Analysis of genomic aberrations associated with the clinicopathological parameters of rectal cancer by array-based comparative genomic hybridization

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Abstract. The aim of the present study was to screen and identify the chromosomal aberrations that are correlated with clinicopathological characteristics of rectal cancer using array-based comparative genomic hybridization (array-CGH). Forty-eight fresh frozen tumor tissues of rectal carcinoma were analyzed by array-CGH. The results showed that most frequent gains included 8q24.3, 20q11.21-q13.32, 20q13.33 and losses in 8p23.3-p12, 17p13.1-p12 and 18q11.2-q23 were noted. Fourteen amplifications and seven homozygous deletions were identified in the rectal cancer samples. Losses of 4p16.1-p15.31, 8p21.1-p12 and gains of 7p12.3-p12.1 and 13q33.1-q34 were associated with positive lymph node status and advanced clinical stage (stages III and IV). The 17q24.2-25.3 gain was more frequent in patients with distant metastasis. Integrated analysis indicated that overexpression of PDP1, TRIB1, C13orf27, FOXA2, PMEPA1 and PHACTR3 was associated with gains, and underexpression of FHOD, SMAD4 and BCL2 was associated with losses. Pathway enrichment analysis showed that pathways of nitrogen metabolism, oxidative phosphorylation, cell cycle, maturity onset diabetes of young, cytokine-cytokine receptor interaction, MAPK signaling pathway and dentatorubropallidoluysian atrophy were influenced by copy number changes.

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Introduction

Colorectal cancer (CRC) is a common malignant tumor worldwide, and over 1.2 million new cases and 608,700 deaths were estimated to have occurred in 2008 (1). The incidence of CRC in China has increased rapidly since the 1980s (2,3). Currently, CRC is the fifth leading cause of cancer-related deaths (4).

CRC can be divided into two types based on genetic abnormalities (5,6). The major type is the chromosomal instability phenotype, which consists of more than 85% of CRCs and is characterized by frequent chromosomal imbalances. The minor type is the microsatellite instability phenotype, which exhibits microsatellite instability owing to DNA replication errors and comprises <15% of CRCs. The genomic instability of the two types can lead to DNA copy number aberrations.

Cancers occur as a result of the accumulation of genetic alterations that are associated with carcinogenesis (5,7). Thus, the study of the cancer genome by high-resolution and high-throughput technology, for example array-based comparative genomic hybridization (array-CGH), not only has the ability to clarify the relationship between genomic abnormalities and clinicopathological factors, but may also optimize the treatment of patients by using their cancer genome information. Although several DNA copy number aberrations have been reported to have linkage with clinicopathological characteristics of patients with CRC (8-10), the available information is still limited particularly in Chinese patients.

The present study identified frequent DNA copy number changes in rectal cancer samples from Chinese patients and candidate target genes using integrated analysis of the genome and gene expression data of NCI-60 cell lines. We evaluated the genetic changes associated with lymph node metastasis, tumor stage and distant metastasis using the methods of frequency plot comparison together with statistical analysis.

Materials and methods

Study design. This study was conducted to identify genomic changes associated with clinicopathological factors and candidate targets of most frequent gains and losses of rectal cancer. We determined the genetic aberrations in 48 rectal carcinomas

using Agilent 60K Human Genome CGH microarray and screened those linked with clinicopathological characteristics. We then compared the gene expression profiling of CRC cell lines with or without gains of 8q, 13q, 17q, 20p, 20q or losses of 8p, 11q, 18q and identified genes whose expression was linked with DNA copy number changes.

Patients and samples. Fresh tissues from 48 rectal carcinoma patients were collected at the Department of Pathology, Cancer Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China. None of the patients received either irradiation or chemotherapy prior to surgery. All of the samples used in this study were residual specimens after diagnostic sampling. Every patient signed a separate informed consent form for sampling and research. Ethics approval was obtained from the Ethics Committee of Cancer Institute and Hospital, Chinese Academy of Medical Sciences. Representative tumor regions were excised by experienced pathologists. The clinicopathologic characteristics of the patients are summarized in Table I.

Genomic DNA extraction. Genomic DNA was isolated from tumor tissues using the Qiagen DNeasy Blood and Tissue kit as described by the manufacturer (Qiagen, Hilden, Germany). Tumor cell content of all the samples was >50% as determined by H&E staining.

Array-based CGH. Array-CGH experiments were performed using standard Agilent protocols (Agilent Technologies, Santa Clara, CA, USA). Commercial human genomic DNA (Promega, Warrington, UK) was used as reference. For each CGH hybridization, 400 ng of reference genomic DNA and the same amount of tumor DNA were digested with *Alu*I and *RSA*I restriction enzymes (Promega). The digested reference DNA fragments were labeled with cyanine 3-dUTP and the tumor DNA with cyanine 5-dUTP (Agilent Technologies). After clean-up, reference and tumor DNA probes were mixed and hybridized onto an Agilent 60K human genome CGH microarray (Agilent Technologies) for 24 h. Washing, scanning and data extraction procedures were performed following standard protocols.

Microarray data analysis. Microarray data were analyzed using Agilent Genomic Workbench (Agilent Technologies), CGH ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) and MD-SeeGH (www.flintbox.ca). Agilent Genomic Workbench was used to calculate the log2ratio for every probe and identified genomic aberrations. Mean Log2ratio of all probes in a chromosome region between 0.25 and 0.75 was classified as a genomic gain, >0.75 as high-level DNA amplification, <-0.25 as a hemizygous loss, and <-0.75 as a homozygous deletion. Pathway enrichment analyses were performed by CGH ArrayTools.

Integration analysis of DNA copy number and gene expression data of the NCI 60 cell lines. The DNA copy number and gene expression data of the NCI 60 cell lines were obtained from CellMiner (http://discover.nci.nih.gov/cellminer). We selected the data sets of aCGH Agilent 44K and Agilent mRNA for analysis. The genetic changes of seven CRC cell lines (including colo205, HCT_116, HCT_15, KM12, HCC_2998,

Table I. Clinicopathological characteristics of the rectal cancer patients in the array-CGH study.

Patient no.	Gender	Age (years)	TNM	pStage
1	M	67	T3N1M0	III B
2	M	58	T3N1M0	III B
3	M	43	T4N2M1	III C
4	F	63	T3N2M0	III C
5	F	69	T4N2M0	III C
6	F	52	T3N2M0	III C
7	M	56	T3N2M1	III C
8	F	47	T4N2M0	III C
9	F	61	T4N2M0	III C
10	M	41	T3N2M1	III C
11	M	58	T3N2M1	IV
12	F	62	T3N1M1	IV
13	F	36	T3N0M1	IV
14	M	59	T3N0M1	IV
15	M	49	T3N1M1	IV
16	M	53	T3N2M1	IV
17	M	75	T3N2M1	IV
18	F	79	T3N2M0	III C
19	F	22	T4N2M0	III C
20	F	70	T4N2M0	III C
21	M	77	T4N2M0	III C
22	M	36	T3N1M0	III B
23	M	49	T3N0M0	II A
24	M	45	T4N0M0	II B
25	M	61	T3N0M0	II A
26	M	51	T3N0M0	II A
27	F	66	T3N0M1	II A
28	M	59	T3N0M1	II A
29	M	45	T3N0M0	II A
30	M	72	T3N0M0	II A
31	M	63	T3N0M0	II A
32	M	58	T3N0M0	II A
33	M	68	T3N0M0	II A
34	M	63	T3N0M0	II A
35	M	63	T3N0M0	II A
36	M	55	T3N0M0	II A
37	M	42	T3N0M0	II A
38	F	56	T3N0M0	II A
39	F	52	T4N0M0	II B
40	M	45	T4N0M0	II B
41	M	47	T4N0M0	II B
42	M	62	T4N0M0	II B
43	M	64	T3N1M0	III B
44	F	53	T4N1M0	III B
45	F	59	T3N1M0	III B
46	F	49	T3N1M0	III B
47	F	39	T3N1M0	III B
48	M	63	T3N1M1	III B

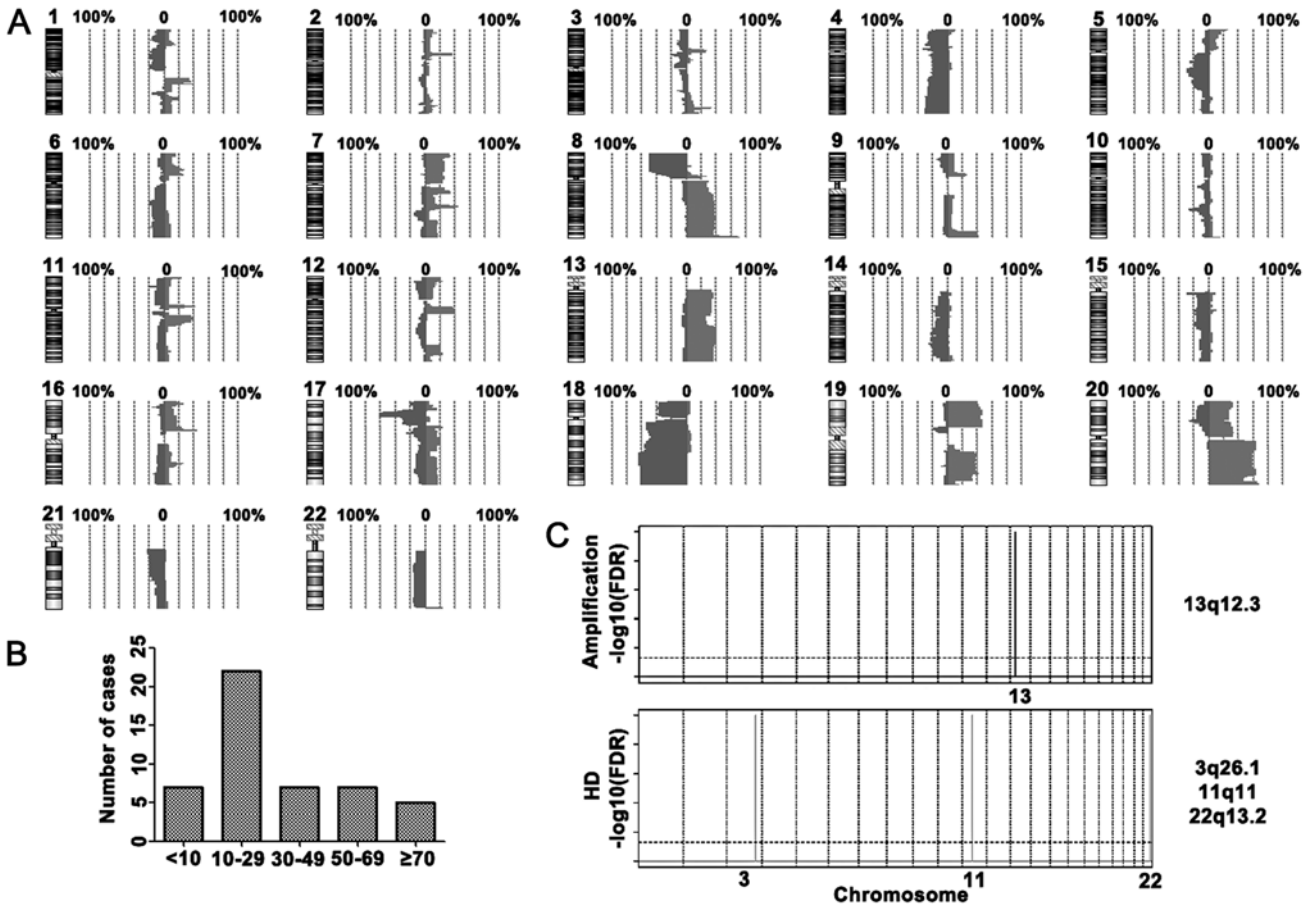

Figure 1. Genomic profiling of rectal cancer in the array-CGH assay. (A) Frequency plot of rectal cancer. Right of 0-axis, gains; left of 0-axis, losses. (B) Number of genomic aberrations; x-axis, number of aberrations; y-axis, number of cases. (C) Amplification and homozygous deletions identified by GISTIC analysis.

HT29 and SW_620) were analyzed and divided into the gain/loss group and no change group. The differentially expressed genes between the two groups were indentified with a cutoff of a 2-fold change using GeneSpring GX (Agilent Technologies).

Oncomine data analysis. The mRNA expression of genes which had a >5-fold change in expression between the gain/loss CRC cell lines and the no change cell lines was analyzed using Oncomine database (https://www.oncomine.org/resource/login.html). Details of standardized normalization techniques and statistical methods can be found on the the Oncomine website. The data of the interested genes in different types of cancer were collected and then their expression status in CRC was analyzed.

Statistical analysis. The Chi-square test was used to analyze the significance of correlation between genomic aberrations and clinical factors. Differences were considered significant at $P<0.05$.

Results

Recurrent copy number alterations in rectal carcinoma detected by array-CGH. Forty eight samples of rectal carcinoma were analyzed in this study and all of them had genomic changes. The most frequent genomic aberrations were gains of 8q24.3, 20q11.21-q13.32, 20q13.33, and losses of 8p23.3-p12, 17p13.1-p12 and 18q11.2-q23 (Fig. 1A). High-level amplifications were detected at 14 chromosome regions including 7p22.3-p21.3, 7q22.1, 8p11.21, 8p11.23, 8q22.1, 8q24.3, 13q12.2, 13q14.2-q14.3, 13q31.3, 16p11.2, 19p13.2, 20p11.23, 20q11.21-q11.23 and 20q13.33. Homozygous deletions were identified in 5q14.3, 8p23.3-p21.2, 15q11.2, 16p13.2, 17p13.1-p12, 18q21.2 and 20p12.1 (Table II). After analyzing the number of changes in rectal cancer, we found that nearly half of cases in the array-CGH study had 10 to 29 genetic alterations (Fig. 1B). GISTIC analysis showed that a copy number increase of MTUS2 (13q12.3) and decrease of C3orf57 (3q26.1), SPRYD5 (11q11), OR5W2 (11q11) and MKL1 (22q13.2) were significant in the rectal cancer cases (Fig. 1C).

Genomic changes associated with lymph node metastasis, tumor stage and distant metastasis. We compared the frequencies of genomic aberrations in the rectal cancer patients subdivided accoding to cases with or without lymph node metastasis, with tumor stages II or III-IV, and with or without distant metastasis using MD-SeeGH software. The results showed that gains of 7p, 13q and losses of 4p, 4q and 8p were more frequent in the rectal cancers with lymph node metastasis (Fig. 2A). The frequencies of 7p, 13q gain and 4q loss were higher in stage III-IV cases when compared with the frequencies in stage II cases (Fig. 2B). The largest differences were detected in copy number changes of 4q and 17q, with more frequent 4q loss and 17q gain in the cases with distant

Table II. High-level amplifications and homozygous deletions in the rectal cancer cases.

Changes	Cytoband	Start	End	No. of probes	No. of cases	Candidates
Amp	7q22.1	99852752	100767476	42	5	MUC17
	7p22.3-p21.3	524935	7428910	128	3	FSCN1
	8q24.3	145210837	145782038	24	11	FOXH1
	8q22.1	95061529	97342088	45	4	CDH17
	8p11.23	39378051	39461834	3	8	ADAM5P, ADAM3A
	8p11.21	42816942	42849186	3	4	THAP1, RNF170
	13q12.2	27095352	27439560	11	4	CDX2
	13q14.2-q14.3	46790942	51899157	117	4	ALG11
	13q31.3	91075476	91176960	4	4	GPC5
	16p11.2	29890929	31412127	78	4	MAPK3
	19p13.2	11141557	11548932	29	3	CNN1
	20q11.21-q11.23	29501535	35014201	155	10	
	20q13.33	60008660	62320720	85	6	PTK6, TNFRSF6B
	20p11.23	18692429	20864752	42	3	
HD	5q14.3	87722621	90788169	48	3	MEF2C
	8p23.3-p21.2	211611	27199611	467	3	DLC1, PCM1
	15q11.2	18835660	20010618	12	5	
	16p13.2	6492886	6860972	9	3	A2BP1
	17p13.1-p12	11089880	15073870	69	3	MAP2K4
	18q21.2	46764796	47107764	9	3	SMAD4
	18q21.2	48023000	48423627	8	3	DCC
	20p12.1	14772372	14939552	4	4	MACROD2

metastasis (Fig. 2C). We analyzed these candidate genomic regions with clinical factors by Chi-square test, and found that losses of 4p16.1-p15.31, 8p21.1-p12 and gains of 7p12.3-p12.1 and 13q33.1-q34 were associated with positive lymph node metastasis and advanced clinical stage (stages III and IV). Moreover, loss of 4q34.3-q35.1 was linked only with advanced stage (stages III and IV). We also found that the patients with distant metastasis had more frequent 17q24.2-25.3 gain (Table III).

Candidate target genes of gains and losses in rectal carcinoma. We performed an integrated analysis of the array-CGH dataset and the gene expression profiling dataset of CRC cells of NCI-60 to identify the candidate target genes of genomic gains and losses. The expression level of CA2, PDP1, ANGPT1, LOC346887, MAL2, NOV, TRIB1 and ZNF572 was higher in cell lines with an 8q gain than without. We also analyzed candidate target genes of gains in 13q, 17q, 20p and 20q and of loss in 18q (Table IV) and found that PDP1 (8q), TRIB1 (8q), C13orf27 (13q), FOXA2 (20p), PMEPA1 (20q) and PHACTR3 (20q) were overexpressed not only in CRC but also in other types of cancers (Table V). Three genes in 18q (FHOD, SMAD4 and BCL2) presented underexpression in several types of cancers including CRC (Table V).

Pathways enriched for copy number alterations. Pathway enrichment analysis using KEGG database was applied to the CGH data, and we found four pathways enriched in genes with gain and three pathways enriched in genes with loss. The genomic gains in rectal carcinoma changed the pathways of nitrogen metabolism, oxidative phosphorylation, cell cycle and maturity onset diabetes of young. However, cytokine-cytokine receptor interaction, MAPK signaling pathway, and dentatorubropallidoluysian atrophy (DRPLA) pathways were changed by the genomic losses (Table VI).

Discussion

The biological properties of cancers are different in patients presenting with different clinical parameters such as invasive depth, lymph node metastasis, distant metastasis, differentiation and clinical stage. Thus, the optimal treatment should be based on an individual cancer. Biomarkers can improve the accuracy of determining the clinical parameters that are predictors of prognosis and indicators of a response to treatment.

By applying array-CGH to rectal carcinoma samples of Chinese parients, we screened the genomic aberrations associated with clinical parameters using frequency comparison. The results showed that losses of 4p16.1-p15.31, 8p21.1-p12 and gains of 7p12.3-p12.1 and 13q33.1-q34 were associated with positive lymph node metastasis and advanced clinical stage (stages III and IV). Loss of 4q34.3-q35.1 was a marker of advanced stage (stages III and IV). We also found that the patients with distant metastasis had a more frequent gain in 17q24.2-25.3. Chromosome 4 was found to be the most frequent loss region in cancers including cervical, esophageal, lung, head and neck, gastric and CRC (11-16). The incidence of 4q loss was much higher in pulmonary metastatic tissues when compared

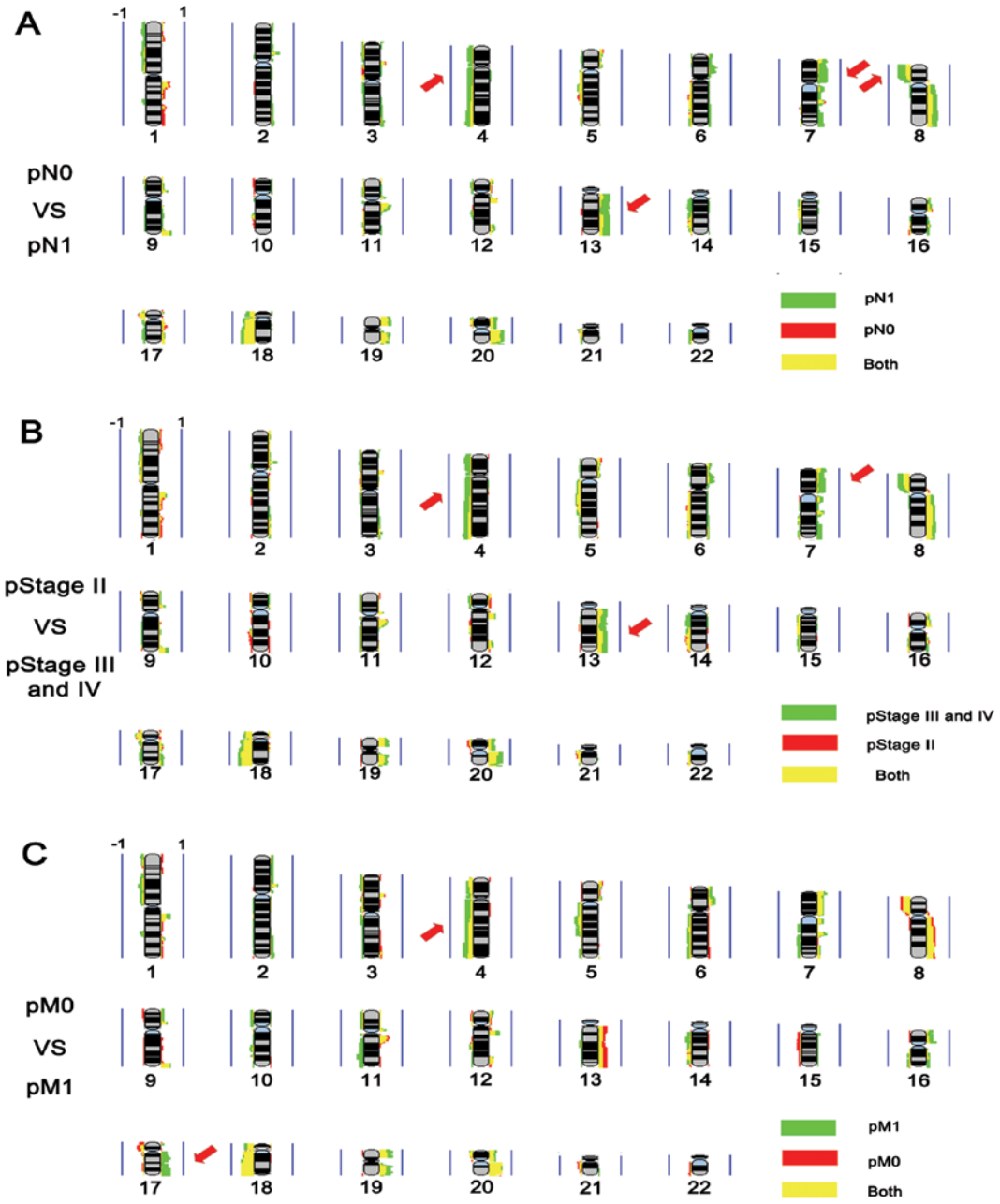

Figure 2. Frequency plot comparison. (A) Frequency plot comparison between pN1 and pN0. Red, pN0 group; green, pN1 group; yellow, shared by two groups. (B) Frequency plot comparison between pStage II and pStage III-IV. Red, pStage II group; green, pStage III-IV group; yellow, shared by two groups. (C) Frequency plot comparison between pM1 and pM0. Red, pM0 group; green, pM1 group; yellow, shared by two groups. The presentation is per array probe; gains are represented by the colors on the right, and losses are represented by the colors on the left. Vertical blue line represents 100% of the samples. Red arrows highlight the chromosomal areas with different frequency in two groups.

with primary cancer tissues and LOH of the D4S1534 locus (4q) in primary tissues was significantly linked with liver metastasis (17,18). Our results also suggested that loss of 4q in primary rectal tissues was a candidate predictor of lymph node metastasis. This indicates that target genes of the loss of 4q play important roles in lymphatic invasion and tumor progression, to which further investigation should be addressed.

The correlation of 17q with clinicopathological factors of CRC was not explicit. Diep *et al* (7) found that gain of 17q was correlated with the transition from a primary tumor to liver metastasis, while Knosel *et al* (16) reported that more deletion at 17q were observed in lung metastasis than primary tumors (19). Our results showed that the gain of 17q was more frequent in rectal cancer patients with distant metastasis when compared with patients without metastasis. Additional independent validation assays should be performed to reveal the correlation of 17q and metastasis.

Our results revealed that the alteration in expression of PDP1 (8q), TRIB1 (8q), C13orf27 (13q), FOXA2 (20p), PMEPA1 (20q), PHACTR3 (20q), FHOD (18q), SMAD4 (18q) and BCL2 (18q) occurred in CRC and other types of cancer, with a consistent copy number increase or decrease. To date, there is no report concerning the function of PDP1, C13orf27 and FHOD in cancer. Our results indicate the need to study these genes in rectal carcinogenesis. TRIB1 is a mammalian homolog of tribbles, an evolutionarily conserved *Drosophila*

Table III. Genomic aberrations linked with clinicopathological characteristics of the rectal cases.

		pN status			Distant metastasis			Stage		
Cytoband	Change	Positive	Negative	P-value	Positive	Negative	P-value	Stage II	Stages III and IV	P-value
4p16.1-p15.31	Loss	9	2	0.036	4	7	0.430	1	10	0.013
	No loss	17	20		9	28		19	18	
4q34.3-q35.1	Loss	11	4	0.072	6	9	0.175	3	12	0.040
	No loss	15	18		7	26		17	16	
7p12.3-p12.1	Gain	12	1	0.001	4	9	0.726	1	12	0.004
	No gain	14	21		9	26		19	16	
8p21.1-p12	Loss	16	5	0.007	5	16	0.653	5	16	0.027
	No loss	10	17		8	19		15	12	
13q33.1-q34	Gain	13	3	0.008	3	13	0.358	3	13	0.023
	No gain	13	19		10	22		17	15	
17q24.2-q25.3	Gain	4	4	0.796	6	2	0.001	2	6	0.295
	No gain	22	18		7	33		18	22	

Table IV. Candidate target genes of gains and losses in rectal cancer.

Change	Cytoband	Genes (>5-fold change)
Gain	8q	CA2, PDP1, ANGPT1, LOC346887, MAL2, NOV, TRIB1, ZNF572
	13q	OXGR1, PCDH9, EFNB2, C13orf27, LMO7, ARL11
	17q	TTYH2, RAB37, MXRA7, SLC26A11, MGAT5B
	20p	FOXA2, C20orf56, SLC24A3, CHGB, C20orf194, NRSN2
	20q	PMEPA1, PCK1, SULF2, LOC100240735, WFDC2, PHACTR3
Loss	8p	Not found
	11q	Not found
	18q	FHOD3, PSTPIP2, SMAD4, MBD2, BCL2, ST8SIA5

Table V. Candidate targets in the Oncomine database.

		Colorectal cancer		
Cytoband	Gene	Up	Down	Change in other cancers
8q gain	PDP1	8	0	Cervical cancer, gastic cancer, head and neck cancer, kidney cancer, leukemia, lymphoma, melanoma, pancreatic cancer
	TRIB1	1	0	Brain and CNS cancer, breast cancer, esophageal cancer, head and neck cancer, leukemia, lymphoma, melanoma, prostate cancer
	ZNF572	2	0	No
13q gain	OXGR1	4	1	No
	C13orf27	5	0	Cervical cancer
20p gain	FOXA2	6	1	Esophageal cancer
20q gain	PMEPA1	9	0	Bladder cancer, breast cancer, esophageal cancer, gastric cancer, head and neck cancer, lung cancer, pancreatic cancer
	PHACTR3	2	0	Brain and CNS cancer, pancreatic cancer
18q loss	FHOD3	0	1	Bladder cancer, brain and CNS cancer, breast cancer, kidney cancer, prostate cancer
	SMAD4	0	2	Lymphoma
	BCL2	0	14	Bladder cancer, brain and CNS cancer, breast cancer, head and neck cancer, leukemia, lymphoma, ovarian cancer, prostate cancer, sarcoma

Table VI. Pathways enriched in the array-CGH data.

Aberration	Pathway	Description	No. of genes	Gene symbol	P-value
Gain	hsa00910	Nitrogen metabolism	22	CA6, CTH, CA14, GLUL, GLS, CPS1, AMT, ASNS, CA8, CA13, CA1, CA3, CA2, CA9, GLUD1, GLS2, HAL, CA12, CA7, CA5A, CA4, CA11	0.003
Gain	hsa00190	Oxidative phosphorylation	109	SDHB, NDUFS5, ATP6V0B, UQCRH, ATP5F1, NDUFS2, SDHC, ATP6V1G3, ATP6V1C2, COX7A2L	0.017
Gain	hsa04110	Cell cycle	108	MAD2L2, SFN, HDAC1, CDC20, CDKN2C, ORC1, GADD45A, CDC7, CDC14A, TGFB2	0.025
Gain	hsa04950	Maturity onset diabetes of the young	24	PKLR, NR5A2, NEUROD1, SLC2A2, HES1, NKX6-1, GCK, BHLHA15, PAX4, MNX1, HNF4G, MAFA, NEUROG3, HHEX, PAX6, IAPP, HNF1A, PDX1, ONECUT1, HNF1B, FOXA3, NKX2-2, FOXA2, HNF4A	0.044
Loss	hsa04060	Cytokine-cytokine receptor interaction	240	TNFRSF18, TNFRSF4, TNFRSF14, TNFRSF25, TNFRSF9, TNFRSF8, TNFRSF1B, IL22RA1, IL28RA, CSF3R	0.001
Loss	hsa04010	MAPK signaling pathway	256	CASP9, PLA2G2E, PLA2G2A, PLA2G5, PLA2G2D, PLA2G2F, CDC42, STMN1, RPS6KA1, MAP3K6	0.011
Loss	hsa05050	Dentatorubropallidoluysian atrophy (DRPLA)	14	RERE, CASP8, MAGI1, CASP3, MAGI2, WWP1, CASP7, CASP1, GAPDH, ATN1, WWP2, BAIAP2, INSR, ITCH	0.038

protein family that regulates protein degradation. In myeloid leukemogenesis, TRIB1 was found to be overexpressed and was a key mediator between the RTK-MAPK pathway and the C/EBP transcription factor (20,21). FOXA2 was found to function as a suppressor of tumor metastasis by inhibition of epithelial-to-mesenchymal transition (EMT). Loss of FOXA2 expression due to epigenetic silencing was frequent in lung cancer (22,23). PMEPA1 is a TGF-β inducible gene and encodes an NEDD4 E3 ubiquitin ligase binding protein. PMEPA1 was found to be overexpressed in prostate, breast, renal cell, stomach and rectal carcinomas (24-26). PHACTR3 was identified as a PP1-binding protein and was selectively expressed in the brain. PHACTR3 was found to be overexpressed in 20% of non-small cell lung cancer (NSCLC), and was associated with reduced survival time of patients. In advanced neoplasia the methylation level of PHACTR3 was 70-fold higher than that in normal colon mucosa, and the sensitivity and specificity in stool assay were 55 and 95%, respectively (27). Loss of SMAD4 expression was reported as a predictor of liver metastasis in CRC, and patients with reduced SMAD4 expression presented with a poor prognosis (28-30). Transgenic expression of SMAD4 was found to significantly reduce the oncogenic potential of SW620 and MC38 cell lines (31). Overall, these genes may be the target genes of genomic gains and losses in rectal cancer. Further research should be addressed to elucidate the roles of the candidate genes in rectal carcinogenesis.

In summary, the genomic aberrations identified in the present study can be suggested as candidate biomarkers with which to predict the clinical outcome of patients with rectal carcinoma and may be expected to serve to individualize the treatment of rectal cancer. Our study identified several candidate target genes of the most common gains and losses in rectal cancer, and our findings provide information to explore the role of these genes in the development and progression of rectal cancer.

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