Tissue gene mutation profiles in patients with colorectal cancer and their clinical implications

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Abstract. Colorectal cancer (CRC) is one of the most common types of cancer in the world, and targeted therapy is frequently used in the clinical management of the disease. A complete and accurate picture of tissue gene mutations is therefore critical. Tissue specimens from 117 patients with CRC were used for high throughput DNA next-generation sequencing (NGS) analysis. Hotspots from 50 genes frequently associated with the development and progression of solid tumors were targeted for sequencing. Characterization of tissue gene mutations was performed; the tissue mutation positive rates of *KRAS*, *KIT*, *PIK3CA*, *MET* and *EGFR* were 52.1, 19.7, 29.9, 15.4 and 14.5%, respectively. The mutation positive rates of *TP53*, *APC*, *CDKN2A*, *STK11* and *FBXW7* were 65.8, 39.3, 32.5, 19.7 and 19.7%, respectively. The most frequent *KRAS* mutations were G12A/C/D/S/V, accounting for 61.2% of all *KRAS* mutations. The most frequent *TP53* mutations were R273C/G/H/L, accounting for 8.5% of all *TP53* mutations. The most frequent *APC* mutation was E1554fs, accounting for 19.7% of all *APC* mutations. *IDH1* R132C/H, *KIT* M541L, *MET* N375S, and *SMAD4* R361C/H were also frequently identified. *TP53* mutations were more common in patients ≥60 years old (P<0.05), and *IDH1* mutations were more common in male patients (P<0.05). NGS 50 gene panel sequencing provides a comprehensive tissue gene mutation profile which may significantly improve clinical management.

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Introduction

Colorectal cancer (CRC) is the fourth most common type of cancer in terms of incidence and the third leading cause of cancer-associated death in the world (1). In the last decade the incidence and the mortality rates of CRC have decreased in the US and some European countries; however, both the incidence and the mortality rates of CRC in China have increased (2,3). The yearly global burden of CRC is estimated to be 2.2 million new cases and 1.1 million deaths by 2030 (2).

The majority of cases of CRC cases are sporadic, and hereditary CRC accounts for ~5%. CRC is a heterogeneous disease, and several oncogenes and tumor suppressor genes, such as *TP53*, *KRAS*, *APC* and *CDKN2A*, are involved in its development (4-6). Gene mutations are the basis for selection of targeted agents for the treatment of various types of malignancy. There are targeted drugs, such as anti-EGFR agents and anti-VEGF/VEGFR agents, that are used in combination with surgery and chemotherapy (4,7,8). Therefore, accurate identification of gene mutations in patients with CRC is critical for selecting therapeutic agents, and predicting efficacy and prognosis.

High throughput DNA sequencing (NGS) technology has been widely used in research as well as clinically. NGS provides sequencing data in quantities orders of magnitude higher than Sanger sequencing, and with much greater sensitivity (9). NGS has been used for detection of gene mutations in CRC using DNA from tissue sections, blood and stool (10-15). Pooling of large quantities of data of gene mutations from tumor tissues and determining their associations with the clinicopathological characteristics may provide important information regarding the pathogenesis of CRC, and thus novel approaches for clinical intervention. To further study the mutational spectrum, mutation hotspots of genes and their clinical associations in patients with CRC in a southeastern Chinese population, NGS

panel sequencing of specimens from 117 patients diagnosed with CRC was performed, and the significance, genetics and associations with the clinicopathological data were assessed.

Materials and methods

Patients and specimen. All patients were hospitalized at Taizhou People's Hospital (Taizhou, China) between January 2011 and December 2013. Among the 117 patients enrolled, 78 patients were male, and 39 patients were female. The median age was 65 years old (range, 58-71). Diagnosis of CRC was performed independently by two pathologists based on histological examination. Sections of tumor tissues were sent to Beijing Genomics Institute (BGI) for NGS. The present study was approved by the Ethical Committee on Medical Research of the Taizhou People's Hospital. Written informed consent was obtained from all study subjects Tissue sections from 117 patients with CRC were analyzed using NGS. The clinicopathological data are summarized in Table SI. Tumor-Node-Metastasis staging was performed as reported previously (16). As in non-small cell lung cancer, patients with a low abundance of *EGFR* mutations may still benefit from EGFR inhibitors (17,18), thus two cutoff values for tissue gene mutation abundances were used, 5 and 0.5%. The objective was not to miss any mutations with a low prevalence, but still sufficient for beneficial results from targeted therapy.

NGS and data analysis. Tissue sections were used for genomic DNA extraction using a kit from Amoy Diagnostics, Co., Ltd. according to the manufacturer's protocol. Only tumor cell-rich regions identified by pathologists were used for DNA extraction. NGS library construction and NGS were performed by BGI. The targeted gene regions were amplified by multiplex PCR using genomic DNA from tissue sections as the template and reagents from the Ion AmpliSeq™ Cancer Hotspot Panel v2 kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The amplified target regions were used for NGS library construction using the Fast cfDNA Library Prep Set for MGI kit (CoWin Biosciences) according to the manufacturer's protocol. NGS was performed on a MGISEQ-2000RS platform using the proprietary sequencing kit (BGI). Speedseq (version 0.1.2: Quinlan Lab) was used for data mapping, and hg19 was used as the human reference genome. Strelka (version 2.9.2; Illumina, Inc.) was used for variant calling. For all sequencing data, Q30 sequences were >85%. The average read depth was 10,000x. The minimal read depth for variant calling was 2,000x.

Statistical analysis. Differences between rates were compared using a χ^2 test. Odds ratio (OR) analysis was performed using MedCalc (medcalc.org/calc/odds_ratio.php). $P<0.05$ was considered to indicate a statistically significant difference. Population data from Chinese Millionome Database (db.cngb.org/cmdb/) were used for comparison.

Results

Mutation rates of common genes. Tissue gene mutation positive rates are summarized in Table I. *KRAS*, *KIT*, *PIK3CA*, *MET* and *EGFR* were among the most frequently mutated driver genes, and *TP53*, *APC*, *CDKN2A*, *STK11* and *FBXW7* were the most frequently mutated tumor suppressor genes (Table I). For the majority of patients with *KRAS*, *TP53* or *APC* mutations, the tissue mutation frequencies were >5%, and for the majority of patients with *EGFR*, *BRAF* and *CDKN2A* mutations, the tissue mutation frequencies were <5% (Table I).

Table I. Mutation occurrence of genes in CRC.

A, Driver genes

	0.5% as cutoff		5% as cutoff	
Gene	Positive cases	Positive rate, %	Positive cases	Positive rate, %
KRAS	61	52.1	50	42.7
KIT	23	19.7	11	9.4
PIK3CA	35	29.9	19	16.2
MET	18	15.4	10	8.5
EGFR	17	14.5	1	0.9
BRAF	14	12	6	5.1
IDH1	14	12	1	0.9
PDGFR3	10	8.5	0	0
CTNNB1	8	6.8	5	4.2
IDH2	8	6.8	0	0
HER2	6	5.1	1	0.9
SMO	6	5.1	1	0.9

B, Tumor suppressor genes

	0.5% as cutoff		5% as cutoff	
Gene	Positive cases	Positive rate, %	Positive cases	Positive rate, %
TP53	77	65.8	56	47.9
APC	46	39.3	34	29.1
CDKN2A	38	32.5	1	0.9
STK11	23	19.7	11	9.4
FBXW7	23	19.7	8	6.8
PTEN	19	16.2	2	1.7
SMAD4	14	12	3	2.6
MLH1	12	10.3	7	6
VHL	10	8.5	2	1.7
ATM	9	7.7	2	1.7
RB1	7	6	0	0
HNF1A	7	6	0	0
SMARCB1	6	5.1	2	1.7

Mutation spectrum and hotspots. The mutation spectrum was analyzed, and the results are summarized in Tables II and III. *IDH1* R132C/H, *KIT* M541L, *KRAS* G12A/C/D/S/V, *MET* N375S and *SMAD4* R361C/H were some of the more prominent mutation hotspots (Tables II and III). *BRAF* V600 mutations accounted for 40% of all *BRAF* mutations (Table III).

Table II. Spectrum of mutations in tumor suppressor genes.

Gene	Exon	Mutations	Positive cases, n	Relative frequency[a] (%)
APC	Exon16	E1554fs	12	19.7
		K1462fs	5	8.2
		Q886X	5	8.2
		R876X	5	8.2
		Others	34	55.7
CDKN2A	Exon2	D125N	12	22.6
		R124H	8	15.1
		D108N	6	11.3
		V106M	6	11.3
		R128W	5	9.4
		Others	16	30.2
FBXW7	Exon8	R385C/H	10	34.5
	Exon9	R266C	6	20.7
	Exon4	R278X	6	20.7
	Exon9	Others	7	24.1
PTEN	Exon6	R173C/H	5	26.3
		Others	14	73.7
SMAD4	Exon9	R361C/H[b]	13	86.7
	Exon3	A118V	2	13.3
STK11	Exon8	F354L	10	37
	Exon4	D194N	6	22.2
	Exon4	E199K	6	22.2
	Exon6	Others	5	18.5
TP53	Exon4	R273C/G/H/L	19	8.5
	Exon1	R175C/H	14	6.3
	Exon2	R213Q/X	14	6.3
	Exon3	R248Q/W	12	5.4
	Exon2	R196Q/X	11	4.9
	Exon4	R280S	11	4.9
		Others	143	63.8

[a]Relative to total number of mutations in the same gene; [b]Hotspot mutation.

Table III. Spectrum of mutations in driver genes.

Gene	Exon	Mutations	Positive cases, n	Relative frequency[a] (%)
BRAF	exon15	p.V600E/M	6	40
		Others	9	60
EGFR	Exon19	p.A743T	5	22.7
	Exon18	p.G721S	4	18.2
	Exon19	p.746del	3	13.6
		Others	10	45.5
IDH1	Exon4	p.R132C/H[b]	13	86.7
		Others	2	13.3
KIT	Exon10	p.M541L[b]	19	79.2
		Others	5	20.8
KRAS	Exon2	p.G12A/C/D/S/V[b]	41	61.2
	Exon2	p.G13D	13	19.4
	Exon4	p.A146T	5	7.5
	Exon2/3	Others	8	11.9
MET	Exon2	p.N375S[b]	17	94.4
	Exon14	p.R988C	1	5.6
PIK3CA	Exon10	p.E545G/K	12	27.9
	Exon21	p.H1047L/R	8	18.6
	Exon21	p.R1023Q	8	18.6
	Exon2	p.R88Q	6	14
		Others	9	20.9

[a]Relative to total number of mutations in the same gene; [b]Hotspot mutation.

Common synonymous variants. *HRAS* H27H (rs12628) and *PDGFRA* V824V (rs2228230) are synonymous variants, but were present in patients with CRC at high frequencies. The variant rate of *HRAS* H27H in CRC patients was 90/117 (76.9%; OR 5.206, P<0.001. The OR for *PDGFRA* V824V was 1.310, but this was not statistically significant (Table IV).

Associations between gene mutations and clinicopathological data. Clinicopathological data and their associations with gene mutations were assessed. The majority of patients in the present study were ≥60 years, and *TP53* mutations were more frequent in patients >60 years old (P<0.05, Table V). The majority of patients in the study were male, and *IDH1* mutations were more frequent in male patients (P<0.05, Table V). Patients with earlier stages of cancer (TNM stages I and stage II) more frequently had a cancer of the rectum as opposed to the colon (P<0.05, Table V). Advanced TNM stage (stage IV) was associated with an increased rate of lymph node metastasis (P<0.05, Table V). There were no other significant associations observed. Gene mutation data are summarized in Table SII.

Discussion

Accurate identification of gene mutations in CRC is critical for selecting the optimum therapeutic agents, and for predicting efficacy and prognosis. The accumulation of gene mutation data also provides important information regarding the pathogenesis of the disease and sheds light on potential novel approaches for clinical management (19-22). NGS covers considerably more genes and for much larger sequencing regions, allowing for greater sequencing sensitivity compared with Sanger sequencin or PCR tests. NGS panel studies also have the advantage of sequencing hot spot regions of a few dozen to a few hundred genes with established implications in clinical diagnosis or targeted therapy, whilst maintaining high sequencing depth compared with whole exome sequencing or whole genome sequencing. Higher sequencing depths translates to higher detection sensitivity and reliability. In CRC studies, panels of 22-genes and 50-genes are commonly used (11,23).

KRAS and *NRAS* mutation tests are mandatory for anti-EGFR therapy (Cetuximab, Panitumumab) (24). *KRAS*, *BRAF* and *PIK3CA* mutation tests are useful for predicting the efficacy of anti-EGFR agents and anti-angiogenic agents (Aflibercept,

Table IV. Frequent synonymous variants identified in the patients with colorectal cancer.

Gene	Variant	SNP	Variant rate	OR[b]	95% CI	P-value
HRAS	p.H27H	rs12628	90/117	5.206	3.369-8.044	$1x10^{-15}$[a]
PDGFRA	p.V824V	rs2228230	36/117	1.31	0.882-1.947	>0.05

P<0.05, [a]P<0.001. [b]Based on the highest possible estimated variant rate in the population from the Chinese Millionome Database CMDB.

Table V. Associations between clinicopathological data and gene mutation data.

Group	Cases (%)	Association with	Associations
Age		*TP53* mutation	
14-59	36 (31.03%)		13
≥60	80 (68.97%)		64[a]
Sex		*IDH1* mutation	
Male	78 (66.67%)		13[a]
Female	39 (33.33%)		1
Location		TNM I/II stage	
Rectum	63 (53.85)		13[a]
Colon	54 (46.15%)		1
TNM stage			
I/II/III	42 (36.21%)	LN metastasis	11
IV	74 (63.79%)		42[a]

[a]P<0.05. TNM, Tumor-Node-Metastasis staging system.

Bevacizumab, Ramucirumab, Regorafenib) (7,8,25). BRAF inhibitor Dabrafenib and MEK inhibitor Trametinib were used to treat patients with CRC who harbored a *BRAF* V600E mutation, and achieved positive results (26). In the present study, 12% of patients harbored *BRAF* mutations, and 40% of *BRAF* mutations were V600 mutations. *PIK3CA* gene encodes a PI3K catalytic subunit. The efficacy of PI3K inhibitors and mTOR inhibitors, approved for treatment of certain types of cancer or are in clinical trials, has yet to be assessed in patients with CRC (27). Regorafenib is an inhibitor of multiple tyrosine kinases, including BRAF, KIT, VEGFR and PDGFR, and used for the treatment of patients with metastatic CRC (28-30). *MET* N375S was found as a hotspot of mutations in the present study. The mutation may confer resistance to MET inhibition (31). The treatment of patients with CRC with MET inhibitors has yet to show promising results (32). Furthermore, *MET* mutations and amplifications may result in resistance to anti-EGFR and anti-BRAF therapy (33). *IDH1* R132C/H were also mutation hotspots in the present study. The IDH1 inhibitor Ivosidenib achieved promising results in the treatment of patients with acute leukemia carrying *IDH1* mutations (34). However, there are no reports regarding the use of this agent in CRC in clinical trials, to the best of our knowledge. *HER2* mutations, such as L755S and V842I, may activate the HER2 kinase domain. Mutant HER2 may sensitize CRC towards Trastuzumab and irreversible HER inhibition (Aftinib, Neratinib, or Dacomitinib) (35). All the 6 cases with *HER2* mutations in the present study carried a V842I mutation.

TP53 gene mutations are frequently identified in solid tumors including CRC (36), and in the present study 65.8% of patients with CRC harbored *TP53* mutations. APR-246 was designed to restore the function of mutant p53 (37). There are multiple ongoing clinical trials involving APR-246 in melanoma, ovarian cancer and hematological malignancies, but not in CRC as of yet, although the agent was shown to exhibit anti-tumor effects on CRC cells *in vitro* (38). Mutant APC or CTNNB1 activates the WNT/β-catenin signaling pathway and multiple agents targeting this pathway are in clinical trials for treatment of various types of cancer, including CRC (39). Although germline mutations in *CDKN2A* increase an individual's susceptibility to CRC (40,41), in the present study, the mutation abundance was <5% in most cases, indicative of the somatic nature. The gene product of *CDKN2A* or p16 protein acts as an inhibitor of CDK4, and *CDKN2A* mutations are common in tumors (42). Clinical trials with CDK4 inhibitor Abemaciclib or Palbociclib in solid tumors including CRC achieved some positive results (43,44). Mutations in *FBXW7* were also commonly identified in the present study. The *FBXW7* mutations are a negative prognostic factor for metastatic CRC (45).

The 50-gene panel used in the present study also covers certain causative genes for common hereditary CRC syndromes, such as *APC* for familial adenomatous polyposis, MLH1 for Lynch syndrome, *PTEN* for Cowden syndrome, *SMAD4* for Juvenile polyposis syndrome, and *STK11* for Peutz-Jeghers syndrome (46-48). A common characteristic of these syndromes is early-onset CRC (46,47). The tissue mutation frequencies of *PTEN*, *SMAD4* and *STK11* genes in most cases were <5% in the present study, indicative of the somatic nature of these mutations. When a certain hereditary CRC syndrome is suspected based on clinical manifestations or NGS screening data, verification by Sanger sequencing using DNA from the peripheral blood is recommended. *MLH1* is the most common gene of mismatch repair genes that are involved in microsatellite instability, that is present in up to 15% of patients with CRC. *MLH1* mutation and microsatellite instability provide important guides for CRC chemotherapy and immunotherapy in clinical settings (49,50).

NGS 50-gene panel sequencing provides comprehensive tissue gene mutation profiles. For most patients with CRC, one or several gene mutations are identified. In the present study, novel mutation spectrums and hotspots of genes commonly involved in CRC were identified, as well as some tentative and potentially relevant associations between genotypes and phenotypes. Further studies are required to investigate the

associations between gene variants in tumor tissues and gene variants identified through liquid biopsy, with an expanded gene panel, with an emphasis on biomarkers for immunotherapy. Mutations of driver genes or tumor suppressor genes may provide critical information for target gene therapy as well as prognosis. A thorough understanding of the significance of the gene mutations is important in the clinical management of patients with CRC.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

JY, ML, HY, KZ conceived and designed the project. CZ, XZ, SL, HL performed collection and quality control of clinical information, specimens and tissue sections. JY assisted with specimen processing and data collection. KZ analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Taizhou People's Hospital (Taizhou, China). All subjects provided informed consent to participate in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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