STEATMENT

BY PROF. DR. PETRANKA TROYANOVA, DM,

Professor of the scientific specialty "Oncology", Professional field 7.1. Medicine, Sphere of higher education 7. Health and sports,

at the Department of Nuclear Medicine, Radiation Therapy and Medical Oncology, Faculty of Medicine, Medical University - Sofia,

member of the scientific jury according to order № P-109-110 / 11.03.2022 of the Rector of the Medical University "Prof. Dr. Paraskev Stoyanov "- Varna

About: Public defense of the dissertation of Dr. Rostislav Radoslavov Manev for the award of educational and scientific degree "Doctor" in the Sphere of higher education 7. Health and Sports, Professional field 7.1. Medicine, in the scientific specialty "Oncology", with the topic of the dissertation "SINGLE NUCLEOTIDE POLYMORPHISMS IN THE GENES FOR NON-CODING RNA AS DIAGNOSTIC AND PROGNOSTIC MARKERS IN PATIENTS WITH METASTATIC COLORECTAL CANCER"

I. Brief data on the candidate's career development

Dr. Rostislav Manev was born on May 31, 1991. In 2016 he graduated from the Medical University "Prof. Dr. Paraskev Stoyanov "- Varna.

Since 2016 he is a specializing physician at the University Hospital "St. Marina" - Varna in the clinical specialty "Medical Oncology".

From 2017 to this day, Dr. Manev is an assistant at the Department of Propaedeutics of Internal Medicine and the Department of Oncology and conducts practical classes in the discipline "Oncology".

In 2018 by order of the Rector of the Medical University "Prof. Dr. Paraskev Stoyanov "- Varna Dr. Manev is enrolled as a PhD student in full-time education at the Department of Propaedeutics of Internal Medicine (later the Department of Oncology) at the Faculty of Medicine, MU-Varna with supervisors Assoc. Prof. Nikolay Tsonev, Ph.D., and Assoc. Prof. Maria Radanova, Ph.D.

Dr. Manev has a strong affinity for research. He has participated in national and international scientific events. In connection with the developed dissertation, Dr. Manev has presented 11 scientific publications, 8 of which in refereed publications with impact factor.

Dr. Manev's documents meet the legal requirements for the development of the academic staff of Law on the development of the academic staff in the Republic of Bulgarian and the Rules for the development of the academic staff in the Medical University "Prof. Dr. P. Stoyanov "- Varna. They are well arranged and contain sufficient evidence.

II. Relevance of the dissertation

In recent years, the incidence of colorectal cancer (CRC) has increased dramatically, and the development of diagnostic methods, surgical treatment and the application of individual algorithms for antitumor treatment have significantly improved the quality of life and overall survival in patients with this disease.

Over the past three decades, extensive genome-associated studies have examined the importance of single nucleotide polymorphisms (SNPs) as factors that are directly related to the risk of developing solid tumors with different primary sites, including CRC. SNPs are a common genetic variation that can reflect different functional processes affecting individual susceptibility to certain cancers, and thus SNPs can be considered as a biomarker for predicting cancer risk. Single nucleotide polymorphisms in microribonucleic acid (miRNA) genes - miR-SNPs are able to modulate miRNA expression and thus affect the risk of cancer, treatment efficacy and patient prognosis.

Microribonucleic acids (miRNAs) are RNA molecules with a length of about 22 nucleotides that play an important role in various biological processes, such as embryonic development, cell differentiation, proliferation, apoptosis, insulin secretion and oncogenesis and others. MiRNAs do not encode proteins, but have an important function in regulating gene expression. An increasing number of studies show that miRNAs play an important role in the development of cancer by regulating the expression of proto-oncogenes or tumor suppressor genes. Recent studies have shown different levels of miRNA expression in biological products such as tissues and serum in cancer patients and healthy individuals.

Current studies on the potential diagnostic, predictive and prognostic role of miR-SNPs are few in number, and for the Bulgarian population have not been conducted so far. That is why the research on the presence of functional genetic variants in the genes of non-coding RNAs is especially important and makes the developed dissertation extremely interesting and relevant.

III. Characteristics and evaluation of the dissertation

The dissertation contains 127 standard pages and is illustrated with 40 tables and 35 figures. The literature includes 257 literature sources, of which 3 are in Cyrillic and 254 in Latin.

The aim of the study is correctly defined and gives the main directions of the research work - to identify new diagnostic and prognostic molecular biological biomarkers in Bulgarian patients diagnosed with CRC in metastatic stage by examining the presence of five selected single nucleotide polymorphisms (SNPs) in genes. encoding microRNAs - rs7372209 in the microRNA gene-26a-1, rs2910164 in the microRNA gene-146a, rs2682818 in the microRNA gene-618, rs353293 in the promoter region of the microRNA gene cluster -143 and microRNA145 and rs322931 in the microRNA gene-181b.

To achieve this goal, 7 tasks are formulated accurately and clearly:

- To construct and characterize in detail a Bulgarian cohort for testing for the presence of five selected SNPs, selecting patients with CRC in metastatic stage, conducted first-line treatment, to stratify by demographic and clinicopathological indicators, to monitor response to therapy and select and characterize a control group of healthy individuals similar in demographics to patients.
- To investigate the allelic distribution and genotypic frequency of the five selected SNPs in the selected cohort of patients with metastatic colorectal cancer.
- To compare the allelic and genotypic frequencies of the selected five SNPs in the Bulgarian group of healthy individuals with the available data for other cohorts.
- To look for an association between the carrier of a certain genotype / allele of the studied five SNPs in the micro-RNA genes and the ability to predict the risk of developing CRC.
- To look for an association between the carrier of a certain genotype / allele of the five studied SNPs in the micro-RNA genes and the overall survival in the patients.
- To compare the plasma levels of micro-RNAs whose genes contain the tested SNPs, in patients with mRNA and in the healthy control group.
- To look for an association between the plasma expression levels of micro-RNAs whose genes contain the tested SNPs and the overall survival in patients.

In the dissertation work a detailed, analytical literature review is made, covering the most modern developments, which proves the relevance of the topic and the usefulness of its development. So far, the available studies examining the importance of single nucleotide polymorphisms (SNPs) as factors directly related to the risk of developing CRC are relatively few and limited, so new studies are needed to analyze the association between the studied SNPs with CRC.

In the developed dissertation work are applied modern clinical, diagnostic, epidemiological and statistical methods, which are sufficient to solve the tasks to achieve the scientific goal. The study was approved by the Commission on Ethics of Research (KENI) of the Medical University "Prof. Dr. Paraskev Stoyanov "-Varna.

The study included 101 patients with inoperable, metastatic colorectal cancer who received first-line fluoropyrimidine-based chemotherapy or in combination with anti-VEGF or anti-EGFR targeted therapy at the University Hospital "St. Marina" -Varna, staged with CT or PET/CT before of treatment, with adequate laboratory parameters such as PKK, renal and hepatic function and ECOG PS - performance status <or = 2, as well as a control group of 90 and healthy volunteers corresponding to patients by sex and age, without direct relatives with a history of colon cancer and clinical evidence of another disease. Patients with CRC were followed up for 36 months. During follow-up, patients were restated every 3 months (12 weeks) using imaging methods (CT and PET / CT).

Medical records, routine clinical trials and biological markers, as well as applied treatment were analyzed. The main focus of the dissertation is the study for the presence of single nucleotide polymorphisms in the genes for non-coding miRNAs as diagnostic and prognostic biomarkers in selected patients, in connection with which specific research methods are applied such as: ***DNA extraction*** - from patients' plasma and healthy controls using the DNeasy Blood & Tissue Kit; ***SNP genotyping*** - TaqMan® Assays SNP Genotyping (ThermoFisher Scientific) to determine the presence of polymorphisms in the genes for selected microRNAs and PCR analysis (Real Time PCR System, Applied Biosystems, 7500); ***Isolation of RNA*** from plasma for the study of micro-RNAs - used kit Macherey-Nagel, Düren, Germany; ***Examination of the expression levels of circulating miRNAs***, including: ***cDNA synthesis*** - RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, Massachusetts, USA) and ***qPCR reaction*** - quantitative PCR analysis was performed with AmplifyMe SG Universal Mix (AM02, BLIRT, Poland), on the ABI 7500 Real Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA). ***Genetic models*** of inheritance were used to analyze the association of the studied SNPs with CRC (A - variant allele, B - wild type) - Alleles (A / B), Recessive (AA / BB + AB), Dominant (AA + AB / BB)), Codominant (AB / BB; AA / BB), Superdominant (AB / BB + AA) Additive (2.AA + AB / BB).

SNPs studied in the micro-RNA genes:

SNPs	Localization	In genes for micro-RNAs
rs7372209 (T>C)	chr3:37969217	micro- RNA -26a-1
rs2910164 (G>C)	chr5:160485411	micro - RNA -146a
rs2682818 (A>C)	chr12:80935757	micro - RNA -618
rs353293 (G>A)	chr5:149427663	micro - RNA -143/145
rs322931 (G>A)	chr1:199050726	micro - RNA PHK-181b

With statistical software package SPSS for Windows, v.21. An analysis of the data that are potentially important in terms of diagnosis and prognosis of CRC has been performed.

The results of the research are described in detail and precisely and are illustrated with tables and figures, following the set tasks.

On the basis of the received reliable and representative data an in-depth discussion was developed and the respective conclusions were formulated:

1. When comparing healthy individuals from the Bulgarian and other European cohorts, a similar frequency distribution of the studied polymorphisms was found.
2. Heterozygous individuals with rs2910164 - miRNA-146a are characterized by a low risk of developing the disease in the over-dominant genetic model.
3. The polymorphism rs2682818 - miR-618 also appears to be protective against CRC in both heterozygous genetic models - codominant and superdominant.
4. Carriers of the dominant A allele in homozygous state with rs353293 - miRNA-143/145 are characterized by a high risk of CRC.
5. Carriers of the TT genotype with rs7372209 have significantly longer overall survival (OS) than patients with TC and CC genotypes.
6. Patients homozygous for the A allele (AA) of rs353293 - miRNA-143/145 also had a longer overall survival (OS) than patients carrying the G allele.
7. TT rs7372209 genotype was assessed as a risk factor for the development of CRC in the right colon.
8. Plasma studies of miRNA-26a-1, miRNA-146a, miRNA-618, and miRNA-181b are of diagnostic value because they distinguish patients with metastatic CRC from healthy controls.
9. The polymorphism studied for the first time in patients with CRC rs322931 – miR-181b was not associated with disease risk and prognosis.
10. Plasma levels of the six siRNAs studied in patients with metastatic CRC were not affected by carrier of a particular genotype in any of the polymorphisms included in the study.

The presented conclusion is logical and justifies the conclusions of the dissertation. The study compared alleles and frequencies and the genotypic distribution of five selected SNPs (rs7372209, rs2910164, rs2682818, rs353293 and rs322931) in the mRNA genes among a Bulgarian group of healthy individuals with European healthy cohorts. The analysis shows a similar frequency distribution of the studied polymorphisms in the two groups. An association with the risk of CRC development was established for three of the studied polymorphisms in the Bulgarian cohort of patients with metastatic CRC (rs2910164 in the miRNA-146a gene, rs2682818 in the miR-618 gene and rs353293 in the promoter region of the miRNA-143 gene cluster and miRNA-145). For two of the studied polymorphisms, a statistically significant association was found with overall patient survival (TT genotype for rs7372209-miR-26a-1 polymorphism and AA genotype for rs353293 in the promoter region of the miRNA-143 and miRNA-145 gene cluster).

In the course of the study, the level of plasma, in patients and healthy controls, of siRNAs in whose genes the polymorphisms studied in the study were located was assessed. Four of them showed different expression between healthy controls and selected patients with high specificity and sensitivity - miRNA-26a-1, miR-146a, miRNA-618 and miRNA-181b.

This study is the first in Bulgaria to study and prove the association of single nucleotide polymorphisms in genes for non-coding RNAs as potential biomarkers that could predict the risk of developing the disease and have a potential relationship with the prognosis of CRC.

The contributions of the dissertation are significant, with theoretical and applied significance:
1. For the first time in Bulgaria data on the allelic frequency and genotypic distribution of polymorphisms in the genes for non-coding miRNAs among healthy individuals have been obtained.
2. For the first time in Bulgaria, data were obtained on the role of rs2910164 – miR-146a, rs2682818 – miR-618 and rs353293 – miR-143 / miR-145 as potential diagnostic biomarkers for CRC patients.
3. For the first time in Bulgaria, data were obtained on the role of rs7372209 – miR-26a-1 and rs353293 – miR-143 / miR-145 as potential biomarkers of prognostic significance for CRC patients.

4. rs322931 – miR-181b was studied for the first time in patients with CRC.
5. For the first time, data were obtained confirming that the A rs353293 allele in the homozygous state is a risk factor for CRC and in the Caucasian cohort.
6. For the first time, data were obtained confirming that the AC rs2682818 genotype is associated with a low risk of developing CRC in the Caucasian cohort.
7. Association of the TT rs7372209 genotype with a longer mean OS has been reported for the first time in patients with metastatic CRC.
8. For the first time, an association of the AA rs353293 genotype with a longer mean OS has been reported in patients with metastatic CRC.
9. The importance of rs2910164-miRNA-146a as a polymorphism associated with the risk of developing CRC has been confirmed.
10. For the first time, the expression levels of miR-26a-1, miR-618, miR-181b were examined in connection with the presence of a certain polymorphism (rs7372209, rs2682818 and rs322931, respectively).

IV. CONCLUSION

The dissertation work developed by Dr. Rostislav Radoslav Manev "SINGLE NUCLEOTIDE POLYMORPHISMS IN THE GENES FOR NON-CODING RNA AS DIAGNOSTIC AND PROGNOSTIC MARKERS IN PATIENTS WITH METASTATIC COLORECTAL CANCER" covers and exceeds all scientometric requirements of Law on the development of the academic staff in the Republic of Bulgarian and the Rules for the development of the academic staff in the Medical University "Prof. Dr. P. Stoyanov "- Varna for the award of educational and scientific degree "Doctor ".

This, as well as the presented scientific production, are grounds for convincingly giving my positive assessment and recommending to the members of the esteemed scientific jury to award the educational and scientific degree "Doctor" to Dr. Rostislav Radoslavov Manev.

May 4, 2022
Varna

Prepared the statement:
PROF. DR. P. TROYANOVA, DM,