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ORIGINAL ARTICLE

Molecular Biological Significance of Cell Cycle Proteins in Colon Cancer Bargained by Gene Mutations And DNA/RNA Viruses

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Abstract

Genetic mutations in BRCA1/2 and hMSH2 genes, as well as viral infections, have been implicated in colon cancer (CC) development and progression.
Aim: To evaluate the molecular characteristics of cell cycle proteins (p53, bcl-2, NF1, pRb1) in colon cancer cases burdened by gene mutations (BRCA1/2, hMSH2) and DNA/RNA viruses.
Methods: A molecular biological and immunological study was performed: cycle proteins NF1, p53, bcl-2, pRb1, viruses (HSV1/2, HHV6, CMV, VEB, HCV, HBV, HVP), BRCA 1/2 genes, hMSH2 gene.
Results: The number of mutations in the BRCA1/2, hMSH2 genes in blood samples from individuals with CC was 2.04%, while the frequency of changes in the hMSH2 gene was 4.17%, which is lower than the frequency of mutations in the same genes in tumor tissue samples – 7.98% (p=0.003). The occurrence of BRCA1/2 gene mutations among women showed their dependence in CC with exons of the hMSH2 gene. Viruses isolated in tumor tissue: HSV 1/2 – 86.8%, HHV6 – 25.0%, CMV – 10.3%, HCV – 4.4%, HBV – 2.94%, HVP – 4, 1%, VEB – 19.1%, mixed inertia – 11.76%. Proteins p53, bcl-2, pRb1, and NF1 in intestinal tissue did not depend on the age and gender of patients.
Conclusion: This study provides compelling evidence for the role of cell cycle proteins as diagnostic markers in colon cancer. The levels of p53, bcl-2, pRb1, and NF1 were significantly elevated in Blood and tumor tissue of colon cancer patients compared to healthy controls.

Keywords: Tumor; Cell cycle; Mutations; Viruses; Colon cancer

1 Introduction

Understanding the molecular mechanisms underlying colon cancer (CC) pathogenesis is essential for the development of targeted therapies and improved patient outcomes. Genetic mutations in BRCA1/2 and hMSH2 genes, as well as viral infections, have been implicated in CC development and progression. How-

ever, the interplay between these factors and their impact on cell cycle protein expression remains poorly understood.

Every year, about a million new cases of colon tumors are diagnosed worldwide, causing death in half of patients. In economically developed countries, colon cancer/colorectal cancer (CC/CRC) are common diseases, in the structure of which the incidence of colorectal cancer is 58.7/100 thousand and 28.8/100 thousand of the population, respectively, and the five-year survival rate – 60% (in countries with limited resources is less than 40%) [1].

According to research results, in Russia, the total annual economic losses due to mortality from cancer have increased from 6.5 billion US dollars in the period from 2001-2005. to 8.1 billion US dollars in 2011-2015, which amounted to 0.24% of the annual gross domestic product (GDP). According to experts, by 2030 this figure will also remain at a high level and amount to 7.5 billion US dollars (0.14% of GDP) [2].

In Belarus, about 50 thousand patients are diagnosed with malignant neoplasms for the first time every year. Although the country is part of a group of countries with relatively low incidence rates of CC, which differ little from neighboring countries, over the past decade the incidence of CC/CRC has tripled [3]. Every year, about 2.5 thousand new cases of colon tumors and 1.9 thousand cases of rectal cancer are detected, 35% of them are diagnosed at stages III and IV. The five-year survival rate of patients, depending on the stage of the disease, varies from 60.6 to 14.5% [4].

The time required for a cell to undergo cell division and the appearance of two daughter cells form the cell cycle. Tumor cells have a shorter life cycle and typically fewer cells are in the resting phase. The exponential growth of the tumor is followed by a "plateau", where the death and formation of daughter cells are approximately at the same level. A subpopulation of cells in a neoplasm often has the properties of stem cells that can enter the proliferation stage. The cellular kinetics of cancer is an important factor: specific oncogenes that regulate the cell cycle may be important for diagnosis, choice of therapy, and determination of disease prognosis [3].

The cause of the development of malignant cells can be DNA breakdowns that change the number or function of protein structures that regulate cell growth, cell division, and DNA repair. Of the approximately 19 million new cancer cases (GLOBOCAN), 15.7% are due to various types of infections. Moreover, the role in infectious carcinogenesis in 51.2-64.2% belongs to various DNA/RNA viruses [4]. Genes involved in normal cell division and DNA repair are important for identifying disrupted growth signals and DNA damage in cells. If genes, as a result of hereditary or acquired mutations, cease to function, the system for detecting DNA damage becomes ineffective, and cells with spontaneous genetic mutations survive and begin to multiply, leading to tumor development [5].

The process of programmed cell death is one of the main mechanisms for the destruction of defective (damaged, mutant, infected) cells [6]. Protein bcl-2 is an anti-apoptotic protein of the mitochondrial apoptosis pathway regulated by p53 [7]. The p53 protein is a product of the tumor suppressor gene TP53 and a transcription factor that performs a suppressor function, which results in cell cycle arrest [8]. The NF1 gene belongs to the group of "cell cycle guardians" and encodes neurofibromin, which regulates the activity of Ras proteins (RAS mutations are found in 15% of human tumors). The pRb1 protein is a regulator of cell proliferation and encodes a protein that regulates the cell cycle by inhibiting DNA replication [9].

The degree of participation of cell cycle proteins in cell division depends on the phase of the cycle, which allows us to consider them as possible diagnostic markers in tumor processes [10].

An analysis of studies in the field of molecular biology of the cell cycle during malignant transformation has shown the contradictory nature of the relationship between cell cycle markers and various mechanisms of oncogenesis, which has become one of the reasons for the establishment of associative relationships between various viruses and mutations of various genes in colon cancer.

The aim of this study is to evaluate the molecular characteristics of cell cycle proteins (p53, bcl-2, NF1, pRb1) in colon cancer (CC) cases burdened by gene mutations (BRCA1/2, hMSH2) and DNA/RNA viruses.

2 Materials and methods

The material for the study was tissue and serum samples from patients with CC: colorectal cancer, colon cancer, rectosigmoid junction cancer. Time to establish diagnosis: from 6 months. up to 13 years old. The age of the subjects at the time of diagnosis ranged from 39 to 87 years; median age (Me) – 61.8±13.7 years, lower quartile (Q25) – 52 years, upper quartile (Q75) – 72 years. The diagnosis of cancer was confirmed by morphological methods (pathomorphological laboratory of the Grodno Regional Clinical Pathological Bureau) in accordance with the International Histological Classification.

Tissue samples (paraffin blocks) from the archives of the Grodno Regional Clinical Pathological Bureau – n=106 and blood samples from patients at the regional oncology clinic (n=53) were studied. The study in samples of intestinal tissue extract was carried out in two zones: in the tumor zone and an area of tissue

with "no criteria for malignancy" – not morphologically described (n=34). The studied samples of native tissue (NT) of the large intestine (not changed, not modified, retaining the structure inherent in a living cell, lack of a malignant process - cancer) were obtained during surgical interventions (n=31). Among the subjects with intestinal tumors, there were 45 women (42.5%) and 61 men (57.5%). The tumor was most often localized in the rectum (54/50.9%), sigmoid colon (8/7.6%), transverse colon (7/6.6%), cecum, and hepatic flexure of the colon (37/34, 9%). The distribution of patients with RTC was carried out in accordance with the TNM International Clinical Classification: T3 – 52.8% (n=56); T4 – 23.6% (n=25), T2 – 16.0% (n=17), T1 – 7.6% (n=8). At the time of diagnosis, 31.4% (n=33) of patients had metastases to regional lymph nodes (N1), and 9.4% (n=10) had distant metastases (M1). The incidence of low-grade tumors (well-differentiated, G1) was 70.8% (75 people), moderate-grade tumors (poorly differentiated, G2) - 17.9% (19 people), high-grade tumors (undifferentiated, G3) – 11.3% (12 people).

The control group was represented by blood samples from 80 practically healthy individuals with no malignant neoplasms and markers of viral infections at the time of examination, who had no relatives with cancer pathology: 46 men (57.5%) and 34 women (42.5%), average age 56.5±8.3 years (minimum 42 years, maximum 68 years).

Groups were identified for the study: group 1 – patients with an established diagnosis of cancer (p1, n=53, study material – blood serum); Group 2 – patients with an established diagnosis of cancer (p2, n=106, study material – tissue); group 3 – samples of native tissue (NT) of the large intestine (p5, n=31), group 4 – control group (pk, n=80, study material – blood serum of practically healthy individuals).

The object of the study were antibodies to proteins NF1, p53, bcl-2, pRb1, DNA/RNA of the genome of the Epstein-Barr virus (EBV, HHV4), hepatitis B (HBV), hepatitis C (HCV), human papilloma (HPV), cytomegalovirus (CMV), herpes simplex type 1/2 (HSV 1/2), herpes virus type 6 (HHV6), BRCA1 gene (exon 2, exon 5, exon 11, exon 20), BRCA2 gene (exon 11) and hMSH2 gene (exon 1-16).

The study of determining the concentration of antibodies to the NF1, p53, bcl-2, and pRB gene proteins was performed by enzyme-linked immunosorbent assay (ELISA) in tissue and serum samples of patients using the FineTest reagent kit produced by Wuhan Fine Biological Technology Co. Ltd" (China) on the enzyme immunoassay analyzer "Mindray 96RA" (China). Serial sections were prepared from tissue blocks in paraffin. In accordance with the standard protocol, tissue samples were prepared for testing using a set of reagents produced by MagneSil Genomic, Fixed System (Promega, USA). Samples of biological material (blood serum) were obtained in a standard way using vacuum systems with a coagulation activator produced by Greiner Bio-One, Austria. The preparation of blood samples for research was carried out in a standardized way: centrifugation (Fenox-24M centrifuge, China) at 3000g for 10 minutes. Serum samples were collected into separate systems in which the study was performed.

476 studies of the DNA/RNA genome of viruses were completed: EBV (HHV4), HBV, HCV, HPV, CMV, hHSV 1/2, and HHV6. A molecular biological study of the BRCA1 (exon 2, 5, 11, 20), BRCA2 (exon 11), and MSH2 (exon 1-16) genes was performed in 143 cases (analysis of 1077 detections).

Isolation of DNA/RNA (PCR) from tissue samples was carried out according to the manufacturer's instructions: test system "Applied Biosystems" (USA), "MagneSil Genomic, Fixed System" (Promega, USA)" (USA), "QIAamp DNA Blood Mini Kit", Qiagen (Germany). Genomic DNA/RNA from blood samples was isolated using DNA/RNA Sorb V kits (Russia) in accordance with the instructions. Amplification of gene exons was carried out using kits from Pronto Diagnostics Ltd. (Israel) for detection of the BRCA gene (exon 5, exon 2 – 185delAG), exon 11 – 4145delA, exon 20 – 5382insC in the BRCA1 gene; exon 11 – 6174delT in the BRCA2 gene) and detection of exons of the hMSH2 gene (MLPA Tests: HNPCC - deletion/duplication - Diagnosis of Hereditary Nonpolyposis Colorectal Cancer (HNPCC), Pronto Diagnostics Ltd. Amplification of DNA/RNA viruses was carried out according to a given protocol in automatic mode using a thermal cycler "RotorGene" (Germany), test systems "Amplisens" (Russia), additional control of the quantitative and qualitative characteristics of DNA/RNA - using a spectrophotometer "BioPhotometer Plus" (Eppendorf, Germany). Statistical data processing was carried out using the standard package of applied statistical programs SPSS. The difference between the studied parameters was considered significant at $p < 0.05$. Among the methods of mathematical processing, the following were utilized: studying the distribution type and obtaining numerical characteristics, including the calculation of mean (M), standard deviation, the 75th percentile (Q75), the median , and the median absolute deviation (Me); identification of response to influence in a two-sample task, including t-tests, Mann-Whitney U test, and Wilcoxon test (Z); Heal method; ROC analysis (Area under ROC curve – AUC); multiple linear regression models; Pearson correlation coefficient (r), and Spearman non-parametric correlation analysis (R).

3 Results

Established concentrations of antibodies to the receptors of the cell cycle proteins p53, bcl-2, pRb1, and NF1 in blood serum samples from practically healthy individuals in the population, in blood samples and tissue extracts of individuals with diagnosed CC and native tissue samples are presented in Table 1.

The established concentrations of antibodies to the proteins p53, bcl-2, pRb1, and NF1 in samples of native tissue (colon tissue of individuals in the absence of morphologically proven CC) were assessed as reference values, which were for cell cycle proteins: p53 - 23.91±8.39 ng/ml; bcl-2 – 35.04±10.14 ng/ml; pRb1 – 4.58±0.58 ng/ml; NF1 – 4397.84±664.48 pg/ml. It is worth noting that in our previous study, which examined native tissue samples, similar results were obtained. The level of protein concentrations p53, bcl-2, pRb1, and NF1 in blood serum samples and tissue extract samples of individuals with CC were significantly higher than in the blood serum of practically healthy individuals (Table 2). Thus, a correlation can be traced between the expression indicator of the pRb1 protein, which has the function of preventing the progression of excessive cell growth by inhibiting the cell cycle, from the data of the control group to the indicators of the apoptosis regulator bcl-2, the transcription factor p53 and the "guardian of the cell cycle" NF1, as in blood serum patients with cancer, as well as in tumor tissue samples. Noteworthy is the lack of correlation between cell cycle proteins in native tissue samples and in blood serum samples from the control group, which can be regarded as the presence of control of "biochemical switches" and transitions between different phases of the cell cycle: switches support the orderly development of the cell cycle and probably act, as checkpoints to ensure the correct completion of each phase before moving on to the next phase. Among individuals in the control group who had no relatives with cancer, the incidence of mutations in the BRCA1, BRCA2 and hMSH2 genes was 1.25% for BRCA1 (exon 20) and 1.25% for hMSH2 (exon 11). No BRCA2 gene mutations were found in controls.

The occurrence of mutations in the studied genes in tissue and blood samples of persons with an established diagnosis of CC is presented in Table 3.

Table 1: Concentrations of antibodies to receptors of cell cycle proteins p53, bcl-2, pRb1, and NF1 in blood serum and tissue samples from patients with CC and control.

Group	Indicator (sample)	M	m	Min	Max	P value
Healthy (p_K)	p53, ng/mL	2,54	2,18	0,90	17,30	p_K-1=0,073
	bcl-2, ng/mL	8,01	6,11	1,01	30,90	p_K-2=0,000001
	pRb1, ng/mL	0,31	0,09	0,18	0,51	p_K-3=0,00001
	NF1, pg/mL	1077,86	132,47	840,00	1285,00	p_K-1=0,0000001
Group 1 – CCserum (p1)	p53, ng/mL	4,99	4,13	0,85	21,05	p_K-2=0,0000001
	bcl-2, ng/mL	36,19	14,42	14,60	70,80	p_K-3=0,0000001
	pRb1, ng/mL	4,14	3,83	0,330	22,20	p_K-1=0,000934
	NF1, pg/mL	3836,62	3573,95	655,00	15155,00	p_K-2=0,0000001
Group 2 – CCtissue (p2)	p53, ng/mL	26,08	14,98	4,25	74,00	p_K-3=0,0000001
	bcl-2, ng/mL	32,69	11,36	4,90	46,01	p_K-1=0,000131
	pRb1, ng/mL	7,36	1,96	4,50	14,50	p_K-2=0,0000001
	NF1, pg/mL	4093,40	942,64	3350,00	6750,00	p_K-3=0,000001
Group 3 – NT (p3)	p53, ng/mL	23,91	8,39	8,90	36,50	p1-2=0,000001
	bcl-2, ng/mL	35,04	10,14	13,20	49,00	p1-3=0,000001
	pRb1, ng/mL	4,58	0,58	3,40	6,80	p1-2=0,0052
						p2-3=0,000001

Note: NT – native tissue, unaltered tissue sample; unmodified, retaining the structure inherent in a living cell, no colon cancer; M: mean; m: median; min: minimum; max: maximum. Statistical analysis was conducted using the Mann-Whitney U test and Wilcoxon test (Z) for p-values <0.05

Table 2: Correlation Analysis of Cell Cycle Proteins in Blood and Tissue Samples.

Protein	Sample Type	Correlated Protein	Correlation Coefficient (r)	p-value
p53	blood	NF1	0.680	0.0001
bcl-2	blood	pRb1	0.395	0.006
pRb1	blood	bcl-2	0.395	0.006
pRb1	control	bcl-2 (tissue)	-0.385	0.003
pRb1	control	p53 (tissue)	-0.432	0.0021
NF1	blood	pRb1 (tissue)	-0.418	0.002
p53	tissue	bcl-2 (tissue)	0.469	0.003
p53	tissue	pRb1 (control)	-0.432	0.002
bcl-2	tissue	pRb1 (control)	-0.385	0.002
pRb1	tissue	NF1 (serum)	-0.418	0.002

Note: The Spearman and Kendall analyses were utilized

Table 3: Frequency of detection of BRCA1/2 and hMSH2 gene mutations in tissue and blood samples from individuals with CC.

Gene , exon	n	Mutations, tissue		Mutations, blood	
		quantity	%	quantity	%
BRCA 1 exon 5	129	1*	0,77	0**	0,00
BRCA exon 2	129	2*	1,55	0**	0,00
BRCA 1 exon 20	129	2*	1,55	2**	1,55
BRCA 1 exon 11	129	1*	0,77	1**	0,78
BRCA 2 exon 11	129	2*	1,55	1**	0,78
hMSH2, exon 1	27	8	29,6	1	3,70
hMSH2, exon 2	27	6	22,2	0	0,00
hMSH2, exon 3	27	4	14,8	0	0,00
hMSH2, exon 4	27	0	0,0	0	0,00
hMSH2, exon 5	27	0	0,0	0	0,00
hMSH2, exon 6	27	14	51,9	4	14,81
hMSH2, exon 7	27	4	14,8	0	0,00
hMSH2, exon 8	27	10	37,0	1	3,70
hMSH2, exon 9	27	17	63,0	0	0,00
hMSH2, exon 10	27	4	14,8	3	11,11
hMSH2, exon 11	27	8	29,6	8	29,63
hMSH2, exon 12	27	1	3,7	1	3,70
hMSH2, exon 13	27	0	0,0	0	0,00
hMSH2, exon 14	27	2	7,4	0	0,00
hMSH2, exon 15	27	0	0,0	0	0,00
hMSH2, exon 16	27	0	0,0	0	0,00

Note:* - tissue samples of female persons (n=28 – female persons, of the total number of samples studied).

The total number of mutations in tissue samples from CC was 86/7.98% of the total number of studies (n=1077). An assessment of 432 studies of the hMSH2 gene (1-16 exons) in tissue samples from RTC showed that the incidence of mutations was 78/18.05%. The number of changes in the hMSH2 gene varied: the greatest in exon 9 (17/63%), followed by exon 6 (14/51.9%), exon 8 (10/37%), exon 1 and exon 11 (8/29 each, 6%), exon 2 (6/22.2%), exon 3 and exon 10 (4/14.8% each), exon 14 (2/7.4%), exon 12 (1/3.7%). No changes were found in other studied exons of the gene.

Mutations of the BRCA1 and BRCA2 genes in tissue samples from patients with cancer were detected with a frequency of 0.77% (BRCA1: exon 5 and 11) to 1.55% (BRCA1: exon 2 and 20; BRCA2: exon 11) and only in females (n=8). The proportion of detected mutations from all studied samples of individuals diagnosed with cancer was 1.24% of the total number of studies (n=645) or 28.5% of the studied samples from female individuals diagnosed with cancer (n=28).

The total number of mutations in blood samples from CC was 22/2.04% of the total number of studies (n=1077). When assessing genetic changes in blood samples from individuals diagnosed with cancer, it was found that mutations in the exons of the hMSH2 gene were found in 18 cases (4.17%): exon 11 of the gene (8/29.63%), exon 6 (4/14, 81%), exon 10 (3/11.11%), exon 1, exon 8 and exon 12 (1/3.7% each). No changes were detected in other exons of the hMSH2 gene studied.

Analysis of detected mutations of the hMSH2 gene in the blood, in comparison with tissue samples, showed that the number of changes in the hMSH2 gene in tissue samples with CC is higher than in the blood: 86/7.98% and 22/2.04% ($p<0.05$).

When conducting an exploratory and correlation analysis of the occurrence of the studied mutations in tumor tissue along the exons of the hMSH2 gene, it was established that the component of exon 10, exon 11 and exon 12 with the identified mutational characteristics in the blood (the probabilistic hereditary nature of the changes) provides variation in the tissue (Table 4).

In all cases, in tumor tissue samples, exon 6 of the hMSH2 gene was isolated as the main component of the tumor process (power – 0.921). Multiple regression analysis of the predicted value of the age of probable development of CC in carriers of the hMSH2 gene mutation (exon 6) was established for 58.5 ± 3.7 years (–95.0% IS = 50.7 years; +95.0% IS = 66, 3 years; t=15.538; p=0.00001). In tissue samples from patients diagnosed with cancer, BRCA1/2 gene mutations were detected with a frequency of 0.78% to 1.55% and only in 4 females (Table 3). The proportion of detected mutations from all studied samples of individuals diagnosed with cancer was 0.62% of the total number of studies (n=645), or 14.24% of the studied samples of female individuals diagnosed with cancer (n=28).

Mutations of the BRCA1 and BRCA2 genes in the blood of women were found in 4/3.1% of cases of all studied samples of persons diagnosed with CC.

Analysis of the occurrence of BRCA1/2 gene mutations in colon cancer among women showed significant rank correlations with the hMSH2 gene: exon 11 of the BRCA2 gene and exon 5 of the BRCA1 gene (rs=0.705; p<0.05), exon 2 of the BRCA1 gene (rs=0.493 ; p<0.05) and exon 14 of the hMSH2 gene and exon 2 of the BRCA1 gene (rs=0.460; rs=0.461; p<0.05).

As the main component of the tumor, in women with CC, the value of gene mutation is distributed according to power (p<0.05): 1st power (0.932) – exon 11 of the BRCA2 gene; 2nd (0.627) – exon 5 of the BRCA1 gene; 3rd (0.295) – exon 2 of the BRCA1 gene. The occurrence of DNA/RNA viruses in tissue samples of persons with an established diagnosis of RTC is presented in Table 5.

The mixed persistence of viruses during cancer in tumor tissue was represented by 8 combinations: 1 case each in men (EBV + HHV6; HSV type 1/2 + EBV; HSV type 1/2 + HHV6; HCV + HHV6); 3 cases of CMV+EBV combination (2 men, 1 woman); 1 case in a man CMV+EBV+HHV6. A number of reliable relationships with DNA/RNA viruses have been established between genetic changes in tumor tissue and blood samples of persons with CC (Table 6).

Taking into account the traceable relationship between the expression of the studied cell cycle proteins (healthy-tumor) and the estimated occurrence of mutations of the BRCA1/2, hMSH2 and DNA/RNA viruses in the studied groups, an analysis of possible dependencies between the level of expression of the proteins of the p53, bcl-2, pRb1 genes was performed, NF1, gene mutations and DNA/RNA viruses. Analysis of viruses and cell cycle proteins in individuals diagnosed with cancer revealed significant correlations between them: hepatitis B and p53 (blood serum) – r=0.364, p=0.003; EBV and NF1 (tissue) – r=0.371, p=0.003.

This fact attracts attention in persons with CMV carriage, in which EBV is almost always present in the body, affecting the concentration level of "cell cycle guardians" - NF1 (p = 0.003). At the same time, in patients with CC infected with hepatitis viruses (HBV, HCV), mixed persistence of HPV and HHV6 and increased activity of the cell cycle regulating protein p53 (p = 0.003) were noted. An assessment of the correlations between the levels of p53, bcl-2, pRb1 and NF1 and mutations of the BRCA1, BRCA2, and hMSH2 genes (exon 1-16) also revealed significant relationships: BRCA2 gene (exon 11) and p53 (control) – r=0.448, p=0.002; hMSH2 gene (exon 1) and p53 (CC, tissue) – r=0.453, p=0.002; hMSH2 gene (exon 3) and p53 (CC, tissue) – r=0.388, p=0.002; hMSH2 gene (exon 8) and p53 (CC, tissue) – r=0.404, p=0.001; hMSH2 gene (exon 14) and bcl-2 (control) – r=-0.417, p=0.002; hMSH2 gene (exon 14) and pRb1 (control): r=-0.428, p=0.002.

As in the case of viral DNA/RNA, positive and negative correlations in groups are shown. The BRCA2 genes (exon 11) and the hMSH2 gene (exon 1, 3, 8, 14) drew attention to themselves, when the presence of mutations in them promotes an increase in the expression of cell cycle proteins p53, bcl-2 and pRb1, as in the control group (practically healthy face, p=0.002), and among persons with CC (tissue, p=0.001). The presence of mutations in the hMSH2 gene was accompanied by the expression of pRb1 and bcl-2 proteins in healthy individuals (p=0.002). The obtained result of the expression of cell cycle proteins in practically healthy individuals in the population and individuals with CC (0.77% in carriers of the BRCA2 exon 11 gene mutation; 18.6% in carriers of the hMSH2 exon 1,2,8,14 gene mutation) indicates the need to include "healthy » persons who carry mutations of these genes are at a possible risk of developing cancer.

Next, the dependence of the influence of the expression level of proteins p53, bcl-2, pRb1, and NF1 in the studied samples on the gender and age of patients in the study groups was assessed. Dependent expression of p53, bcl-2, pRb1, and NF1 in the extract of tumor-affected colon tissue, blood serum samples from individuals with CC and native tissue with the gender of patients was not established (Spearman), p>0.05: p53 (healthy), r= -0.045; bcl-2 (healthy), r=-0.027; pRb1 (healthy), r=0.349; NF1 (healthy), r=-0.217; p53 (CC, tissue), r=-0.195; bcl-2 (CC, tissue), r=-0.164; pRb1 (CC, tissue), r=0.164; NF1 (CC, tissue), r=0.180; p53 (NT), r=0.203; bcl-2 (NT), r=0.170; pRb1 (NT), r=0.171; NF1 (NT), r=0.063; p53 (CC, serum), r=-0.251; bcl-2 (CC, serum), r=-0.020; pRb1 (CC, serum), r=-0.041; NF1 (CC, serum), r=-0.056.

A similar pattern of expression of cell cycle proteins p53, bcl-2, pRb1, and NF1 was observed in the extract of tumor-affected colon tissue, blood serum samples from individuals with CC and native tissue with the age of patients - no reliable correlations were established (Spearman, Kendall's Tau correlation), p>0.05: p53 (healthy), r=-0.157; bcl-2 (healthy), r=0.267; pRb1 (healthy), r=0.103; NF1 (healthy), r=0.122; p53 (CC, tissue), r=0.169; bcl-2 (CC, tissue), r=0.109; pRb1 (CC, tissue), r=0.074; NF1 (CC, tissue), r=-0.208; p53 (NT), r=-0.294; bcl-2 (NT), r=0.115; NF1 (NT), r=0.113; p53 (CC, serum), r=0.020; bcl-2 (CC, serum), r=-0.172; pRb1 (CC, serum), r=0.126; NF1 (CC, serum), r=0.026.

The dependence on the age of patients was established only for the protein of the pRb1 gene in samples of native tissue, when protein expression is observed at a younger age: r = -0.358; p=0.002. When analyzing the levels of protein concentrations in samples of

blood serum and tumor tissue with 100% confirmation of the diagnosis according to ICD-10, no significant relationship was established with either age (Median Test, p=0.303) or gender (Median, Test, p=0.322) , Table 7.

There were no significant correlations between the level of expression of cell cycle proteins in the extract of tumor-affected colon tissue and the age of the patients (Spearman R=0.101, p=0.44). There was also no connection between the expression of the studied proteins in the tissue and the gender of patients with CC (Mann - Whitney U Test - p = 0.711).

Based on the analysis of the dependence of the proteins p53, bcl-2, pRb1 and NF1 in tissue samples of CC, NT and blood serum of the examined patients, it can be argued that their concentration in the tissue does not depend on the location of the tumor, age and gender of the patients. The assessed dependencies also allow us to assert that there is no influence of age and gender on cell cycle parameters (NF1, p53, bcl-2, and pRb1) in individuals diagnosed with cancer, with the exception of the pRb1 protein in native tissue in young people. Since the concentration level of cell cycle proteins NF1, p53, bcl-2 and pR1 in tissue and blood serum samples does not correlate with the gender and age of patients, it is advisable to determine cell cycle proteins in non-tumor tissue and blood serum regardless of gender and age. These indicators can be regarded as diagnostic markers of possible tumor pathology and the potential risk of developing the disease.

The results obtained were assessed for their consistency with generally accepted diagnostic tumor markers. To quantify the information content, a comparative analysis of the areas under the ROC curves was used. It is generally accepted that the curve area coefficient lying in the range of 0.9-1 should be considered as an indicator of the highest information content of the diagnostic method; in the range of 0.8-0.9 – good information content; in the range of 0.7-0.8 – satisfactory , in the range of 0.6-0.7 - mediocre, and below - useless classification. ROC analysis allows you to select the optimal cut-off value and evaluate the predictive ability of the study.

ROC curves or error curves were constructed for the dependence of the proportion of correct positive classifications on the proportion of false positive classifications when varying the threshold of the decision rule in CC and healthy individuals in the population (Figure 1).

Table 4: Correlation analysis of exon combinations in blood and tissue mutations

Analysis	Exon Combination	Correlation Coefficient (r)	p-value
Analysis of Mutations in Blood	Exons 11 and 3	0.414	<0.05
	Exons 11 and 6	0.463	<0.05
	Exons 11 and 10	0.414	<0.05
	Exons 10 and 6	0.402	<0.05
	Exons 10 and 12	0.470	<0.05
	Exons 12 and 14	0.693	<0.05
	Exon 1 with exon 3	0.643	<0.05
Analysis of Mutations in Tissue	Exon 1 with exon 7	0.529	<0.05
	Exon 6 with exon 7	0.402	<0.05
	Exon 6 with exon 8	0.586	<0.05
	Exon 6 with exon 9	0.489	<0.05
	Exon 8 with exons 6	0.586	<0.05
	Exon 8 with exons 9	0.429	<0.05

Note: Pearson correlation coefficient (r) and Spearman non-parametric correlation analysis (R).

Table 5: Correlation analysis of exon combinations in blood and tissue mutations

Viruses	Total	Female		Male		Total,%
		n	%	n	%	
HPC	68	0	0	0	0	0
HSV 1/2	68	48	70,6	11	16,2	86,8
CMV	68	4	5,9	3	4,4	10,3
EBV	68	3	4,4	10	14,7	19,1
HBV	68	1	1,47	1	1,47	2,94
HCV	68	0	0	3	4,4	4,4
HHV6	68	3	4,4	14	20,6	25,0
Mixed-persistence	8	1	12,5	7	87,5	11,76*

Note:* - regarding all tissue samples with CC

Table 6: Analysis of Relationships Between Cell Cycle Protein Expression, Gene Mutations, and DNA/RNA Viruses in Colorectal Cancer Patients

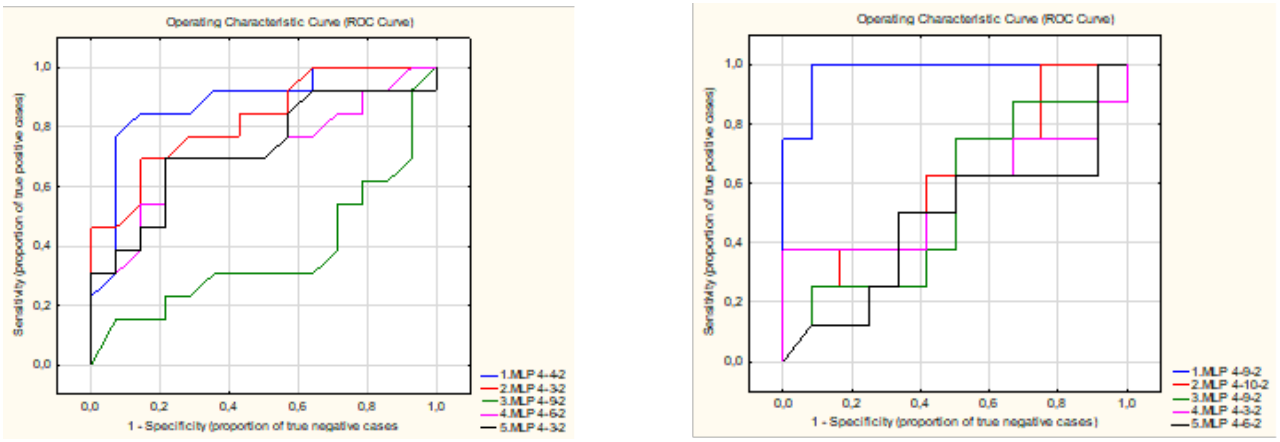
Genetic Mutations and DNA/RNA Viruses in Tumor Tissue	Correlation (r)	p-value
BRCA1 gene exon 5 mutations: HSV types 1/2	0.265	<0.05
BRCA1 gene exon 2 mutations: HPV16/18 types	0.702	<0.05
BRCA1 gene exon 2 mutations: HPV31,33,35,39,45,51,52,56,58,59 types	0.486	<0.05
BRCA1 gene exon 20 mutations: HHV6 type	0.292	<0.05
BRCA2 gene exon 11 mutations: HPV16/18 types	0.702	<0.05
BRCA2 gene exon 11 mutations: HPV31,33,35,39,45,51,52,56,58,59 types	0.486	<0.05
hMSH2 gene exon 10 mutations: HBV	0.470	<0.05
Presence of EBV: CMV	0.454	<0.05
Presence of HHV6: HCV	0.419	<0.05
Presence of HCV: HPV31,33,35,39,45,51,52,56,58,59 types	0.327	<0.05

Note: Pearson correlation coefficient (r) and Spearman non-parametric correlation analysis (R)

Table 7: Correlations between the expression level of cell cycle proteins p53, bcl-2, pRb1 and NF1 and age, $p > 0.05$

Age / Gender	Age, gender	bcl-2, ng/ml	pRb1, ng/ml	NF1, pg/ml
Age, gender	1,000	0,286	-0,214	-0,021
bcl-2, ng/ml	0,186	1,000	-0,325	0,068
pRb1, ng/ml	-0,214	-0,324	1,000	-0,208
NF1, pg/ml	-0,012	0,079	-0,188	1,000

Note: Pearson correlation coefficient (r) and Spearman non-parametric correlation analysis (R)



(a) **(b)**

Figure 1: ROC curves of the dependence of the proportion of correct positive classifications on the proportion of false positive classifications of the level of cell cycle proteins p53, bcl-2, pRb1, and NF1 in colon cancer (a) and in apparently healthy individuals in the population (b).

It was established that the coefficient of the area under the curve for cell cycle proteins: A. for CC:

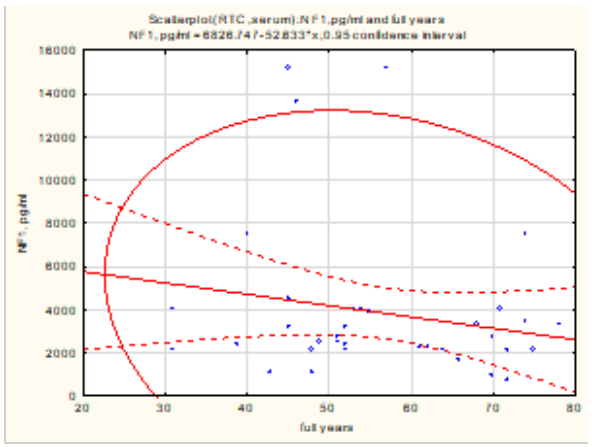
1. p53: sensitivity – 66.8%, specificity – 48.0% (mediocre);
2. bcl-2: sensitivity – 82.7%, specificity – 45.0% (good);
3. pRb1: sensitivity – 88.2%, specificity – 49.3% (good);
4. NF1: sensitivity – 71.4%, specificity – 71.4% (satisfactory).

B. in practically healthy individuals in the population:

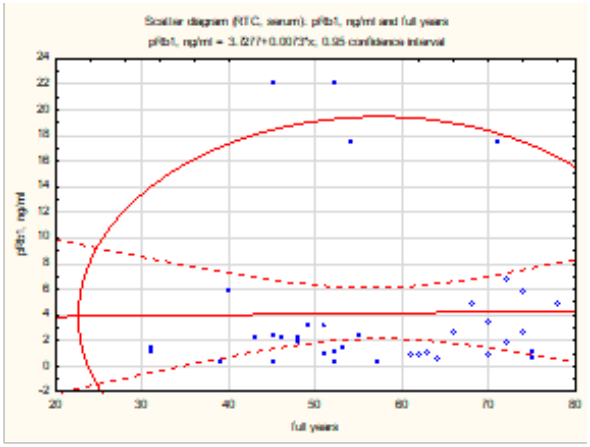
1. p53: sensitivity – 55.2%, specificity – 40.0% (useless);
2. bcl-2: sensitivity – 61.9%, specificity – 39.9% (mediocre);
3. pRb1: sensitivity – 75.5%, specificity – 74.9% (satisfactory);
4. NF1: sensitivity – 72.4%, specificity – 50.1% (satisfactory).

Thus, the established diagnostic informativeness of the cell cycle proteins p53, bcl-2, pRb1 and NF1, as markers of possible tumor pathology and the potential risk of developing the disease, was: for cancer - pRb1 / bcl-2 / NF1 – good / satisfactory, in practically healthy individuals in the population – pRb1 / NF1 – satisfactory. Based on the results of multiple regression of cell cycle proteins, taking into account the age groups of individuals with an established diagnosis of cancer, the predicted value of their concentration for individuals with malignant processes in the large intestine was estimated (Figure 2).

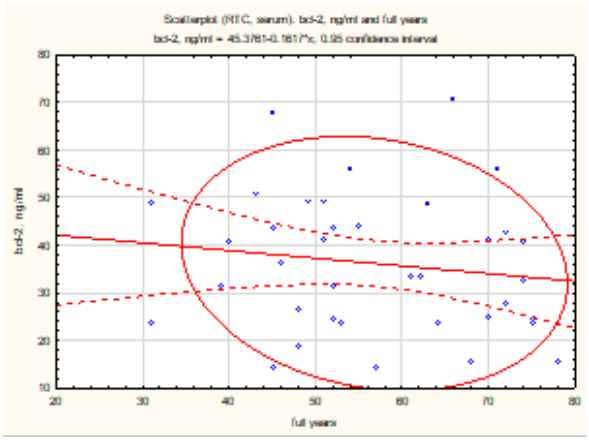
The predicted value of NF1 cell cycle protein concentrations for individuals with CC in the blood serum was: for the age group of 52 years - 4,089.83 pg/ml; for the age group of 72 years – 3,037.17 pg/ml, t=2.57, p=0.01. The predicted value of cell cycle protein pRb1 concentrations for individuals with CC in the blood serum was: for the age group of 52 years – 4.11 ng/ml; for the age group of 72 years – 4.25 ng/ml, t=1.84, p=0.04. The predicted value of cell cycle protein bcl-2 concentrations for individuals with CC in the blood serum was: for the age group of 52 years – 36.96 ng/ml; for the age group of 72 years – 33.73 ng/ml, t=4.202, p=0.0002. The predicted value of cell cycle protein p53 concentrations for individuals with CC in the blood serum was: for the age group of 52 years – 5.24 ng/ml; for the age group of 72 years – 4.20 ng/ml, t=1.720, p=0.05.



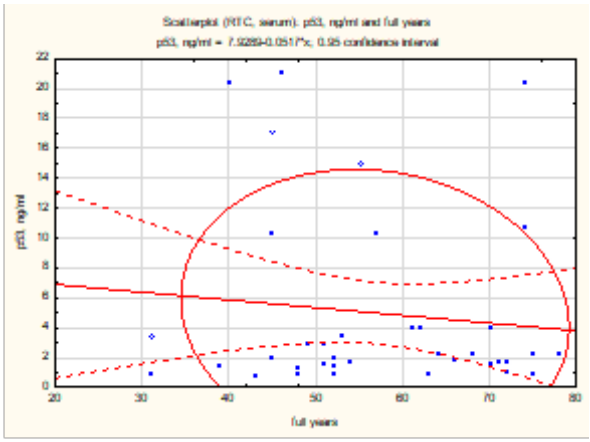
(a) NF1



(b) pRb1



(c) bcl-2



(d) p53

Figure 2: The predicted value of cell cycle protein concentrations for individuals with malignant CC processes in blood serum.

4 Discussion

Among gastrointestinal tumors, the incidence and mortality of colon cancer remain high. In economically developed countries, colon cancer/colorectal cancer (CC/CRC) are common diseases, in the structure of which the incidence of colorectal cancer is 58.7/100 thousand and 28.8/100 thousand of the population, respectively, and the five-year survival rate – 60% (in countries with limited resources is less than 40%).

In Belarus, about 50 thousand patients are diagnosed with malignant neoplasms for the first time every year. Although the country is part of a group of countries with relatively low incidence rates of CRC, which differ little from neighboring countries, over the past decade the incidence of CRC/CRC has tripled . Every year, about 2.5 thousand new cases of colon tumors and 1.9 thousand cases of rectal cancer are detected, 35% of them are diagnosed at stages III and IV. The five-year survival rate of patients, depending on the stage of the disease, varies from 60.6 to 14.5% [4].

When studying the etiopathogenesis of colon cancer, an important role is played by chronic infections (viruses) and genes associated with a high risk of cancer development (BRCA1, BRCA2, hMSH2 and hMLH1), which increase the risk of cancer development by 10 times. significant role. Cell cycle proteins during malignant transformation have a significant relationship with various mechanisms of oncogenesis.

The results of the study confirmed the role of cell cycle proteins p53, bcl-2, pRb1 and NF1 as diagnostic markers in the process of clarifying the diagnosis of cancer. The established limits for the concentration of antibodies to p53, bcl-2, pRb1 and NF1 in the blood serum of healthy individuals differed significantly from similar indicators in patients with CC. The degree of influence on the function of genes presents in the genome of DNA/RNA viruses and mutations (BRCA 1/2, hMSH2) suggested different degrees of oncogenicity of viruses and the hereditary nature of the disease.

The number of mutations in the BRCA1/2, hMSH2 genes in blood samples from individuals with CC is 2.04%, while the frequency of changes in the hMSH2 gene (exon 1-16) is 4.17%, which is significantly lower than the frequency of detected mutations in the same genes in tumor tissue samples – 7.98% (p=0.003), with the frequency of genetic changes in the hMSH2 gene (exon 1-16) equal to 18.05% (p=0.001).

The occurrence of BRCA1/2 gene mutations among women showed their dependence in CC with exons of the hMSH2 gene ($p<0.05$): the presence of mutations in the BRCA1/2 genes (BRCA2 exon 11, BRCA1 exon 5, exon 2) in women should be considered as potential risk of developing RTC. In all cases of detection of mutations in tumor tissue samples, exon 6 of the hMSH2 gene was isolated as the main component of the tumor process (power - 0.921). The predicted age of probable development of CC in carriers of the hMSH2 gene mutation (exon 6) was 58.5±3.7 years (p=0.00001).

Expression of cell cycle proteins in practically healthy individuals in the population and individuals with CC (0.77% in carriers of the BRCA2 gene mutation exon 11; 18.6% in carriers of the hMSH2 gene mutation exon 1,2,8,14) indicates the need to include “healthy" individuals who carry mutations of these genes are at risk of developing colon cancer.

Evaluation of p53, bcl-2, pRb1, and NF1 as diagnostic markers of possible tumor pathology and potential risk of disease development made it possible to establish their informativeness, as markers of possible tumor pathology and the potential risk of developing the disease, was: for CC - pRb1 / bcl-2 / NF1 - good/satisfactory, for practically healthy individuals in the population – pRb1 / NF1 – satisfactory.

Associations of cell cycle proteins, the degree of their influence on the regulatory function of genes and DNA damage, taking into account DNA/RNA viruses and mutations present in the genome (BRCA 1/2, hMSH2), suggest different degrees of oncogenic danger of viruses: high oncogenic risk - CMV/HSV 1/2 (in the presence of a BRCA 1 gene mutation), EBV (in case of CMV infection), average oncogenic risk - HBV/HCV (in the presence of a BRCA 1/2 gene mutation in women); HCV (if infected with HHV6).

Analysis of the dependencies of bcl-2, p53, pRb1, and NF1 showed the relationship between the expression of pRb1 proteins and the apoptosis regulator bcl-2, the transcription factor p53 and NF1, both in blood serum samples and in tissue samples. The absence of correlations between proteins in native tissue samples and blood serum samples from the control group can be regarded as the presence of control of “biochemical switches” and transitions between different phases of the cell cycle: switches support the orderly development of the cell cycle and probably act as checkpoints that guarantee correct completion each phase of the cell cycle. Analysis of protein expression of the p53, bcl-2, pRb1 and NF1 genes (serum, tumor tissue) did not establish a significant relationship with either age or gender ($p>0.73$). Determination of their concentration can be used for diagnostic studies, in which clinical examination methods do not reveal signs of a neoplasm.

5 Conclusions

This study provides compelling evidence for the role of cell cycle proteins as diagnostic markers in colon cancer. The levels of p53, bcl-2, pRb1, and NF1 were significantly elevated in Blood and tumor tissue of

colon cancer patients compared to healthy controls. Additionally, mutations in the BRCA1/2 and hMSH2 genes, especially hMSH2 exon 6, were strongly associated with colon cancer risk. Viral DNA/RNA, particularly from high-risk viruses like CMV and HSV, also correlated with perturbations in cell cycle protein expression.

Importantly, the expression of these cell cycle proteins was independent of patient age and gender, supporting their potential as widely applicable diagnostic markers. The high sensitivity and specificity of pRb1 and NF1 further validate their utility for detecting possible precancerous changes. Taken together, these findings underscore the integral involvement of genetic mutations, viral infections, and cell cycle disruptions in colon oncogenesis. They provide a rationale for screening approaches that combine assays for viral load, genetic risk variants, and sentinel cell cycle proteins to enable earlier cancer detection and personalized prevention strategies.

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