Original paper

Analysis of the multigene molecular panel for colorectal cancer in patients under 50 years of age – the preliminary results of the Polish department

Analiza wielogenowego panelu molekularnego raka jelita grubego u chorych do 50. roku życia – wstępne wyniki polskiego ośrodka

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Key words: colorectal cancer, genomic era, next-generation-sequencing, personalised medicine.

Słowa kluczowe: rak jelita grubego, era genomu, sekwencjonowanie nowej generacji, medycyna spersonalizowana.

Abstract

Introduction: Colorectal cancer (CRC), one of the most common cancers, is a major public health issue globally, especially in Western countries. In Poland, both in men and women, CRC is a cancer with a high incidence and occupies the 2nd–3rd position among morbidity and deaths. Because of milestones in molecular analysis and deeper insight into molecular pathways, personalised treatment is now possible. Next-generation sequencing (NGS) technology enables panel detection of many genes and therefore identification of people with cancer-predisposing mutations.
Aim of the research: In the current study we analysed molecular studies of CRC in patients ≤ 50 years of age.
Material and methods: We qualified patients without prior radio- and chemotherapy into the inclusion criteria. We isolated DNA from FFPE. We used the Illumina Hot-Spot Cancer Panel containing 50 genes (700 amplicons).
Results: The median of age was 43 years. The female : male ratio was 1 : 1. We recorded the following mutation frequencies: TP53 76%, APC 57%, KRAS 43%, NRAS 29%, SMAD4 9%, PIK3CA 14%, and FBXW7 5%. We noted the co-occurrence APC/KRAS/TP53 mutation in 20% of patients.
Conclusions: Co-occurrence of mutations was found in 86% of patients, most often 2 or 3. IDH1 was found only in patients with a better prognosis, while the TP53, APC, and KRAS mutations occurred significantly more often in patients with a worse prognosis.

Streszczenie

Wprowadzenie: Rak jelita grubego (CRC), jeden z najczęściej występujących nowotworów, stanowi poważny problem zdrowia publicznego na świecie, zwłaszcza w krajach zachodnich. W Polsce, zarówno u mężczyzn, jak i u kobiet, CRC jest nowotworem o dużej zapadalności i zajmuje 2. oraz 3. miejsce pod względem zachorowalności i zgonów. Dzięki kamieniom milowym w analizie molekularnej i głębszemu wglądowi w szlaki molekularne możliwe jest obecnie spersonalizowane leczenie. Technologia sekwencjonowania nowej generacji (NGS) pozwala na panelową detekcję wielu genów, a tym samym identyfikację osób z mutacjami predysponującymi do nowotworów.
Cel pracy: W niniejszym badaniu poddano analizie badania molekularne CRC u pacjentów ≤ 50. roku życia.
Materiał i metody: Do kryteriów włączenia zakwalifikowano pacjentów niepoddanych wcześniej radio- i chemioterapii. Wyizolowano DNA z FFPE. Użyto panelu Illumina Hot-Spot Cancer Panel zawierającego 50 genów (700 amplikonów).
Wyniki: Mediana wieku wynosiła 43 lata. Stosunek liczby kobiet do liczby mężczyzn wynosił 1 : 1. Odnotowano następujące częstości mutacji: TP53 76%, APC 57%, KRAS 43%, NRAS 29%, SMAD4 9%, PIK3CA 14% i FBXW7 5%. Stwierdzono współwystępowanie APC/mutacja KRAS/TP53 u 20% pacjentów.
Wnioski: Współwystępowanie mutacji wykazano u 86% chorych, najczęściej u 2 lub 3 chorych. IDH1 stwierdzono jedynie u chorych z lepszym rokowaniem, natomiast mutacje TP53, APC i KRAS występowały istotnie częściej u chorych z gorszym rokowaniem.

Introduction

Colorectal cancer (CRC) is one of the most common cancers and constitutes a serious medical problem worldwide, especially in Western countries. In Poland, both in men and women, CRC is a cancer with a high incidence and occupies the 2nd–3rd position among morbidities and deaths [1]. The pathogenesis of the CRC is combined; in sporadic cancer the diet, genetic burden, and inflammatory bowel disease are frequently highlighted [2, 3].

Widely implemented screening and molecular testing could lead to diagnosis of precancers or low-stage cancer, which essentially improves survival [4]. NGS has provided a significant step forward in personalised medicine (PM) [5, 6]. Despite significant increases in our knowledge of genetics, NGS additionally provides a more complete picture of the cancer landscape and the discovery of cancer development pathways. This provides greater insight into the mutational processes that occur in cancer, increasing our understanding of the biology of the disease [7].

Targeted sequencing in NGS technology involves sequencing a set of genes of interest. Compared to whole genome and whole exome sequencing, this method reduces the cost per sample and allows multiple samples to be tested simultaneously [8, 9]. Increased target sequencing depth has the additional advantage of detecting somatic variants at very low allele frequencies. In this study, we used a panel of colorectal cancer-associated genes to determine the somatic mutation landscape in a cohort of tumour samples from patients of various ages undergoing surgery for colorectal cancer at various stages of its development [10].

Aim of the research

The study purpose was to analyse the molecular landscape of colorectal cancer in Polish patients ≤ 50 years of age.

Material and methods

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

Patient samples

Patients up to 50 years of age (n = 21), who had been operated on for primary NOS adenocarcinoma in stages II–IV, without previous radio- and chemotherapy, with DNA eligible for next-generation sequencing were qualified for the study. In the study group there were 10 men and 11 women.

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) with a QuantiFluo ONE dsDNA Kit.

Library preparation and sequencing

The amplicon-based analysis included hotspot regions of 50 oncogenes and tumour suppressor genes. The 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 ~2800 clinically relevant mutations. The amplification was performed using multiplex PCR method (HotSpot Panel v2), and the DNA template used for reaction was diluted for final concentration ~30 ng/rxn. The adapters ligation was performed using AmpliSeq™ CD Indexes Set A for Illumina®. The purification of amplified DNA fragments was performed using a magnetic-based DNA purification approach. The product of each sample was used as a template for the second amplification step, which amplifies the product with sequencing primers. After 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 for the equimolar pooling of all sample libraries for subsequent sequencing. The fragment size distribution of each library was analysed using automated gel electrophoresis using Genomic DNA KIT (4150 TapeStation System, Agilent, Santa Clara, CA, United States).

Sequencing: Products were analysed by next-generation sequencing (NGS) using the Illumina platform MiSeq Dx. NGS was performed using a MiSeq Reagent Micro Kit v2 (300-cycle) (Illumina, San Diego, CA, USA). Indexed DNA library concentrations were quantified using a fluorometric method QuantiFluo ONE dsDNA Kit (Quantus® Fluorometer (Promega, Madison, WI, USA) and normalised to 4 nM using Low TE and pool to 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 0.2 N NaOH. The library was diluted using pre-chilled HT1 buffer at a final concentration of 20 pM. Finally, the 9 pM library was spiked in 5% of PhiX Control v2 (Illumina, San Diego, CA, USA), which provides quality control for cluster generation, sequencing, and alignment.

Statistical analysis

The clinic-pathological features of the assessed patients were analysed using SPSS software package

(version 22). Continuous variables were expressed as mean ± SD and range, while categorical variables were expressed as percentages.

Results

Clinical features

Patients were categorised by age, sex, histological type, and staging (Table 1).

There was no statistically significant difference between the average age of men and women, which was 43 years. The female : male ratio was 1 : 1.

The study group was dominated by patients with a higher degree of clinical advancement and therefore with a worse prognosis.

Regarding the histological features, adenocarcinoma NOS was the most predominant subtype reported in 100% (p-value < 0.001).

Detected somatic mutations in our data set

Distribution of mutations in all patients was presented in Table 2.

The most common mutations in the studied group of patients were as follows: TP53 – occurred in 16 patients, APC – occurred in 12 patients, KRAS – occurred in 9 patients, NRAS – occurred in 6 patients, PIK3CA – occurred in 3 patients, FBXW7, IDH1, PTEN, GNAS, ATM – occurred in 1 patient. No statistically significant difference was found in the frequency of mutations between women and men, $p > 0.05$. Mutations occurred with similar frequency in both sexes.

The most common mutation, occurring in almost three-quarters of patients, was TP53. Interestingly, 29% of patients had a rare NRAS mutation.

Co-occurrence of mutations

Only 3 patients had a single mutation, which was TP53. Over 86% of patients presented multigene abnormality, where 2 to 5 or more mutations occurred the most commonly: co-occurrence of 2 mutations – 43% of patients, co-occurrence of 3 mutations – 29% of patients, co-occurrence of 4 mutations – 10% of patients, co-occurrence of 5 or more mutations – 5% of patients.

The most common co-occurring mutation was TP53- KRAS-APC.

Distribution of mutations in groups of patients

Distribution of mutations in patients with better and worse prognoses was presented in Table 3.

TP53, APC, KRAS, and NRAS mutations occurred significantly more often in patients with a worse prognosis. IDH1 mutation was found only in patients with a better prognosis. FBXW7, PTEN, BRAF, GNAS, and ATM mutations were found only in patients with a worse prognosis.

Table 1. Clinicopathological features of the studied groups

Parameter	CRC	*N* = 21
Age:		
Median	43	
Range	31–50	
Gender:		
Male		10
Female		11
Histological type adenocarcinoma NOS (100%)		
Grade:		
I		3
IIA		1
IIC		1
IIIA		1
IIIB		6
IIIC		2
IVA		3
IVB		4

Table 2. Distribution of mutations in all patients

Type of mutation	Frequency of mutations
TP53	16
APC	12
KRAS	9
NRAS	6
PIK3CA	3
SMAD4	2
BRAF	2
FBXW7	1
IDH1	1
PTEN	1
GNAS	1
ATM	1

Conclusions

The most common mutation, occurring in almost three-quarters of patients, was TP53. 29% of patients had a rare NRAS mutation. Over 86% of patients presented multigene abnormality. The most common co-occurring mutation was TP53-KRAS-APC, which occurred in patients with a worse prognosis.

Table 3. Distribution of mutations in patients with better and worse prognoses

Type of mutation	Patients with better prognosis (I, IIA)	Patients with worse prognosis (IIIA, IIIB, IIIC, IVA, IVB)
TP53	2	13
APC	3	9
KRAS	1	7
NRAS	1	5
PIK3CA	2	1
FBXW7	0	1
SMAD4	1	1
IDH1	1	0
PTEN	0	1
BRAF	0	2
GNAS	0	1
ATM	0	1

Discussion

CRC is caused by mutations in oncogenes, tumour suppressor genes, and genes related to DNA repair mechanisms [11]. In colorectal cancer, various types of genomic changes, such as point mutations, genomic rearrangements, gene fusions, or chromosomal copy number changes, can initiate and contribute to disease progression [12]. The advent of a new DNA sequencing technology, next-generation sequencing (NGS), has revolutionised the speed and efficiency of cataloguing cancer-related genomic changes. This advanced technology is currently being used to better understand the molecular mechanism underlying colorectal cancer and to detect clinically relevant genetic biomarkers for screening diagnostics and personalised treatment [8, 9].

Personalised medicine is becoming an indispensable tool, and it is necessary to conduct an in-depth analysis of the cancer characteristics in each patient to select the most appropriate treatment.

The aim of the study was to identify common pathogenic somatic mutations in Polish patients under 50 years of age using NGS technology.

In our patients under 50 years of age, the most common pathogenic somatic mutations were located in the TP53, APC, and KRAS genes. Weinberg, Wang, and Chang achieved similar results [13–15].

Over 86% of patients presented multigene abnormality, and the most common co-occurring mutation was TP53-KRAS-APC, which occurred in patients with a worse prognosis.

In our studies, similarly to Hanna's, most of the significantly recurrent mutations were observed in known cancer-related genes, such as *APC, TP53, KRAS, PIK-3CA, FBXW7, SMAD4*, and *NRAS* [16].

The genomic changes associated with colorectal carcinogenesis are more complex than previously assumed, and therefore the complete set of oncogenic factors associated with colorectal carcinogenesis remains to be discovered [17–19].

The progress of CRC genomic analysis can be summarised in 3 aspects: 1) genetic screening, 2) progress in understanding colorectal carcinogenesis, and 3) identification of new types of mutations in CRC genomes [8–10]. A comprehensive collection of somatic genomic alterations associated with CRC will advance the understanding of colorectal carcinogenesis at the pathway level [3].

Conclusions

NGS technology deepens our knowledge of CRC genomes, and the knowledge gained leads to better diagnosis and personalised therapies for the treatment of CRC [8, 9].

The results obtained in our study may constitute the basis for a new generation of genetic screening tests in the age group ≤ 50 years.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest

The authors declare no conflict of interest.

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