Individual tumorigenesis pathways of sporadic colorectal adenocarcinomas are associated with the biological behavior of tumors

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Clinicopathologic features of sporadic colorectal adenocarcinomas were compared using integrated data from 244 patients subjected to curative resection. Individual steps in the tumorigenesis pathway, that is, adenomatosis polyposis coli (APC), Wnt-activated, base excision repair mutations, mismatch repair defects, RAF-mediated, transforming growth factor (TGF)-β-suppressed, bone morphogenic protein (BMP)-suppressed, and p53 alterations, were examined in terms of genetic and epigenetic changes, as well as protein expression. Genetic and molecular alterations of right colon cancers were distinct from those of left colon and rectal cancers. Rectal cancers showed the attenuated phenotype of left colon cancers. Tumors most frequently displayed either TGF-β- or BMP-suppressed alterations (81.2%), followed by RAF-mediated alterations (78.6%), and mismatch repair defects (38.4%), constituting a total of 24 integrated pathways. Tumors lacking *APC* mutations or carrying the *RAF* alteration (V600E) were frequently associated with lymphovascular invasion and lymph node metastasis ($P < 0.05$). Poorly differentiated or mucinous adenocarcinomas were generally associated with high level microsatellite instability, Axin2 suppression, TGF-β1 or BMPR1A suppression, loss of heterozygosity of *D18S46* or *D18S474*, and absence of base excision repair mutations ($P < 0.0001$–0.05). Early tumor recurrence was significantly correlated with lack of *APC* mutations ($P = 0.036$). Moreover, tumors that concurrently displayed APC/Wnt-activated, TGF-β/BMP-suppressed, and p53 alterations were significantly predisposed to early recurrence ($P = 0.026$). Our data clearly indicate that particular steps or pathways of colorectal tumorigenesis are closely associated with characteristic clinicopathologic features that, in turn, determine biological behavior, such as tumor growth, invasion, and recurrence. (*Cancer Sci* 2008; 99: 1348–1354)

The classical pathway of colorectal tumorigenesis constitutes stepwise or consecutive genetic and molecular changes, commencing with adenomatosis polyposis coli (*APC*) alterations through *RAS* family genes, and concluding with *p53*.[1] Although this model provides a simplified explanation of pathogenesis, numerous crossover or alternative changes also occur in human colorectal cancer. These stepwise alterations are consistently observed in only 6.6% of all colorectal cancers.[2] Two types of molecular and biological characteristics have been identified in hereditary colorectal cancer.[3] Hereditary non-polyposis colorectal cancer is associated with microsatellite instability (MSI), right-sided location, diploid DNA, transforming growth factor-β receptor 2 (*TGFBR2*) and Bc1-2-associated X protein mutations, and indolent behavior. In contrast, familial adenomatous polyposis displays chromosomal instability, left-sided location, aneuploid DNA, and pathogenic mutations in *APC*, *KRAS*, and *p53*, along with aggressive behavior. Previous investigations of sporadic hereditary colorectal cancer have disclosed various concurrent molecular and genetic changes, that is, APC and Wnt-activated mutations, mismatch repair (MMR) defects, and base excision repair (BER), RAF-mediated, transforming growth factor (TGF)-β- and bone morphogenic protein (BMP)-suppressed, and p53 alterations.[3–5] These mutations are interconnected to generate diverse pathways of tumorigenesis and progression. For example, one study showed that both *TGFBR2* mutations and MSI are approximately two- to threefold more prevalent in tumors displaying normal *p53*, *APC*, and *RAS*, compared to those with alterations in these genes.[6]

In view of the several conflicting reports to date, single genetic or epigenetic events associated with clinicopathologic outcome should be interpreted with caution. An earlier study on patients receiving adjuvant chemotherapy reported a non-significant improvement in the 5-year overall survival in high level MSI (MSI-H) cancers.[7] However, patients diagnosed with MSI-H in combination with *TGFBR2* mutations displayed a significant survival advantage. The presence of larger invasive tumors in compound heterozygotes suggests that APC and TGF-β1 act synergistically to protect against intestinal cancer.[8] Therefore, an innovative approach is required to compile the genetic and molecular alterations of the separate steps, either with regard to functional cascades or phenotypic characteristics. In the present study, we examine known genetic and molecular changes, and determine the associated integrative tumorigenesis pathways in sporadic colorectal adenocarcinoma, accounting for approximately 80% of all occurrences. Discrete steps and pathways are additionally compared in terms of biological behavior. Our comprehensive and molecular pathogenesis findings could be further applied to individualize treatment for the disease.

Materials and Methods

Patients and tissue samples. In total, 224 sporadic colorectal cancer patients subjected to curative resection were consecutively and prospectively enrolled at the Asan Medical Center (Seoul, Korea) (Table 1). All neoplastic tissues, confirmed by histologic identification, and peripheral blood lymphocytes from patients were available for genetic and molecular analyses. Normal colonic mucosa was simultaneously obtained at least 5 cm from the tumor border. Samples were acquired with informed consent, and the study was conducted with the approval of the Institutional Review Board for Human Genetic and Genomic Research, in accordance with the Declaration of Helsinki. Patients were monitored postoperatively every 6 months with our protocols, including clinical examination,

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Table 1. Clinicopathologic features according to primary tumor location†

	Right colon (*n* = 73)	Left colon (*n* = 72)	Rectum (*n* = 79)	*P*
Male/Female	43/30	44/28	44/35	0.793
Age (years), mean ± SD	58 ± 11	60 ± 11	59 ± 11	0.366
AJCC stage‡, I/II/III/IV	3/40/19/11	4/36/21/11	13/26/34/6	0.005
Preop. s-CEA, ≤6 / >6 ng/mL	59/14	53/19	63/16	0.524
Tumor size§, ≤4 / >4 cm	13/60	23/49	18/61	0.130
Differentiation, WD + MD/PD + muc	56/17	64/8	71/8	0.042
Growth, expanding/infiltrative	68/5	62/10	71/8	0.377
Synchronous adenoma (+)	18	27	25	0.248
Lymphovascular invasion (+)	20	15	22	0.550
Recurrence (+)	13	16	12	0.532

†Right colon, cecum–splenic flexure of transverse colon; left colon, splenic flexure of transverse colon–sigmoid colon. ‡Cancer staging according to the American Joint Committee on Cancer.[9] §The largest diameter of tumor. (+), yes; MD/PD, moderately/poorly differentiated; muc, mucinous; Preop. s-CEA, preoperative level of serum carcinoembryonic antigen; WD, well differentiated.

common blood chemistry, serum carcinoembryonic antigen, chest radiography, and abdominal and pelvic computed tomography. Recurrences, including distant metastases, occurred in 41 of the 224 patients (18.3%) during a mean follow-up period of 30 months (12–40 months).

Detection of MSI and loss of heterozygosity (LOH). The MSI status of tumors was determined based on the Bethesda panel (*BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250*). Polymerase chain reaction (PCR) products were run on an ABI Prism 310 DNA Sequencer (Applied Biosystems, Foster City, CA), and analyzed using GeneScan 3.1 software, according to the manufacturer's instructions. Tumors with two or more unstable markers were classified as MSI-H, whereas those with no or one unstable marker were classified as microsatellite stable or low level MSI. Additional LOH or MSI were assayed for *D18S46* and *D18S474* on an automated denaturing high-performance liquid chromatography (WAVE; Transgenomic, Omaha, NE), using an established protocol.[10] *D18S46* and *D18S474* are the closest markers to Drosophila Mothers against decapentaplegic proteins (*SMAD*)*4* (0.1 Mb).

Mutation analysis. We examined eight genes involved in the key steps of colorectal tumorigenesis (*APC*, catenin β-1 [*CTNNB1*], *RAS*, *RAF*, *TGFB1*, and *p53* for somatic mutations, and Mut Y homolog [*MYH*] and 8-oxoguanine DNA glycosylase [*OGG1*] for germline mutations). 'Hot spots' or possible pathogenic mutation sites were identified for all genes, except *APC*, specifically, *CTNNB1* exon 3, *BER* (*MYH* codons 324 and 359, *OGG1* c.1–18 and c.1–23 as possible pathogenic changes in Korean patients), *RAS* exons 12 and 13, *RAF* codon 600, *TGFB1* exons 5–7, and *p53* exons 5–8.[11–15] Automated denaturing high-performance liquid chromatography was used to detect mutations in *APC* exons 1–14, *CTNNB1*, and *TGFB1*, using established protocols and specific primers, whereas single-strand conformation polymorphism PCR was used for *MYH*, *OGG1*, and *p53*. Restriction fragment length polymorphism PCR was applied to analyze mutations in *RAS* exons 12 and 13, as well as *RAF* codon 600. We used the protein truncation test to identify the translation-terminating mutation in *APC* exon 15, using four primer pairs designed in our laboratory (sequence details available on request).

CpG methylation analysis. The methylation status of human Mut-L homolog 1 (*hMLH1*), O^6-methylguanine-DNA methyltransferase (*MGMT*), and *APC* in tumors was determined by methylation-specific PCR with the EZ DNA methylation kit (Zymo Research, Orange, CA). To assay for *hMLH1* silencing, we analyzed the 3′ small region close to the transcription start site, which is invariably associated with the absence of hMLH1 expression in colorectal tumors, in addition to the 5′-promoter region.[15] PCR products obtained using specific primer pairs were electrophoresed on a 3% agarose gel. The following unmethylated and methylated bands were distinguished: 152 bp and 123 bp for the 5′ promoter site of *hMLH1*; 232 bp and 122 bp for the 3′ small site of *hMLH1*; 108 bp and 98 bp for the 5′ promoter site of *APC*; and 93 bp and 81 bp for the 5′-promoter site of *MGMT*.[13,16]

Immunohistochemistry. Paraffin-embedded tissue cores of carcinomas were applied to construct tissue microarrays, and arrayed onto recipient paraffin blocks using a precision instrument (Beecher Instruments, Sun Prairie, WI). Tissue array blocks containing core cylinders were subjected to immune staining based on the labeled streptavidin–biotin method, using a Dako LSAB kit (Dako, Carpinteria, CA) and monoclonal antibodies to 10 protein markers (sources available on request). The protein markers included Wnt-associated (β-catenin, Axin2, and glycogen synthase kinase 3β), mismatch repair proteins (hMLH1 and human Mut-LS homolog 2 [hMSH2]), RAF-mediated (mitogen-activated protein kinase [MAPK] kinase [MEK]), TGF-β (TGFB1 and TGFB2), BMP (BMPR1A), and altered p53. All samples displaying negative immune staining were repeatedly assayed. Negative staining was mainly categorized into two groups, no to weak staining (≤10%) and no staining. The former group included glycogen synthase kinase 3β, hMLH1 and hMSH2 (nuclear), and p53, whereas the latter group contained Axin2, MEK, TGFB1, TGFB2, and BMPR1A. For β-catenin, no or cytoplasmic staining was interpreted as negative, and nuclear staining as positive. All hematoxylin–eosin and immunohistochemical staining results were confirmed by two separate pathologists.

Integration of individual colorectal tumorigenesis pathways. Colorectal tumorigenesis was separated into seven consecutive steps, specifically, APC, Wnt-activated, MMR defects, RAF-mediated, TGF-β-mediated, BMP-suppressed, and p53 alterations, as validated from previous studies.[12–15,17,18] Ultimately, *BER* mutations were eliminated, as their functional relevance remains to be established.

Statistical analysis. Genetic and molecular changes were compared with each other or various clinicopathologic parameters by cross-table analysis using Fisher's exact test or Pearson's χ^2-test, depending on statistical validity. Numerical variables of independent groups were examined using the unpaired Student's *t*-test or ANOVA with least-squares deviation verification. The significance level was adjusted at 5% for each analysis, and all calculations were carried out using SPSS software (version 13; SPSS, Chicago, IL).

Results

Genetic and molecular changes in relation to tumor location. Colorectal tumorigenesis pathways associated with distinct genetic and molecular changes were compared in tumors

Table 2. Genetic and molecular alterations significantly associated with colorectal tumorigenesis in different primary tumor locations†

Alterations	Location of cancer†, altered/all cases (%)			*P*
	Right, R	Left, L	Rectum, P	R *versus* P/R *versus* L/L *versus* P
APC	55/73 (75)	48/72 (67)	37/79 (47)	<0.0001/0.166/0.011
Mutations	53/73 (73)	44/72 (61)	27/79 (34)	<0.0001/0.098/0.001
5′-methylation	8/73 (11)	11/72 (15)	15/79 (19)	0.124/0.3/0.35
Wnt-activated‡	60/73 (82)	53/72 (79)	51/79 (65)	0.011/0.401/0.035
β-catenin, nuclear	21/72 (29)	22/72 (31)	23/78 (30)	0.555/0.5/0.514
GSK-3β suppressed	10/70 (14)	16/72 (22)	11/78 (14)	0.579/0.157/0.14
Axin2 suppressed	51/72 (71)	40/72 (56)	41/78 (53)	0.016/0.042/0.42
BER mutations§	3/73 (4)	10/72 (14)	9/79 (11)	0.085/0.039/0.414
MMR	46/73 (63)	20/72 (28)	20/79 (25)	<0.0001/<0.0001/0.437
MLH1/MSH2, suppressed	17/73 (23)	4/72 (6)	7/79 (9)	0.013/0.002/0.322
MSI-H	14/73 (19)	1/72 (1)	5/79 (6)	0.015/<0.0001/0.128
MLH1 5′- or 3′-methylation	36/73 (49)	17/72 (24)	12/79 (15)	<0.0001/0.001/0.135
RAF-mediated¶	69/73 (95)	52/72 (72)	55/79 (70)	<0.0001/<0.0001/0.432
RAF mutation	10/73 (14)	1/72 (1)	0	0.001/0.005/0.293
RAS mutations	27/73 (37)	14/72 (19)	14/79 (18)	0.006/0.015/0.474
MEK suppressed	61/73 (84)	45/72 (63)	51/78 (65)	0.009/0.004/0.422
TGF-β‡	17/72 (24)	13/72 (18)	20/78 (26)	0.461/0.269/0.178
TGF-β1 suppressed	13/72 (18)	9/72 (13)	15/78 (19)	0.511/0.244/0.184
TGF-β2 suppressed	5/71 (7)	4/71 (6)	4/78 (5)	0.624/0.731/0.891
BMP	62/73 (85)	58/72 (81)	57/78 (73)	0.056/0.317/0.187
BMPR1A suppressed	55/73 (75)	50/72 (69)	46/78 (59)	0.024/0.271/0.122
LOH, *D18S46/D18S474*	29/73 (40)	22/72 (31)	18/79 (23)	0.019/0.163/0.185
p53	37/73 (51)	49/72 (68)	58/79 (73)	0.003/0.025/0.293
Mutations, exons 5–8	17/73 (23)	23/72 (32)	24/79 (30)	0.212/0.163/0.487
Altered p53 overexpressed	30/73 (41)	48/72 (67)	54/79 (68)	0.001/0.002/0.481

†Right colon, cecum–splenic flexure of transverse colon; left colon, splenic flexure of transverse colon–sigmoid colon. ‡*CTNNB1* and *TGFB1* mutations were identified in one tumor, and omitted from the analysis. §*MYH* codons 324 and 359, *OGG1* c. 1–18 and *c.* 1–23 as possible pathogenic changes in Korean patients.[12] ¶Mutations of V600E in *RAF* and exons 12 or 13 in *RAS*. APC, adenomatosis polyposis coli; BER, base excision repair; BMP, bone morphogenetic protein; GSK, glycogen synthase kinase; LOH, loss of heterozygosity; MEK, MAPK kinase; MLH, Mut-L homolog; MMR, mismatch repair; MSH, Mut-S homolog; MSI-H, high-level microsatellite instability; TGF, transforming growth factor.

from different locations (Table 2). APC, Wnt-activated, and BMP-suppressed alterations were considerably more frequent in right colon cancers than rectal cancers. Moreover, APC and Wnt-activated alterations were more common in left colon cancers than rectal cancers. MMR defects and RAF-mediated alterations were predominant in right colon cancers, whereas p53-mediated alterations were more frequent in cancers of the left colon or rectum. The 5′-promoter methylation of *MGMT* was more regularly observed in left colon cancers, compared to rectal cancers (43% *versus* 19%, $P = 0.001$).

Genetic and molecular changes associated with MMR defects. MMR defects were compared with respect to individual alterations (Table 3). *APC* mutations and Axin2 or TGF-β1 suppression were frequently reported in tumors lacking *MLH1* or *MSH2* suppression. *RAS* mutations were generally identified in tumors with *MLH1* 5′- or 3′-methylation, whereas the *RAF* V600E mutation was threefold more prevalent in tumors with *MLH1* 5′-methylation. BMPR1A-suppressed alterations or LOH of *D18S46* and *D18S474* correlated significantly with MSI-H tumors. Altered p53 expression or mutations of *p53* exons 5 and 7 were more frequent in tumors with MMR defects.

Integrative tumorigenesis pathways. During colorectal tumorigenesis, tumors most frequently underwent either TGF-β- or BMP-suppressed alterations (81.2%), followed by RAF-mediated mutations (78.6%), and MMR defects (38.4%). TGF-β- or BMP-suppressed alteration was more frequent in tumors with either APC or Wnt-activated alterations than those without it (85.7% *versus* 59.5%, $P < 0.0023$). APC or Wnt-activated and p53 alterations occurred in 55.4% of the tumors, whereas MMR defects or RAF-mediated and p53 alterations occurred in 52.7% of tumors. Assembly of the consecutive or functionally linked tumorigenesis steps, specifically, APC-Wnt-activated (AW), MMR defects (M), RAF-mediated (R), TGF-β-BMP-suppressed (TB), and p53-associated (P) alterations, led to the identification of 24 different pathways (Fig. 1). In total, 31 of the 224 tumors (13.8%) underwent all five steps, but one tumor did not undergo any of the five steps.

Genetic and molecular alterations associated with clinicopathologic features (Table 4). Tumors lacking *APC* mutations were predisposed to more frequent lymphovascular invasion, lymph node metastasis, and early recurrence, compared with those carrying *APC* mutations ($P = 0.015$, 0.027, and 0.036, respectively). Lymphovascular invasion and lymph node metastasis were more frequent in tumors with the *RAF* mutation (V600E) ($P < 0.034$ and 0.039, respectively), whereas lymphovascular invasion was lower in tumors with LOH of *D18S46* or *D18S474* ($P = 0.02$). Poorly differentiated or mucinous adenocarcinomas were generally associated with MSI-H, Axin2, TGF-β1, or BMPR1A suppression, LOH of *D18S46* or *D18S474*, and absence of *BER* mutations ($P < 0.0001$–0.05). Large (largest diameter >4 cm) and advanced (T3 +4) tumors were markedly correlated with MSI-H and *MLH1* 5′- or 3′-methylation and MEK suppression, respectively ($P < 0.05$–0.001). Synchronous adenomas were less frequent in tumors with 5′-methylation of *APC* and BMPR1A-suppressed alterations ($P < 0.05$–0.01).

Molecular pathway association with clinicopathologic features. Early recurrence was predominantly observed in tumors

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Table 3. Mismatch repair (MMR) defects associated with alterations of genes or proteins involved in colorectal tumorigenesis

Alterations	% of tumors with respective MMR status								
	MLH1 or MSH2 expression			MSI			*MLH1* 5′- or 3′-methylation		
	–	+	*P*	+	–	*P*	+	–	*P*
APC									
Mutations	71	53	0.050	70	54	0.126	55	55	0.558
5′-methylation	25	14	0.106	20	15	0.529	17	15	0.39
Wnt-activated[†]									
β-catenin, nuclear	14	32	0.056	25	30	0.421	26	31	0.280
GSK-3β suppressed	11	18	0.397	5	18	0.159	14	19	0.337
Axin2 suppressed	82	56	0.006	70	58	0.223	66	57	0.123
BER mutations[‡]	4	11	0.335	0	10	0.122	6	11	0.238
RAF-mediated									
RAF mutation[§]	11	4	0.129	10	4	0.298	9	3	0.063
RAS mutations[§]	11	27	0.069	15	26	0.227	34	21	0.031
MEK suppressed	82	69	0.106	80	70	0.324	75	68	0.189
TGF-β[†]									
TGF-β1 suppressed	32	14	0.024	30	15	0.092	15	17	0.455
TGF-β2 suppressed	7	6	0.767	5	6	0.856	6	6	0.861
BMP									
BMPR1A suppressed	82	66	0.059	90	66	0.025	72	66	0.218
LOH, *D18S46/D18S474*	46	29	0.048	55	28	0.016	37	28	0.134
p53									
Mutations, exons 5–8	4	32	0.012	5	31	0.014	19	33	0.022
Altered p53 overexpressed	39	62	0.021	25	62	0.011	52	62	0.128

[†]*CTNNB1* and *TGFB1* mutations were identified in one tumor, respectively, and omitted from the analysis. [‡]*MYH* codons 324 and 359, *OGG1* c. 1–18 and *c*. 1–23 as possible pathogenic changes in Korean patients.[12] [§]Mutations of V600E in *RAF* and exons 12 or 13 in *RAS*. +, yes; –, no; APC, adenomatosis polyposis coli; BER, base excision repair; BMP, bone morphogenetic protein; GSK, glycogen synthase kinase; LOH, loss of heterozygosity; MEK, MAPK kinase; MLH, Mut-L homolog; MSH, Mut-S homolog; MSI, microsatellite instability; TGF, transforming growth factor.

Table 4. Genes or proteins involved in colorectal tumorigenesis associated with clinicopathologic features

Alterations	Clinicopathologic parameters, % of tumor with alterations (*P*-value symbol[†])						
	T, 1 + 2/3 + 4	N, 0/1 + 2	Size, S/L	Adenoma, –/+	WD + MD/PD + muc	LVI, –/+	Recurrence, –/+
APC							
Mutation	58/55	61/47 (1)	57/55	56/53	55/58	60/42 (1)	59/42 (1)
5′-methylation	12/16	12/19	9/17	18/9 (1)	14/21	14/18	15/17
Wnt-activated[‡]							
β-catenin, nuclear	35/29	32/26	30/30	30/30	29/34	32/29	28/40
GSK-3β suppressed	17/15	17/17	13/18	18/13	15/26	17/18	16/23
Axin2 suppressed	62/59	58/61	54/61	61/56	56/81 (2)	59/60	58/65
BER mutations[§]	4/11	9/12	15/8	10/10	12/0 (1)	10/9	11/5
MMR							
MLH1/MSH2 suppressed	12/13	14/11	6/15	14/10	11/21	14/9	14/15
MSI-H	12/9	9/8	2/11 (1)	10/7	5/30 (3)	9/9	9/5
MLH1 5′ or 3′-methylation	19/30	33/23	13/34 (2)	31/24	28/33	31/25	29/29
RAF-mediated[§]							
RAF mutation	0/6	2/8 (1)	4/5	5/4	5/6	3/11 (1)	4/10
RAS mutations	27/24	26/22	20/26	26/21	26/15	24/26	24/27
MEK suppressed	50/73 (1)	67/75	61/73	69/74	70/75	70/72	69/35
TGF-β[¶]							
TGF-β1 suppressed	19/16	15/19	15/17	18/15	15/29 (1)	16/18	16/21
TGF-β2 suppressed	12/5	6/5	9/5	6/6	6/7	4/11	5/11
BMP							
BMPR1A suppressed	69/68	66/71	65/69	73/56 (2)	65/84 (1)	66/72	67/73
LOH, *D18S46/D18S474*	27/31	35/25	22/34	34/23	28/46 (1)	35/19 (1)	31/32
p53							
Mutations, exons 5–8	39/27	29/28	33/27	27/31	29/24	26/37	27/37
Altered p53 overexpressed	65/58	61/57	63/58	58/61	61/49	60/56	59/61

[†]*P*-value symbol: 1, <0.01–0.05; 2, <0.001–0.01; 3, <0.0001–0.001; other columns not significant. [‡]*CTNNB1* and *TGFB1* mutations were identified in one tumor, and omitted from the analysis. [§]*MYH* codons 324 and 359, *OGG1* c. 1–18 and *c*. 1–23 as possible pathogenic changes in Korean patients.[11] [¶]Mutations of V600E in *RAF* and exons 12 or 13 in *RAS*. +, yes; –, no; Adenoma, synchronous adenoma; APC, adenomatosis polyposis coli; BER, base excision repair; BMP, bone morphogenetic protein; GSK, glycogen synthase kinase; L, >4 cm of the largest diameter of tumor; LVI, lymphovascular invasion of tumors; LOH, loss of heterozygosity; MD/PD, moderately differentiated/poorly differentiated; MEK, MAPK kinase; MLH, Mut-L homolog; MMR, mismatch repair; MSH, Mut-S homolog; MSI-H, high-level microsatellite instability; muc, mucinous; N and T, primary tumor and lymph node; cancer stage according to the American Joint Committee on Cancer;[9] S and L ≤4 cm and >4 cm of the largest diameter of tumor; TGF, transforming growth factor; WD, well differentiated.

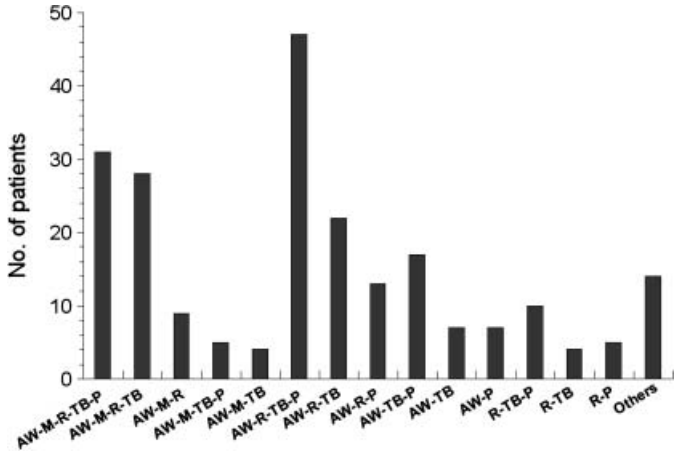

Fig. 1. Integrative tumorigenesis pathways obtained from the assembly of consecutive or functionally linked tumorigenesis steps in sporadic colorectal adenocarcinomas. AW, adenomatosis polyposis coli/Wnt-inactivated; M, mismatch repair defects; Others, other 10 pathways identified in equal or less than two patients; P, p53-associated alterations; R, RAF-mediated; TB, transforming growth factor-β/bone morphogenetic protein-suppressed.

concurrently undergoing three specific steps of colorectal tumorigenesis, that is, APC/Wnt-activated, TGF-β/BMP-suppressed and p53 alterations, regardless of MMR defects and RAF-mediated alterations ($P = 0.026$). However, both APC/Wnt-activated and TGF-β/BMP-suppressed alterations were significantly associated with poorly differentiated or mucinous tumors and lower levels of accompanying synchronous adenoma, irrespective of MMR defects, RAF-mediated alterations, and p53 alterations ($P = 0.003$ and 0.002, respectively).

Discussion

In our study, genetic and molecular alterations of right colon cancers were distinct from those of left colon and rectal cancers. Moreover, rectal cancer showed the attenuated phenotype of left colon cancer, with similarities in MMR defects and RAF-mediated and p53 alterations. Interestingly, APC and Wnt-activated alterations were more frequent in colon cancers than rectal cancers, a point not identified in the previous studies. The traditional adenoma–carcinoma sequence, which accounts for more than two-thirds of all colorectal cancers, is triggered by mutations in *APC* or *CTNNB1* that activate Wnt signaling.[19,20] Alternatively, MMR defects of left colon cancer (mainly involving promoter methylation of *hMLH1* and *MGMT*) differ from those of right colon and rectal cancers. Promoter methylation-associated silencing of *MGMT* triggers another mutator pathway that is more prevalent than MSI-H.[21] MMR defects and RAF-mediated alterations were predominantly identified in right colon cancers, and p53-mediated alterations in left colon or rectal cancers. In MSI-H tumors, the presence of *TGFBR2* correlated positively with the methylator phenotype, *RAF* alterations, and location in the right colon.[22,23] The predominant p53-mediated alterations in left colorectal cancers are consistent with earlier reports.[23–25]

APC mutations were associated with MMR defects, possibly related to frequent alterations of the key growth regulation genes in MMR-deficient cells, such as neurofibromatosis 1 (*NF1*), *APC*, *p53*, and *Ras*.[26] In contrast, a previous report showed that 77% of tumors expressing MMR display Wnt activation triggered by *APC* mutations.[27] Conversely, frequent *AXIN2* mutations were observed in colorectal cancers with defective MMR, confirming our data.[28] Based on these findings, we propose that MMR defects are closely associated with TGF-β and BMP suppression. In MSI-H colon cancers, activities of both TGF-β and activin, mediated by the same intracellular SMAD proteins, are abrogated due to frameshift mutations in their type II receptor.[29] *RAS* mutation were correlated with *hMLH1* 5′-methylation, in contrast to other studies that reported no association.[30] Consistent with earlier reports, altered p53 expression was inversely correlated with MSI-H in our experiments.[25,31] Together with the connection between these alterations and tumor locations, our data strongly indicate that the two steps, MMR defects and *p53* alterations, are mutually exclusive in colorectal tumorigenesis.

A few studies have explored the integration of canonical steps related to colorectal tumorigenesis. In one investigation, only 6.6% of tumors contained mutations in *APC*, *RAS*, and *p53*, with 38.7% of tumors displaying alterations in only one of these genes.[2] Another study examining mutations and promoter methylation in five genes of the Wnt signaling cascade (APC, β-catenin, Axin2, transcription factor 4 [TCF4], and WNT1 inducible signaling pathway protein 3 [WISP3]) and three genes indirectly affecting this pathway (cadhcrin1 type 1 [*CDH1*], phosphatase and tensin homolog [*PTEN*], tumor protein p53 [*TP53*]) revealed alterations in more than 90% of all samples.[15] In our analyses, tumors most frequently underwent TGF-β- or BMP-suppressed alterations, closely associated with APC or Wnt-activated alteration. During malignant transformation of colon epithelial cells, acquisition of resistance to this pathway occurs commonly, suggesting a significant pathogenic role in colon cancer formation.[32,33] Approximately half of the tumors underwent either APC/Wnt/p53-mediated or MMR/RAF/p53-mediated alterations. Thus, the TGF–BMP pathway with the comprehensive APC or MMR cascade might play pivotal roles in colorectal tumorigenesis. However, our data require further functional validation in terms of the respective pathways involved.

Interestingly, tumors with no APC alterations were predisposed to aggressive biological behavior, including lymph node metastasis, lymphovascular invasion, and early recurrence. Loss of normal APC is thought to contribute to all processes governing tumor tissues, specifically, proliferation, migration, apoptosis, and differentiation.[34] Accordingly, *APC* mutations could confer diminished proliferation and migration properties to tumor cells, thus decreasing biological aggressiveness. Conversely, the *RAF* V600E mutation, associated with lymph node metastasis and lymphovascular invasion, is one of the major causes for aberrant activation of the mitogen-activated protein kinase pathway in human cancers.[35] A large multicenter study reported that *RAF* V600E is correlated with extra-thyroidal invasion, lymph node metastasis, and advanced disease stages in papillary thyroid cancer.[36] Another recent study indicated a significant association of chromosome LOH of *D18S46* or *D18S474* and SMAD4 inactivation with lymph node metastasis of colorectal cancer, consistent with our finding.[37] We additionally considered a compound pathway associated with early recurrence in tumors with APC/Wnt-activated alterations in concurrence with TGF–BMP suppression and p53 mutations. In a previous investigation using *Apc* and *Tgfbr2* knockout mice, loss of Tgfbr2 in intestinal epithelial cells promoted the invasion and malignant transformation of tumor cells initiated by *Apc* mutation.[38] Another mouse study reported a significant increase in entire chromosome gains and losses among adenomas with both Apc and p53 alterations ($Apc^{+/1638N}Tp53^{-/-}$), and possible implications in tumor progression.[39]

In our samples, tumor growth was closely associated with MMR defects and MEK suppression. The MMR system is a component in signaling events that activates the cell cycle checkpoint or apoptosis.[40] Disruption of the RAF/MEK/ERK (mitogen-activated protein kinase) kinase pathway, either

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through *RAS* or *RAF* mutations, might also contribute to colorectal tumorigenesis through upregulation of antiapoptotic activity.[41] Synchronous adenoma, possibly explaining the adenoma–carcinoma continuum, was inversely correlated with suppression of BMPR1A involved in the BMP pathway directly regulating SMAD1, 5, and 8. Although the earliest loss of pSMAD1, 5, and 8 nuclear staining in the region of high-grade dysplasia/carcinoma *in situ* within adenoma has been shown,[42] the oncological significance of synchronous adenoma remains to be determined. In our experiments, poorly differentiated or mucinous adenocarcinomas were more frequent with Axin2 or TGF–BMP suppression and MSI-H, whereas well or moderately differentiated tumors were associated exclusively with *BER* mutations. Axin2 suppression enhances Wnt activation, affecting the dedifferentiation of epithelial cells.[43] Moreover, a close relationship between poorly differentiated tumors and MSI-H or TGF-β suppression has been reported.[6,24,25] The significant correlation between MSI-H and BMP-suppressed tumors additionally explains the similarities in differentiation. BER protects genomic DNA from oxidative stress, but its contribution to cellular differentiation remains to be determined. Our results show that several pathways involving specific gene alterations are significantly linked with differentiation and synchronous adenoma.

In conclusion, particular steps or pathways of colorectal tumorigenesis are closely associated with characteristic clinicopathologic features that, in turn, determine biological behavior, such as tumor growth, invasion, and recurrence. The integrative tumorigenesis pathways of individual patients might thus present an opportunity to select recurrence-prone candidates for strict follow-up and adjuvant treatment after curative operations.

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