

Site specific genetic differences in colorectal cancer via Next-Generation-Sequencing using a multigene panel



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AIM: Next-generation sequencing (NGS) has been proposed as a comprehensive and efficient genomic profiling tool to guide personalized therapy for colorectal cancer. This study aimed to review the site-specific difference and the potential benefits of actionable mutation panel for colorectal cancer in relation to the clinicopathological features.

MATERIAL AND METHODS: One hundred and six patients who underwent colorectal surgery with curative or palliative intent for histopathologically confirmed carcinoma between June 2016 and June 2018 were identified from a prospectively maintained database. Formalin-fixed, paraffin-embedded tumor tissues were analyzed for actionable variants in 11 genes via NGS (EGFR, ALK, KRAS, NRAS, KIT, BRAF, PDGFRA, ERBB2, ERBB3, ESRI, and RAF1).

RESULTS: Most of the primary tumors were in the rectum (49 patients; 46.2%) followed by the right colon (32 patients; 30.1%) and left colon (25 patients; 23.5%), respectively. Of sequenced cases, 43 KRAS mutations, 7 EGFR mutations, 6 NRAS mutations, 6 BRAF mutations, 3 KIT mutations, 1 ERBB2 mutation, 1 PDGFRA mutation, and 1 RAF1 mutation were identified in 106 patients. The frequency of mutations is mostly concentrated on the right colon group. The highest drug resistance observed in all patients was against Cetuximab and Panitumumab, and the highest drug resistance was found in the right colon group (53.1%).

CONCLUSIONS: The utility of actionable multigene panel revealed the value of a well-designed next-generation sequencing workflow in the practical use of clinical outcomes via the prediction of responsiveness to therapeutic agents or indications for novel treatment modalities in addition to prognosis estimate.

KEY WORDS: Colorectal Cancer, Drug Resistance, Next-Generation Sequencing

Introduction

Colorectal cancer is the third most frequently diagnosed cancer and is the second leading cause of cancer-related deaths. Approximately 1,9 million patients were newly diagnosed with colorectal cancer, and 935,000 deaths were due to colorectal carcinoma in 2020¹. Despite many cutting-edge chemotherapeutic agents and surgi-

cal techniques, the 5-year survival rates of colorectal carcinoma are still lower than expected. Of all diagnosed colorectal cancer patients, approximately 64.4% may have a 5-year survival, and this rate decreases to 14.2% with metastatic disease². It is well known that 20% of patients have distant metastatic disease at the time of diagnosis³.

In the last decade, a lot of new molecules or drugs specific to genetic mutations in etiopathogenesis at the molecular level related to solid tumors have been introduced⁴. Within the patient-specific treatment, specific genes in the specific cell signalling pathways came to be the target of potential therapies. These therapies have improved overall survival (OS) from 6 to around 20-24 months on metastatic colorectal cancer⁵.

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The Human Genome Project was completed after the majority of the human DNA sequence was identified using Sanger sequencing and fluorescence-based electrophoresis technologies. Subsequently, massive parallel sequencing methods called next-generation sequencing (NGS) began to be developed. Whole-genome and whole exon sequencing with NGS technologies can be determined. In addition, a large number of genes in the etiology of genetic heterogenic diseases can be sequenced simultaneously with targeted NGS panels. Nowadays, NGS technology has been using in the classification of many cancers and the determination of effective drugs in cancer treatment ^{6,7}.

In this study, we detected a genetic mutation profile in 106 colorectal cancer samples from Turkish patients via NGS. The panel we used consisted of 11 genes that are commonly used in cancer risk. Our study is expected to identify genetic mutations in colorectal cancer and provide clues about the polymorphism that varies according to anatomical localization.

Material and methods

One hundred and six patients who underwent colorectal surgery with curative or palliative intent for histopathologically confirmed carcinoma between June 2016 and June 2018 on department of colorectal surgery of Cukurova University were identified from a prospectively maintained database. Formalin-fixed paraffin-embedded tumor tissues were analyzed for actionable variants via NGS.

SAMPLING

Tumor samples from all patients for whom molecular testing was requested by staff surgeons were analyzed in daily routine practice. Data were prospectively collected. DNA was extracted from FFPE tumor samples, after macro-dissection of the tumor area, using the QIAamp FFPE tissue kit (Qiagen, Hildenberg, Germany). The hematoxylin and eosin-stained slide from the same block, previously reviewed by a pathologist who determined the tumor area and evaluated the tumor percentage and tumor necrosis percentage, was used as a guide for the somatic variant interpretation. The DNA obtained was quantified using the Qubit[®] fluorometer in combination with the Qubit[®] dsDNA HS assay kit (Life Technologies, Gent, Belgium).

NEXT-GENERATION SEQUENCING

Optimized NGS workflow was performed as previously described ^{8,9}. Briefly, 40 ng of DNA was enriched by Polymerase Chain Reaction (PCR) in order to sequence

targeted hotspot regions in 11 genes including *EGFR*, *ALK*, *KRAS*, *NRAS*, *KIT*, *BRAF*, *PDGFRA*, *ERBB2*, *ERBB3*, *ESR1*, and *RAF1*. Then samples were tagged with sample-specific barcodes and libraries prepared for the sequencing step. Quality control (QC) had been done at the end of each step by capillary gel electrophoresis. At the end of the workflow, prepared libraries next-generation sequenced with GeneReader NGS system (Qiagen, Hildenberg, Germany). The raw data and QC of sequenced data were evaluated before variant interpretation. Samples with eligible sequencing data went through somatic variants analysis by using QCI-A software (Qiagen, Hildenberg, Germany). All sequenced samples had at least 500× coverage. In the variant list obtained, we considered a variant as authentic if the variant coverage was at least 500× which enable to detection of the somatic variant with lower frequency ^{8,9}. Bioinformatics analyses had been performed for all actionable variants in QCI-I bioinformatics tool.

STATISTICAL ANALYSIS

Data were analyzed with the statistical package program SPSS v 20.0 (IBM Co., Armonk, NY, USA). Categorical measurements were summarized as numbers and percentages, and continuous measurements as mean deviation and minimum-maximum. The conformity of the variables to the normal distribution was examined using one of the analytical methods. Chi-square test was used to compare categorical. Student's t-test and Mann-Whitney U test were used to compare continuous variables between groups. Results are reported as mean SD, median, number (n), and percent (%). P-value <0.05 was considered significant.

Results

DEMOGRAPHIC AND OPERATIVE DATA

A total of 106 patients who underwent colorectal surgery with curative or palliative intent for histopathologically confirmed carcinoma were included in the study. Table I shows the demographical, pathological, and surgical characteristics of the patients. There were 70 male and 36 female patients. The mean age was 58.5±11.5 years. The mean postoperative hospital stay was 7.7±4.9 days. 42.4% percent of patients had Stage III disease and 19.7% had stage IV disease. Most of the primary tumors were in the rectum (49 patients; 46.2%) followed by the right colon (32 patients; 30.1%) and left colon (25 patients; 23.5%), respectively. Forty-one patients underwent low anterior resection, 26 right hemicolectomy, 12 left hemicolectomy, 10 anterior resection, 8 abdominoperineal resection, 6 total abdominal colectomy, and 3 total proctocolectomy.

TABLE I - Demographical, pathological and surgical characteristics of the patients

		Total (n:106) (%)	Left (n:25) (%)	Rectum (n:49) (%)	Right (n:32) (%)	p
Gender	Male	70 (66%)	17 (68%)	34 (69.4%)	19 (59.4%)	0.63
	Female	36 (34%)	8 (32%)	15 (30.6%)	13 (40.6%)	
Age (years) (±SD) (range)		58.5±11.5(23-82)	59.3±8.2 (38-69)	57.4±11.4 (23-76)	59.5±13.8 (27-82)	0.68
Postoperative length of stay (days)(±SD) (range)		7.7±4.9(3-36)	8.8±7.4 (3-36)	7.1±4.1 (4-23)	7.7±3.3 (4-14)	0.36
ASA score	1	39 (36.8)	10 (40%)	16 (32.7%)	13 (40.6%)	0.41
	2	50 (47.1%)	9 (36%)	26 (53.1%)	15 (46.9%)	
	3	15 (14.1)	6 (24%)	5 (10.2%)	4 (12.5%)	
	4	2 (1.9%)	0 (0%)	2 (4.1%)	0 (0%)	
Stage	0	2 (1.8%)	1 (4%)	1 (2%)	0 (0%)	0.01
	1	3 (2.8%)	1 (4%)	2 (4.1%)	0 (0%)	
	2A	10 (9.4%)	3 (12%)	3 (6.1%)	4 (12.5%)	
	2B	25 (23.5)	6 (24%)	6 (12.2%)	13 (40.6%)	
	3A	5 (4.7%)	0 (0%)	5 (10.2%)	0 (0%)	
	3B	24 (22.6%)	8 (32%)	10 (20.4%)	6 (18.8%)	
	3C	16 (15.1%)	2 (8%)	13 (26.5%)	1 (3.1%)	
	4A	19 (17.9)	4 (16%)	9 (18.4%)	6 (18.8%)	
	4B	2 (1.8%)	0 (0%)	0 (0%)	2 (6.2%)	
	MSI statuses	MSId	8 (7.5%)	2 (8%)	2 (4%)	
Surgery	Abdominoperineal resection	8 (7.5%)	0 (0%)	8 (16.3%)	0 (0%)	NA
	Anterior resection	10 (9.4%)	9 (36%)	1 (2%)	0 (0%)	
	Low anterior resection	41 (38.7%)	3 (12%)	38 (77.5%)	0 (0%)	
	Right hemicolectomy	26 (24.5%)	0 (0%)	0 (0%)	26 (81.2%)	
	Left hemicolectomy	12 (11.3%)	12 (48%)	0 (0%)	0 (0%)	
	Total abdominal colectomy	6 (5.7%)	0 (0%)	0 (0%)	6 (18.8%)	
	Total proctocolectomy	3 (2.8%)	1 (4%)	2 (4%)	0 (0%)	

OVERVIEW OF IDENTIFIED VARIANTS

Table II gives the number of mutational variants and drug resistance/sensitivity status in relation to the tumor side. The number of mutations per tumor ranged from 0 to 4. In the majority of the cases (n=53; 50%), only 1 or 2 mutations were detected. In 51 cases (48.1%), no mutations were detected in any of the analyzed regions. Fig. 1 show the distribution of detected variant mutations. Accordingly, single mutation was detected in 46.9% (n: 15) of right colon cancers, in 44% (n: 11) of left colon cancers, and in 38.8% (n: 19) of

rectal cancers. Two mutations were detected in 15.6% (n: 5) of right colon cancers, followed by 8% (n: 2) of left colon cancers and 2% (n: 1) of rectal cancers. Accordingly, two mutations were most common in the right colon. In addition, only in the right colon 3 mutations and 4 mutations were detected. However, no statistically significant correlation was found between the number of mutations and the location of tumor (p = 0.11). Drug resistance was found in 31.3% (n: 10) of right colon cancers, 16% (n: 4) of left colon cancers, and 8.2% (n: 4) of rectal cancers. Accordingly, drug resistance was most common in right colon cancers. The results

TABLE II - Number of mutational variants and drug resistance or sensitivity status in relation to the tumor location

		Total (n:106) (%)	Left (n:25) (%)	Rectum (n:49) (%)	Right (n:32) (%)	p
Number of mutational variants	0	51 (48.1%)	12 (48%)	29 (59.2%)	10 (31.3%)	0.11
	1	45 (42.4%)	11 (44%)	19 (38.8%)	15 (46.9%)	
	2	8 (7.5%)	2 (8%)	1 (2%)	5 (15.6%)	
	3	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)	
	4	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)	
Drug resistance or sensitivity status	Resistance	18 (16.9%)	4 (16%)	4 (8.2%)	10 (31.3%)	0.03
	Sensitivity	2 (1.8%)	0 (0%)	0 (0%)	2 (6.3%)	
	N/A	51 (48.1%)	12 (48%)	29 (59.2%)	10 (31.3%)	
	None	35 (33%)	9 (36%)	16 (32.7%)	10 (31.3%)	

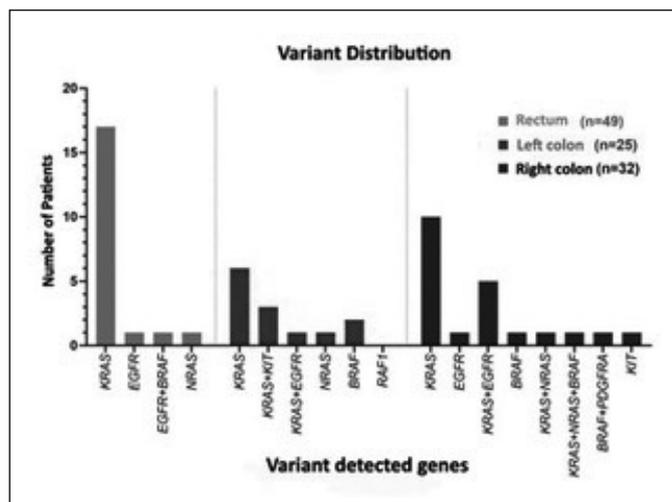


Fig. 1: Distribution of detected variant mutations.

of the right colon group were statistically significant in terms of resistance/sensitivity status ($p = 0.03$). Table III summarizes the types of single or multiple mutations according to the location of tumor. Of sequenced cases, 43 KRAS mutations, 7 EGFR mutations, 6 NRAS mutations, 6 BRAF mutations, 3 KIT mutations, 1 ERBB2 mutation, 1 PDGFRA mutation, and 1 RAF1 mutation were identified in 106 patients. The detection of mutations by NGS showed high sensitivity. Fifty-five cases (51.8%) showed at least one mutation. The most frequent single mutation was found in KRAS (41 patients; 38.6%) and BRAF (5 patients; 4.7%) irrespective of the tumor localization. EGFR, NRAS, and

KIT were the most frequent second mutations (4 patients, 3.7%; 2 patients, 1.8%; and 2 patients, 1.8%, respectively). The third mutation was determined only in KRAS (1 patient, 0.94%) and NRAS (1 patient, 0.94%). As for the fourth mutation, only NRAS was detected in the right colon group (1 patient, 0.94%). When the three groups were compared, no statistically significant difference was found in terms of single, triple, or quadruple mutation frequency ($p > 0.05$). However, the increase in the frequency of the second mutation in the right colon group was statistically significant ($p = 0.02$).

Genetic polymorphism was detected in 2 patients. Incidental FAP was detected in one of these patients. BRAF p. P632S variant was found to be associated with a high risk of metastasis in colorectal cancer in a patient. EGFR R521K mutation of unknown prognostic significance was detected in another patient. Since synchronous colon cancer and adrenal metastasis were detected in this patient at the time of diagnosis, it was thought that this gene might be a poor prognostic indicator. Drug resistance status and their frequencies are shown in Table IV according to the tumor location. Overall, drug resistance was detected in 53.1% (n: 17) of the right colon group, 38.8% (n: 19) of the rectum group, and 48% (n: 12) of the left colon group. Accordingly, the highest drug resistance was in the right colon group. The more common drug resistance was against Cetuximab and Panitumumab (17 patients, 34.7% of rectum cancer, 11 patients, 34.4% of right colon cancer, and 5 patients, 20% of left colon cancer, respectively).

TABLE III - Types of single or multiple mutation according to the location of tumor

		Total (n:106) (%)	Left(n:25) (%)	Rectum (n:49) (%)	Right (n:32) (%)	p*
1 Mutation(51.8%)	BRAF	5 (4.7%)	2 (8%)	1 (2%)	2 (6.3%)	0.47
	EGFR	3 (2.8%)	1 (4%)	1 (2%)	1 (3.1%)	
	ERBB2	1 (0.94%)	0 (0%)	1 (2%)	0 (0%)	
	KIT	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)	
	KRAS	41 (38.6%)	9 (36%)	16 (32.7%)	16 (50%)	
	NRAS	2 (1.8%)	1 (4%)	1 (2%)	0 (0%)	
	PDGFRA	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)	
	RAF1	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)	
	None	51 (48.1%)	12 (48%)	29 (59.2%)	10 (31.3%)	
	2 Mutation(9.4%)	BRAF	1 (0.94%)	0 (0%)	0 (0%)	
EGFR		4 (3.7%)	0 (0%)	1 (2%)	3 (9.4%)	
KIT		2 (1.8%)	2 (8%)	0 (0%)	0 (0%)	
KRAS		1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)	
NRAS		2 (1.8%)	0 (0%)	0 (0%)	2 (6.3%)	
None		96 (90.5%)	23 (92%)	48 (98%)	25 (78.1%)	
3 Mutation(1.8%)		KRAS	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)
NRAS	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)		
4 Mutation(0.94%)	None	104 (98.1%)	25 (100%)	49 (100%)	30 (93.8%)	0.31
	NRAS	1 (0.94%)	0 (0%)	0 (0%)	1 (3.1%)	
	None	105 (99%)	25 (100%)	49 (100%)	31 (96.9%)	

TABLE IV - Drug resistance status and their frequencies

Side	Drugs	Number of case (%)
Right colon (n:17)	Cetuximab	1 (3.1%)
	Panitumumab	1 (3.1%)
	Cetuximab+Panitumumab	11 (34.4%)
	Carboplatin/Paclitaxel and Erlotinib	3 (9.4%)
	EGFR TK	1 (3.1%)
Left Colon (n:12)	Cetuximab	2 (8%)
	Panitumumab	2 (8%)
	Cetuximab+Panitumumab	5 (20%)
	Cetuximab, Carboplatin/Paclitaxel and Erlotinib	1 (4%)
	Cetuximab, Carboplatin/Paclitaxel and Panitumumab	2 (8%)
Rectum (n:19)	Cetuximab	2 (4.1%)
	Cetuximab+Panitumumab	17 (34.7%)

Discussion

In this study, it was shown that the mutation profile in the right colon was different from the mutation profile in the left colon and rectum by NGS. Although not statistically significant, NGS results may explain why right colon cancers behave more aggressively oncologically. With the present study, this situation was analyzed with both the mutation profile and the number of mutations. This study provides important information on drug resistance and susceptibility to modify therapy. The identified polymorphisms provide an idea about increased risk of CRC and prognostic markers. In particular, follow-up protocols for patients with negative prognostic factors can thus be made more closely.

NGS technology has revolutionized the analysis of genomic mutations in cancer tissues⁹. In recent years, this technology has become more accessible and has led to extensive studies on cancers¹⁰. NGS results have improved our understanding of tumorigenesis pathways, providing a rational basis for drug development and individualized treatments¹¹. As a result, the number of specific gene aberrations tests is increasing rapidly.

In this study, we report genetic analysis of 106 CRC patients using targeted NGS. The molecular profile of CRC tumors reported in this study is similar to that reported in the literature¹²⁻¹⁸. Most mutations were KRAS (n:43), EGFR (n: 7), NRAS (n: 6), and BRAF (n: 6). We observed that the frequency of mutations ranging from one to four was mostly concentrated in the right colon group.

Identification of mutational profile helps guide adjuvant therapy on CRC patients. According to international guidelines, the presence of a RAS mutation is a contraindication to anti-EGFR therapy^{19,20}. Nevertheless, the patients with any known KRAS mutation (exon 2, 3, 4) or NRAS mutation (exon 2, 3, 4) should not be treated with either cetuximab or panitumumab²¹⁻²³. BRAF V600E mutation makes response to panitumumab or cetuximab highly unlikely unless given with a

BRAF inhibitor²⁴⁻²⁶. BRAF mutation is a strong negative prognostic biomarker and evidence is accumulating that patients with a BRAF mutant tumor do not benefit from anti-EGFR therapy^{19,27}. Other than these known genetic mutations, the discovery of particular mutations in rare genes can cause a change in the treatment by including the patient in a clinical trial or medical need program. In our study, the highest drug resistance was observed against Cetuximab and Panitumumab. The distribution of genetic changes detected in patients in terms of drug resistance and tumor localization, we can say that the highest drug resistance was found in the right colon group (53.1%).

NGS systems have started to take place in routine clinical applications in the last five years and it has come to the forefront especially in the field of cancer in terms of patient treatment. Planning of adjuvant treatment protocols considering the results of the molecular genetic examination and close follow-up of patients identified as high-risk may be associated with better oncologic outcomes. Using NGS systems, individualized treatment is possible with clinical diagnosis and determination of correlation. Planning of adjuvant treatment protocols for molecular investigations may be associated with better oncologic outcomes. Closer follow-up of patients identified as high-risk is required. It is a more valuable recommendation in patients at risk of familial colorectal cancer (polymorphisms).

Conclusion

The utility of actionable multigene panel revealed the value of a well-designed NGS workflow in the practical use of clinical outcomes via the prediction of responsiveness to therapeutic agents or indications for novel treatment modalities in addition to prognosis estimate. This study provides how NGS can drive advances that bring us closer to precision oncology and how it is increasingly used to guide personalized treatment deci-

sion in order to realize the ultimate goal of medicine in oncological practice.

Riassunto

OBIETTIVO: Il sequenziamento di nuova generazione (NGS) è stato proposto come uno strumento di profilazione genomica completo ed efficiente per guidare la terapia personalizzata per il cancro del colon-retto. Questo studio mirava a rivedere la differenza sito-specifica e i potenziali benefici del pannello di mutazione attuabile per il cancro del colon-retto in relazione alle caratteristiche clinicopatologiche.

MATERIALE E METODI: 16 pazienti sottoposti a chirurgia coloretale con intento curativo o palliativo per carcinoma istopatologico confermato tra giugno 2016 e giugno 2018 sono stati identificati da un database mantenuto in modo prospettico. I tessuti tumorali fissati in formalina e inclusi in paraffina sono stati analizzati per le varianti utilizzabili in 11 geni tramite NGS (EGFR, ALK, KRAS, NRAS, KIT, BRAF, PDGFRA, ERBB2, ERBB3, ESR1 e RAF1).

RISULTATI: La maggior parte dei tumori primari erano nel retto (49 pazienti; 46,2%) seguito rispettivamente dal colon destro (32 pazienti; 30,1%) e sinistro (25 pazienti; 23,5%). Dei casi sequenziati, 43 mutazioni KRAS, 7 mutazioni EGFR, 6 mutazioni NRAS, 6 mutazioni BRAF, 3 mutazioni KIT, 1 mutazione ERBB2, 1 mutazione PDGFRA e 1 mutazione RAF1 sono state identificate in 106 pazienti. La frequenza delle mutazioni è per lo più concentrata sul gruppo del colon destro. La più alta resistenza ai farmaci osservata in tutti i pazienti è stata contro Cetuximab e Panitumumab e la più alta resistenza ai farmaci è stata riscontrata nel gruppo del colon destro (53,1%).

CONCLUSIONI: L'utilità di un pannello multigene attuabile ha rivelato il valore di un flusso di lavoro di sequenziamento di nuova generazione ben progettato nell'uso pratico dei risultati clinici attraverso la previsione della reattività agli agenti terapeutici o indicazioni per nuove modalità di trattamento oltre alla stima della prognosi.

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