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Differences in the Molecular Landscape of Colorectal Cancer in Old and Young Patients

Kozlowska-Geller M, Strzelecka A, Nawacki L, Wawszczak-Kasza M, Gluszek S and Lewitowicz P*

Medical College, The Jan Kochanowski University in Kielce, Poland

Abstract

Introduction: Colorectal cancer is a major global public health issue, especially in Western countries. The application of Next-Generation Sequencing (NGS) technology has facilitated multigene panel analysis and is used to identify individuals with cancer-predisposing gene variants.

Aim: We analyzed the molecular differences in colorectal cancer in patients younger than 50 years and older than 50 years.

Material and Methods: To the inclusion criteria We used no previous radio- or chemotherapy or DNA eligible for next-generation sequencing. The patients were divided into two groups: up to 50 years of age and over 50 years of age. DNA was isolated from FFPE tissue. We used a hot spot cancer panel (Illumina) harboring 50 genes (700 amplicons).

Results: The median age of the young and older patients was 43 and 63 years, respectively. The female-to-male ratio was 1.2:1. We observed a following mutation frequency (≤ 50/>50): TP53 76/64%, APC 57/45%, KRAS 43/73%, NRAS 29/0%, SMAD4 9/15%, PIK3CA 14/33%, FBXW7 5/15%. We noted the co-occurrence APC/KRAS/TP53 mutation in 20% of patients. KRAS mutations were significantly more common in elderly patients (p=0.001).

Conclusion: Almost half of the respondents (46%) presented multigene abnormalities, with three to five or more mutations being the most common. IDH1 and CTBX1 are found only in patients with a better prognosis when the TP53 mutation is almost twice as common in patients with worse prognosis. Interestingly is the fact that 29% of the young have NRAS mutations, the old do not have it at all, p=0.021.

Keywords: Genomic era; Next-Generation-Sequencing (NGS); Personalized medicine

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Correspondence:
Monika Kozlowska-Geller, Medical College, The Jan Kochanowski University in Kielce, Aleja IX Wieków Kielc 19A, 25-317 Kielce, Poland,

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Introduction

Colorectal Cancer (CRC), one of the most common cancers, is a major global public health issue, especially in Western countries [1]. In Poland, both in men and women, CRC has a high incidence and occupies the 2nd to 3rd position among morbidity and deaths [2]. The pathogenesis of CRC has also been combined. Diet, genetic burden, and inflammatory bowel disease are frequently highlighted in sporadic cancer [1].

Targeted sequencing is an NGS technology (Next-Generation Sequencing) used to sequence a set of genes of interest. Compared to whole genome and whole exome sequencing, this method has the advantage of reducing the cost per sample, increasing depth, and running multiple samples simultaneously [3]. The increased depth of target sequencing has an additional advantage, even over targeted PCR-based techniques, for detecting somatic variants at very low allele frequencies [4]. In this study, we used a panel of colorectal cancer-associated genes to determine the somatic mutation landscape in a cohort of tumor samples from patients of various ages undergoing surgery for colorectal cancer at various stages of development.

Widely implemented screening and molecular testing could lead to the diagnosis of precancers or low-stage cancer, which essentially improves survival [5]. Despite tremendous growth in our understanding of genetics, NGS has the added advantage of providing a more comprehensive picture of the cancer landscape and uncovering cancer development pathways [3-6]. This provides more in-depth insights into the mutational processes functioning in various types of cancers, which eventually enhances our understanding of the biology of the disease and leads to better patient management and genetic screening [5].

Aim

The purpose of this study was to analyze the molecular differences in the CRC base according to patient age.

Material and Methods

The research was carried out in the Department of Clinical and Experimental Pathology of the Jan Kochanowski University in Kielce.

Inclusion and exclusion criteria

This study included patients with colorectal cancer with a histopathological diagnosis of NOS adenocarcinoma. We excluded other histopathological types because of the various molecular pathways. Moreover, only patients without a history of radiotherapy and chemotherapy were included in the study. DNA eligible for next-generation sequencing qualified for this study. DNA eligible for next-generation sequencing qualified for this study.

The patients were divided into two groups: Patients up to 50 years of age n=21 and patients - 50 n=33. The study group comprised 29 men and 25 females.

DNA extraction

Cancer genomic DNA was extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tissue using MagCore automatic extraction Kit number 405 (MagCore, RBC Bioscience, New Taipei, Taiwan) according to the manufacturer's instructions. The purified DNA was quantified using a Quantus® Fluorometer (Promega, Madison, WI, USA) and the QuantiFluo ONE dsDNA Kit.

Library preparation and sequencing

Amplicon-based analysis included hotspot regions of 50 oncogenes and tumor suppressor genes. Library preparation for NGS analysis was performed using the AmpliSeq Library PLUS for Illumina® assay kit (San Diego, CA, USA) according to the manufacturer's instructions. (AmpliSeq for Illumina Cancer HotSpot Panel v2 Reference Guide). The assay generated a library of 207 gene-specific amplicons and approximately 2800 clinically relevant mutations. Amplification was performed using the multiplex PCR method (HotSpot Panel v2), and the DNA template used for the reaction was diluted to a final concentration of approximately 30 ng/rxn. The adapters ligation was performed using AmpliSeq CD Indexes Set A for Illumina. The amplified DNA fragments were purified using a magnetic-based DNA purification approach. The product of each sample was used as a template for the second amplification step, in which the product was amplified using sequencing primers. After the final amplification, each tagged amplicon library was purified using NucleoMag® NGS Clean-up and Size Select beads (Machery-Nagel GmbH& Co., Düren, Germany). Each library was qualified using the QuantiFluo® ONE dsDNA System (Promega, USA) to allow equimolar pooling of all sample libraries for subsequent sequencing. The fragment size distribution of each library was analyzed by automated gel electrophoresis using Genomic DNA KIT (4150 TapeStation System, Agilent, Santa Clara, CA, United States).

Sequencing

Products were analyzed by Next-Generation Sequencing (NGS) using the Illumina MiSeq Dx platform. NGS was performed using the MiSeq Reagent Micro Kit v2 (300-cycle) (Illumina, San Diego, CA, USA). Indexed DNA library concentrations were quantified using the fluorometric method QuantiFluo ONE dsDNA Kit (Quantus® Fluorometer (Promega, Madison, WI, USA), normalized to 4 nM using Low TE, and pooled to the final library according to the manufacturer's instructions (Protocol A, MiSeq System, Denature and Dilute Libraries Guide, Illumina). The library was denatured using 5 µL of 4 nM library and 5 µL of 0.2 N NaOH. The library was diluted with pre-chilled HT1 buffer to a final concentration of 20 pM. Finally, the 9 pM library was spiked with 5% of PhiX Control v2 (Illumina, San Diego, CA, USA), which provides quality control for cluster generation, sequencing, and alignment.

Statistical analysis

Clinicopathological features of the patients were analyzed using the SPSS software package (version 22). Continuous variables are expressed as mean ± SD and range, while categorical variables are expressed as percentages. Comparisons between groups were analyzed using the $\chi 2$ test or Fisher's exact test, when appropriate for categorical variables, and by Mann–Whitney test or Student's *t*-test when appropriate for continuous variables. p-value was set significant when p-value ≤ 0.05.

Results

Clinical features

As shown in Table 1, patients were divided into two groups: patients ≤ 50 and patients > 50 and categorized by age, sex, histological type and staging.

The median age of the patients aged ≤ 50 years was 43 years, and the >50 ones 63. The female-to-male ratio was 1.2:1. There were no significant differences in the mean age and sex between the groups. However, younger patients had significantly more advanced-stage cancer than older patients, where less advanced cancer predominated (p< alpha). Regarding the histological features, the adenocarcinoma NOS was the most predominant subtype reported in 100% (p<0.001).

The Detected somatic mutations in our data set

As shown in the Figure 1 and Table 2 the most common mutations in the studied group of patients were TP53 (64%; 35 out of 54), KRAS (60%; 33/54), APC (51%; 28/54), PIK3CA (25%; 14/54), SMAD4 (13%; 7/54), NRAS (11%; 6/54), FBXW7 (11%; 6/54), BRAF (7%; 4/54).

According to the Table 3 in the ≤ 50 group (n=21), TP53 (76%; 16

Table 1: Clinicopathological features of the studied groups.

	CRC n=54	≤ 50 n=21	>50 n=33
Age			
Median (Range)	55 (31-92)	43 (31-50)	63 (51-92)
Gender			
Male	29 (54%)	10	19
Female	25 (46%)	11	14
Histological Type	**Adenocarcinoma NOS (100%)**		
Grade			
I	9		
IIA	14		
IIIA	2		
IIIB	10		
IIIC	5		
IVA	6		
IVB	8		

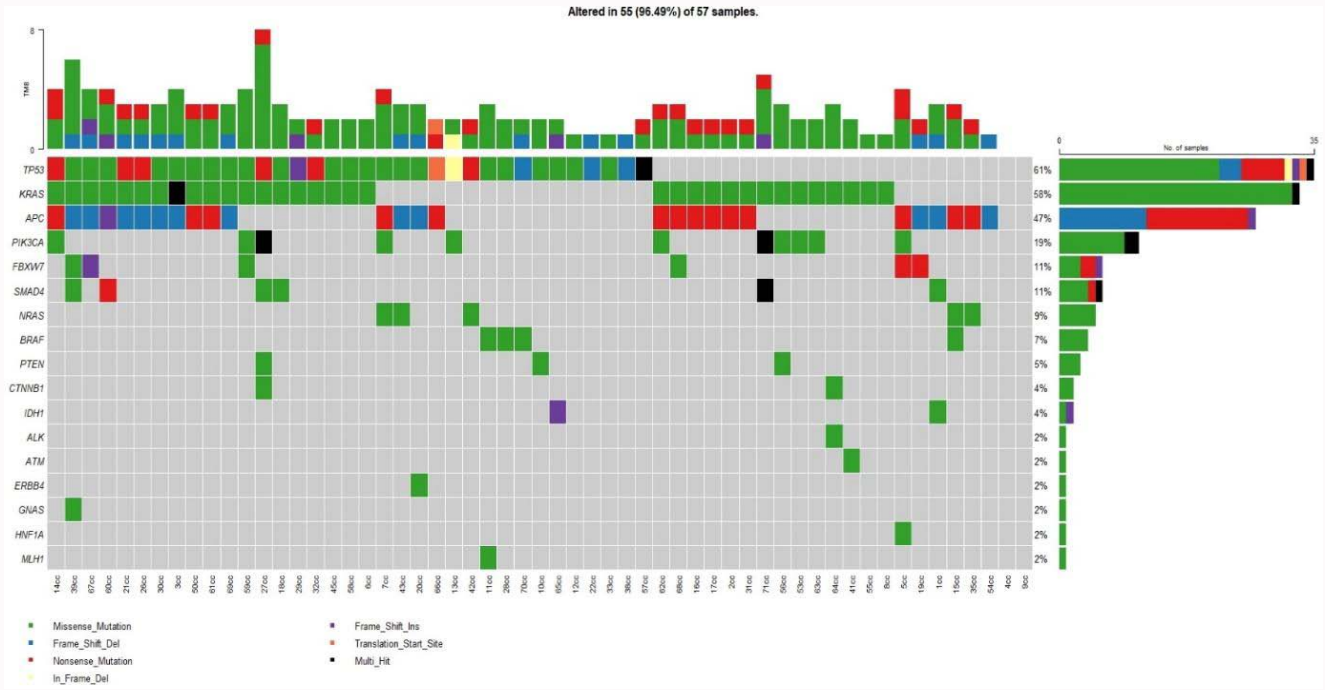

Figure 1: Oncoplot displays the somatic mutations distribution of the top highly mutated genes.

Table 2: Distribution of mutations in all patients (n=54).

Type of mutation	%	n
TP53	64	35
KRAS	60	33
APC	51	28
PIK3CA	25	14
SMAD 4	13	7
NRAS	11	6
FBXW7	11	6
BRAF	7	4

out of 21), APC (57%; 12/21), KRAS (43%; 9/21), NRAS (29%; 6/21), PIK3CA (14%; 3/21), FBXW7 (5%; 1/21) were the top mutated genes.

In the >50 group (n=33), KRAS (73%; 24 out of 33), TP53 (64%; 21/33), APC (45%; 15/33), PIK3CA (33%; 11/33), SMAD4 (15%; 5/33), FBXW7 (15%; 5/33) were the top mutated genes.

We observed a following mutation frequency (≤ 50/>50): TP53 76/64%, APC 57/45%, KRAS 43/73%, NRAS 29/0%, SMAD4 9/15%, PIK3CA 14/33%, FBXW7 5/15%.

In patients aged >50 years, the most common mutation was KRAS, which affected 3/4 of the patients, while KRAS in ≤ 50 people appeared in every second person.

In ≤ 50 patients, the most common mutation, occurring in 3/4 of patients, was the TP53 mutation; in >50 patients, TP53 affected 64% of patients.

KRAS is significantly more common in the >50, p=0.001.

29% of the ≤ 50 have NRAS mutation, the >50 do not have it at all, p=0.021.

As it results from the Figure 2 almost half of the respondents

Table 3: Distribution of mutations in groups: ≤ 50, >50.

Type of mutation	≤ 50 (n)	>50 (n)	p	
APC	12	15	0.557	p>α
KRAS	21 (9)	24	0.001	p<α
TP53	16	21	0.383	p>α
PIK3CA	3	11	0.202	p>α
FBXW7	1	5	0.386	p>α
HNF1A	0	1	1	p>α
SMAD4	2	5	0.693	p>α
ERBB4	0	1	1	p>α
PTEN	1	2	0.839	p>α
BRAF	2	2	0.638	p>α
CTNNB1	0	2	0.516	p>α
ALK	0	1	1	p>α
IDH1	1	1	1	p>α
NRAS	6	0	0.021	p<α
GNAS	1	0	0.389	p>α
ATM	1	0	0.389	p>α
Patients (n)	21	33		

(47%) presented multigene abnormalities, with three to five or more mutations occurring the most commonly:

- co-occurrence of three mutations – 28% of patients

- co-occurrence of four mutations – 13% of patients

- Co-occurrence of five or more mutations: 6% of patients.

However, it has not been shown that the co-occurrence of multiple mutations predominates in any age group.

According to the Table 4, TP53 mutations are almost twice as

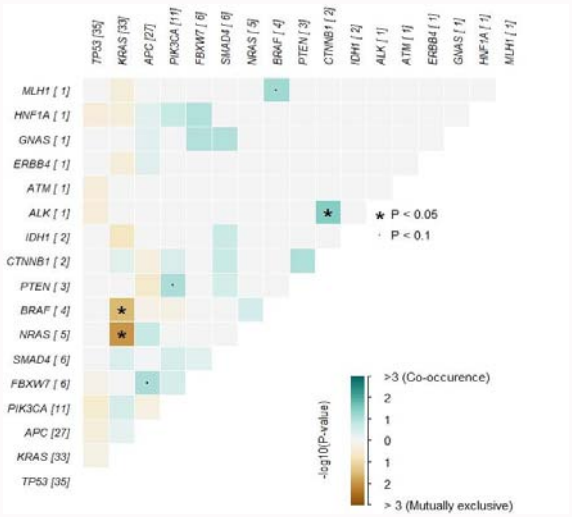

Figure 2: Co-occurrence of mutations in the studied group of patients.

Table 4: Distribution of mutations in groups of patients with better and worse prognosis.

Type of mutation	Patients with better prognosis (I, IIA)	Patients with worse prognosis (IIIA, IIIB, IIIC, IVA, IVB)
KRAS	17	15
APC	16	13
TP53	13	23
PIK3CA	9	6
FBXW7	3	3
SMAD4	3	4
IDH1	2	0
NRAS	2	4
CTBX1	2	0
HNF1A	1	0
PTEN	1	2
ALK	1	0
BRAF	1	3
ERBB4	0	1
GNAS	0	1
ATM	0	1

common (1,8) in patients with worse prognosis.

IDH1, CTBX1, HNF1A, and ALK mutations were found only in patients with better prognosis.

ERBB4, GNAS, and ATM mutations were found only in patients with worse prognosis.

Discussion

CRC is caused by mutations in oncogenes, tumor suppressor genes, and genes related to DNA repair mechanisms [7]. Next-Generation Sequencing (NGS) has become a promising approach for detecting somatic mutations in tumors [8].

The results obtained in these studies can set the foundation for a new generation of genetic screening tests in different age groups: ≤ 50 and >50 years.

The incidence of Colorectal Cancer in Young Adults (CRCYAs) is increasing globally, and it is now the third leading cause of cancer-related deaths among adults under 50 years of age. The rising incidence is attributed to various emerging risk factors such as genetics, lifestyle factors, and microbiome profiles [9].

As the majority of YO-CRC studies utilize an age threshold of 50 years, which is currently the inflection point for the age-dependent change in CRC incidence and the age at which most countries initiate CRC screening for the average-risk population, we used the threshold of <50 years of age to define YO-CRC in this Primer [10].

In the context of treatment, personalized medicine is rapidly becoming an indispensable tool. Thus, it is necessary to perform an in-depth analysis of the tumor features of each patient to determine the most appropriate treatment [5].

Wang et al., in a large group of 648 patients, found the frequency of the following mutations [6]: TP53 (52.82%), KRAS (46.68%), APC (24.09%), PIK3CA (18.94%), SMAD4 (9.47%), BRAF (6.15%), FBXW7 (5.32%), and NRAS (4.15%), other less frequently mutated genes were also identified.

The results of our research coincide with those of the Chinese, as the frequency of CRC mutations is similar: TP53 (64%), KRAS (60%), APC (51%), PIK3CA (25%), SMAD4 (13%), FBXW7 (11%), NRAS (11%), BRAF (7%).

Although the overall incidence of CRC has been decreasing worldwide, there has been an increase in the trend in young age in the last few decades. This observed disparity in the incidence trend between different ages could be due to the increased exposure to risk factors such as body weight, alcohol, smoking, physical inactivity, and red meat-rich diet in younger individuals [9-12].

Molecular and clinicopathological differences were not as significant among different age groups of CRC patients as previously suspected, which was confirmed by Chang's large study in Taiwan on 1,475 CRC patients [11]. We observed the following mutation frequencies (≤ 50/>50): TP53 76/64%, APC 57/45%, KRAS 43/73%, NRAS 29/0%, SMAD4 9/15%, PIK3CA 14/33%, FBXW7 5/15%, which supports Chang's theory that there are no significant differences in CRC mutations between young and old patients, except for KRAS, where the difference between young and old was statistically significant p<0.05, and NRAS, which did not occur at all in the elderly.

According to Chatsirisupachai et al., all TP53 and CTNNB1 mutations are more common in younger colorectal cancer patients, while APC, KRAS, and BRAF V600 mutations are higher in older patients [12], which is partially confirmed in our research, because in patients >50, the most common mutation was KRAS and it affected 3/4 of older patients, while in patients ≤ 50, the most common mutation was TP53 and affected ¾ of younger patients.

Most colorectal cancer cases are typically diagnosed in adults over 50 years of age [13,14]. However, mounting evidence from the past decade has shown that the incidence of colorectal cancer is increasing among young adults [9-12]. Colorectal cancer is now the third leading cause of cancer-related deaths among young adults aged less than 50 years [9].

These observations highlight the importance of early detection, diagnosis, and personalized treatment of CRCYA [9,10].

A recent retrospective review of approximately 36,000 colorectal cancer patients comparing genetic characteristics and different age groups showed that patients with early onset disease were more likely to be microsatellite unstable and have CTNNB1 and ATM mutations and CIMP hypermethylation [9,11].

In our study in the ≤ 50 group, TP53 (76%), APC (57%), KRAS (43%), NRAS (29%), PIK3CA (14%), FBXW7 (5%) were the top mutated genes.

In the >50, KRAS (73%), TP53 (64%), APC (45%), PIK3CA (33%), SMAD4 (15%), FBXW7 (15%) were the top mutated genes.

In our study Hanna's, most of the significantly recurrent mutations were observed in known cancer-related genes such as APC, TP53, KRAS, PIK3CA, FBXW7, SMAD4, and NRAS [15].

According to Lipsyc, Kalady, Shen NRAS, KRAS, BRAF, and PIK3CA are associated with poor prognosis and survival. In our study, TP53 occurred twice as often in patients with worse prognosis [15-17].

NRAS mutations may promote tumorigenesis during colorectal inflammation [16]. This may explain the fact that in our study, NRAS mutation was detected only in young patients, not in old patients.

Recent studies have demonstrated that IDH1 mutations are associated with younger age, better prognosis, and better response to treatment [18,19]. In our study, we noticed that the IDH1 mutation appeared only in patients with better prognosis.

Cancer research has begun to use this innovative and highly performing method, and interesting results have started to appear in colorectal cancer analysis. Analysis of the results unveiled relevant perspectives aiding in evaluating the response to therapies.

Currently, the challenge is to exploit this advanced technology to better understand the underlying molecular mechanism of colorectal carcinogenesis and to identify clinically relevant genetic biomarkers for diagnosis and personalized therapeutics [20,21].

Conclusions

1. Molecular and clinicopathological differences were not as significant among the different age groups of CRC patients as previously suspected, except for KRAS, where the difference between young and old CRC patients was statistically significant $p<0.05$, and NRAS, which did not occur at all in the old.

2. In young patients, the most common mutation, occurring in ¾ of the patients, was the TP53 mutation, while in old patients, the most common mutation was KRAS, which affected ¾ of the old patients.

3. Almost half of the respondents (46%) presented multigene abnormalities, with three to five or more mutations occurring the most commonly.

4. IDH1, CTBX1, HNF1A and ALK mutations are found only in patients with a better prognosis when the TP53 mutation is almost twice as common (1,8) in patients with worse prognosis.

5. Interestingly is the fact that 29% of the young have NRAS mutations, the old do not have it at all, $p=0.021$.

6. We concluded that this targeted NGS assay is suitable for clinical practice, and our findings could help in the diagnosis and prognosis of Polish patients with CRC.

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