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BASIC RESEARCH

Gene expression profiling: Canonical molecular changes and clinicopathological features in sporadic colorectal cancers

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Abstract

AIM: To investigate alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, possibly determining at-risk populations and predicting responses to treatment.

METHODS: Using microarray gene-expression analysis, we analyzed patterns of gene expression relative to canonical molecular changes and clinicopathological features in 84 sporadic colorectal cancer patients, standardized by tumor location. Subsets of differentially expressed genes were confirmed by real-time reverse-transcript polymerase chain reaction (RT-PCR).

RESULTS: The largest number of genes identified as being differentially expressed was by tumor location, and the next largest number by lymphovascular or neural invasion of tumor cells and by mismatch repair (MMR) defects. Amongst biological processes, the immune response was significantly implicated in entire molecular changes observed during colorectal tumorigenesis ($P < 0.001$). Amongst 47 differentially expressed genes, seven (*PISD*, *NIBP*, *BAI2*, *STOML1*, *MRPL21*, *MRPL16*, and *MKKS*) were newly found to correlate with tumorigenesis and tumor growth. Most location-associated molecular changes had distinct effects on gene expression, but the effects of the latter were sometimes contradictory.

CONCLUSION: We show that several differentially expressed genes were associated with canonical molecular changes in sporadic colorectal cancers, possibly constituting alternative or subordinate pathways of tumorigenesis. As tumor location was the dominant factor influencing differential gene expression, location-specific analysis may identify location-associated pathways and enhance the accuracy of class prediction.

Key words: Colorectal adenocarcinomas; Sporadic; Gene expression; Profiling; Tumorigenesis

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INTRODUCTION

Analysis of genetic alterations in hereditary colorectal cancers have identified several molecular changes, including those involving APC-Wnt signaling, mismatch repair (MMR) defects, RAF cascades, and p53 alterations[1-3]. The pattern of molecular changes observed in hereditary colon cancers suggested a stepwise model for colorectal tumorigenesis. About 80% of colorectal cancers, however, are sporadic, and the pattern of genetic alterations observed in hereditary tumors has been consistently observed in only a small number of sporadic tumors[1]. These findings suggest the existence of alternative or subordinate and crossover pathways of colorectal tumorigenesis.

The APC protein is thought to contribute to all processes governing tumor tissues, including proliferation, migration, apoptosis, and differentiation[4]. Loss of APC

function leads to intracellular β-catenin stabilization, the key component of canonical Wnt signaling, and constitutive signaling of β-catenin within the nucleus[5,6]. The current model of colon tumorigenesis suggests that MMR defects cause tumors primarily through two mechanisms, mutations in tumor suppressor gene pathways and inappropriate apoptosis[7]. Sporadic colorectal cancers with MMR defects, including almost all those with BRAF mutations, are thought to arise through the CpG island methylator phenotype (CIMP) associated with methylation of MLH1[3]. These alterations initiate cellular processes directed towards either proliferation or differentiation, depending on signal intensity and duration[8]. Alternatively, RAS mutations may be early events in the adenoma-carcinoma sequence, and *RAF* alterations may be related to the progression and development of de novo colorectal cancer[9].

The p53 pathway is ubiquitously lost in human cancers, either by *p53* mutations, observed in 60% of tumors, or by loss of cell signaling upstream and downstream of p53 in the 40% of cancers expressing wild-type p53[10]. Following disruption of $p21^{WAF1}$, p53 expression is enhanced because of p53 stabilization, which correlates with the increased expression of the tumor suppressor $p14^{ARF}$, an inhibitor of the ubiquitin ligase activity of MDM2[11]. Apart from these molecular changes, however, little is known about crossover pathways between APC-Wnt signaling and MMR or RAF alterations. *APC* and *RAS* mutations have been shown to be synergistic in promoting β-catenin nuclear translocation, thus enhancing canonical Wnt signal transduction[12]. Moreover, APC was shown to regulate cellular proliferation and transformation induced by the activation of both RAS and β-catenin signaling[13].

To identify alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, we assessed gene expression patterns, relative to canonical molecular changes and clinicopathological features in patients with colorectal tumors. Individual steps and pathways were sorted into various biological processes. We also performed location-specific analysis to determine whether this exercise might improve the accuracy of class prediction. Our results may also be used to determine at-risk populations and to predict responses to treatment.

MATERIALS AND METHODS

Patients and tissue samples

We prospectively enrolled 84 consecutive patients with sporadic colorectal cancer scheduled to undergo curative resection between 2006 and 2007 at the Asan Medical Center (Seoul, Korea) (Table 1). Tumors were standardized by location, and samples of tumor and normal colonic mucosa, taken at least 5 cm from the tumor borders, were obtained at the time of surgery. The tissue samples were snap-frozen in liquid nitrogen. Total RNA was extracted using RNeasy RNA extraction kits (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions, and DNA was extracted from lymphocytes and tumors using standard methods. Cancer staging was determined by imaging studies and operative findings with histological diagnosis according to the American Joint Committee on Cancer (6th ed., 2001). Our sample size was determined for competent cluster analysis using an efficient annealing algorithm with error rates of < 10%. All patients provided written informed consent, and the study protocol was approved by the Institutional Review Board for Human Genetic and Genomic Research, in accordance with the Declaration of Helsinki.

Clinicopathological features and molecular changes in colorectal tumorigenesis

Methods of representative molecular changes in tumor tissues, including *APC* mutations, Wnt-activated alterations, MMR defects, RAF-mediated changes, and p53 alterations have been described using different samples[14]. Briefly, *APC* mutations were assessed throughout all exons and introns, whereas Wnt-activated alterations were assessed by immune staining for β-catenin, Axin2, GSK3β, and E-cadherin. The search for MMR alterations included microsatellite instability (MSI) assays using the Bethesda panel, assays of methylation status at the 5'-promoter site and the 3'-small site of *hMLH1*, and immune staining for hMLH1 and hMSH2. We assessed RAF-mediated alterations by determining *BRAF* codon 600 mutations, mutations in *KRAS* exons 12 and 13, and immune staining for MEK. Alterations in *p53* were assessed by immune staining for altered p53. Crossover was defined when a tumor carried both APC/Wnt-activated changes and MMR defects or RAF-mediated alterations.

cDNA microarray and data analyses

The 21k cDNA microarray chips were prepared using Korean Unigene Information (KUGI) cDNA clones (http://kugi.kribb.re.kr/) and Incyte Human 10k cDNA clones. The PCR products of each clone were spotted on type-7 glass slides using an Array Spotter Generation Ⅲ (Amersham Pharmacia, Piscataway, NJ, USA). Aliquots of tumor and non-tumor RNAs (20 mg respectively) were used as templates for the synthesis of cDNA, labeled with Cy5 or Cy3, respectively, using SuperScript Ⅱ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 2 h at 42℃. The two labeled cDNAs were mixed, filtered through Microcon YM-30 filters (Millipore, Bedford, MA, USA) to exclude unincorporated dNTPs, and hybridized to the microarray slides at 50℃ overnight using a 3DNA Array 50 kit (Genisphere Inc., Hatfield, PA, USA). After hybridization, each microarray was washed twice with 2 × SSC with 0.2% (w/v) SDS at room temperature for 5 min, and finally with 95% (v/v) ethanol at room temperature for 1 min. The slides were scanned using a ScanArray 5000 Scanner (Axon Instruments, Union City, CA, USA), and scanned images were analyzed using the GenePix Pro 4.0 program (Axon Instruments). The raw data were normalized using the print-tip Lowess method available in the OLIN package

Table 1 Clinicopathological features relative to location of sporadic colorectal cancers

Clinicopathologic features	Tumor location[1] (No. of patients)			*P*
	R (*n* = 27)	L (*n* = 29)	P (*n* = 28)	
Male/Female	18/9	15/14	20/8	0.273
Age	62 ± 7	60 ± 12	62 ± 10	0.646
AJCC stage[2], Ⅰ/Ⅱ/Ⅲ/Ⅳ	4/13/6/4	4/15/6/4	4/10/9/5	0.926
Tumor differentiation, WD +	22/5	29/0	24/4	0.021 (R *vs* L)
MD/PD + muc				0.052 (L *vs* P)
Synchronous adenoma, -/+	18/9	23/6	14/14	0.052 (L *vs* P)
LVN invasion, -/+	15/12	22/7	20/8	0.236

[1]R: Cecum-splenic flexure of transverse colon; L: Splenic flexure of transverse colon-sigmoid colon; P: Rectum. [2]According to the American Joint Committee on Cancer (6th ed., 2001). WD, MD, PD, and muc, well-, moderately-, poorly-differentiated, and mucinous. LVN: Lymphovascular or neural invasion of tumor cells.

of the Bioconductor project (http://www.bioconductor.org)[15]. Missing values were imputed using the k-nearest neighbor method (available at the GEPAS web service: http://gepas.bioinfo.cipf.es/cgi-bin/preprocess/). The raw data have been deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo/) under the accession number GSE10982.

Quantitative reverse-transcript polymerase chain reaction (RT-PCR)

Total cellular RNA (5 μg) was reverse transcribed into cDNA using SuperScript Ⅱ (Invitrogen). Real-time (RT)-PCR was performed using the Exicycler Quantitative Thermal Block (Bioneer, Daejeon, Korea). The RT-PCR reaction product (100 ng) was amplified in a 15 μL reaction volume with 2 × SYBR Premix EX *Taq* (Takara, Shiga, Japan). Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Following an initial denaturation at 95℃ for 1 min, the amplification protocol consisted of 45 cycles of denaturation at 95℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s, followed by a final extension step of 72℃ for 10 min. The β-actin protein was used as an internal control. Relative quantification of each mRNA was analyzed by the comparative threshold cycle (TC) method.

Parametric analysis of gene set enrichment (PAGE)

We applied the PAGE method to identify significant changes in expression of gene sets[16]. Diverse categories of gene sets included molecular changes associated with colorectal tumorigenesis, namely cell cycle and apoptosis pathways, receptor protein tyrosine kinase signaling, Wnt/cadherin signaling, DNA MMR, and TGF-β signaling pathway. They were prepared from Affymetrix annotation files (http://www.affymetrix.com/netaffy) and annotation files were downloaded from the Source web service (http://genome-www5.stanford.edu/cgi-bin/source/sourceBatchSearch). Gene sets identified by gene ontology (GO) protocols included those involved in various biological processes, genes responsible for cellular components and molecular functions, genes defined by chromosomal locations, genes related by InterPro domains, and genes involved in distinct metabolic pathways. Pathway information was obtained from the BioCarta (http://www.biocarta.com) and KEGG (http://www.genome.ad.jp/kegg/) databases. Publications on differentially expressed genes were accessed to understand gene effects on biological functions and tumorigenesis, using PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez).

Statistical analysis

The associations of molecular changes and clinicopathologic features with tumor location were examined by cross-table analysis using Fisher's exact or Pearson's χ^2 tests with their significance level at 5%. The statistical significance of between-group comparisons was analyzed using Student's *t*-test and *Q*-values were calculated from corresponding *P*-values to control the false discovery rate (FDR) that may occur when testing multiple hypotheses[17]. Differential gene expression between tumors and normal epithelia were deemed to be significant at $P < 0.01$ for initial screening and $P < 0.001$ for individual gene candidates. Class prediction was examined using the BRB-Array Tools package (version 3.6) available at http://linus.nci.nih.gov/BRB-ArrayTools.html. All computations were performed using R statistical programming language (http://cran.r-project.org/) and the Bioconductor packages.

RESULTS

Differentially expressed genes relative to molecular changes and clinicopathological features

Assays for genes differentially expressed relative to molecular changes and clinicopathological features (Tables 2 and 3) showed that tumor location was associated with the highest numbers of differentially expressed genes. When we compared the right colon with the left colon and rectum taken together, we found that 1628 genes were differentially expressed, and when we compared the right colon with the left colon and rectum considered separately, we found that 1263 genes were differentially expressed. The next greatest extent of differential gene expression was seen when lymphovascular or neural invasion (LVI) of tumor cells occurred, and an analysis by defects in MMR yielded the next largest differentially expressed gene set. The differentially expressed genes significantly associated with canonical tumorigenesis and tumor progression are

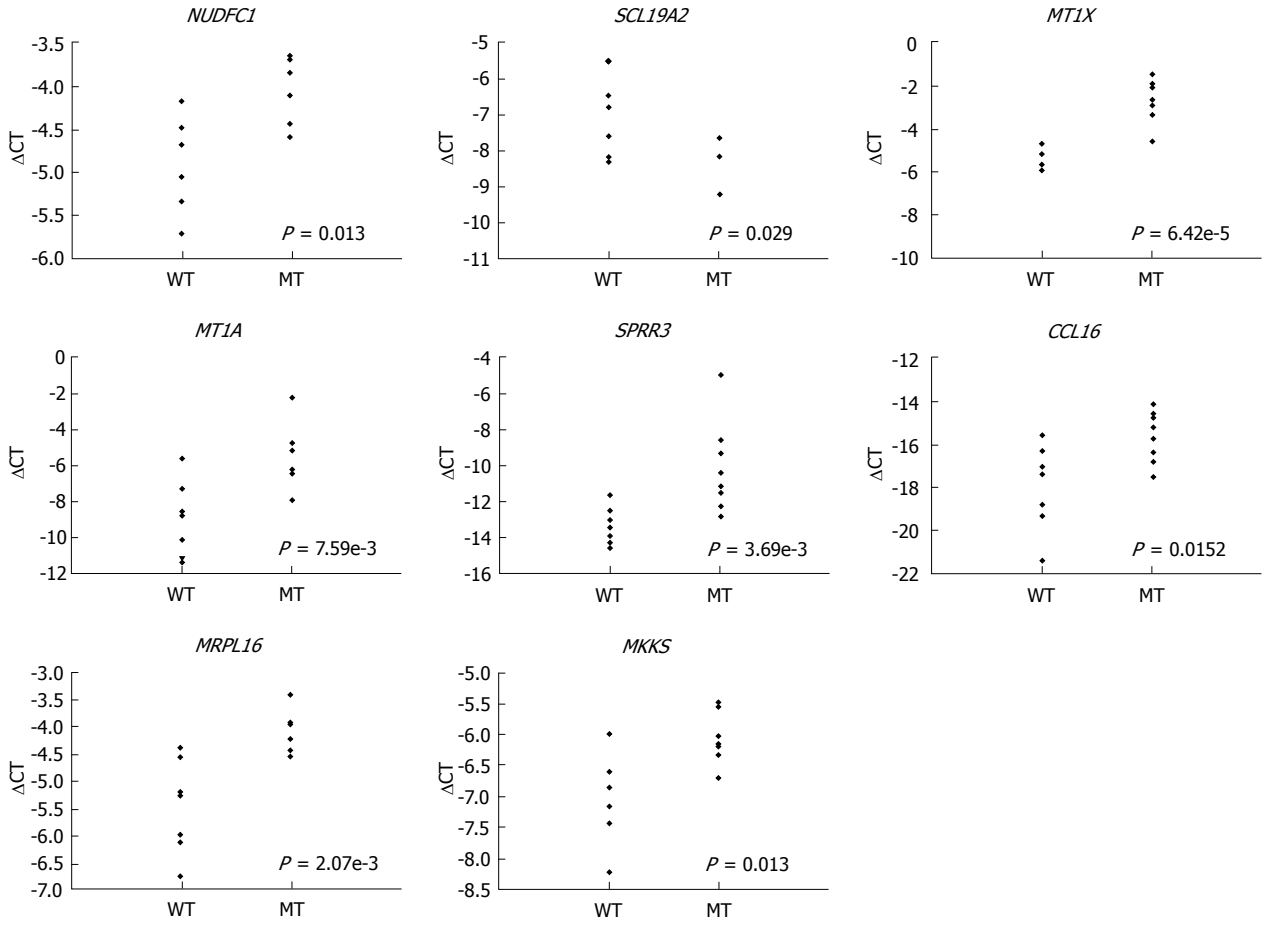

Figure 1 Quantitative RT-PCR of selected genes associated with molecular changes and clinicopathological features from microarray gene expression data. These were *NUDFC1* and *SCL19A2* (with *APC* mutations), *MT1X* and *MT1A* (with MMR defects), *SPRR3* (with crossover), *CCL16* (with lymphovascular or neural invasion), and *MRPL16* and *MKKS* (with synchronous adenoma). Genes differentially expressed between two groups were selected and their expression patterns measured using RT-PCR. WT: Without molecular or clinicopathological changes; MT: Molecular or clinicopathological changes. *P*-values from unpaired *t*-tests are shown.

collectively shown in Table 3. The differential expression of several candidate and novel genes was confirmed by real time RT-PCR (Figure 1).

Gene sets associated with APC and Wnt pathways

APC mutations are related to expression of constituents of the extracellular matrix (ECM) and to formation of the axonemal dynein complex, whereas Wnt-associated alterations are associated with the immune response, ECM formation, and filopodium expression. In addition, changes in pyruvate and arginine/proline metabolism have been associated with *APC* mutations; whereas alterations in G-protein receptor binding, the activities of various chemokines, phosphatase binding efficiency, and glycolysis/gluconeogenesis rates are associated with mutations in *Wnt*. We found that upregulation of three genes (*CDH7*, *DYRK1A*, *PISD*) and downregulation of one (*SLC19A2*) were associated with APC mutations, whereas upregulation of two genes (*PRAF2*, *CD99L2*) and downregulation of one (*FOXF1*) were associated with Wnt-activated changes ($P < 0.001$).

Gene set alterations associated with the MMR and RAF pathways

Biologically, MMR defects affect the immune response (including antigen processing), chromosome functions, and cytoskeleton structure, whereas RAF-mediated alterations are related to thyroid hormone generation and cytoplasmic effects. Cadmium and copper ion binding, MHC class Ⅱ receptor activity, and fatty acid metabolism have been associated with MMR defects, and protein dimerization activity with RAF-mediated alterations. We found that four upregulated genes (*MT1X*, *MT1A*, *SST*, *TDG*) and three downregulated genes (*HMGB1*, *SUGT1*, *VTI1B*) were associated with MMR defects, and that two were upregulated (*PPP1R13L*, *CAST*) and one was downregulated (*RAB22A*) in association with RAF-mediated alterations ($P < 0.001$).

Gene set alterations associated with p53 and crossover pathways

Alterations in p53 have been associated with the immune response (including antigen processing), ECM structure, and sensory perception, whereas crossover was related to cell cycle stage and protein localization. MHC class I receptor activity, oxidoreductase activity, and glycolysis were associated with p53 alterations, and protein kinase binding and renyltransferase activity were associated with crossover. No upregulated but three downregulated genes (*HLA-F*, *XRCC3*, *CCDC24*)

Table 2 Number of differentially expressed genes in terms of molecular changes and clinicopathological features

Parameters	No. of patients (missing)	No. of differentially expressed genes (P < 0.01), total (up/down)
Molecular changes[1], -/+		
APC mutations	55/27 (2)	83 (41/42)
Wnt-activated	45/38 (1)	82 (37/45)
MMR defects	70/14	238 (122/116)
RAF-mediated	58/26	108 (59/49)
Altered p53 expression	24/59 (1)	125 (57/68)
Crossover	64/19 (1)	92 (44/48)
Clinicopathologic features		
Tumor location[2], R/L + P	27/57	1628 (936/692)
R/L/P	27/29/28	1263
AJCC stage[3], Ⅰ + Ⅱ/Ⅲ + Ⅳ	50/34	195 (103/92)
Tumor differentiation, WD + MD/PD + muc	75/9	151 (69/82)
Synchronous adenoma, -/+	55/29	152 (92/60)
LVN invasion, -/+	57/27	279 (147/132)

[1]Wnt-activated: explored by β-catenin assay, AXIN2 measurement, and GSK-3β immune staining; MMR defect: analyzed by MSI assay, MLH1 5'-promoter measurement or 3'-methylation, and MLH1 or MSH2 immune staining; RAF-mediated alterations: assayed by detection of mutations in *BRAF* V600E and *KRAS* exons 12 and 13, and MEK immune staining; Crossover: When a tumor carried both APC/Wnt-mediated alterations and MMR defects or RAF-mediated alterations. [2]R: Caecum-splenic flexure of transverse colon; L: Splenic flexure of transverse colon-sigmoid colon; P: Rectum. [3]According to the American Joint Committee on Cancer (6th ed., 2001). WD, MD, PD, and muc, well-, moderately-, poorly-differentiated, and mucinous; MMR: Mismatch repair.

were associated with p53 alterations, whereas four upregulated genes (*NID2*, *EGLN3*, *NIBP*, *SPRR3*) and three downregulated genes (*ITIH1*, *CFH*, *ABI3BP*), were associated with crossover ($P < 0.001$).

Tumor location-specific analysis shows distinct patterns of gene expression

As colon tumor location had a very marked effect on differential gene expression, genes differentially expressed as a result of particular molecular changes and clinicopathological features may be concealed by tumor location. Interestingly, the number of genes differentially expressed as a result of tumor location increased slightly in association with several clinicopathological variables, although the sample size was much smaller (about one-third, data not shown) than those of other tumor sets. More importantly, most molecular changes had distinct location-associated effects on gene expression, but these were sometimes accompanied by contradictory effects. For example, p53 alterations inhibited the expression of antigen presentation-related genes only in right colon cancers, but had no effect in left colon or rectal cancers (Figure 2). After separation into classes showing various molecular and clinicopathological characteristics, the accuracy of binary outcome prediction was estimated using seven different machine learning algorithms available at BRBArrayTools. As expected, the best prediction accuracy (85%-94%) was achieved by tumor location (Table 4), followed by MMR defects (76%-83%). Most of the other molecular and clinicopathological variables had prediction accuracies < 75%. The prediction accuracies for each variable were

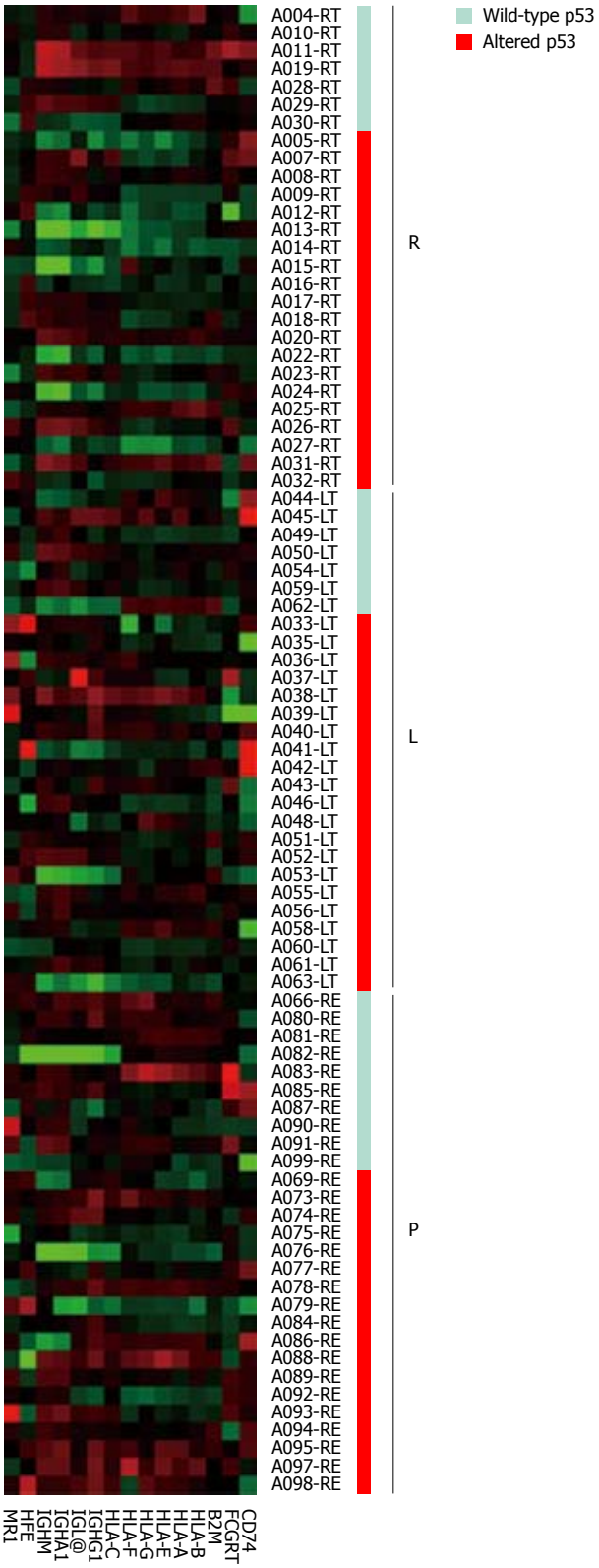

Figure 2 Pattern of expression of genes in the "antigen presentation, endogenous antigen" gene set as distinguished by tumor location and p53 status. Protein p53 alterations in the ascending colon coordinately decreased the expression of genes in the gene set whereas p53 alterations in the descending colon or rectum had no effect on gene expression. R: Cecum-splenic flexure of transverse colon; L: Splenic flexure of transverse colon-sigmoid colon; P: Rectum.

Table 3 Differential gene expression associated with molecular changes and clinicopathological features[1]

Parameters	Symbol	Name	Log_2 fold changes	Unadjusted *P*
APC mutations	*CDH7*	Cadherin 7, type 2	0.419885	0.00033
	DYRK1A	DS tyr-(Y)-phosphorylation regulated kinase 1A	0.326912	0.000343
	SLC19A2	Solute carrier family 19 member 2	-0.48574	0.000427
	PISD	Phosphatidylserine decarboxylase	0.272127	0.000545
	NDUFC1	NADH dehydrogenase 1, subcomplex unknown	0.539581	0.000572
Wnt-activated alterations	*PRAF2*	PRA1 domain family, member 2	0.620619	0.000205
	FOXF1	Forkhead box F1	-0.93359	0.000524
	CD99L2	CD99 molecule-like 2	0.753489	0.000772
MMR defects	*HMGB1*	High-mobility group box 1	-0.38141	3.74E-06
	MT1X	Metallothionein 1X	0.965429	0.000252
	MT1A	Metallothionein 1A	1.17894	0.000351
	SUGT1	SGT1, G2 allele of SKP1 (S. cerevisiae)	-0.34799	0.00039
	VTI1B	Vesicle transport with t-SNAREs homolog 1B (yeast)	-0.28513	0.000435
	SST	Somatostatin	1.24407	0.000564
	TDG	Thymine-DNA glycosylase	0.479829	0.000946
RAF-mediated alterations	*RAB22A*	RAB22A, member RAS oncogene family	-0.36017	0.000289
	PPP1R13L	Protein phosphatase 1, regulatory subunit 13 like	0.823379	0.000614
	CAST	Calpastatin	0.285773	0.000872
Altered p53 expression	*HLA-F*	Major histocompatibility complex, class I, F	-0.55645	0.000429
	XRCC3	XRCC in Chinese hamster cells 3	-0.26678	0.000588
	CCDC24	Coiled-coil domain containing 24	-0.28995	0.000996
Crossover[2]	*NID2*	Nidogen 2 (osteonidogen)	0.938223	0.000214
	EGLN3	egl nine homolog 3 (C. elegans)	0.740933	0.000375
	ITIH1	Inter-alpha (globulin) inhibitor H1	-0.34777	0.0004
	CFH	Complement factor H	-0.33826	0.000542
	ABI3BP	ABI gene family, member 3 binding protein	-0.82558	0.000637
	NIBP	NIK and IKK binding protein	0.649949	0.000688
	SPRR3	Small praline-rich protein 3	0.99738	0.000946
AJCC stage[3]	*PNPT1*	Polyribonucleotide nucleotidyltransferase 1	0.716381	4.94E-06
	BAI2	Brain-specific angiogenesis inhibitor 2	0.545369	1.57E-05
	ADCY1	Adenylate cyclase 1 (brain)	-0.43437	1.97E-05
	VEGFC	Vascular endothelial growth factor C	0.329341	9.11E-05
	ATAD3B	ATPase family, AAA domain containing 3B	-0.40978	0.000108
	CAP1	CAP, adenylate cyclase-associated protein 1 (yeast)	-0.66905	0.000405
	RPS6KA6	Ribosomal protein S6 kinase, 90kDa, polypeptide 6	0.301665	0.000586
	FGF5	Fibroblast growth factor 5	0.464162	0.000701
LVN invasion	*MMP12*	Matrix metallopeptidase 12 (macrophage elastase)	-0.69015	7.76E-05
	RAP1GDS1	RAP1, GTP-GDP dissociation stimulator 1	-0.60949	9.63E-05
	STOML1	Stomatin (EPB72)-like 1	-0.39407	0.000255
	CCL16	Chemokine (C-C motif) ligand 16	0.475838	0.000542
	NOTCH3	Notch homolog 3 (Drosophila)	0.592567	0.000679
	DHPS	Deoxyhypusine synthase	-0.33131	0.000965
Synchronous adenoma	*PARP2*	Poly (ADP-ribose) polymerase family, member 2	0.573461	6.00E-05
	MRPL21	Mitochondrial ribosomal protein L21	0.243204	0.000149
	MRPL16	Mitochondrial ribosomal protein L16	0.267004	0.000432
	MKKS	McKusick-Kaufman syndrome	0.439584	0.000447
	LHX2	LIM homeobox 2	0.663108	0.000782

[1]Differential gene expression in tumor tissues compared to normal epithelia was examined in terms of molecular changes and clinicopathological features, as was considered to be significant when $P < 0.001$; [2]When a tumor carried both APC/Wnt-mediated alterations and MMR defects or RAF-mediated alterations; [3]According to the American Joint Committee on Cancer (6th ed., 2001).

also examined after restricting the analysis by the three tumor locations mentioned above. As expected, location-specific analysis generally increased the prediction accuracy significantly (Figure 3), especially in the case of variables associated with synchronous adenoma, tumor stage, RAF-mediated changes, and Wnt-activated alterations. Accuracy, however, decreased when variables associated with *APC* mutations, p53 alterations, and crossover, were analyzed.

Clinicopathological features correlated with genomic alterations

Biologically, we found that tumor stage was related to antigen presentation, cell adhesion and migration, bone mineralization, and epithelial cell differentiation, and that lymphovascular or neural invasion was related to cell adhesion, immune response, and sensory perception. We also found that serine-type enzyme activities, high-density lipoprotein binding, pancreatic RNase activity, and glycolysis/gluconeogenesis were related to tumor stage, and that various structural molecules, hormones, serine-type enzyme activities, phosphate transport, the metabolism of the ECM and related molecules, and high density lipoprotein binding were related to lymphovascular or neural invasion. Synchronous adenoma was related to protein biosynthesis, ribosomal

Table 4 Class prediction accuracies (%) relative to molecular changes and clinicopathological features

Parameters	Genes[1]	CCP	DLDA	1-NN	3-NN	NC	SVM	BCCP
Tumor location	620	85	86	92	90	86	94	94
Synchronous adenoma	12	58	60	64	63	57	67	65
Tumor stage	22	62	58	58	60	61	54	60
APC mutations	12	72	72	57	72	67	73	67
LVN invasion	27	70	69	57	60	68	64	68
MMR defects	44	76	77	82	82	76	81	83
p53 alterations	8	65	70	61	73	66	72	79
RAF-mediated alterations	3	36	35	43	51	36	42	69
Wnt-activated alterations	9	54	54	61	61	58	49	54

[1]Number of classifier genes. CCP: Compound covariate predictor; DLDA: Diagonal linear discriminant analysis; 1-NN: One nearest-neighbor; 3-NN: Three nearest-neighbor; NC: Nearest centroid; SVM: Support vector machine; BCCP: Bayesian compound covariate predictor; LVN: Lymphovascular or neural.

proteins, and MHC class I receptor activity. We found that five genes (*PNPT1*, *BAI2*, *VEGFC*, *RPS6K6A*, *FGF5*) were upregulated and three (*ADCY1*, *ATAD3B*, *CAP1*) were downregulated in association with tumor stages; that two genes (*CCL16*, *NOTCH3*) and four genes (*MMP12*, *RAP1GDS1*, *STOML1*, *DHPS*) were up- or down-regulated, respectively, in association with lymphovascular or neural invasion, and that five genes (*PARP2*, *MRPL21*, *MRPL16*, *MKKS*, *LHX2*) were upregulated but no gene was downregulated in association with synchronous adenoma ($P < 0.001$).

DISCUSSION

Distinctive molecular changes, such as *APC* mutations and MMR defects, are respectively associated with two types of hereditary colorectal cancers, familial adenomatous polyposis and hereditary non-polyposis colorectal cancer. Although these hereditary tumors constitute fewer than 5%-8% of all colorectal cancers, the molecular changes identified in hereditary tumors are important in sporadic colorectal cancers[14,18,19]. The tumor suppressor APC is the major regulator of canonical Wnt signaling; these two proteins form a multi-protein complex encompassing kinases such as GSK-3β, CK1, and Axins, to prevent colorectal tumorigenesis[5]. Mutations in the oncogenes *RAF* and *RAS* are closely associated with MMR defects, and may act as alternative tumor-initiating steps that synergize with DNA methylation and occur within the context of serrated polyps[19,20]. The p53 protein, which normally induces G1 cell cycle arrest to facilitate DNA repair during replication, cannot induce cell cycle arrest when mutated in later stages of the adenoma-carcinoma sequence, thus leading to cell proliferation[21].

In our study, ECM interactions and the immune response were down- and up-regulated in tumors with *APC* mutations and Wnt-activated alterations, respectively. Gene set analysis earlier showed that the structural motif of osteopontin mediated critical cell-matrix and cell-cell signaling whose transcriptional regulation involves multiple pathways including Wnt/β-catenin/APC/GSK-3β/Tcf-4[22]. Expression of the E-cadherin β-catenin was observed in dendritic cells and loss of E-cadherin adhesion triggered a functionally distinct pathway of maturation linked more closely to the maintenance of tolerance than to the initiation of immunity[23]. In $Apc^{Min/+}$ mice, in which *APC* mutations are upregulated, dietary arginine increased colon tumorigenesis[24]. Amongst the eight genes we identified that were associated with *APC* mutations and Wnt activation, we found that one, *PISD*, was a novel gene upregulated in tumor cells with these alterations. Phosphatidylserine decarboxylation may provide a functionally important source of phosphatidylethanolamine in mitochondria[25].

We found that MMR defects correlated positively with an enhanced immune response and metal ion binding, whereas RAF alterations correlated with activation of cellular processes and thyroid hormone generation. Many tumor-infiltrating lymphocytes are present in MSI+ tumors, along with activated CD8+ cytotoxic T cells[26,27]. Furthermore, tumor-specific peptides generated by MSI may be involved in anti-tumor immune responses and may be useful in the diagnosis and treatment of patients with MSI+ colorectal cancers[27]. The deleterious effects of Cd2+ reported to date include generation of reactive oxygen species, inhibition of DNA repair, depletion of glutathione, and alteration of apoptosis[28]. In contrast, RAS/RAF/MEK/ERK-transduced signals can initiate cellular processes directed towards either proliferation or differentiation, depending on signal intensity and duration[29]. *RAF* mutations are associated with advanced clinical stages and early recurrence in patients with papillary thyroid cancer[30]. Amongst the genes up-regulated by MMR defects are the metallothionein genes, including *MT1X* and *MT1A*, which are expressed differentially in various tissues, during several developmental stages, and in response to metals, steroids, and stress[30]. Several of these genes, including *MT1X*, were overexpressed in MSI+ colorectal and gastric cancers[31].

We found that alterations in p53 downregulated immune responses and ascorbic acid binding. Anti-p53 IgG has been detected in the sera of subjects with various types of cancer, indicating induction of anti-p53 CD4+ Th cells[32]. Ascorbic acid can block the effects of TNF-α on endothelial cell proliferation and apoptosis

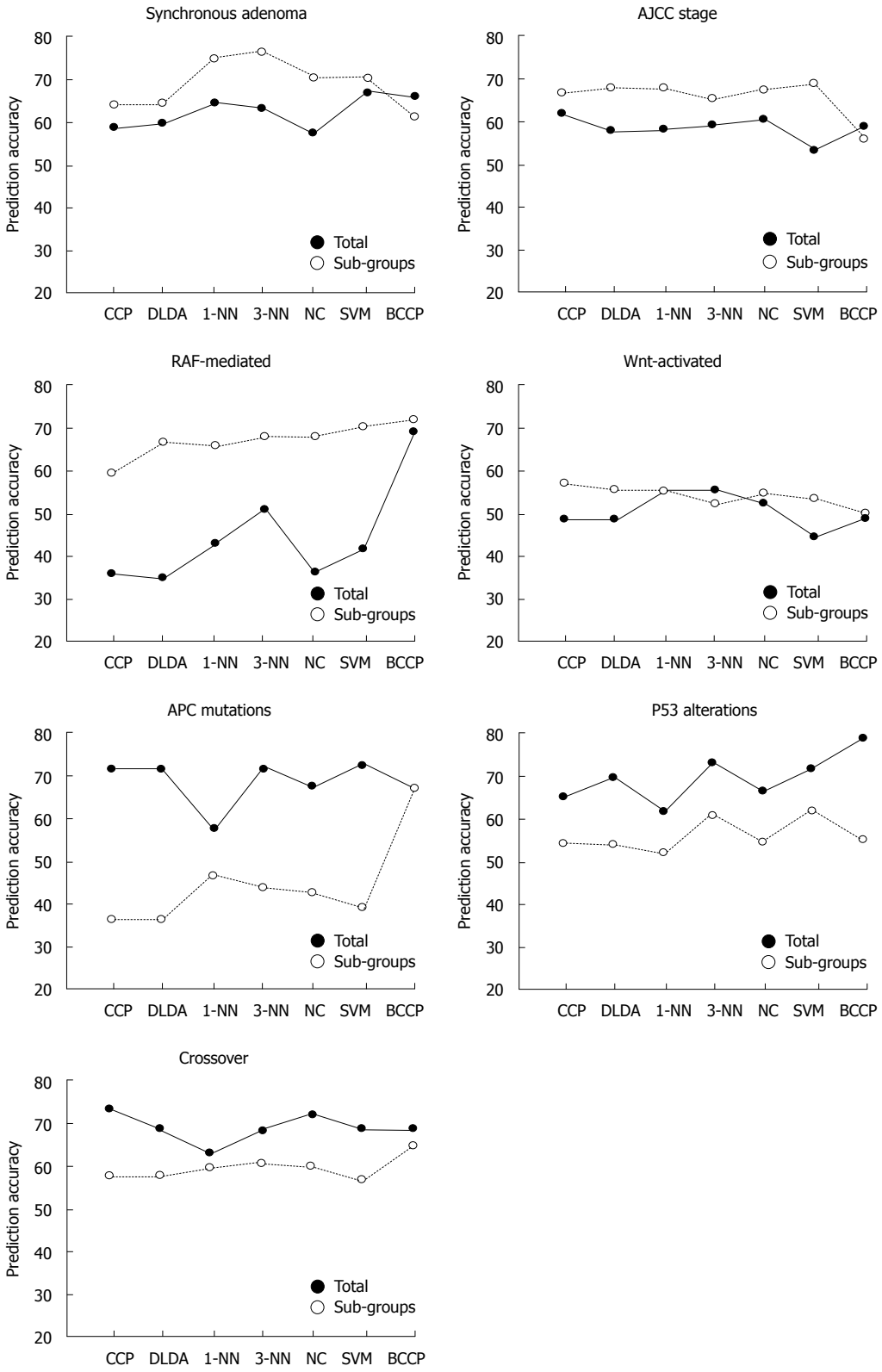

Figure 3 Accuracy of class prediction increases with tumor location-specific analysis. Samples were divided into three subgroups corresponding to three tumor locations (right colon, left colon, and rectum). Class prediction was performed using either all samples or samples within each subgroup. For tumor location-specific analysis, the results of class prediction (true or false) from each of the three locations were combined to calculate the overall prediction accuracy. Ten genetic or clinicopathological parameters were analyzed.

by inhibiting TNF-α-induced p53 expression and Rb hypophosphorylation, as well as by promoting collagen IV production[33]. We also observed that the crossover pathway between APC/Wnt-activated and MMR defects or RAF-mediated alterations, which has rarely been observed in human colorectal cancers, was associated with cell cycle and protein localization. Recently, mice carrying compound *Apc* and *Ras* mutations were characterized as having a striking increase in intestinal tumor multiplicity and progression, compared with *Apc*-only mutant animals[12]. Amongst the seven genes we identified as associated with the crossover pathway, one, *NIBP*, was a novel gene upregulated in tumor cells with these alterations. NIBP has been reported to enhance the cytokine-induced NF-κB signaling pathway by interacting with NIK and IKKβ[34], which may activate the TNF-induced invasive activity of tumor cells.

Embryologically, the right and left colon has different

origins, the midgut and hindgut, respectively, and is supplied by different circulation and innervation[35]. We found that tumor location was the dominant factor for differential gene expression in colorectal cancers. Thus, location-specific analysis may more precisely discriminate between alterations in gene expression caused by canonical molecular changes. The dependence of gene expression differences on tumor location has been reported previously[36-39]. The dominant expression pattern has been shown to be consistent with different embryonic origins and a second pattern reveals a gradual change from the caecum to the rectum[39]. We found that the prediction accuracy by tumor location-specific analysis was increased using analyses by synchronous adenoma, tumor stage, and RAF-mediated and Wnt-activated alterations, but decreased by analyses using *APC* mutations and p53 alterations. These findings suggest that *APC* mutations and p53 alterations may affect tumorigenesis as initiators and terminators, respectively, along the entire colon. In the absence of *APC* mutations and p53 alterations, however, synchronous adenoma, tumor stage, RAF-mediated changes, and Wnt-activated alterations may determine tumorigenesis at different locations. In addition, we found that several biological processes were affected differently by tumor location, often in opposite senses. One of the most significantly altered biological processes was the immune response. We observed that genes involved in the immune response were coordinately downregulated in left colon cancers with p53 alterations but not in right colon or rectal cancers. The same trend was observed with *APC* mutations, but the opposite trend was observed with MMR defects. Location-specific analysis also allowed the prediction of gene class by expression profiling in 6 of 10 parameters in our analysis, and in agreement with previous findings[38]. Gene expression profiling has been used to predict metastasis or recurrence in patients with stage Ⅱ colon cancer, thus enhancing the selection of chemosensitive patients for adjuvant chemotherapy[40,41]. Our finding that distinct molecular pathways of tumorigenesis occur in right and left colon cancers suggests that prediction of responsiveness to adjuvant therapy will benefit from location stratification.

In our study, both tumor stage and lymphovascular or neural invasion were associated with antigen presentation, ECM metabolism, and cellular and extracellular processes that determine tumor initiation and progression. Amongst the 14 differentially expressed genes associated with these biological functions, one encodes CCL16, a chemoattractant for monocytes and lymphocytes that can increase tumor rejection, antigen presentation by macrophages, T cell cytotoxicity, and the angiogenic activity of vascular endothelial cells[42]. *BAI2* and *STOML1* are novel genes, upregulated in advanced cancers and downregulated in lymphovascular and neural tumor invasion, respectively. Human BAI2, probably a G-protein-coupled receptor in the brain, participates in the early stages of neovascularization of the cerebral cortex after ischemia[43]. The stomatin homolog (UNC-24) of *C. elegans*, a protein similar to the human stomatin homolog STOML1 (SLP-1), is required for normal locomotor response to volatile anesthetics and contains a region of sequence homologous to the nonspecific lipid transfer protein[44].

As the traditional adenoma-carcinoma sequence, which is instigated in adenomas (or aberrant crypt foci) by the APC-Wnt signaling pathway, accounts for more than two-thirds of all colorectal cancers[3], we examined the molecular association of tumorigenesis in patients with synchronous adenoma. We found that three novel genes, *MRPL21*, *MRPL16*, and *MKKS*, were upregulated in tumors with synchronous adenoma. A mitochondrial ribosomal protein, MRPL21, arrests the cell cycle by increasing p21WAF1/CIP1 and p27Kip1 levels under growth inhibitory conditions[45]. The *MRPL16* gene originated *via* duplication of a pre-existing mitochondrial ribosomal protein gene as well as by recruitment of some DNA sequence from outside of the mitoribosomal genome[46]. McKusick-Kaufman syndrome (MKKS) is a human developmental anomaly syndrome featuring hydrometrocolpos, postaxial polydactyly, and congenital heart disease[47]. In protein biosynthesis, MKKS is similar in function to type Ⅱ chaperonins, which are responsible for folding a wide range of proteins[48].

In conclusion, we found that the differential expression of 47 genes was associated with canonical molecular changes and clinicopathological characteristics of sporadic colorectal cancers, possibly constituting alternative or subordinate pathways of tumorigenesis and tumor growth. Currently, the seven novel genes of our study that correlate with tumorigenesis and tumor growth, are functionally assessed to be possible candidates as diagnostic or therapeutic targets for colorectal cancers. Amongst these biological processes, the immune response was uniformly involved in all molecular changes, that is, APC/Wnt-activated alterations, changes arising from MMR defects, RAF-mediated changes, and p53-caused alterations. As tumor location was the dominant factor for differential gene expression in colorectal cancers, location-specific analysis may precisely discriminate particular gene expression profiles and enhance the accuracy of tumor class prediction.

COMMENTS

Background

Although various molecular changes have been identified in colorectal cancers, a clear pattern is detected in only 6.6% of these tumors, indicating the need to identify alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth.

Research frontiers

To identify alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, this study assessed gene expression patterns, relative to canonical molecular changes and clinicopathological features, in patients with colorectal tumors. Individual steps and pathways were sorted into various biological processes.

Innovations and breakthroughs

The largest number of genes identified as differentially expressed was by tumor location, and the next largest number by lymphovascular or neural invasion of tumor cells and by mismatch repair (MMR) defects. Amongst biological

processes, the immune response was significantly implicated in entire molecular changes observed during colorectal tumorigenesis ($P < 0.001$). Amongst 47 differentially expressed genes, seven (*PISD*, *NIBP*, *BAI2*, *STOML1*, *MRPL21*, *MRPL16*, and *MKKS*) were newly found to correlate with tumorigenesis and tumor growth. Most location-associated molecular changes had distinct effects on gene expression, but the effects of the latter were sometimes contradictory.

Applications

This study found that the differential expression of 47 genes was associated with canonical molecular changes and clinicopathological characteristics of sporadic colorectal cancers, possibly constituting alternative or subordinate pathways of tumorigenesis and tumor growth. The seven novel genes of this study correlate with tumorigenesis and tumor growth and can functionally be assessed as possible candidates for diagnostic or therapeutic targets of colorectal cancers.

Terminology

The cDNA microarray becomes a fundamental tool to gain direct molecular insight into tumorigenesis. Additionally, as phenotypic diversities of cancer occur from genetic alterations, genomic expression profiling might have been recognized as the first step to find useful therapeutic targets.

Peer review

This paper describes alternative or subordinate pathways involved in colorectal tumorigenesis and tumor growth, constituting an individual geno-pathogenesis map for colorectal cancer. As the study strengthened tumor location as a dominant factor for differential gene expression in colorectal cancers, location-specific analysis precisely discriminate particular gene expression profiles, possibly providing individual responses to respective regimen. It's an interesting paper.

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