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Regulation of the metabolite profile by an *APC* gene mutation in colorectal cancer

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Mutation of the *APC* gene occurs during the early stages of colorectal cancer development. To obtain new insights into the mechanisms underlying the aberrant activation of the Wnt pathway that accompanies *APC* mutation, we carried out a gas chromatography–mass spectrometry-based semiquantitative metabolome analysis. *In vitro* experiments comparing SW480 cells expressing normal APC and truncated APC indicated that the levels of metabolites involved in the latter stages of the intracellular tricarboxylic acid cycle, including succinic acid, fumaric acid, and malic acid, were significantly higher in the SW480 cells expressing the truncated APC. In an *in vivo* study, we found that the levels of most amino acids were higher in the non-polyp tissues of $APC^{min/+}$ mice than in the normal tissues of the control mice and the polyp tissues of $APC^{min/+}$ mice. Ribitol, the levels of which were decreased in the polyp lesions of the $APC^{min/+}$ mice and the SW480 cells expressing the truncated APC, reduced the growth of SW480 cells with the APC mutation, but did not affect the growth of SW480 transfectants expressing full-length APC. The level of sarcosine was found to be significantly higher in the polyp tissues of $APC^{min/+}$ mice than in their non-polyp tissues and the normal tissues of the control mice, and the treatment of SW480 cells with 50 μM sarcosine resulted in a significant increase in their growth rate. These findings suggest that *APC* mutation causes changes in energetic metabolite pathways and that these alterations might be involved in the development of colorectal cancer. (*Cancer Sci* 2012; 103: 1010–1021)

Colorectal cancer is one of the most commonly diagnosed cancers, the second leading cause of cancer mortality in the USA, and the third leading cause of cancer mortality in Japan.[1] The National Cancer Center Hospital (Tokyo, Japan) has estimated that the 5-year survival rate of patients with stage I colorectal cancer exceeds 90%, but that survival decreases with advancing disease stage. To reduce the mortality of colorectal cancer, new screening methods, especially for early stage disease, and new medical treatments for late stage disease have been studied worldwide. Most malignant colorectal cancers arise from pre-existing benign adenomas. The incidence of colorectal cancer is associated with the multistep accumulation of mutations in both oncogenes and tumor suppressor genes.[2,3] Mutation of the *APC* gene occurs in the early stages of colorectal cancer,[4] and the gene been recognized as the gatekeeper of colorectal carcinogenesis.[2] Mutations in the *APC* gene have also been implicated in familial adenomatous polyposis (FAP).[5–7] This is an autosomal, dominantly inherited disease that typically causes the development of hundreds to thousands of colorectal adenomas. In addition, 80–90% of patients with sporadic colorectal cancer show *APC* mutations. APC proteins negatively regulate the Wnt pathway by aiding the degradation of β-catenin, whereas *APC* mutation results in the stabilization of β-catenin and activation of the Wnt pathway. Stabilized β-catenin promotes the transcription of Wnt target genes then leads to aberrant cellular proliferation. Recently, it was revealed by proteome analysis that β-catenin activation in mouse liver might affect glucose metabolism,[8] so an investigation of metabolite changes induced by *APC* mutation might help to elucidate the mechanisms of colorectal cancer development.

The human genome had been completely identified by the end of 2003. Proteomics has since been heavily studied. In 2002, Petricoin *et al.*[9] found an effective diagnostic marker for ovarian cancer, including stage I disease, using proteomic analysis. Thus, "clinical proteomics" was developed, and since then many researchers have tried to apply proteomic analysis to the medical field in order to find effective diagnostic markers and elucidate unknown pathological conditions. Furthermore, metabolomics, which is the comprehensive study of low molecular weight metabolites, has also been developed. In clinical research using metabolomics, Sreekumar *et al.*[10] revealed, using a combination of the high-throughput liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry (GCMS), that sarcosine is a potentially important metabolic intermediary factor for prostate cancer cell invasion and aggressivity. In addition, the value of metabolomics for research on diagnostic biomarkers and/or the elucidation of the pathological conditions of lung cancer, gastroenterological cancer, pancreatic cancer, and inflammatory bowel disease has also been shown.[11–17]

In this study, we used $APC^{min/+}$ mice and human colorectal cancer cell-implanted nude mice. APC multiple intestinal neoplasia (APC^{min}) was the first heritable mutant *APC* allele to be induced in mice.[18] $APC^{min/+}$ mice develop numerous intestinal lesions that resemble human FAP and are useful models for investigating malignant transformation in colon tumorigenesis.[19] It is also well-known that nude mice implanted with human tumors maintain not only their histological appearance but also their functional mechanisms. *In vitro* experiments were carried out using SW480 human epithelial colorectal cancer cells expressing truncated APC, and SW480 transfectants expressing full-length APC. Metabolomic analyses of $APC^{min/+}$ mice, human colorectal cancer cell-implanted nude mice, SW480 cells, and SW480 transfectants were carried out by GCMS, and the relationships between colorectal cancer, *APC* mutation, and metabolite levels were evaluated.

Materials and Methods

Cell culture and treatment. SW480 cells expressing truncated APC and SW480 transfectants expressing full-length APC were kindly donated by Dr. Maree C. Faux (Ludwig Institute for Cancer Research, Melbourne, Australia).[20] The culture medium consisted of RPMI-1640 medium supplemented with

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10% FBS, 1% penicillin–streptomycin, and 1.25 mg/mL G418. The cells were grown at 5×10^4/well in 96-well plates for 24 h in medium then exposed to arabitol, inositol, ribitol, L-proline, isoleucine, 2-hydroxybutyric acid, 3-hydroxypropanoic acid, and sarcosine. After 24 h, 10 μL of the solution provided with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) were added to each well of the plate in order to evaluate cell growth.

Animal and experimental design. All treatments in this study were approved by the Institutional Animal Care and Use Committee and carried out in accordance with the Kobe University Animal Experimentation Regulations. Female 6–8-week-old C57BL/6J mice were purchased from Clea Japan (Shizuoka, Japan). $APC^{min/+}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). $APC^{min/+}$ mice with a C57BL/6J background were maintained by breeding male mice that were heterozygous for the Min allele with female wild-type C57BL/6J mice. All mice were housed and bred at the Animal Unit of the Kobe University School of Medicine (Kobe, Japan) in a specific pathogen-free facility under an approved experimental protocol. The mice were killed, and their blood was collected by cardiac puncture followed by separation of their serum by centrifugation at 3000*g* for 10 min at 4°C. Intestinal polyps and non-polyp regions of the small intestine were obtained from $APC^{min/+}$ mice using a microscope. Normal small intestinal tissues were collected from the wild-type C57BL/6J mice.

BALB/cAJc1-nu/nu mice were purchased from Clea Japan. Human epithelial colon cancer HT-29 cells, SW480 cells expressing truncated APC, and the SW480 transfectants expressing full-length APC were implanted s.c. into male 6-week-old nude mice at 2×10^6 cells per mouse. After the enlargement of implanted tumors was recognized, the mice were killed and their blood collected by cardiac puncture followed by serum separation.

Histological examination. The intestinal tissue of the mice was dissected and fixed with 10% formalin, then the paraffin-embedded tissue was sliced at 5 μm and stained with H&E in a blinded manner. The sections were observed using a microscope (BX51; Olympus, Tokyo, Japan).

Sample collection and preparation. The extraction of low molecular weight metabolites from serum and the pretreatment procedure used for GCMS measurement were carried out according to the methods described in our previous reports.[11–16,21]

The collected intestinal polyps and non-polyp and normal tissues were stored at −80°C until use. To extract low molecular weight metabolites, 10 mg of the tissues was transferred to a clean tube and homogenized in 1000 μL of a solvent mixture (MeOH : H_2O : $CHCl_3$ = 2.5:1:1). Then 10 μL of 0.5 mg/mL 2-isopropylmalic acid (Sigma-Aldrich, St. Louis, MO, USA) was added to each tube, and the mixture was mixed well. The mixture was subsequently shaken at 1200 rpm for 30 min at 37°C, before being centrifuged at 16 000*g* for 3 min at 4°C. The resultant supernatant (900 μL) was separately collected in a clean tube. Then 450 μL $CHCl_3$ was added to the supernatant before it was centrifuged at 16 000*g* for 3 min at 4°C, and 500 μL of the resultant supernatant was separately collected in a clean tube. Distilled water (200 μL) was added to the collected supernatant before it was centrifuged at 16 000*g* for 3 min at 4°C, and 500 μL of the resultant supernatant was separately collected in a clean tube. The collected supernatant was then lyophilized using a freeze dryer before oximation and derivatization. The oximation and subsequent derivatization for GCMS measurement were carried out according to the method described in our previous reports.[11–16,21]

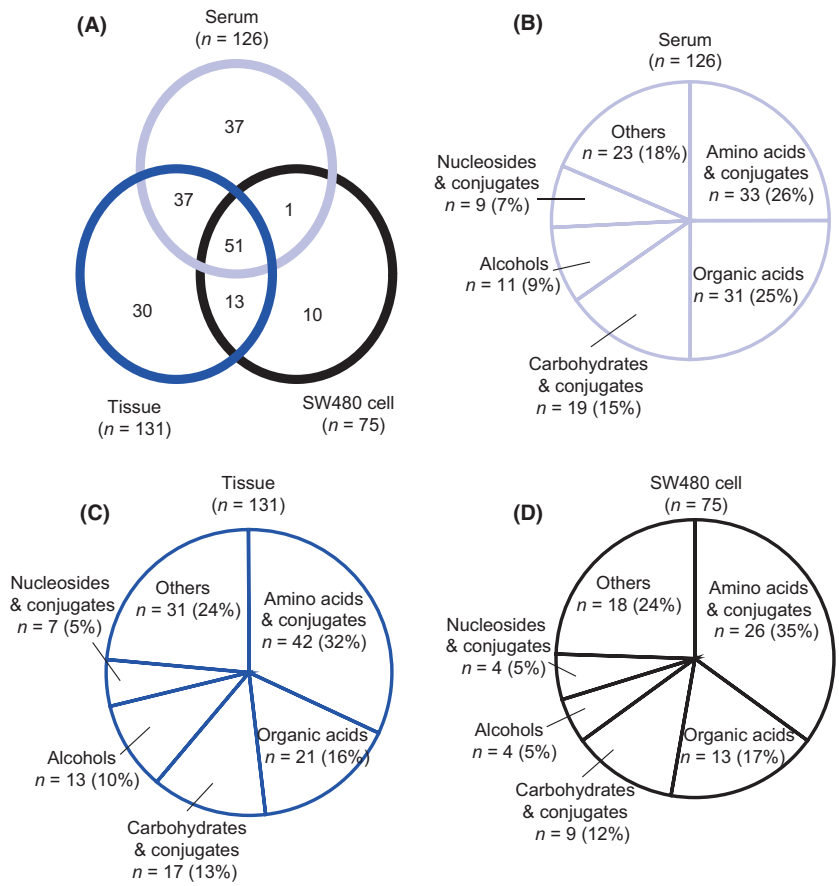

Fig. 1. Metabolomic profiling of $APC^{min/+}$ mice and SW480 human epithelial colorectal cancer cells. (A) Venn diagram based on the metabolites detected in sera and tissues of $APC^{min/+}$ mice, corresponding control mice, and SW480 cells. The circle graph represents the distribution of chemical classes for the metabolites detected in sera (B), tissues (C), and SW480 cells (D). Others include amines, miscellaneous, heterocyclic molecules, ketones, and aldehydes. The values in parentheses indicate the percentage of total.

The SW480 cells and SW480 transfectants at 70–80% confluence were washed twice with Krebs–Ringer HEPES buffer and collected. The cells were washed twice with Krebs–Ringer HEPES buffer before being counted and collected in a clean tube, then washed with H_2O and lyophilized using a freeze dryer. The extraction of low molecular weight metabolites and the pretreatment procedure used for GCMS measurement were carried out according to the methods described in our previous reports.[11–16,21]

Analysis using GC/MS and data processing. According to the method described in a previous report,[21] GC/MS analysis was carried out using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleed/MS, 30 m × 0.25 mm [inner diameter], film thickness 0.25 μm; Agilent Technologies, Palo Alto, CA, USA). Data processing was carried out using MetAlign software (Wageningen UR, http://www.pri.wur.nl/UK/products/MetAlign/) and in-house analytical software (AIoutput), as described in a previous report.[22] For semiquantification, the peak height of each ion was calculated and normalized using the peak height of 2-isopropylmalic acid as an internal standard. In GC/MS analysis, multiple peaks are sometimes detected for a particular metabolite due to trimethysilyl (TMS) derivatization, isomeric

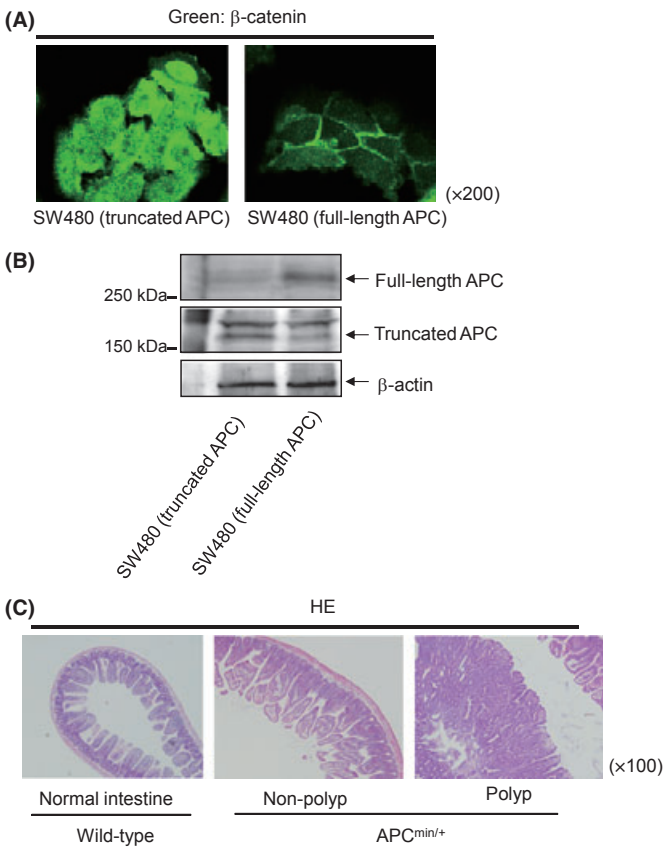

Fig. 2. Mutation of the *APC* gene causes the development of adenoma in the digestive tract. (A) SW480 colorectal cancer cells expressing truncated APC and SW480 transfectants expressing full-length APC were subjected to immunohistochemical analysis with anti-β-catenin antibody. Representative images are shown. Green, β-catenin. Magnification, ×200. (B) SW480 cells expressing truncated APC and SW480 transfectants expressing full-length APC were subjected to Western blot analysis with anti-APC antibody and anti-β-actin antibody. Representative images are shown. (C) Small intestinal tissue was collected from $APC^{min/+}$ mice with non-polyp and polyp lesions and C57BL/6J mice and subjected to H&E staining. Representative images are shown. Magnification, ×100.

Table 1. Metabolites that displayed significantly altered serum levels between $APC^{min/+}$ mice and wild-type mice

	Chemical class	Fold induction	*P*-value
		$APC^{min/+}$/control	$APC^{min/+}$ versus control
1-Hexadecanol	Alcohols and polyols	4.56	<0.00001*
2-Aminobutyric acid	Amino acids	2.74	0.00081*
2-Dehydro-D-gluconate	Hydroxy acids	1.21	0.04400*
2′-Deoxyuridine	Nucleoside analogues	2.41	0.01200*
3,4-Dihydroxymandelic acid	Hydroxy acids	1.27	0.00240*
3-Hydroxyisovaleric acid	Hydroxy acids	1.40	0.04700*
4-Hydroxymandelate	Aromatic acids	3.05	0.07700*
Adenine	Purines and purine derivatives	2.01	0.00010*
Alanine	Amino acids	2.01	0.00010*
Anthranilic acid	Amino acids	2.75	0.00045*
Arabinose-5-phosphate	Carbohydrates	0.24	0.00230*
Arabitol	Alcohols and polyols	0.27	0.00006*
Ascorbic acid	Hydroxy acids	5.27	0.00011*
α-Sorbopyranose (or fructose)	Carbohydrates	0.25	0.01600*
Asparagine	Amino acids	1.98	0.00220*
β-Alanine	Amino acids	0.49	0.00071*
Benzoic acid	Aromatic acids	3.03	<0.00001*
β-Glutamic acid	Amino acids	0.86	0.04800*
β-Lactose	Carbohydrates	2.06	0.00260*
Citrulline	Amino acids	2.24	0.00019*
Coniferyl aldehyde	Hydroxy acids	17.90	0.00023*
Cysteine + cystine	Amino acids	1.79	0.00058*
Dopa	Catecholamines and derivatives	0.50	0.02100*
Fructose	Carbohydrates	0.60	0.00032*
Fructose-6-phosphate	Sugar phosphates	0.63	0.02400*
Galactosamine	Carbohydrates	4.40	<0.00001*
Galactose	Carbohydrates	0.78	0.00330*
Galacturonic acid	Hydroxy acids	0.56	0.00024*
Glucarate	Dicarboxylic acids	1.54	0.00150*
Glucosamine	Aminoglycosides	1.78	0.00440*
Glucuronate	Carbohydrates	0.57	0.00058*
Glutamine	Amino acids	1.35	0.03300*
Glyceric acid	Hydroxy acids	0.75	0.00300*
Glycerol	Alcohols and polyols	0.61	0.00037*
Glycolic acid	Hydroxy acids	0.41	<0.00001*
Histidine	Amino acids	2.01	0.00140*
Homoserine	Amino acids	3.91	<0.00001*
Hydroxybutyrate	Hydroxy acids	2.57	0.00110*
Inosine	Nucleoside analogues	0.35	0.02300*
Ketoisoleucine	Keto-acids	0.37	<0.00001*
Ketovaline	Keto-acids	0.65	0.00048*
Lauric acid	Fatty acids	1.47	0.00210*
Lysine	Amino acids	3.07	0.00002*
Lyxose (or xylose)	Carbohydrates	1.52	0.000011*
Malonic acid	Dicarboxylic acids	1.87	0.00550*

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Table 1 (continued)

	Chemical class	Fold induction	*P*-value
		$APC^{min/+}$/ control	$APC^{min/+}$ versus control
Mannose	Carbohydrates	1.21	0.02900*
N-acetyl-D-glucosamine	Carbohydrates	0.60	0.04300*
N-caprylic acid	Fatty acids	5.57	<0.00001*
Nonanoic acid (C9)	Amino acids	1.90	0.00240*
O-phosphoethanolamine	Acyl phosphates	0.57	0.02700*
Ornithine	Amino acids	3.07	0.00007*
Orotic acid	Pyrimidines and derivatives	3.65	<0.00001*
Palmitoleate	Fatty acids	0.20	<0.00001*
Pantothenate	Amino alcohols	1.54	0.00620*
Phenylalanine	Aromatic acids	1.34	0.02900*
Proline	Amino acids	2.68	0.00013*
Putrescine	Polyamines	1.20	0.02700*
Ribitol	Alcohols and polyols	0.44	0.00077*
Ribose	Carbohydrates	0.28	<0.00001*
Ribulose-5-phosphate	Sugar phosphates	0.17	0.00068*
Saccharopine	Amino acids	1.39	0.03200*
Sarcosine	Amino acids	5.92	0.00130*
Thymine	Pyrimidines and derivatives	3.19	0.00220*
Trans-4-hydroxy-L-proline	Amino acids	2.07	0.00310*
Tryptophan	Amino acids	0.37	0.00150*
Tyrosine	Amino acids	1.46	0.00110*
Uracil	Pyrimidines and derivatives	1.74	0.00033*
Uric acid	Purines and purine derivatives	0.55	0.00930*
Valine	Amino acids	1.34	0.02800*
Xylitol	Alcohols and polyols	0.42	0.00083*
Xylulose	Carbohydrates	0.58	0.00260*

Values are represented as the fold-induction of the peak intensity for the $APC^{min/+}$ mice (n = 7) against that of the corresponding controls (n = 6). All data are represented in Table S1, and the metabolites that showed significantly altered levels are shown in Table 1. APC^{min}, APC multiple intestinal neoplasia. *P*-values were calculated using Student's *t*-test; *$P < 0.05$.

form, etc. In such cases, the peak that most reflected the level of the metabolite was adopted for the semiquantitative evaluation.

Western blot analysis. The extraction of proteins from the cultured cells and the subsequent Western blotting using anti-APC antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin antibody (Sigma-Aldrich) were carried out as described in our previous report.[22]

Statistical analysis. The results are expressed as the mean ± SE. Statistical significance was analyzed using Student's *t*-test, and a level of probability of 0.05 was used as the criterion for significance.

Results

To profile the metabolites changed by the *APC* mutation, we used GCMS-based metabolome analysis, and the relative levels of metabolites were evaluated across the tissues and/or sera of $APC^{min/+}$ mice, SW480 cells, and human colorectal cancer cell-implanted nude mice. As shown in Figure 1(A), the 51 metabolites were shared across the tissues, sera, and cultured cells, and in our analysis system, distribution of the chemical classes for the detected metabolites were similar among sera, tissues, and cultured cells (Fig. 1B–D).

Metabolite profiling of sera and polyps of $APC^{min/+}$ mice. In a histopathological examination with H&E staining (Fig. 2C), adenomatous proliferations characterized by cell dysplasia were noted in the polyp lesions of $APC^{min/+}$ mice as well as in parts of their non-polyp lesions. Low molecular weight metabolites were extracted from the sera and the intestinal tissues of $APC^{min/+}$ mice and the corresponding control mice, and a total of 126 and 131 metabolites were detected in their sera and intestinal tissues, respectively (Fig. 1, Tables S1,S2). Among these detected metabolites, the 88 metabolites were shared across the tissues and sera (Fig. 1A). In the sera of the $APC^{min/+}$ mice, the levels of 44 and 27 metabolites showed significant increases and decreases, respectively, compared with those of the corresponding control mice (Table 1). In the polyp lesions of $APC^{min/+}$ mice, the levels of 25 metabolites were significantly increased and those of 18 metabolites were decreased in comparison to those in the normal intestinal tissue of the corresponding control mice (Table 2). In addition, the levels of 15 and 24 metabolites were significantly increased and decreased, respectively, in the polyp lesions of $APC^{min/+}$ mice compared with those of the non-polyp lesions of the $APC^{min/+}$ mice (Table 2). These results suggest that the metabolite profiles of sera and tissues differ between *APC* mutation mice and the corresponding control mice.

Metabolite profiling in SW480 cells expressing truncated APC and full-length APC. To investigate the effects of *APC* mutation on the levels of intracellular metabolites, SW480 cells expressing truncated APC and full-length APC were used. In an immunohistochemical examination, the expression of full-length APC in SW480 cells led to the localization of β-catenin in the cell membrane (Fig. 2A). Expression of the truncated APC protein in SW480 cells and expression of the full-length APC protein in SW480 transfectants could be also confirmed by Western blotting (Fig. 2B). Low molecular weight metabolites were extracted from SW480 cells and SW480 transfectants, and a total of 75 metabolites were detected (Fig. 1, Table S3). Among these detected metabolites, the 64 metabolites were shared across the tissues of $APC^{min/+}$ mice and SW480 cells (Fig. 1A). In the SW480 cells expressing truncated APC, the levels of 23 and eight metabolites were significantly increased and decreased, respectively, in comparison to those observed in the SW480 transfectants (Table 3). These results suggest that the *APC* mutation causes alterations in the levels of metabolites in the intracellular environment.

Metabolite profiling of the sera of nude mice implanted with HT-29 or SW480 cells. Human epithelial colorectal adenocarcinoma HT-29 cells express a truncated *APC* gene.[23] Nude mice were implanted s.c. with HT-29 cells, SW480 cells expressing the truncated APC, and SW480 transfectants expressing the full-length APC. After the enlargement of the implanted tumor, the mice were killed and their blood obtained. A total of 95 and 84 metabolites were detected in sera of HT-29 cells and SW480 cells implanted nude mice, respectively (Tables S4,S5). In the sera of the HT-29 cell-implanted mice, the levels of 16 and 17 metabolites were significantly increased and decreased, respectively, in comparison with those of the corresponding control mice (Table 4). In addition, the levels of seven and four metabolites were significantly increased and decreased, respectively, in SW480 cells expressing the truncated APC in comparison to the full-length APC (Table 5).

Changes in amino acid levels induced by *APC* gene mutation. The changes in the amino acid compositions of the sera and

Table 2. Metabolites that showed significantly altered tissue levels between APC$^{min/+}$ mice and wild-type mice

	Chemical class	Fold induction			*P*-value		
		Polyp/ control	Polyp/ non-polyp	Non-polyp/ control	Polyp versus control	Polyp versus non-polyp	Non-polyp versus control
1,5-Anhydro-D-glucitol	Carbohydrates	0.41	1.35	0.30	0.00120*	0.20000	0.00011*
1,6-Anhydroglucose	Carbohydrates	2.18	1.60	1.36	0.02900*	0.17000	0.33000
1-Methylhistamine	Cyclic amines	2.59	1.91	1.35	0.08500	0.17000	0.04100*
2,3-Bisphospho-glycerate	Acyl phosphates	0.28	0.55	0.52	0.27000	0.04700*	0.46000
2-Aminobutyric acid	Amino acids	9.70	3.60	2.69	<0.00001*	<0.00001*	0.00540*
2-Hydroxypyridine	Hydroxy acids	1.41	0.74	1.90	0.06200	0.04100*	0.00710*
3-Hydroxy-butyrate	Hydroxy acids	2.47	1.43	1.73	0.00015*	0.04700*	0.04100*
4-Aminobutyric acid	Amino acids	1.22	0.57	2.14	0.13000	0.00250*	0.00077*
4-Hydroxybenzyl alcohol	Alcohols and polyols	1.26	0.72	1.76	0.12000	0.06000	0.01100*
5-Hydroxyindoleacetate	Indoles and indole derivatives	2.38	1.42	1.67	0.03200*	0.23000	0.01200*
Adenine	Purines and purine derivatives	1.95	1.65	1.18	0.02800*	0.05300	0.40000
Arabitol	Alcohols and polyols	0.82	0.64	1.29	0.32000	0.03800*	0.09800
Ascorbic acid	Hydroxy acids	3.91	5.80	0.67	0.01400*	0.00290*	0.59000
α-Sorbopyranose (or fructose)	Carbohydrates	1.77	1.26	1.40	0.00210*	0.14000	0.05400
β-Alanine	Amino acids	1.30	1.06	1.22	0.02400*	0.56000	0.20000
β-Glutamic acid	Amino acids	0.23	0.29	0.79	0.00003*	0.00330*	0.20000
Ciliatine	Amino acids	0.12	0.16	0.75	0.00330*	<0.00001*	0.32000
Citric acid + isocitric acid	Tricarboxylic acids	8.18	4.60	1.78	0.16000	0.21000	0.02600*
Cystathionine	Amino acids	14.67	5.34	2.75	<0.00001*	0.00001*	0.01300*
Cysteine + cystine	Amino acids	0.44	0.44	0.99	0.00480*	0.00100*	0.97000
Dimethylbenzimidazole	Heterocyclic aromatic organic compound	3.34	2.13	1.57	0.00250*	0.02100*	0.19000
Fructose	Carbohydrates	1.82	1.27	1.43	0.00160*	0.12000	0.04000*
Fructose-6-phosphate	Sugar phosphates	3.67	1.32	2.78	0.01400*	0.42000	0.01200*
Fucose	Carbohydrates	2.34	1.52	1.55	<0.00001*	0.00450*	0.02600*
Fumaric acid	Dicarboxylic acids	0.65	0.62	1.05	0.01000*	0.06100	0.81000
Galactitol	Alcohols and polyols	2.90	1.00	2.90	0.02100*	1.00000	0.08900
Galactosamine	Carbohydrates	0.68	0.59	1.14	0.17000	0.00058*	0.56000
Galactose	Carbohydrates	0.78	0.59	1.32	0.19000	0.01800*	0.17000
Galacturonic acid	Hydroxy acids	0.61	0.71	0.87	0.05000	0.04400*	0.52000
Glucosamine	Aminoglycosides	3.22	1.25	2.59	0.27000	0.79000	0.27000
Glucose	Carbohydrates	0.53	0.61	0.88	0.01600*	0.04900*	0.56000
Glucuronate	Carbohydrates	0.53	0.62	0.86	0.01300*	0.03800*	0.51000
Glutaric acid	Dicarboxylic acids	0.79	0.49	1.61	0.16000	0.00260*	0.00750*
Glyceraldehyde	Alcohols and polyols	0.88	0.68	1.30	0.50000	0.04600*	0.22000
Glyceric acid	Hydroxy acids	1.20	0.84	1.43	0.10000	0.21000	0.02000*
Glycerol	Alcohols and polyols	0.45	0.68	0.66	0.00007*	0.09800	0.03700*
Homoserine	Amino acids	9.43	2.95	3.20	<0.00001*	<0.00001*	0.00007*
Hypotaurine	Amino acids	2.53	2.61	0.97	0.01600*	0.01400*	0.90000
Hypoxanthine	Purines and purine derivatives	3.49	6.80	0.51	0.00007*	0.00001*	0.08900
Inositol	Alcohols and polyols	0.77	0.65	1.18	0.01800*	0.02400*	0.30000
Isoleucine	Amino acids	0.88	0.66	1.33	0.52000	0.02700*	0.17000
Kojic acid	Hydroxy acids	2.90	1.74	1.67	0.00260*	0.02200*	0.13000
Malic acid	Dicarboxylic acids	0.69	0.62	1.10	0.00210*	0.04700*	0.63000
N-α-acetyl-L-lysine	Amino acids	0.59	0.55	1.07	0.07000	0.03500*	0.79000
N-acetyl-D-glucosamine	Carbohydrates	1.16	0.79	1.47	0.43000	0.17000	0.01500*
O-phosphoethanolamine	Acyl phosphates	2.43	1.05	2.33	0.00250*	0.85000	0.01400*
Ornithine	Amino acids	0.56	0.58	0.98	0.00700*	0.01400*	0.89000
Orotic acid	Pyrimidines and derivatives	3.35	1.51	2.22	0.00008*	0.03400*	0.00260*
Oxalate	Dicarboxylic acids	1.20	0.77	1.55	0.15000	0.09200	0.01300*
Pantothenate	Amino alcohols	0.50	0.47	1.07	0.00470*	0.00087*	0.70000
Putrescine	Polyamines	14.01	13.63	1.03	0.04700*	0.04700*	0.95000
Rhamnose	Carbohydrates	2.80	1.37	2.05	<0.00001*	0.08700	0.01600*
Ribose	Carbohydrates	0.42	0.42	1.00	0.00120*	0.00150*	1.00000
Ribulose	Carbohydrates	0.65	0.57	1.13	0.01600*	0.01000*	0.43000
Sarcosine	Amino acids	10.44	2.08	5.01	<0.00001*	0.00075*	<0.00001*
Sorbitol	Alcohols and polyols	0.62	0.71	0.86	0.04100*	0.08100	0.49000
Succinic acid (or aldehyde)	Dicarboxylic acids	0.53	0.63	0.85	0.03600*	0.09800	0.55000
Tagatose	Carbohydrates	2.73	1.95	1.40	0.00030*	0.00630*	0.18000
Trans-4-hydroxy-L-proline	Amino acids	4.30	2.00	2.15	<0.00001*	0.00011*	0.00100*
Uric acid	Purines and purine derivatives	0.73	0.37	1.95	0.03300*	0.00039*	0.00200*
Xylitol	Alcohols and polyols	0.45	0.52	0.86	0.00160*	0.00620*	0.42000

Values are represented as the fold-induction of the peak intensity among polyps (n = 7) and non-polyp tissues (n = 7) of the APC$^{min/+}$ mice, and the corresponding control intestinal tissues (n = 6) of the wild mice. All data are represented in Table S2, and the metabolites that showed significantly altered levels are shown in Table 2. APC^{min}, APC multiple intestinal neoplasia. *P*-values were calculated using Student's *t*-test; *$P < 0.05$.

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Table 3. Metabolites that showed significantly altered levels between SW480 colorectal cancer cells expressing truncated APC and those expressing full-length APC

	Chemical class	Fold induction	*P*-value
		Truncated/ full-length APC	Truncated versus full-length APC
1,5-Anhydro-D-glucitol	Carbohydrates	3.1500	0.03200*
1,6-Anhydroglucose	Carbohydrates	1.7500	0.02900*
2-Aminopimelic acid	Dicarboxylic acids	3.0100	0.00088*
2-Hydroxypyridine	Hydroxy acids	1.4900	0.03000*
6-Hydroxynicotinic acid	Aromatic acids	0.3900	0.02500*
Acetoacetic acid	Keto-acids	6.3100	0.00005*
Alanine	Amino acids	1.4800	0.00100*
Arabitol	Alcohols and polyols	0.5200	0.04100*
Asparagine	Amino acids	3.7400	0.00200*
Aspartic acid	Amino acids	1.8700	0.01200*
Citric acid + isocitric acid	Tricarboxylic acids	2.7600	0.00077*
Creatinine	Amino ketones	2.2500	0.00120*
Cystathionine	Amino acids	3.9600	0.00046*
Fumaric acid	Dicarboxylic acids	2.1000	0.00920*
Glycolic acid	Hydroxy acids	1.6300	0.00089*
Gulcono-1,4-lactone	Carbohydrates	2.1800	0.00009*
Inositol	Alcohols and polyols	0.4600	0.00002*
Kojic acid	Hydroxy acids	2.4900	0.01500*
Malic acid	Dicarboxylic acids	5.3300	<0.00001*
Meso-erythritol	Alcohols and polyols	0.7200	0.00200*
Nicotinamide	Cyclic amines	0.7400	0.01000*
Nicotinic acid	Amino acids	0.0017	<0.00001*
N-methylethanolamine	Amino alcohols	1.3000	0.03500*
Proline	Amino acids	0.4800	0.00006*
Putrescine	Polyamines	1.7200	0.00087*
Pyroglutamic acid	Amino acids	2.9600	0.00021*
Pyruvate + oxalacetic acid	Keto-acids	1.8400	0.04700*
Ribitol	Alcohols and polyols	0.2400	0.00001*
Succinic acid (or aldehyde)	Dicarboxylic acids	18.4600	0.00008*
Tagatose	Carbohydrates	1.6900	0.00530*
Thymine	Pyrimidines and derivatives	2.5800	0.00020*

Values are represented as the fold-induction of the peak intensity for SW480 cells expressing truncated APC (n = 5) against that for SW480 transfectants expressing full-length APC (n = 5). All data are represented in Table S3, and the metabolites that showed significantly altered levels are shown in Table 3. *P*-values were calculated using Student's *t*-test; *$P < 0.05$.

intestinal tissues of mice and SW480 cells were examined. In our experimental conditions, 18 amino acids were detected in the tissues and sera of $APC^{min/+}$ mice and sera of SW480 cell-implanted nude mice. Seventeen amino acids were detected in the sera of HT-29 cell-implanted nude mice, and 14 amino acids were detected in SW480 cells (Tables 6,7,S1–S5). The levels of 10 amino acids were significantly increased in the sera of $APC^{min/+}$ mice than in the corresponding control mice, but the serum level of tryptophan in the $APC^{min/+}$ mice was only significantly lower than that of the control mice (Table 6). In addition, 10 of the 17 amino acids showed significantly increased serum levels in the HT-29 cell-implanted nude mice compared with the control mice, and no amino acids displayed significant decreases (Table 4). There were no significant changes in the serum levels of amino acids between mice implanted with SW480 cells with truncated APC and full-length APC. In contrast, the levels of four amino acids in the serum of nude mice implanted with SW480 cells expressing truncated APC or full-length APC were significantly lower than that of the control nude mice (Table 6). In the intestinal tissue, the levels of most amino acids in the non-polyp tissue of the $APC^{min/+}$ mice were higher than those in the corresponding control mice and the polyps of the $APC^{min/+}$ mice (Table 7). In the SW480 cells expressing truncated APC, the levels of most amino acids were similar or modestly increased compared with those of the SW480 cells expressing full-length APC (Table 7). The level of cysteine + cystine in the polyps of the $APC^{min/+}$ mice was significantly lower than those in the non-polyp tissue of the $APC^{min/+}$ mice and the normal intestinal tissue of the corresponding control mice (Table 2).

Changes in levels of metabolites associated with the tricarboxylic acid (TCA) cycle induced by *APC* gene mutation. The changes in the levels of metabolites involved in the TCA cycle induced by APC mutation were evaluated. In our experimental conditions, citric acid + isocitric acid, succinic acid, fumaric acid, and malic acid were detected. In the polyp tissues of the $APC^{min/+}$ mice, the levels of citric acid and isocitric acid were higher than in non-polyp tissues and those of the control mice (Table 7). In the SW480 cells expressing truncated APC, the levels of all of these TCA cycle-related metabolites were significantly increased compared to those of the SW480 cells expressing full-length APC (Table 7), indicating that the *APC* mutation might cause the increases in TCA cycle-related metabolite levels to gain more energy. In the sera of the $APC^{min/+}$ mice and SW480 cell-implanted nude mice, there were no significant changes in the serum levels of TCA cycle-related metabolites compared with those of the control mice (Table 6). In contrast, in the sera of the HT-29 cell-implanted nude mice, the levels of all of these metabolites were significantly decreased compared with those of the control mice (Table 4).

Changes in proliferation of SW480 cells treated with metabolites. Next, we evaluated whether the metabolites whose levels were altered by *APC* mutation affect tumor proliferation. The levels of proline, arabitol, inositol, ribitol, isoleucine, 2-hydroxybutyric acid, and 3-hydroxypropanic acid tended to be decreased in the polyp lesions of the $APC^{min/+}$ mice and SW480 cells expressing truncated APC. The sarcosine level was significantly increased in the polyp lesions of $APC^{min/+}$ mice and tended to be increased in SW480 cells expressing truncated APC (Tables 2,3,S2,S3). Therefore, the growth rate of SW480 cells that had been treated with three different concentrations of each metabolite was investigated. In SW480 cells expressing full-length APC, the addition of 1 mM inositol significantly increased their proliferation rate (Fig. 3A). The addition of 1 or 10 mM ribitol significantly decreased the proliferation rate of SW480 cells expressing truncated APC (Fig. 3B). The addition of 50 μM sarcosine significantly promoted the growth rate of SW480 cells expressing truncated APC (Fig. 3E). Both 2-hydroxybutyric acid and 3-hydroxypropanic acid had similar effects on SW480 cell proliferation at each concentration (Fig. 3C,D). In addition, L-proline (0.6–1.8 mM), arabitol (0.1–10 mM), and isoleucine (0.6–1.8 mM) did not significantly affect the rate of cell growth (data not shown). These results suggest that some of the metabolites

Table 4. Metabolites that showed significantly altered serum levels in HT-29 cell-implanted nude mice

	Chemical class	Fold induction	*P*-value
		HT-29-implanted mice/control	HT-29-implanted mice versus control
2-Aminoethanol	Amino alcohols	0.77	0.00026*
Acetoacetic acid	Keto-acids	0.54	0.00066*
Acetylsalicylic acid	Salicylates	1.84	0.02800*
Aspartic acid	Amino acids	1.39	0.03000*
β-Alanine	Amino acids	0.68	0.00930*
Citric acid + isocitric acid	Tricarboxylic acids	0.73	0.00260*
Citrulline	Amino acids	1.59	0.00140*
Fumaric acid	Dicarboxylic acids	0.47	0.00015*
Glucose	Carbohydrates	0.88	0.04200*
Glyceric acid	Hydroxy acids	0.78	0.00410*
Glycerol	Alcohols and polyols	0.75	0.00001*
Hippurate	Amino alcohols	1.68	0.02000*
Histidine	Amino acids	1.45	0.00710*
Homocysteine	Amino acids	0.64	0.03100*
Hydrocinnamate	Aromatic acids	1.94	0.01800*
Hypoxanthine	Purines and purine derivatives	0.38	0.02500*
Isoleucine	Amino acids	1.48	0.00014*
Lactic acid	Hydroxy acids	0.84	0.00530*
Lysine	Amino acids	1.20	0.02100*
Malic acid	Dicarboxylic acids	0.44	0.00009*
Ornithine	Amino acids	2.00	0.00029*
Phenylalanine	Amino acids	1.52	0.00031*
Proline	Amino acids	1.62	0.00780*
Pyruvate + Oxalacetic acid	Keto-acids	0.58	0.00021*
Ribose	Carbohydrates	0.63	0.00007*
Ribulose	Carbohydrates	0.55	0.00023*
Serine	Amino acids	1.37	0.03100*
Succinic acid(or aldehyde)	Dicarboxylic acids	0.62	0.00310*
Tagatose (or psicose)	Carbohydrates	2.11	0.00090*
Threonine	Amino acids	1.76	0.00210*
Trans-4-hydroxy-L-proline	Amino acids	1.59	0.02400*
Urea	Amino ketones	0.88	0.04200*
Valine	Amino acids	1.40	0.00053*

Values are represented as the fold-induction of the peak intensity for HT-29 cell-implanted nude mice (n = 9) against that of the corresponding controls (n = 12). All data are represented in Table S4, and the metabolites that showed significantly altered levels are shown in Table 4. *P*-values were calculated using Student's *t*-test; *$P < 0.05$.

whose levels are altered by *APC* mutation regulate the tumor environment and proliferation.

Discussion

To obtain new insights into the mechanisms underlying the aberrant activation of the Wnt pathway that accompanies *APC* mutation, we carried out a GCMS-based semiquantitative metabolome analysis using *in vitro* and *in vivo* experiments. In intestinal tissue, *APC* mutation accompanied by activation of the Wnt pathway seems to cause alterations in the levels of various metabolites. In $APC^{min/+}$ mice, which display the same histological findings as human colorectal adenomas (Fig. 2C), differences in the metabolite profiles of polyp and non-polyp regions were observed (Table 2). The serum levels of various metabolites in $APC^{min/+}$ mice were significantly altered compared with those of the corresponding control mice (Tables 1, S1). In addition, in nude mice that had been implanted with HT-29 cells and SW480 cells, the serum levels of various metabolites were markedly altered compared with those of the control nude mice (Tables 4,5), and an *in vitro* comparison between SW480 cells expressing truncated APC and those expressing normal APC showed variations in the levels of metabolites between the two cell types (Table 3). These results indicate that the *APC* mutation in intestinal tissue and/or intestinal epithelial cells causes alterations in the metabolome by activation of the Wnt pathway.

As expected from the notion that the tumor microenvironment induces many changes in energetic metabolite pathways in order to gain sufficient energy for cell proliferation, the levels of intermediates involved in the latter stages of the intracellular TCA cycle, including succinic acid, fumaric acid, and malic acid, were significantly higher in SW480 cells expressing truncated APC than in SW480 transfectants expressing full-length APC (Tables 3,7). Hirayama *et al.*[17] showed that the levels of three TCA metabolites, succinic acid, fumaric acid, and malic acid, were significantly higher in colorectal tumor tissues and that the active use of fumarate respiration by cancer cells may provide a feasible and intriguing explanation for the accumulation of fumaric acid and succinic acid. Some parasites and bacteria are able to synthesize ATP in anaerobic conditions by producing fumaric acid, which accepts electrons from succinic acid.[24] This process is known as fumarate respiration, and the pattern of changes in the levels of TCA cycle intermediates observed in the SW480 cells with truncated APC was also indicative of fumarate respiration. On the contrary, no significant increases in the levels of these TCA cycle intermediates were observed in the polyp regions of $APC^{min/+}$ mice

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Table 5. Metabolites detected in the serum of SW480 cells with truncated APC and SW480 transfectants with full-length APC implanted in nude mice

	Chemical class	Fold induction (SW480 cell-implanted nude mice)			*P*-value		
		Mutation/ full-length	Full-length/ control	Mutation/ control	Mutation versus full	Full versus control	Mutation versus control
2-Hydroxypyridine	Hydroxy acids	0.35	1.77	0.62	0.0190*	0.04600*	0.0540
Alanine	Amino acids	1.02	0.72	0.74	0.8500	0.00800*	0.0440*
Arabitol	Amino acids	0.55	3.67	2.01	0.0440*	0.00046*	0.0100*
α-Sorbopyranose (or fructose)	Carbohydrates	0.49	1.08	0.52	0.0350*	0.56000	0.0210*
Asparagine	Amino acids	0.81	0.82	0.67	0.2200	0.13000	0.0260*
Coniferyl alcohol	Alcohols and polyols	1.25	0.67	0.84	0.0170*	0.00180*	0.0880
Glucose	Carbohydrates	1.23	0.87	1.08	0.0052*	0.07700	0.2400
Histidine	Amino acids	0.90	0.77	0.69	0.3600	0.00510*	0.0046*
Ketoisoleucine	Keto-acids	1.18	0.63	0.75	0.3300	0.00300*	0.0390*
Lactic acid	Hydroxy acids	1.01	0.70	0.70	0.9100	0.00290*	0.0110*
Lauric acid	Fatty acids	1.02	1.09	1.11	0.5900	0.02900*	0.0021*
Lysine	Amino acids	0.89	0.82	0.73	0.2700	0.03400	0.0032*
Malic acid	Dicarboxylic acids	0.75	0.89	0.66	0.2600	0.49000	0.0690
n-Caprylic acid	Fatty acids	0.42	1.82	0.77	0.0140*	0.06600	0.6700
Pyruvate + Oxalacetic acid	Keto-acids	0.89	0.76	0.68	0.4100	0.08000	0.0480*
Rhamnose	Carbohydrates	2.07	0.57	1.18	0.0340*	0.16000	0.6100
Ribitol	Alcohols and polyols	0.27	1.70	0.46	0.0180*	0.12000	0.0970
Ribose	Carbohydrates	0.66	1.34	0.88	0.0019*	0.03500*	0.4800
Succinic acid (or aldehyde)	Dicarboxylic acids	0.87	0.83	0.72	0.3700	0.21000	0.0480*
Tagatose	Carbohydrates	1.15	0.75	0.86	0.0380*	0.34000	0.6300
Taurine	Amino acids	0.65	1.86	1.21	0.0420*	0.02500*	0.5700
Uracil	Pyrimidines and derivatives	1.11	0.69	0.76	0.0640	0.00310*	0.0220*
Xylose	Carbohydrates	0.99	0.43	0.43	0.9700	0.02100*	0.0360*

Values are represented as the fold-induction of the peak intensity among nude mice implanted with SW480 cells with truncated APC ($n = 4$), SW480 transfectants full-length APC ($n = 5$), and the corresponding control nude mice ($n = 6$). All data are represented in Table S5, and the metabolites that showed significantly altered levels are shown in Table 5. *P*-values were calculated using Student's *t*-test; $*P < 0.05$.

(i.e., adenoma). It was shown that an anthelminthic, pyrvinium pamoate, which is an inhibitor of fumarate respiration, inhibited the growth of the human colon cancer cell line WiDr and the human pancreatic cancer cell line PANC-1.[25] These findings suggest that *APC* mutation causes changes in energetic metabolite pathways, and such regulation might lead to the discovery of therapeutic strategies for colorectal carcinoma.

Amino acid profiles also seem to be important for understanding *APC* mutation and the subsequent Wnt pathway activation. Hirayama *et al.*[17] reported that the levels of most amino acids, except glutamine, were significantly higher in colorectal cancer tissues than in normal colon tissues, indicating that a relationship exists between colorectal cancer and amino acids. In this study, the levels of most amino acids tended to be higher in the non-polyp tissues of the $APC^{min/+}$ mice than in the normal tissues of the control mice (Table 7). The amino acid levels in the polyp tissues of $APC^{min/+}$ mice also tended to be lower than those in non-polyp tissues (Tables 7,S2). These results indicate that *APC* mutation is the first step in colorectal cancer development and causes variations in amino acid profiles, and such changes might lead to the formation of polyps. One of the reasons why the changes in amino acid levels are induced by *APC* mutation is that autophagy might be responsible. Autophagy is a catabolic process involving the degradation of a cell's own components by lysosomal machinery, resulting in the production of peptides and amino acids by protein degradation. The autophagy pathway is downregulated in colorectal tumor tissues,[26] while a mutation in *p53* was found to impair autophagic flux and cause excessive LC3 accumulation upon starvation, culminating in apoptosis.[27] Therefore, the studies about the interrelationship between amino acid profiles and autophagy must be needed to understand the colorectal cancer in detail.

In metabolite profiling, significantly lower levels of lactic acid were observed in the sera of mice implanted with HT-29 cells, SW480 cells, and SW480 transfectants (Tables 4–6). These results differ from observations in previous studies using clinical samples obtained from colorectal cancer patients.[16] The serum levels of lactic acid in colorectal cancer and other cancer patients were higher than in healthy volunteers.[11,28] However, lower serum levels of lactic acid were observed in patients with metastatic colorectal cancer.[29] Gluconeogenesis of metastatic colorectal cancer patients may mask anaerobic dissimilation of glucose at the end stage of cancer,[30] and the condition of human colorectal tumor xenograft model mice may be similar to metastatic colorectal cancer patients. In addition, it could be confirmed that there were differences in metabolite profiling between the sera of nude mice implanted with SW480 cells expressing truncated APC and SW480 transfectants expressing full-length APC (Table 5), suggesting that the presence of tumor in the body would lead to alterations in serum metabolome. Although the number of metabolites with significant differences between the sera of SW480 cell- and SW480 transfectant-implanted nude mice were small, some interesting alterations in serum metabolome were evident. Serum levels of arabitol and ribitol in the SW480 cell-

Table 6. Identified major metabolites in sera of APC$^{min/+}$ mice and SW480 cell-implanted nude mice

Pathway and chemical class	Metabolite	Fold induction (APC$^{min/+}$ mice)	Fold induction (SW480 cell-implanted nude mice)		
		APC$^{min/+}$ mice/ wild mice	Truncated APC/ full-length APC	Full-length APC/control	Truncated APC/control
Glycolysis	Glucose	1.16	1.23*	0.87	1.08
	Fructose-6-phosphate	0.63	n.d.	n.d.	n.d.
	Pyruvate + oxalacetic acid	1.05	0.89	0.76	0.68
	Lactic acid	1.18	1.01	0.70**	0.70*
Tricarboxylic acid cycle	Citric acid + isocitric acid	1.12	0.97	0.96	0.93
	Succinic acid	1.08	0.87	0.83	0.72*
	Fumaric acid	0.76	0.83	0.92	0.76
	Malic acid	0.84	0.75	0.89	0.66
Amino acids (essential)	Histidine	2.04*	0.90	0.77**	0.69**
	Isoleucine	1.39	1.31	0.79	1.04
	Lysine	3.08***	0.89	0.82*	0.73**
	Methionine	1.26	1.11	0.87	0.96
	Phenylalanine	1.34*	1.12	0.87	0.97
	Threonine	1.31	1.16	0.82	0.95
	Tryptophan	0.37*	0.93	1.26	1.17
	Valine	1.34*	1.17	0.85	1.00
Amino acids (non-essential)	Alanine	2.01***	1.02	0.72**	0.74*
	Asparagine	1.98**	0.81	0.82	0.67*
	Aspartic acid	1.08	0.78	1.25	0.97
	Cysteine + cystine	1.79***	1.08	0.90	0.98
	Glutamic acid	0.72	0.70	1.31	0.92
	Glutamine	1.35*	1.00	0.82*	0.81
	Glycine	1.18	1.06	0.86	0.92
	Proline	2.68***	0.98	0.82	0.81
	Serine	1.25	0.99	0.88	0.88
	Tyrosine	1.46**	0.94	0.87	0.82
Monosaccharides	Allose	0.50	n.d.	n.d.	n.d.
	Arabinose	1.21	0.75	1.14	0.86
	Fructose	0.60***	1.03	1.18	1.21
	Fucose	0.85	n.d.	n.d.	n.d.
	Galactose	0.78**	n.d.	n.d.	n.d.
	Glucosamine	1.78**	n.d.	n.d.	n.d.
	Glucose	1.16	1.23*	0.87	1.08
	Glucuronate	0.65***	1.04	0.92	0.95
	Gulcono-1,4-lactone	1.25	n.d.	n.d.	n.d.
	Mannose	1.21*	0.97	0.90	0.87
	Rhamnose	n.d.	2.07*	0.57	1.18
	Ribose	0.28***	0.66**	1.34*	0.88
	Ribulose	n.d.	0.52	1.11	0.57
	Tagatose	n.d.	1.15*	0.75	0.86
	Xylulose	0.58**	n.d.	n.d.	n.d.
	Xylose	1.87	0.99	0.43*	0.43*
Alcohols	2-Aminoethanol	0.95	0.95	0.93	0.88
	Arabitol	0.27***	0.55*	3.67***	2.01*
	Inositol	1.13	0.90	1.03	0.93
	Lyxose(or xlylose)	1.52	n.d.	n.d.	n.d.
	Ribitol	0.45***	0.27*	1.69	0.46
	Xylitol	0.42***	0.88	1.10	0.96
Nucleobases	Adenine	2.01***	n.d.	n.d.	n.d.
	Thymine	3.20**	n.d.	n.d.	n.d.
	Uracil	1.74***	1.11	0.69**	0.76*

Alterations in serum levels of metabolites were shown according to major metabolomic pathways and chemical classes. Values are represented as the fold-induction between two groups shown in Table 6. APC^{min}, APC multiple intestinal neoplasia. *P*-values were calculated using Student's *t*-test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. n.d., not determined.

implanted nude mice were significantly lower than those of the SW480 transfectant-implanted nude mice, and these phenomena were similar to alterations in the intracellular levels of SW480 cells and SW480 transfectants (Tables 5,6). In addition, ribitol could affect the proliferation of SW480 cells with truncated APC (Fig. 3B). These results suggest that a decrease in the level of ribitol in tumor cells by APC mutation might cause the proliferation of tumor cells.

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Table 7. Identified major metabolites in intestinal tissues of APC$^{min/+}$ mice and SW480 cells

Pathway and chemical class	Metabolite	Fold induction (intestinal tissue of APC$^{min/+}$ mice)		Fold induction (SW480 cells)	
		Polyp/ control	Polyp/ non-polyp	Non-polyp/ control	Truncated APC/full-length APC
Glycolysis	Glucose	0.53*	0.61*	0.88	1.29
	Fructose-6-phosphate	3.67*	1.32	2.78*	n.d.
	Pyruvate + oxalacetic acid	1.41	1.15	1.23	1.84*
	Lactic acid	0.96	0.79	1.22	0.88
Tricarboxylic acid cycle	Citric acid + isocitric acid	8.18	4.60	1.78*	2.76***
	Succinic acid	0.53*	0.63	0.85	18.46***
	Fumaric acid	0.65*	0.62	1.05	4.61***
	Malic acid	0.69**	0.63*	1.10	5.33***
Amino acids (essential)	Histidine	0.93	0.69	1.35	n.d.
	Isoleucine	0.88	0.66*	1.33	0.87
	Lysine	0.77	0.64	1.20	0.70
	Methionine	0.78	0.75	1.04	n.d.
	Phenylalanine	0.87	0.73	1.18	1.00
	Threonine	1.01	0.74	1.36	0.97
	Tryptophan	0.92	0.75	1.23	1.49
	Valine	1.01	0.74	1.36	0.93
Amino acids (non-essential)	Alanine	0.91	0.77	1.18	1.48**
	Asparagine	1.01	0.79	1.27	3.74**
	Aspartic acid	1.00	0.76	1.32	1.87*
	Cysteine + cystine	0.44**	0.44***	0.99	n.d.
	Glutamic acid	0.96	0.77	1.25	2.10**
	Glutamine	1.13	0.86	1.32	n.d.
	Glycine	1.10	0.80	1.37	0.83
	Proline	0.95	0.77	1.24	0.48***
	Serine	0.99	0.77	1.28	1.49
	Tyrosine	1.05	0.79	1.34	1.21
Monosaccharides	Arabinose	0.92	0.70	1.31	n.d.
	Fructose	1.82**	1.27	1.43*	1.09
	Fucose	2.34***	1.52**	1.55*	n.d.
	Galactose	n.d.	0.78	0.59*	1.32
	Glucosamine	3.22	1.25	2.59	n.d.
	Glucose	0.53*	0.61*	0.88	1.29
	Glucuronate	0.53*	0.62*	0.86	n.d.
	Gulcono-1,4-lactone	n.d.	n.d.	n.d.	2.18***
	Mannose	0.84	0.75	1.13	n.d.
	Rhamnose	2.80***	1.37	2.05*	n.d.
	Ribose	0.42**	0.42**	1.00	0.99
	Ribulose	0.65	0.57	1.13	0.80
	Tagatose	2.73***	1.95**	1.40	1.69**
	Xylose	0.53	0.56	0.95	n.d.
Alcohols	2-Aminoethanol	0.83	0.79	1.05	0.85
	Inositol	0.77*	0.65*	1.18	0.46***
	Lyxose (or xlylose)	0.57	0.56	1.01	n.d.
	Ribitol	0.84	0.80	1.05	0.24***
	Xylitol	0.45**	0.52**	0.86	n.d.
Nucleobase	Adenine	1.95*	1.65	1.18	0.87
	Cytosine	1.12	0.93	1.19	n.d.
	Thymine	1.28	0.98	1.32	2.58***
	Uracil	1.27	1.02	1.24	1.01

Alterations in tissue and cellular levels of metabolites are shown according to major metabolomic pathways and chemical classes. Values are represented as the fold-induction between two groups shown in Table 7. APC^{min}, APC multiple intestinal neoplasia. *P*-values were calculated using the Student's *t*-test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. n.d., not determined.

Metabolism is the set of chemical reactions occurring in a cell to keep it living, growing, and dividing, indicating that the tumor microenvironment may cause many changes in metabolism to gain more energy. As described above, ribitol was suggested as one metabolite affecting tumor cell proliferation. Ribitol reduced the growth of SW480 cells expressing the *APC* mutation, but it did not affect the growth of SW480 transfectants expressing full-length APC (Fig. 3B). Bello *et al.*[31] revealed that 5-*o*-alkyl 1,4-imino-1,4-dideoxyribitols are active as inhibitors of cancer cell growth, and it was shown that the inhibitory activity depends on the length of the alkyl side chain, although the anticancer activity of ribitol itself has

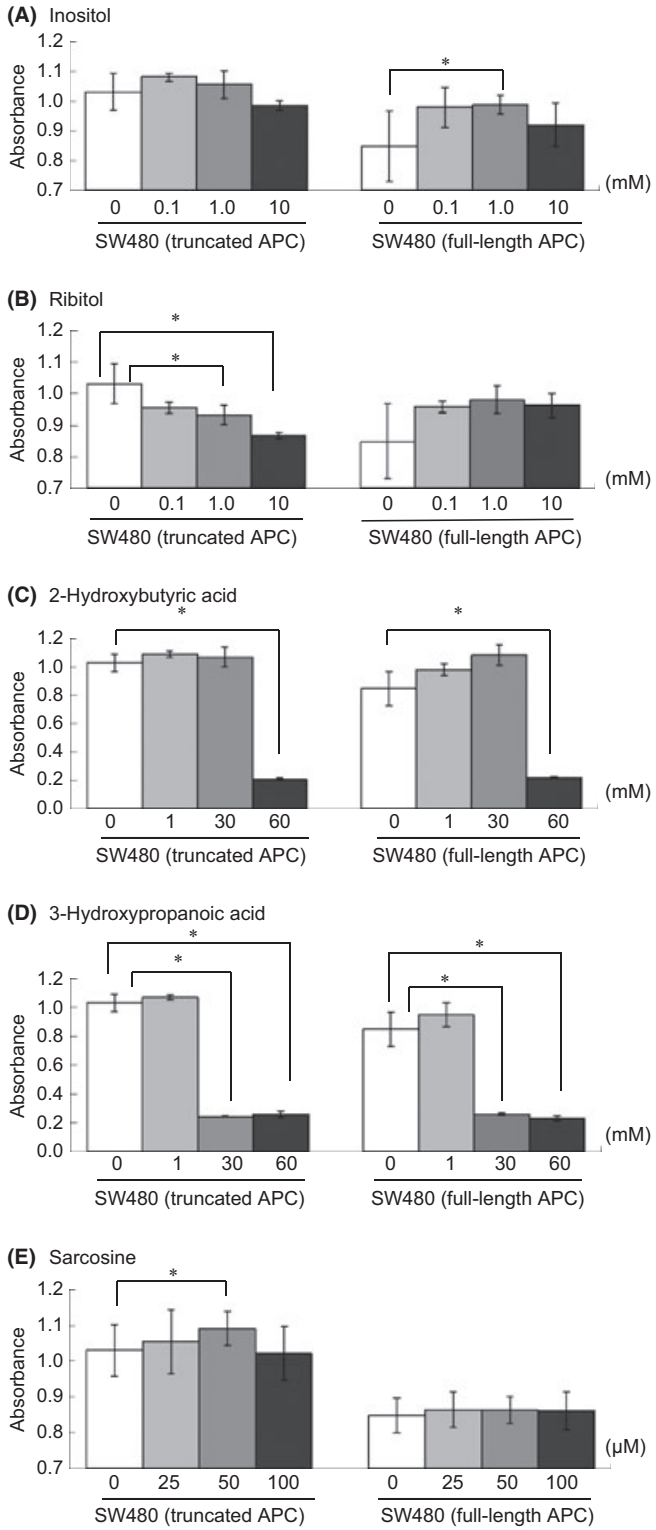

Fig. 3. Effects of target metabolites on the cell growth rates of SW480 colorectal cancer cells and SW480 transfectants. SW480 cells were grown at 5×10^4/well in 96-well plates for 24 h then exposed to the indicated concentrations of inositol (A), ribitol (B), 2-hydroxybutyric acid (C), 3-hydroxypropanoic acid (D), and sarcosine (E). After 24 h, the rate of cell growth was evaluated. Data are shown as the mean ± SD, n = 3–10. Statistical significance was analyzed using Student's t-test, and a level of probability of 0.05 was used as the criterion of significance. *$P < 0.05$. L-proline (0.6–1.8 mM), arabitol (0.1–10 mM), and isoleucine (0.6–1.8 mM) did not significantly affect the rate of cell growth (data not shown).

not been investigated. In contrast, 1 mM inositol significantly increased the proliferation rate of SW480 transfectants expressing full-length APC (Fig. 3A). It was previously reported that the inositol level in colon polyps were significantly decreased and that glycolysis may be critical in early tumorigenesis.[32] Therefore, the action of inositol on the tumor cells may lead to upregulation of glycolysis and cancer cell growth. Treatment of 2-hydroxybutyric acid and 3-hydroxypropanoic acid caused similar effects on SW480 cell proliferation at each concentration (Fig. 3C,D). These results may be due to the cell toxicity of 2-hydroxybutyric acid and 3-hydroxypropanoic acid, and this action would be independent of *APC* mutation in the cells, although their cell toxicity was not reported until now. In this study, we observed interesting behavior of sarcosine, an *N*-methyl derivative of the amino acid glycine. Sreekumar *et al.*[10] revealed that the level of sarcosine was highly increased during prostate cancer progression to metastasis and that it can be detected non-invasively in urine. In our study, we found that the serum levels of sarcosine were significantly higher in $APC^{min/+}$ mice than in corresponding control mice (Table 1), and they were significantly higher in polyp tissues than in non-polyp tissues of $APC^{min/+}$ mice (Table 2). The treatment of SW480 cells expressing truncated APC with 50 μM sarcosine resulted in a significant increase in their rate of growth ($P = 0.043$, Fig. 2E). In a previous study, LNCaP cells, a human prostate cancer cell line, exposed to 25, 50, and 100 μM showed upregulated HER2/neu mRNA expression with the strongest effect being seen at 50 μM,[17] and an increase in the intracellular sarcosine concentration was observed in cells exposed to 50 μM sarcosine.[10] Therefore, cancer cells might be regulated by sarcosine within a small concentration range. In prostate cancer cells, it was reported that the levels of sarcosine might be involved in the regulation of HER2/neu[33] and that they were correlated with the tumor suppressor activity of the transmembrane protein with epidermal growth factor and two follistatin motifs 2,[34] although the relationship between sarcosine and human colorectal cancer remains to be confirmed. Taken together, sarcosine and related molecules might have potential as biomarkers of early colorectal cancer involving *APC* mutation and could serve as targets of new therapeutic interventions.

In conclusion, we analyzed the metabolomic status of cells and tissues with *APC* gene mutation and found that the *APC* gene might regulate amino acid-related pathways and other energy-related metabolomic pathways. It was also suggested that some metabolites might influence cancer cell proliferation. This metabolomic study will aid the discovery of novel biomarkers for early cancer and the development of cancer-specific therapies, and the results from this study increase our understanding of colorectal cancer.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Metabolites detected in the sera of APC$^{min/+}$ mice and wild-type mice.

Table S2. Metabolites detected in the tissues of APC$^{min/+}$ mice and wild-type mice.

Table S3. Metabolites detected in SW480 cells with truncated APC and full-length APC.

Table S4. Metabolites detected in the serum of HT-29 cell-implanted nude mice.

Table S5. Metabolites detected in the serum of SW480 cells with truncated APC- or full-length APC-implanted nude mice.

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