MEDICAL UNIVERSITY

"Prof. Dr. Paraskev Stoyanov" - Varna

DEPARTMENT OF ONCOLOGY

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EXPRESSION OF TUMOR REVERSION MARKERS IN COLORECTAL CANCER

DISSERTATION SUMMARY

FOR THE ACQUSITION OF THE EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR (PhD)"

SUPERVISOR:

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The dissertation contains 117 standard pages and is illustrated with 18 tables and 17 figures. The reference list includes 274 references, 1 in Cyrillic and 273 in Latin.

The dissertation was discussed, approved and directed for defense at the Departmental Council of Oncology of MU-Varna and the decision of the Faculty of Science protocol №..... and according to the order of the Rector of MU-Varna the following scientific jury was elected:

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The public defense of the dissertation will be held on 20.02.2024 at 13:00 in the virtual hall of the electronic platform Webex at MU-Varna at an open meeting of the scientific jury.

Content:

1. Abbreviations used

In Cyrillic:

GIT - gastrointestinal tract

DNA - deoxyribonucleic acid

iRNA - ribonucleic acid information

IHC - immunohistochemistry

CRC - colorectal cancer

RNA - ribonucclic acid

FAP - familial adenomatous polyposis

In Latin:

AJCC - American Joint Committee on Cancer

ASO - antisense oligonucleotide

cAMP-PKA - Cyclic adenosine 3', 5'-monophosphate protein kinase A pathway

CDH1L - Chromodomain helicase/ATPase DNA binding protein 1-like gene

Cdk1 - cyclin-dependent kinase 1

CEA- carginoembryonic antigen

CIMP - CpG Island Methylator Phenotype

CIN - chromosomal instability

CK 7- cytokeratin 7

CK 20 -cytokeratin 20

DCR - disease control rate

DFS - disease-free survival

ECOG - Eastern Cooperative Oncology Group

eIF4E - eukaryotic translation elongation factors 4E

EGFR - epidermal growth factor receptor

Hsp27 - heat shock protein 27

GDP -guanosite diphosphate

GTP - guanosine triphosphate

HCC - hepatocellular carcinoma

HIF1α - hypoxia inducible factor 1α

HNPCC - hereditary nonpolyposis colorectal cancer

HRF - histamine-releasing factor

KRAS - Kirsten rat sarcoma viral oncogene homolog

KREV 1 - protein encoded by the RAP1A gene

lncRNA- long no-coding RNA

LOH - loss of heterozugosity

Mcl-1 - myeloid leukemia cell differentiation protein Mcl-1

miRNAS or miR - micro-RNA

MMR-DNA - mismatch repair genes

mRNA- messeger RNA

MrsB - methionine sulfoxide reductase B

MSI - microsatellite instability

MSI-H - microsatellite instability - high

MSI-L - microsatellite instability - low

MYC - myelocytomatosis oncogene

NcRNA - non-coding RNA

NOTCH - cell signaling system present in most animals

NUMB - a membrane-associated protein related with cell differentiation

OS - overall survival

PI3K - phosphoinositide 3-kinases

PFS - progression-free survival

PTEN - phosphatase and tensin homolog deleted on chromosome 10

PTEN/FOXO3/CCND1 - phosphatase and tensin homolog/forkhead box O3/cyclinD1 signaling pathway

RAP1A - Ras-related protein Rap-1A

RIG-I - retinoic acid-inducible gene I

SIAH - Ubiquitin Protein Ligase

STAT 3 - Signal transducer and activator of transcription 3

TAMs - tumour-associated macrophages

TCTP1 - translationally controlled tumor protein 1

TGF- ß RII - transforming growth factor beta, receptor II

TSAP6 - tumour suppressor activated pathway 6

VEGF - vascular endothelial growth factor

**5`-TOP** - 5`-terminal-oligopyrimidine tract

2. Introduction

Globally, colorectal cancer (CRC) ranks third among human malignancies and accounts for approximately 10% of all malignant solid tumors. CRC is the second most common cause of death from malignancy, responsible for over 700,000 new deaths annually. About 25% of newly diagnosed patients are in clinical stage IV. In about 50% of patients diagnosed at an early stage, disease progression with development of distant metastases is expected. The introduction of screening programs, early diagnosis, treatment and follow-up provides improved overall survival (OS). Today, OS in patients with metastatic disease is ~30 months, almost double that of 20 years ago, yet 5-year survival in clinical stage IV remains below 14%.

The development of CRC is a multistep process that is influenced by a number of endogenous and exogenous factors. In terms of carcinogenesis, CRC is a heterogeneous disease with multiple complex interactions between environmental factors and an individual's genetic predisposition. A genetic predisposition is found in 5-10% of the patient population. These are the individuals in whom some of our well-known inherited syndromes, such as familial adenomatous polyposis (FAP) and Lynch syndrome, are found. Based on family history, CRC can be classified as sporadic, hereditary and familial cancer.

For the first time, Vogelstein and Fearon elucidated the pathogenesis of CRC, presenting their model according to which colon cancer arises from an adenoma that has maligned over time. Today, thanks to molecular biology, there has been a significant advance in elucidating its pathogenesis. The accumulated knowledge of the genomic and epigenomic disorders involved in the earliest stages of carcinogenesis is essential for the

development of an effective therapeutic strategy for patients with CRC. Genetic disorders manifest as such as chromosomal instability (CIN) and microsatellite instability (MSI), while epigenetic disorders are due to hypermethylation of the promoter regions of a number of genes known as CpG islands and this leads to the development of a methylator phenotype in the colorectal epithelium.

Numerous long-term studies on carcinogenesis have largely revealed the main pathways of malignant transformation and the main characteristics that neoplastic cells possess. But all this knowledge also raises another important question. Is the reverse process possible? Are tumor cells capable of losing their malignant potential and what actually constitutes tumor reversion?

Tumor reversion is a complex biological process, occurring under the influence of a number of genetic and epigenetic factors that lead to suppression of the malignant phenotype of the cell, i.e. neoplastic cells lose their malignant characteristics completely or partially. It is crucial to clarify that the process of tumor reversion is different from tumor suppression, which is achieved by targeting certain oncogenic events. In tumor reversion, tumor suppressor genes are not necessarily involved. So far, at least 300 genes are known to be involved in this process, such as SIAH 1, PSEN1, TSAP6 and most notably TPT1/TCTP. A key point in the reprogramming of tumor cells is the reduction of TPT1/TCTP expression.

Translin controlled tumor protein (TCTP) is a protein with a strictly conserved structure that is localized in the cytoplasm and nuclei of all eukaryotic cells (. It participates in a number of fundamental cellular processes such as: DNA repair, protein

synthesis and degradation, stabilization of the dividing spindle, regulation of cell growth, inhibition of apoptosis, and others. Dysregulation of TCTP levels can induce the development of multiple pathological processes, given the key role it plays in maintaining cellular homeostasis, TCTP has been shown to play a significant role in the carcinogenesis of a large number of malignant solid tumors. High expression of TCTP in tumor tissue has been found to be a poor prognostic marker.

The research in this dissertation focused on demonstrating the potential value of TCTP as a novel prognostic biomarker in patients with histologically verified colon cancer.

3. Aim and objectives of the study

3.1 Purpose of the study

The aim of this study was to identify the potential value of TCTP as a novel prognostic biomarker in patients with histologically verified colorectal cancer.

3.2 Tasks of the study

In relation to the so formulated aim of the thesis the following tasks were set:

1. To select patients with histologically verified colorectal cancer who are to be started on systemic drug therapy.

2. To collect the main clinicopathological characteristics of the patients and their response to the systemic drug therapy.

3. To investigate by immunohistochemical methods the nuclear expression levels of TCTP in primary tumor cells of patients with colorectal cancer.

4. To investigate by immunohistochemical methods the cytoplasmic expression levels of TCTP in primary tumor cells of patients with colorectal cancer.

5. To determine correlations between nuclear and cytoplasmic TCTP expression levels in primary tumor cells and various clinicopathological features in selected patients.

6. To analyze the prognostic potential of nuclear and cytoplasmic expression of TCTP in primary tumor in terms of relapse-free survival and progression-free survival in selected patients.

7. To analyze the prognostic potential of nuclear and cytoplasmic expression of TCTP in primary tumor in relation to overall survival in selected patients.

8. To compare the data obtained in the studied patient population with the accumulated data from the world literature.

4. Materials and Methods

4.1 Basis of the dissertation realization

- Clinic of Medical Oncology - University Multiprofile Hospital for Active Treatment "St. Marina”-Varna
- Department of General and Clinical Pathology, Forensic Medicine and Deontology - Medical University-Varna and University Multiprofil Hospital for Active Treatment "St. Marina" - Varna

4.2 Patient population

A retrospective, non-interventional, single-center study was conducted, including a total of 74 participants who received systemic drug therapy for colorectal carcinoma between January

2015 and December 2015. All of them were staged with CT or PET/CT before treatment initiation and had good performance status assessed by ECOG as < 2 (Table 1). All participants met the inclusion criteria of the study and had no exclusion criteria.

Inclusion criteria:

1. Age over 18.

2. Patients with histologically verified CRC who underwent surgical treatment of the primary tumor.

3. The systemic drug therapy should have been carried out in the Clinic of Medical Oncology at UMHAT "St.Marina"-Varna.

Patients who received palliative chemotherapy for metastatic disease or patients who received adjuvant therapy for high-risk recurrence (high-risk stage II or III) were included in this study.

4. ECOG PS - performance status ≤ 2 (Table 1).

5. Absence of serious abnormalities in laboratory parameters such as: PCK, preserved liver and kidney function.

Tab. 1 Assessment of general condition according to the Eastern Cooperative Oncology Group (ECOG) scale, adapted from Robert L. Comis, MD, Group Chair.

Evaluation	ECOG (PS) - performance status
0	The patient is fully active, able to perform all activities from before his illness without limitations
1	There are limitations in physical activity, but the patient is able to perform light or sedentary work (e.g. light housework or office work)
2	Patient is ambulatory and able to take full care of self, but unable to perform work activity; spends > 50% of time awake in upright position
3	Patient is capable of only limited self-care; spends > 50% of time awake in bed
4	Patient is completely unable to care for self; completely confined to bed or chair
5	Death

Exclusion Criteria:

1. ECOG (PS) - performance status > 2 according to AJCC.

2. Patients whose tumor blocks did not allow further immunohistochemical treatment.

3. Missing information in the hospital record, not allowing the determination of key parameters for the study, such as.

4.3 Patient Medical History

Study and follow-up parameters were predefined and retrospectively collected within our study. The following information was collected in an individual medical record for each trial participant:

Demographics:

- *Names (Initials):*
- *ID number/date of birth:*
- *Age:*
- *Gender: male/female*

Medical History:

- *The baseline performance status (PS) of each patient according to the Eastern Cooperative Oncology Group (ECOG) (Table 13)*
- *Presence/absence of comorbidities*

Cancer information:

- *Clinical diagnosis: localization of the primary tumor, TNM staging and degree of differentiation*
- *Date of diagnosis*
- *Date and extent of surgical intervention*
- *Histological result: number, date, number of paraffin blocks*
- *Date of initiation of systemic drug therapy*
- *Type of systemic drug therapy received (adjuvant, palliative)*
- *Molecular pathogenetic analysis of RAS status in metastatic patients*
- *CT and PET/CT results as baseline and restaging studies every 3 months during the course of treatment, RECIST score 1.1.*
- *Survival without progression*
- *Overall survival*

4.4 Specific research methods

4.4.1 Immunohistochemical method of study

An indirect immunoperoxidase method was used for immunohistochemical analysis using the following antibody:

–Anti-TPT1 antibody with catalogue No ABIN701089, manufactured in the USA, polyclonal rabbit antibody against human TPT1, with a dilution of 1:400 and antigen detection at pH 9.0

The study of the tumor tissue biomarker was carried out in the Department of General and Clinical Pathology of St. Varna.

4.4.2 Preparation of biopsy materials for immunohistochemical examination

1. Biopsy materials fixed in neutral formalin and embedded in paraffin blocks were cut into 5-micron-thick sections and placed on slides.

2. The resulting cuts were deparaffinized in descending order of alcohols as follows: ethanol 100% 3 min, ethanol 90% 3 min, ethanol 80% 3 min, ethanol 70% 3 min, xylene 3x10 min. Subsequent washing with running water and placing the sections in distilled water.

3. Antigen retrieval. After cooling, samples were washed at room temperature with diluted FLEX Wash Buffer (20x) for 1-5 min.

4.4.3 Immunohistochemical protocol

The resulting sections were stained according to EnVision FLEX using DAKO Autostainer Plus.

1. Incubation with peroxidase blocking solution (3% H2O2) at room temperature for 5 min to block endogenous peroxidase activity. After incubation, rinse with wash buffer for 5 min.

2. Incubation with the primary antibody (anti-TPT1) at the appropriate dilution (1:400) at room temperature for 20 min.

3. Washing with washing buffer at room temperature for 2x5 min.

4. Incubation with HRP labelled polymer at room temperature for 20 min.

5. Another wash with washing buffer at room temperature for 3x5 min.

6. Incubation of sections with chromogen DAB peroxidase solution under continuous microscopy for 2x5 min.

7. Last flush with wash buffer for 2 min.

8. For 2 min. rinse with distilled water.

9. Counterstaining with Mayer`s hematoxylin for 5 min. for morphological confirmation.

10. Rinse the samples with distilled water for 5 min.

11. Dehydration in reverse ascending order: ethanol 70%, ethanol 80%, ethanol 90%, ethanol 100%. The process is the same duration as dewaxing.

12. Laying the cuts in mounting medium and placing on slide.

4.4.4 A method for reporting TCTP expression levels

Immunohistochemical evaluation was performed by examining 10 fields at the highest magnification (x400). Digital images of the cases were taken using a Leica Aperio ScanScope AT2 device (Aperio Technologies, Vista, CA), and subsequent image analysis was performed with ImageScope V12.1.0.5029 software (Aperio).

Two pathologists independently performed immunohistochemical assessment of TCTP expression levels in the nucleus and cytoplasm of tumor cells using the so-called H-score (histo score). Positive staining is identified when nuclear and cytoplasmic brown staining is detected in tumor cells. The staining intensity was classified in a four-level scale:

– *0 - missing colouring*

–1+ - weak colouring, light yellow

–2+ - saturated colour, deep yellow

–3+ - strong staining, brown

Calculation of the total H-score (histo score) of the tissue samples was performed using the following formula:

H-score (histo score) = [1x (% cells with 1+) + 2x (% cells with 2+) + 3x (% cells with 3+)], ranging from 0 to 300.

In our patient population, the mean cytoplasmic H-score was 180. Based on the obtained mean, patients were divided into two groups for the subsequent statistical analysis, those with low (≤ 180) and those with high (> 180) cytoplasmic H-score. The mean nuclear H-score in the studied samples was 0. Accordingly, patients were stratified again into two groups: those with no nuclear expression of TCTP (H-score=0) and those with nuclear expression of TCTP (H-score>0) (Fig. 1). Following the same protocol, nuclear and cytoplasmic TCTP H-score was assessed in healthy tissue from the resection lines area.

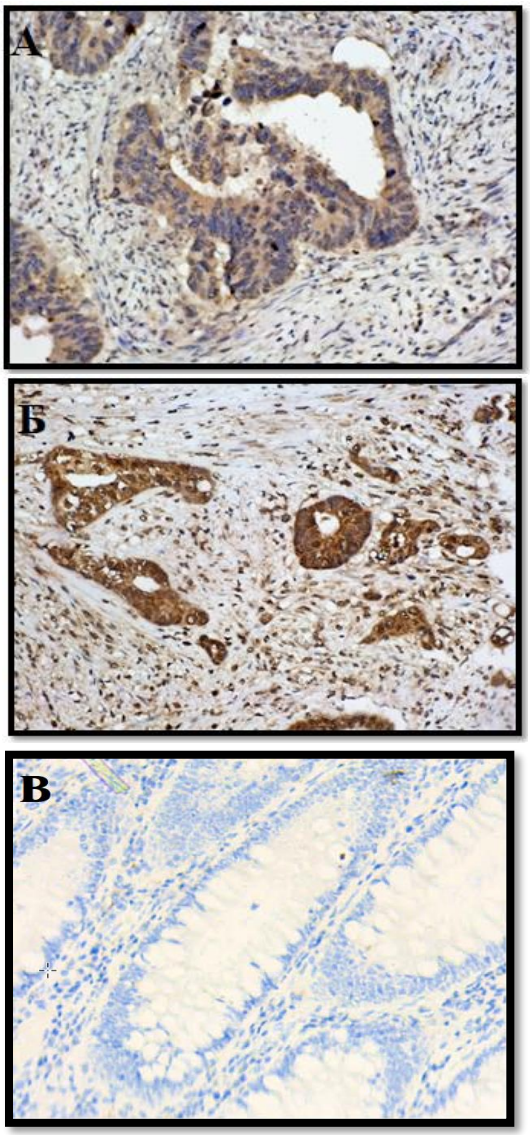

Figure 1. Immunohistochemical staining of TCTP in colorectal cancer. (A) Low nuclear expression of TCTP (B) High nuclear expression of TCTP (C) Healthy colon tissue from resection lines.

4.5 Therapeutic response imaging

All selected patients had systemic drug therapy for CRC. Some of them were treated adjuvantly and others received palliative chemotherapy for metastatic disease. Each patient was staged by imaging before initiation of treatment and regularly every three months during treatment until disease progression was demonstrated according to RECIST 1.1 criteria (Table 2). The imaging modalities used for evaluation were CT of the chest and abdomen, and whole-body PET/CT. In the course of systemic drug therapy using the imaging methods for disease assessment, the following parameters were monitored:

– Disease-free survival (DFS) defined as the time to relapse or death due to any cause in patients who received adjuvant treatment.

– Progression-free survival (PFS) defined as the time elapsed between initiation of first-line therapy for metastatic disease and the occurrence of prognosis or death from any cause.

– Overall survival (OS) defined as the time between initial diagnosis of disease and patient death.

– Disease control rate (DCR) is defined as the number of patients with a complete or partial response, or stable disease after receiving first-line treatment for a placebo.

Table 2 Types of response to therapy according to RECIST 1.1 criteria and adapted EORTC criteria

Type of answer	Adapted EORTC PET/CT criteria	RECIST 1.1
Full answer (CR)	Absence of FDG accumulation in all lesions	Disappearance of all merimi lesions
Partial Response (PR)	Reduction of 25% or less in the sum of SUVmax after	Reduction by 30% or less of the sum of the longest

	more than 1 conducted HT cycle in up to 5 target lesions	target lesion diameters from the baseline measurement
Stable Disease (SD)	Lack of sufficient reduction in the size of the lesions as well as sufficient increase to be judged as disease progression	Lack of CR, PR or PD
Progression of disease (PD)	An increase of 25% or more in total SUVmax or the appearance of FDG accumulation in new lesions (new lesions and/or progression of an existing non-target lesion)	The appearance of one or more new lesions or at least a 20% increase in the size of the sum of the longest diameters of the target lesions compared to the smallest sum relative to the study

4.6 Methods for medical-statistical data processing

Data analysis was performed with the statistical package IBM SPSS for Windows, v.23 (IBM Corp.). All values are presented as mean ± standard deviation (SD). Differences with $p \leq 0.05$ were considered statistically significant. The following statistical methods were used in data processing:

4.6.1 Method of statistical grouping of data

The ordering of attributes is by type and is implemented in variational, interval, categorical, stepwise and time series statistics.

4.6.2 Statistical estimation method

Performed by point estimates (to calculate the mean, median or mode of continuous traits), by interval estimates and by confidence intervals (CI);

4.6.3 Graphical method

Line and plane graphical representations, pie charts, pie-sector diagrams, stereograms, and symbol diagrams were used.

4.6.4 Nonparametric analysis

Pearson's $\chi 2$ (chi-square) test, Mann-Whitney test, Kruskal-Wallis H test, Jonckheere-Terpstra test, Student's t-test.

- *The $\chi 2$ (chi-square) test or Fisher's test was applied to compare nuclear and cytoplasmic TCTP levels in different patient subgroups.*
- *The t-test was used to compare the expression levels of TCTP in tumor cells and healthy colonic epithelial cells from the resection lines.*
- *The Mann-Whitney U test and the Jonckheere-Terpstra test were applied to compare and identify an association between TCTP levels in tumor cells and clinicopathological characteristics of the study group.*

4.6.5 Correlation Analysis

Spearman linear correlation coefficients were determined. The correlation coefficient rho can take values between 0 and -1 for an inverse relationship and between 0 and +1 for an orthogonal relationship. In interpreting the results of the correlation analysis, the following correlation strength scale is used according to the rho value:

– *< 0.19 - very low correlation;*
– *0.19 ÷ 0.39 - low correlation;*
– *0.40 ÷ 0.59 - average correlation;*
– *0.60 ÷ 0.79 - strong correlation;*
– *≥ 0.80 - very strong correlation;*

4.6.6 Survival Analysis

Kaplan-Meier methods were used for this purpose, and differences were assessed by log-rank test;

4.6.7 Cox proportional hazards models

Cox proportional hazards models - to determine HRs and confidence interval 95% CIs - a test to assess the predictive value of an established biomarker.

5. Results

5.1 Clinical and pathological patient stratification

The retrospective study we conducted included 74 patients with histologically verified colon cancer. Each of the participants had received systemic drug therapy for the cancer at the Clinic of Medical Oncology of St. Marina University Hospital - Varna.

The following clinical data were collected for the patients in the course of the study: demographic data (name, sex, age at diagnosis, etc.), localization of the primary tumor, TNM classification, type and duration of systemic drug therapy, general condition according to ECOG (PS).

The pathological data collected were as follows: histological characteristics of the tumor, degree of differentiation, and *RAS* mutational status.

According to the different clinical and pathological parameters, the patients were divided into groups (Table 3).

Table 3 Baseline clinicopathological characteristics of patients

Gender (N, %)	
Men	46 (62.2)
Women	28 (37.8)
Mean age (years) +/- SD	64.9 +/- 9
Tumor localization (N, %)	
Left column	43 (58.2)
Right column	31 (41.8)
Degree of differentiation (N, %)	
Grade 1	0 (0)
Grade 2	62 (83.8)
Grade 3	12 (16.2)
RAS status (N, %)	
Wild type	41 (55.4)
Mutated type	33 (44.6)
Patient groups according to disease stage (N, %)	
Non-metastatic disease	20 (27)
Primary metastatic disease	54 (73)
Stage at diagnosis (N, %)	
Second stage	2
Third stage	18
Fourth stage	54
Total ECOG status (N, %)	
0	46 (62.16)
1	28 (37.84)

Distribution by groups

In the total patient population (n=74), the gender distribution favored men with 62.2% (n=46), compared with 37.8% for women (n=28). The mean age of the selected participants at diagnosis was 64.9 years with a minimum age of 24 years and a maximum age of 82 years (Fig. 2).

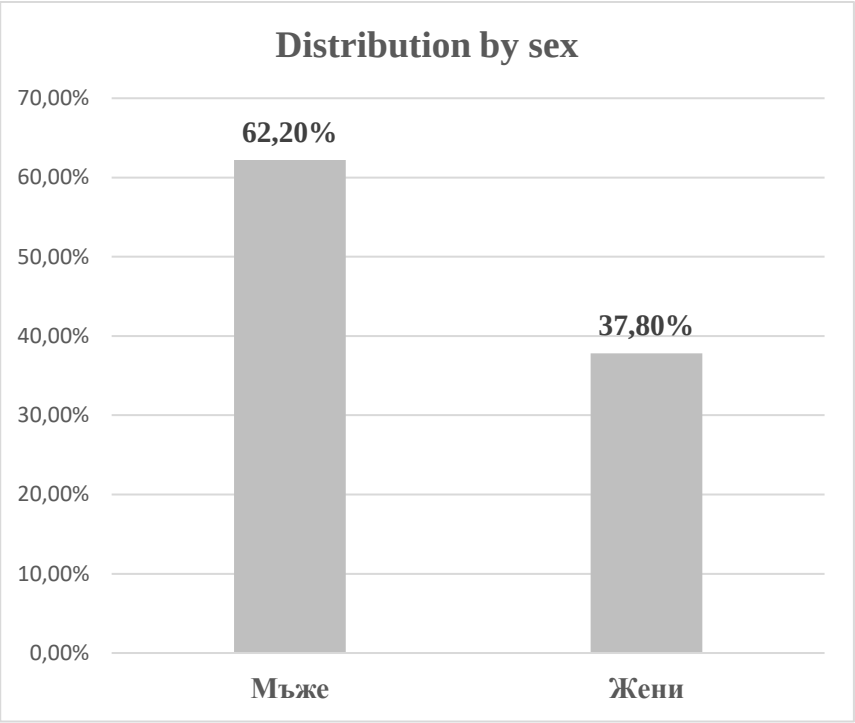

Figure 2. Bar-plane diagram reflecting the distribution of patients by sex (%)

In 58.2% (n=43) of all selected patients, CRC was left-sided and in 41.8% (n=31) it originated from the right column (Fig. 3).

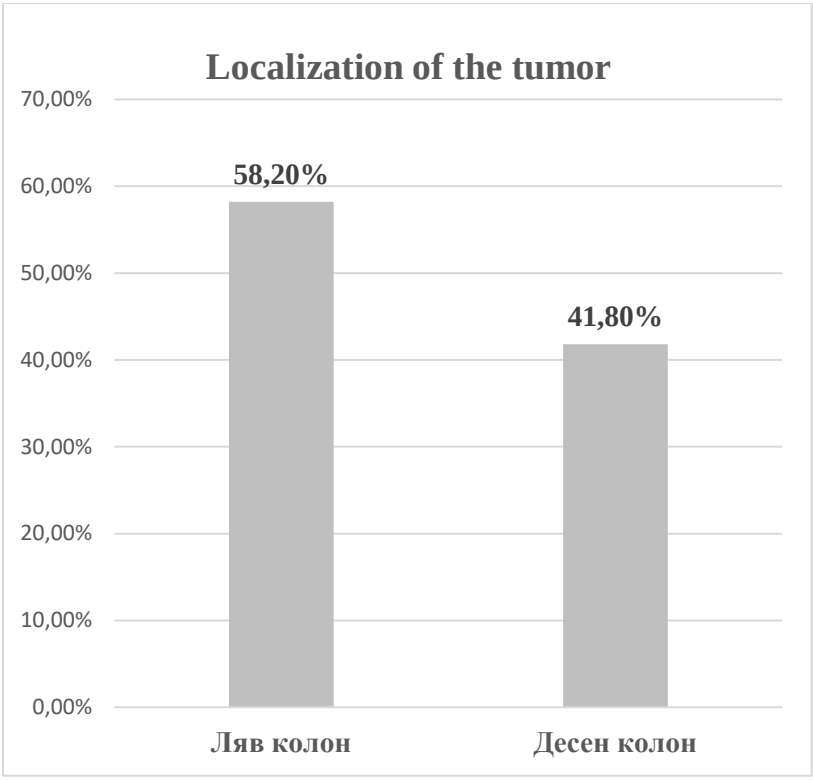

Figure 3. Bar-plane diagram reflecting the distribution of patients according to primary tumor localization (%)

Depending on the grade of tumor differentiation, patients with Grade 2 tumors predominated - 83.8% (n=62) followed by the group of patients with Grade 3 differentiation - 16.2% (n=12) (Fig. 4).

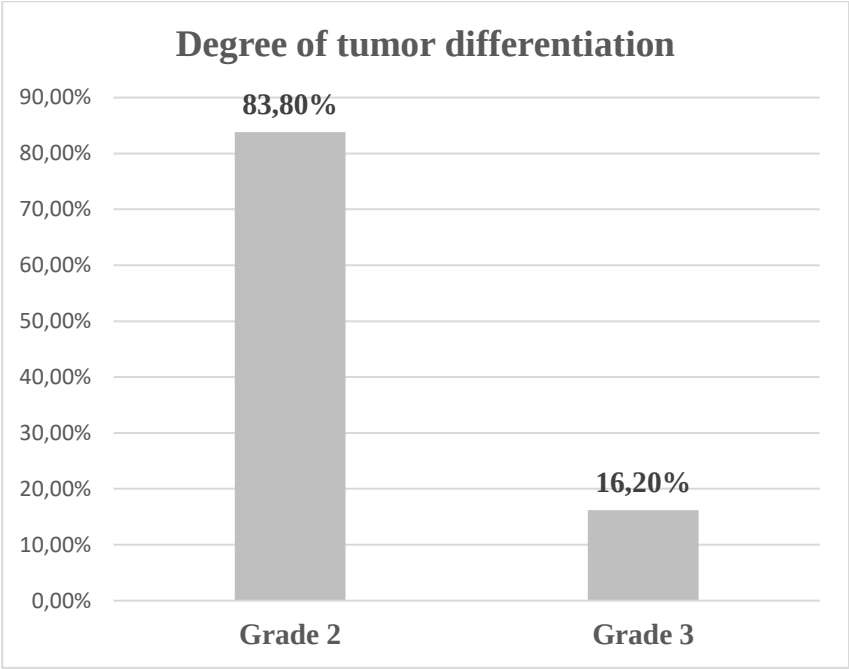

Figure 4. Bar-plane diagram reflecting the distribution of patients according to the degree of tumor differentiation (%)

According to the molecular pathological study of RAS status, the leading number of patients with wild type RAS was 55.4% (n=41), while those with mutant type RAS were 44.6% (n=33) (Fig. 5).

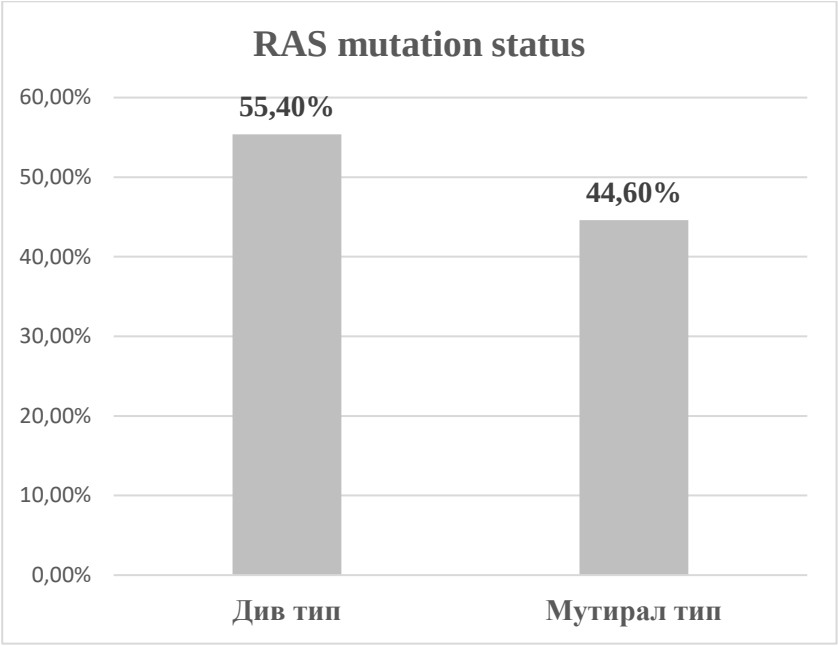

Figure 5. Bar-plane plot reflecting the distribution of patients according to RAS mutation status (%)

The patients included in the present study were divided into two groups, depending on the stage of the disease. The majority of them were diagnosed with primary metastatic disease, 73% (n=54), while 27% (n=20) were diagnosed with primary non-metastatic disease, of which 18 were in clinical stage III and only 2 in clinical stage II. Over the course of the study, 85% (n=17) of the non-metastatic patients progressed to stage IV (Fig. 6).

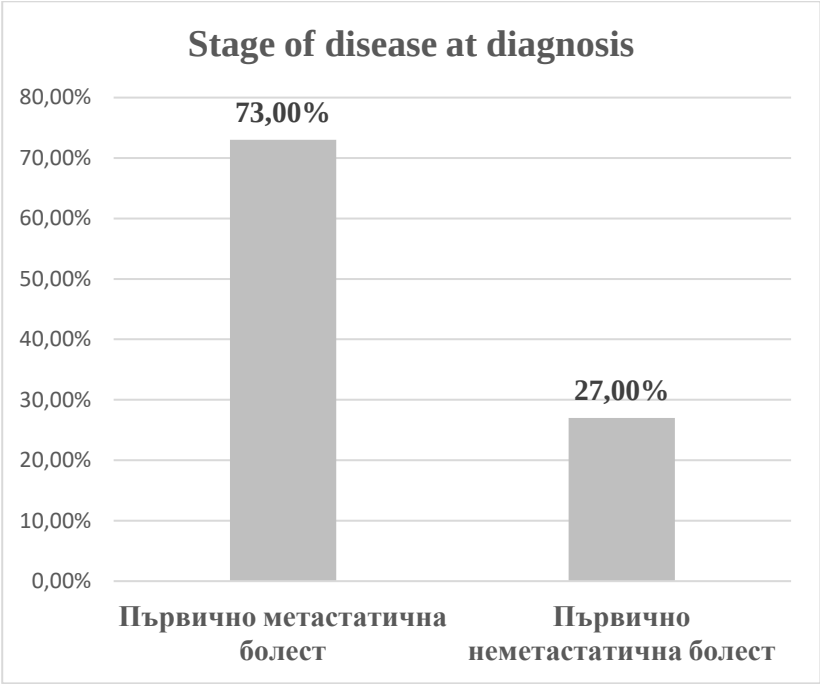

Figure 6. Bar-plane plot showing the distribution of patients according to clinical stage of disease (%)

Patients with a performance status < 2 as assessed by the ECOG Performance Status (PS) scale were included in the trial. The majority of all patients at the initiation of the first cycle of chemotherapy had ECOG 0, 62.16% (n=46), followed by those with ECOG 1, 37.84% % (n=28) (Fig. 7).

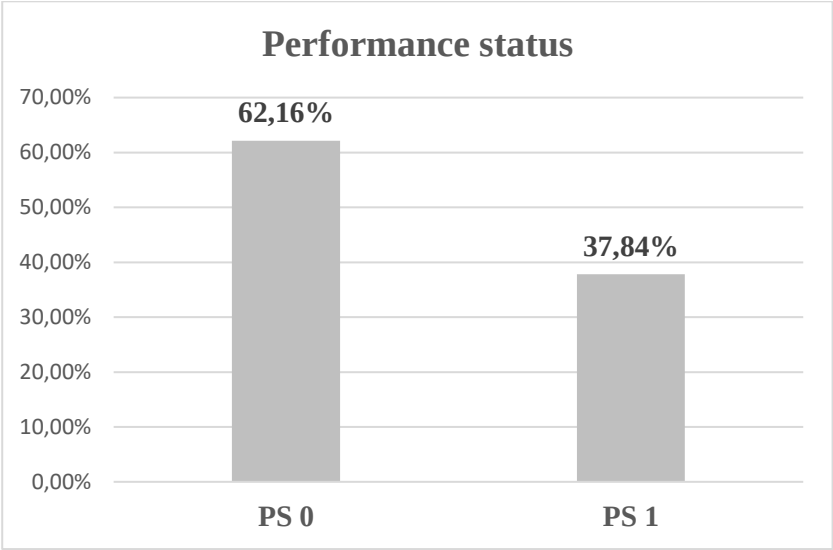

Figure 17. Bar-plane diagram reflecting the distribution of patients according to performance status (%)

5.2 Association between TCTP expression levels, demographic and clinicopathological characteristics in the patient population studied

Applying the statistical method of nonparametric analysis (χ^2 test), no significant correlation was found in the selected group of patients with respect to nuclear and cytoplasmic TCTP expression levels and sex, age, ECOG (PS), tumor differentiation grade, primary tumor localization, stage at diagnosis, and best response to first-line systemic drug therapy (Table 4).

Patients with higher nuclear TCTP H-score showed a trend for a greater number of different metastatic foci at diagnosis ($p = 0.059$, Jonckheere-Terpstra test).

Higher nuclear TCTP H-score was associated with a higher degree of tumor differentiation ($p = 0.049$, Mann-Whitney test) (Fig. 8).

In tumor cells, the mean cytoplasmic and nuclear TCTP H-Score was significantly higher compared with adjacent healthy tissue from the resection line area ($p = 0.004$ and $p < 0.001$; paired samples t-test).

Table 4. Association between TCTP expression levels and clinicopathological characteristics of patients

Clinicopathological characteristics	**Cytoplasmic expression of TCTP**			**Nuclear expression of TCTP**		
	High	**Low**	**P-Value**	**High**	**Low**	**P-Value**
Age			0.056			0.621
≤64	24 (75.0%)	8 (25.0%)		21 (65.6%)	11 (34.4%)	
>64	22 (54.4%)	20 (47.6%)		30 (71.4%)	12 (28.6%)	
Gender			0.323			1
Men	31 (67.4%)	15 (32.6%)		32 (69.6%)	14 (30.4%)	
Women	15 (53.6%)	13 (46.4%)		19 (67.9%)	9 (32.1%)	
ECOG			0.622			0.606
0	30 (65.2%)	16 (34.8%)		33 (71.7%)	13 (28.3%)	
1	16 (57.1%)	12 (42.9%)		18 (64.3%)	10 (35.7%)	
G			0.757			0.173
2	39 (62.9%)	23 (37.1%)		45 (72.6%)	17 (27.4%)	
3	7 (58.3%)	5 (41.7%)		6 (50.0%)	6 (50.0%)	
RAS status			0.630			0.802
WT	24 (58.5%)	17 (41.5%)		29 (70.7%)	12 (29.3%)	
M+	22 (66.7%)	11 (33.3%)		22 (66.7%)	11 (33.3%)	
Localization of the tumor			0.229			0.612
Left column	24 (55.8%)	19 (44.2%)		31 (72.1%)	12 (27.9%)	
Right column	22 (71.0%)	9 (29.0%)		20 (64.5%)	11 (35.5%)	

Patient groups according to stage at diagnosis			0.280			0.266
Nematostat	10 (50%)	10 (50%)		16 (80%)	4 (20%)	
Metastatic	36 (66.7%)	18 (33.3%)		35 (64.8%)	19 (35.2%)	
Stages at diagnosis			0.420*			0.447*
Second stage	1 (50%)	1 (50%)		2 (100%)	0 (0%)	
Third stage	9 (50%)	9 (50%)		14 (77.8%)	4 (22.2%)	
Fourth stage	36 (66.7€)	18 (33.3%)		35 (64,5%)	19 (35.5%)	
Best answer to front line HT			0.277			0.784
CR	1 (33.3%)	2 (66.7%)		3 (100%)	0 (0%)	
PR	6 (85.7%)	1 (14.3%)		5 (71.4%)	2 (28.6%)	
SD	15 (53.6%)	13 (46.4%)		19 (67.9%)	9 (32.1%)	
PD	22 (66.7%)	11 (33.3%)		21 (63.6%)	12 (36.4)	
DCR			0.474			0.613
CR+PR+SD	22 (57.9%)	16 (42.1%)		27 (71.1%)	11 (28.9%)	
PD	22 (66.7%)	11 (33.3%)		21 (63.6%)	12 (36.4%)	

**** Fisher test***

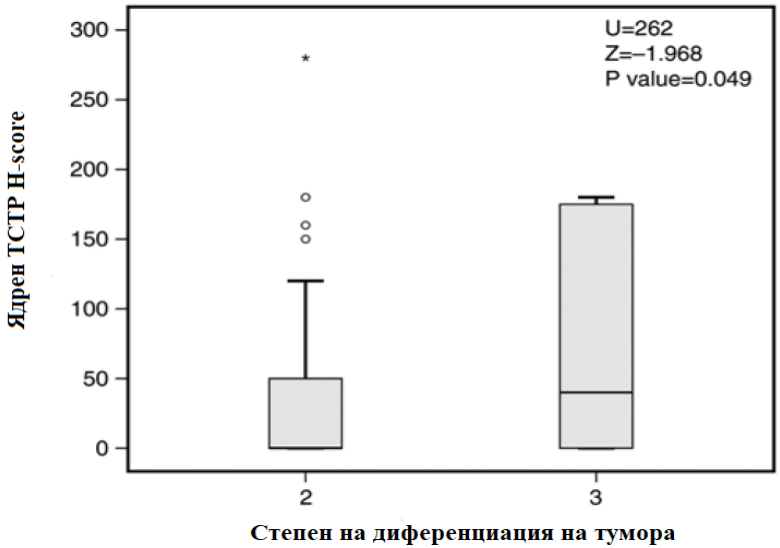

Figure 8. Box plot graph reflecting TCTP H-score in the core in patients with different degrees of CRC differentiation

5.3 Association between TCTP expression levels and survival in the studied patient population

The study found that there was no statistically significant association between cytoplasmic expression levels of TCTP and DFS (p=0.723), PFS (p=0.377) and OS (p=0.990).

Patients who had a negative nuclear TCTP H-score in the primary tumor had statistically significant better clinical outcomes. The PFS for the TCTP-negative group was 7.7 months (95% CI, 5.8-9.5) versus 5.5 months (95% CI, 3.2-7.8) for the TCTP-positive group (p=0.023, Mantel-Cox log-rank) (Fig. 9).

Again, patients with negative nuclear expression of TCTP had significantly higher median overall survival (22.2 months; 95% CI, 16.1-28.3) compared with the positive nuclear

expression group (mean, 13.2 months; 95% CI, 10.1-16.3) (p=0.008, Mantel-Cox log-rank) (Fig. 10) (Table 5).

Univariate Cox regression analysis showed that a positive TCTP H-score was a statistically significant risk factor for worse PFS (HR 1.797; 95% CI, 1.073-3.010; p=0.026) and worse OS (HR 1.995; 95% CI, 1.189-3.348; p=0.009) (Table 6).

In the multivariable Cox regression model, positive nuclear TCTP H-score was an independent risk factor for worse progression-free survival and worse overall survival (Table 7).

Disease-free survival in the non-metastatic group did not differ according to the degree of nuclear expression of TCTP (p=0.813).

One-year overall survival in the patient population with negative nuclear expression of TCTP was 86.3% compared to 56.5% in patients with positive nuclear expression of TSTP (p=0.008). Statistically, there was a significant negative correlation between nuclear TCTP H-score and OS (p= -0.287; p=0.013).

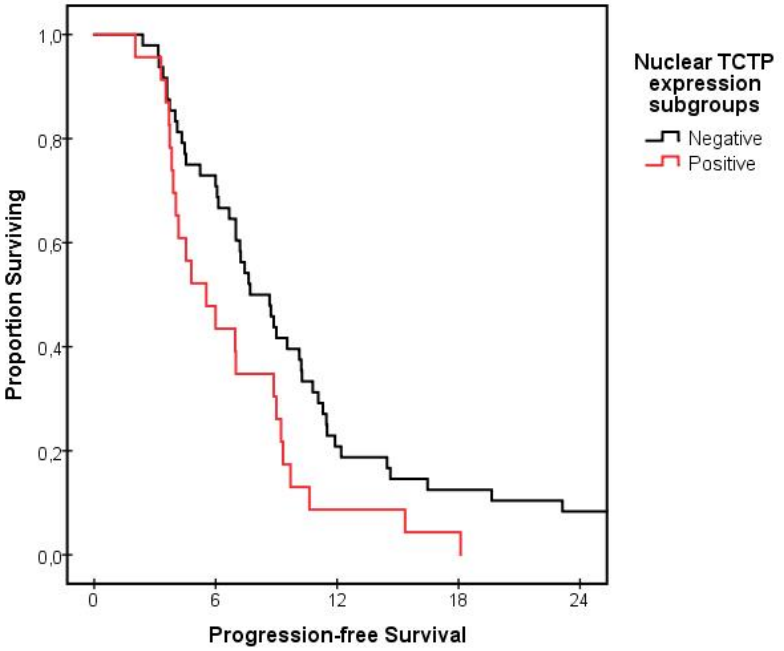

Figure 9: Kaplan-Meier curves of progression-free survival (PFS) distributions in the positive nuclear expression and negative nuclear expression TCTP groups

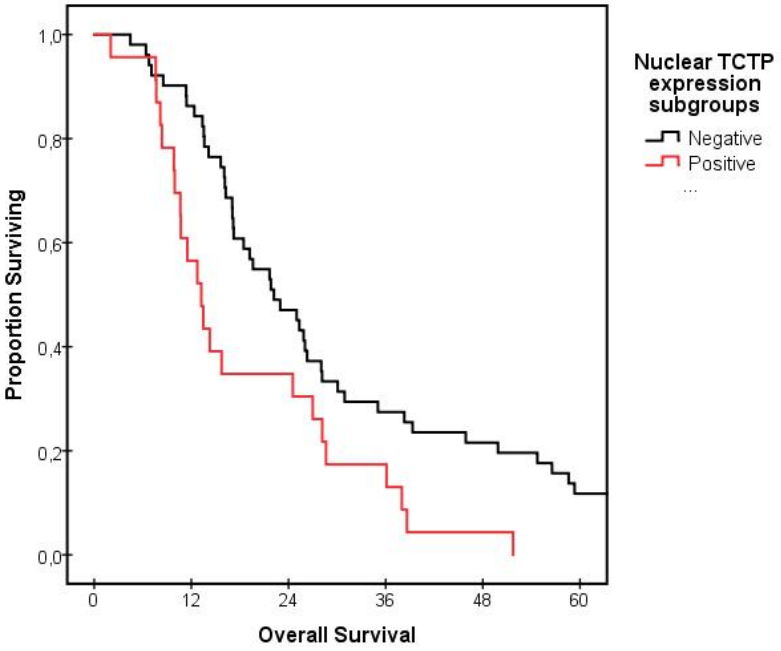

Figure 10. Kaplan-Meier curves of overall survival (OS) distribution in the TCTP positive and TCTP negative nuclear expression groups

Table 5: Mean overall survival and progression-free survival according to nuclear TCTP H-score

Nuclear TTR H-score	PFS			OS		
	Median	95% Security Interval		Median	95% Security Interval	
		Lower limit	Upper limit		Lower limit	Upper limit

Missing expression	7.700	5.852	9.548	22.200	16.069	28.331
Positive expression	5.533	3.238	7.829	13.233	10.155	16.311

Table 6: Univariate analysis to estimate progression-free survival and overall survival

Indicator	**PFS**			**OS**		
	HR	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Age			0.682			0.606
≤64	1	-		1	-	
>64	1.105	0.685-1.782		1.134	0.703-1.828	
Gender			0.326			0.286
Men	1	-		1	-	
Women	1.284	0.779-2.117		1.301	0.802-2.111	
ECOG			0.109			**0.012**
0	1	-		1	-	
1	1.489	0.915-2.423		1.871	1.148-3.047	
G			0.311			**0.006**
2	1	-		1	-	
3	1.387	0.736-2.613		2.491	1.293-4.799	
RAS status			0.485			**0.002**
WT	1	-		1	-	
M+	1.184	0.737-1.900		2.113	1.306-3.420	
Localization of the tumor			0.301			0.154
Left column	1	-		1	-	
Right column	1.287	0.798-2.075		1.422	0.877-2.305	
Stages at diagnosis			0.318			**<0.001**
Nematostat	1	-		1	-	
Metastatic	1.331	0.759-2.334		3.174	1.775-5.673	
TCTP - cytoplasmic expression			0.379			0.990
Low	1	-		1	-	

High	1.249	0.761-2.049		1.003	0.619-1.626	
TCTP - nuclear expression			**0.026**			**0.009**
Low	1	-		1	-	
High	1.797	1.073-3.010		1.995	1.189-3.348	

Table 7: Multivariate analysis to estimate progression-free survival and overall survival

Indicator	**PFS**			**OS**		
	HR	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Gender			0.456			**0.024**
Men	1	-		1	-	
women	1.225	0.719-2.088		1.845	1.085-3.137	
G			0.290			**0.003**
2	1	-		1	-	
3	1.432	0.736-2.785		3.092	1.481-6.456	
RAS			0.809			**0.009**
WT	1	-		1	-	
M+	1.063	0.646-1.750		2.050	1.196-3.512	
ECOG			0.097			**0.043**
0	1	-		1	-	
1	1.570	0.921-2.676		1.703	1.017-2.851	
Stages at diagnosis			0.853			**0.005**
Nematostat	1	-		1	-	
Metastatic	1.060	0.571-1.967		2.352	1.290-4.286	
TCTP - nuclear expression			**0.042**			**0.040**
Low	1	-		1	-	
High	1.743	1.021-2.975		1.799	1.027-3.151	

6. Discussion

Colorectal cancer is the third most common cancer worldwide and the second leading cause of cancer death. Globally, the incidence of CRC is projected to increase, with a worrying trend of increasing incidence among younger patients. Over the past few decades, five-year survival rates have risen to over 60% due to advances in unraveling the underlying pathophysiological mechanisms by which the disease arises and a significant increase in the treatment options available for local and metastatic disease.

TCTP is a multifunctional protein occupying a key regulatory role in a number of cellular processes such as: cell growth, cell cycle progression, protein synthesis, apoptosis, immune response and malignant transformation. This protein has a strictly conserved structure and can be found in all eukaryotic cells, interacting also with a number of other proteins. In addition to its involvement in many normal cellular functions, it underlies the pathogenesis of many diseases.

Numerous studies have established the link between TCTP and oncogenic transformation, with TCTP expression levels in the cells of a number of malignant solid tumours being significantly higher than in the normal cells from which they derive. TCTP blocks p53 transcription and thus inhibits the activation of apoptosis in tumor cells. Inhibiting TCTP expression in neoplastic cells suppresses the malignant phenotype of cancer cells, making them more vulnerable to oxidative and metabolic stress. High levels of TCTP are also associated with chemoresistance. TCTP plays an important role in tumor

progression by inhibiting apoptosis, accelerating exit from mitosis, inducing invasion and metastasis.

Tumor reversion is a biological process in which some neoplastic cells lose their partially or completely malignant phenotype. The discovery of the key molecules involved is intriguing for clinicians because these are the molecules that can be used as targets in cancer treatment. During the reversion process, significantly reduced levels of TCTP are found, which in turn increases interest in it and its use as a potential therapeutic target in treatment. Suppressing TCTP expression levels in CRC cells has been shown to inhibit invasion and migration in vitro, and the development of liver metastases in vivo.

The drastic increase in the number of malignancies worldwide is causing enormous economic and social damage. Classical chemotherapeutic agents remain the mainstay of treatment for most tumours, but they are characterised by numerous side effects such as alopecia, gastrointestinal manifestations and suppression of bone marrow function. Another major problem with them is the development of drug resistance. Therefore, in the last decades, work has been mainly focused on the discovery of new therapeutic agents that are more effective and better tolerated.

CRC is a heterogeneous disease, with treatment outcomes varying in different patient populations and depending on tumor biology (presence of RAS and BRAF mutations, microsatellite instability, etc.). Despite screening programmes, advances in diagnosis and treatment, colorectal cancer, especially at an advanced stage, still remains one of the malignancies with a poor prognosis. Hence, there is a need to identify new prognostic

markers to guide our choice of therapeutic strategy and to suggest the likely outcome of treatment.

The present retrospective study was conducted to evaluate the role of TCTP expression levels as a prognostic factor in patients with histologically verified colorectal cancer. To date, few studies investigating this issue are available.

In this retrospective study, we found significant differences in the survival of CRC patients depending on the expression levels of TCTP in the nuclei of tumor cells from the primary tumor. Despite the similar clinicopathological characteristics (gender, age, ECOG, PS, differentiation grade, RAS status and stage at diagnosis) in the patient population studied, we found that the median PFS and OS were significantly lower in the subgroup with higher nuclear expression of TCTP. Multivariable regression analysis showed that nuclear expression of TCTP was an independent risk factor for worse PFS and OS in the selected patient group. No prognostic value was found with respect to cytoplasmic TCTP levels.

Today, there is considerable interest in the prognostic value of TCTP in various tumor types. Some studies in breast cancer have found that higher expression of TCTP in the nucleus is associated with a higher degree of tumor differentiation and higher expression of Ki-67 as a marker of disease aggressiveness. In another study on hepatocellular carcinoma, high expression of TCTP was shown to be a poor prognostic marker associated with shorter overall survival. In patients with gliomas, it is again confirmed that high TCTP levels are associated with lower differentiation rates and shorter overall survival. Similar results were observed in patients with epithelial ovarian tumors.

To date, the accumulated data on the prognostic value of TCTP in patients with CRC are significantly limited (286). Fisher et al. investigated the role of TCTP iRNA and protein expression levels as a prognostic factor in various tumor types. In their study, there was no association between TCTP irRNA expression and overall survival in patients with colon cancer, but nuclear expression of TCTP was associated with a lower degree of tumor differentiation, as was found in our study. Xiao et al. found higher TCTP expression in lower differentiated tumors and in patients with metastatic disease. They associated elevated TCTP levels with shorter metastasis-free survival.

Since its discovery, the key role of TCTR in both the carcinogenesis of malignant solid tumors and the process of tumor reversion has been known. TCTP expression levels are significantly higher in tumor cells than in revertant cells. TCTP stimulates carcinogenesis by binding to p53 and subsequent inhibition of apoptosis. Inhibition of TCTP function in turn activates apoptosis and reduces tumor cell viability. Suppression of TCTP expression improves response to treatment with 5-fluorouracil and oxaliplatin in colon carcinoma cell lines.

The use of TCTP as a prognostic marker in patients with CRC is promising, but there are some barriers to its implementation into routine clinical practice. First and foremost is the lack of a standardized method for testing its expression levels. Most studies to date have used the immunohistochemical method for measurement, but here there is considerable variability in the antibodies used and the systems used for assessment. This calls for the assessment methods to be standardized to ensure that TCTP expression can be reliably measured and compared across studies. Most studies available to

date are small and retrospective, which in turn necessitates prospective studies with larger patient cohorts to validate TCTP as a prognostic marker.

There are several limitations to our study, the main one being its retrospective nature. Selected patients were followed for a specific time interval, which did not allow us to use information of more recently identified prognostic and predictive markers, such as the presence of BRAF mutations or microsatellite instability. For this reason, we were unable to perform an analysis of the relationship between TCTP expression levels and prognostic markers routinely used in clinical practice. On the other hand, the longer follow-up period allowed us to perform a statistical survival analysis.

In conclusion, our study revealed that the nuclear expression levels of TCTP can be used as a prognostic marker in colon cancer patients. Positive nuclear expression of TCTP was associated with a lower tumor differentiation rate and shorter progression-free survival and overall survival. To the best of our knowledge, our study is the first to identify the value of nuclear TCTP expression as a prognostic marker regarding PFS and OS in patients with histologically verified CRC. Our findings would be useful to identify patients with more aggressive tumors and worse prognosis, and this in turn would be helpful for us to choose an individualized therapeutic strategy.

Conclusion

In summary, the most important contribution of the study in this thesis is the discovery of an association between the expression levels of the tumor reversion biomarker TSTR and survival in patients with histologically verified colorectal cancer. Our retrospective study found that there was an inverse

correlation between nuclear TCTP expression levels in primary tumor cells and progression-free time and overall survival in the patient population. This demonstrates that nuclear H-score of TCTP may serve as an independent prognostic marker regarding PFS and OS in patients with colon cancer. The results we obtained are in agreement with the results of trials conducted worldwide to date. Our study found no significant correlation between TCTP expression levels and clinicopathological characteristics of the patient population.

The process of tumor reversion is an intriguing problem for a number of researchers. Although some of the underlying molecules and pathways are already known, the accumulated data in the world literature are still insufficient. Detailed elucidation of the subtle molecular mechanisms of progression would provide a solid foundation on which to build a better therapeutic strategy in the treatment of patients with malignant solid tumors.

7. Conclusions

1. There was no correlation between nuclear and cytoplasmic expression levels of TCTP in primary tumor cells and some clinicopathological characteristics such as: sex, age, ECOG (PS), primary tumor location, stage at diagnosis.

2. There was a linear correlation between nuclear TCTP expression levels and the higher number of different metastatic foci in stage IV patients.

3. There was a linear relationship between nuclear TCTP expression levels and higher tumor differentiation.

4. An inverse correlation was found between nuclear TCTP expression levels and progression-free survival.

5. An inverse correlation was found between nuclear TCTP expression levels and overall survival.

6. High nuclear TCTP expression levels were found to be an independent prognostic factor for worse progression-free survival and overall survival.

7. There was no correlation between cytoplasmic and nuclear levels of TCTP expression and disease-free survival.

8. Dissertation contributions

1. The potential role of TCTP expression level in the primary tumor as a prognostic factor regarding progression-free survival and overall survival in patients with histologically verified colorectal cancer is reported for the first time worldwide.

2. For the first time in Bulgaria the expression level of TCTP in the primary tumor as a marker of tumor reversion in patients with histologically verified colorectal cancer was investigated.

3. For the first time in Bulgaria, the level of TCTP in the primary tumor and its correlation with various clinicopathological characteristics in patients with histologically verified colorectal carcinoma were investigated.

9. Scientific publications related to the thesis

Publications:

1. "Prognostic value of translationally controlled tumor protein in colon cancer"

Authors *Dragomir Svetozarov Stoyanov,* ***Mariya Penkova****, Nikolay Conev, Ivan Donev*
Publication date: *2023/9/1*
Source:*Molecular and Clinical Oncology*

Issue: 3/2023
***Pages** 1-8*
***Publisher:** Spandidos Publications*

2. "To <u>what extent has Lonsurf ® altered therapeutic response in metastatic colorectal cancer"</u>
***Authors. T.** Panayotova, M**. T. Penkova-Ivanova**, N. Tsonev*
***Publication date:** 2023*
Source.
***Issue:** 3/2023*
***Stations:** 54-60*
***Publisher:**Medic Print Ltd.*
3. "<u>Circulating nucleosomes with posttranslational modifications as epigenetic biomarkers in malignant malignant tumors"</u>
***Authors. M**. M. Panayotova, R. T. Manaev, M. M. Maneva, D. M**. Penkova**, N. Tsonev*
***Publication date:** 2021/4*
Source.
***Issue:** 4/2021*
***Pages:** 94-99*
***Publisher:** MEDINFO EOOD*

<u>Participations:</u>
1. "<u>Translationally controlled tumor protein as a prognostic marker in metastatic colon cancer"</u>
***Authors** Dragomir Svetozarov Stoyanov, **Mariya Penkova**, Nikolay Conev, Ivan Donev*
***Publication date:** 2023/6/1*
***Publication**: refereed with impact factor - Journal of Clinical Oncology*
***Volume**: 41*

***Pages**: e15513-e15513*
***Publisher**: American Society of Clinical Oncology*

2. "Regorafenib and trifluridine/tipiracil efficacy and safety in chemorefractory metastatic colorectal cancer patients: A single Bulgarian centre retrospective study"
***Authors M Penkova,** D Stoyanov, T Panayotova, I Donev, M Petrova, N Conev*
***Publication date:** 2020/7/1*
***Publication:** refereed with impact factor - Annals of Oncology*
***Pages:**S100*
***Publisher:** Elsevier*

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