mode and at five months in positive ionization mode, respectively. Empty and solid circles represent healthy (wild-type) and tumor-bearing (*Apc*^{Min/+}) mice, respectively. The longitudinal variations in the creatinine-normalized urinary excretion of (C) glutamine, (D) proline, (E) Nα-acetyl lysine, and (F) carnitine in healthy and tumor-

bearing mice are shown by dotted and solid lines, respectively. P values (calculated by two-tailed Mann-Whitney test with 95% confidence interval) < 0.05 , < 0.01 and < 0.005 are indicated by '*', '**' and '***', respectively. All values are presented as mean $\pm$ SEM.

Figure 2.

Effect of tumorigenesis on urinary excretion of metabolites related to urea cycle and polyamine metabolism. The longitudinal variations in the creatinine-normalized urinary excretion of (A) *symmetric*-dimethylarginine, (B) *asymmetric*-dimethylarginine, (C) citrulline, (D) spermine, (E) N1-acetylspermidine, and (F) N8-acetylspermidine in healthy and tumor-bearing mice are shown by dotted and solid lines, respectively. P values (calculated by two-tailed Mann-Whitney test with 95% confidence interval) < 0.05, < 0.01 and < 0.005 are indicated by ‘*’, ‘**’ and ‘***’, respectively. All values are presented as mean $\pm$ SEM.

Figure 3.

Effect of tumorigenesis on urinary excretion of metabolites related to nucleic acid metabolism. The longitudinal variations in the creatinine-normalized urinary excretion of (A) xanthosine, (B) inosine, (C) xanthine, (D) cytidine, (E) deoxyuridine, and (F) thymidine in healthy and tumor-bearing mice are shown by dotted and solid lines, respectively. P values (calculated by two-tailed Mann-Whitney test with 95% confidence interval) < 0.05, < 0.01 and < 0.005 are indicated by '*', '**' and '***', respectively. All values are presented as mean $\pm$ SEM.

Figure 4.

Validation of metabolomic biomarkers and evaluation of diagnostic power. (A) Box plots for creatinine-normalized concentrations of carnitine, glutamine, N1-acetylspermidine, N8-acetylspermidine, proline, *asymmetric*-dimethylarginine, *symmetric*-dimethylarginine, spermine, xanthosine and inosine in the urine samples from age-matched non-littermate healthy (N = 6) and tumor-bearing (N = 6) mice from independent validation cohort. ROC curve for tumor diagnosis in the combined cohort (12 wild-type and 12 *Apc*^{Min/+} mice) using biomarkers related to (B) amino acid metabolism (proline, glutamine and N α -acetyllysine), (C) polyamine metabolism (N1-acetylspermidine, N8-acetylspermidine and spermine), (D) nucleic acid metabolism (xanthosine, inosine, deoxyuridine and thymidine) and (E) methylation (*symmetric*-dimethylarginine, *asymmetric*-dimethylarginine and carnitine). Areas under the ROC curve (AUCROC) and accuracy are noted on each plot.

Figure 5.

Effect of colorectal tumorigenesis on tissue metabolome. Box plots showing relative abundance of metabolites related to (A) amino acid metabolism, (B) polyamine metabolism (C) nucleic acid metabolism, and (D) methylation in normal colon mucosa (wild type) and colon tumor (*Apc*^{Min/+}) tissue. The abundance of proline, glutamine, glutamic acid, threonine, arginine, citrulline, ornithine, N1-acetylspermidine, deoxyuridine, thymidine, dimethylarginine and carnitine were elevated while those of xanthine and inosine were depleted. All values presented as fold-change with respect to the average of the abundances of the metabolite in the normal colon epithelium. P values were calculated by two-tailed Mann-Whitney test with 95% confidence interval.

Figure 6.

Effect of colorectal tumorigenesis on the metabolome of human epithelial tissue. Arrow plots showing changes in metabolite abundance between paired colon tumor and adjacent non-tumor tissue. All cases ($N = 23$) with tumors found in rectum and sigmoidal, descending or transverse colon are presented. P values were calculated by two-tailed paired t-test with 95% confidence intervals.

Figure 7.

Coordinate reprogramming of metabolic machinery in colorectal tumorigenesis. (A) Metabolic pathways captured by metabolomic and transcriptomic analysis in this study with metabolite names in red, black or blue indicating increase, no change or decrease, respectively, in abundance in *Apc^{Min/+}* tumor tissue. ‘*’ indicates that the metabolite was significantly elevated in human tumor tissues. Solid red, black or blue boxes indicate that creatinine-normalized excretion of the metabolite was elevated, unchanged or depleted, respectively, in urine of the *Apc^{Min/+}* mice. Dotted box indicates that the metabolite was

either not detected or monitored in *Apc^{Min/+}* urine. FA, Ac-CoA, Na-Ac Lysine, OAA, α -KG, PRRP, SAH, SAM, MTA, SAMA, ADMA, SDMA, NMMA, N1-AcS and N8-AcS represent, fatty acid, acetyl coenzyme A, Na acetyllysine, oxaloacetic acid, α -ketoglutaric acid, 5-phosphoribosyl 1-pyrophosphate, S-adenosylhomocysteine, S-adenosylmethionine, S-methyl-5-thioadenosine, S-adenosylmethioninamine, *asymmetric*-dimethylarginine, *symmetric*-dimethylarginine, N ^{ω} -monomethylarginine, N1-acetylspermidine and N8-acetylspermidine, respectively. Arrows indicate pathways of conversion of metabolites with solid and dotted lines indicating the involvement of single and multiple reactions, respectively. Red, black or blue colors indicate upregulation, no change or downregulation of expression of genes encoding enzymes involved in these pathways in *Apc^{Min/+}* tumor tissue compared to normal colon mucosa, respectively. Dashdotted arrows indicate pathways for biosynthesis of essential amino acids (such as threonine and lysine) that are unannotated in human and mouse. (B) The origin and utility of biomarkers of metabolic reprogramming in early noninvasive diagnosis.