

Clinical Influence of Ploidy and Cancer Stem Cells and Other Parameters in Stage IV Colorectal Cancer

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Abstract. *Background/Aim:* The aim of the present study was the evaluation of the influence of cancer stem cells and other parameters in stage IV colorectal cancer patients. *Materials and Methods:* One hundred patients were retrospectively included in the study and 24 variables were examined for their relation with response to treatment and survival. *Results:* A low ploidy score in the histology of colorectal cancer was associated with improvement of performance status and response to therapy. No significant correlations between the percentage of cancer stem cells from the same tissue and the remaining clinical parameters was revealed. In the multivariate analysis of all the examined parameters in Cox models, independent unfavorable prognostic factors were increased ploidy score, existence of bone metastases, use of epoetin, and existence of side-effects such as anorexia, mucositis, and weight loss. *Conclusion:* Our findings emphasize on the prognostic role of ploidy in advanced colorectal cancer, but further analysis is required to evaluate the role of cancer stem cells.

Colorectal cancer (CRC) is one of the leading causes of death worldwide. The introduction of novel chemotherapeutic agents has allowed targeted therapy and has changed the survival rate

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of patients with metastatic disease. Hence, it is of high priority to precisely determine the prognostic factors of treatment response in patients with metastatic CRC.

Cancer stem cells (CSCs) are of great importance in tumor genesis, progression, metastasis, recurrence, and resistance to chemotherapy. CD133 (protamin 1) is a pentaspan membrane glycoprotein, expressed on CD34⁺ stem and progenitor cells, in endothelial precursors and fetal neural stem cells that is detected by its glycosylated epitope, AC133. CD133 is one of the most commonly used biomarkers for the characterization of stem cells and has a key role in tumor growth and development (1-4).

Chromosomal instability (CIN) is the most common form of genomic instability as it is identified in up to 85% of patients with CRC and is the result of arithmetical and structural alterations of chromosomes. CIN has been proposed as a biomarker of poor prognosis due to its contribution to clonal diversity and tumor growth. It is identified by DNA flow cytometry or image flow cytometry. Based on previous work, a low ploidy score might correlate with greater benefit from palliative chemotherapy. In addition, a number of laboratory parameters, such as C-reactive protein level, no or only mild anemia, and normal albumin level have also been associated with better outcomes (5-7). Based on our clinical interest and previous work on investigation of prognostic factors in metastatic CRC, the present study tried to evaluate the role of cancer stem cells, in association with other molecular, clinical and laboratory parameters, for their correlation with treatment response and survival.

Materials and Methods

Patients. The medical records of 100 patients with histologically-proven CRC, International Union of Cancer Control stage IV (8) between 2000 and 2009 were retrospectively reviewed and their

paraffin-embedded tissue samples from the primary site of CRC were collected. All were consecutive non-elected cases from a single center and all patients were treated outside of clinical trials. No patients were candidates for surgical treatment (either curative or palliative); however, all received palliative chemotherapy according to established protocols.

Chemotherapy regimens were based on single agent leucovorin modulated 5-fluorouracil (5-FU) (Mayo clinic) or combination treatments of 5-FU (De Gramont or simple infusion and leucovorin) with either oxaliplatin or irinotecan, or capecitabine with or without bevacizumab or cetuximab.

Records with complete data (for the parameters used as prognostic factors) were included in the analysis. Follow-up was continued until death from CRC or from any other cause, and patients who remained alive were censored as of January 1, 2015. Overall survival was the primary endpoint. This protocol was approved by the Ethics Committee of the National and Kapodistrian University of Athens on the 5th of April 2013 (approval number: 7315).

Prognostic variables. Twenty-four potential prognostic variables were selected. These included patient-related variables such as age, body surface area (BSA), gender, performance status (PS) according to the Karnofsky performance status scale index (before chemotherapy initiation, and after receiving it) response to therapy, location of distant metastases (lymph nodes, liver, lung, abdominal, pelvic, locoregional disease, bone, skin, adrenal glands) symptoms of fever, side-effects from therapy such as neutropenia, anemia, thrombocytopenia, nausea and vomiting, diarrhea, anorexia, weight loss, mucositis, fatigue; anemia with need for administration of erythropoietin and red blood cells transfusion, and laboratory parameters such as albumin, C-reactive protein (CRP), carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19.9) values.

DNA measurements (ploidy). For DNA measurements, the Feulgen staining technique was applied as previously described (9). The nuclei of Feulgen-stained cells from the histology of the primary colorectal tissue biopsy were evaluated for DNA ploidy using a Nikon eclipse microscope (Nikon, Tokyo, Japan) connected to a Nikon CCD video camera and an IBM Pentium 4/PC cell measurement software (Image Pro Plus v. 5.1; Media Cybernetics Inc., Silver Springs, MD, USA). Areas of the Feulgen-stained sections containing pathological lesions, identified in adjacent stained slides, were selected for DNA content analysis.

A total of 200-300 nuclei with clear boundaries appearing to have no loss of membrane integrity were analyzed in each tissue sample. Cytometric measurements were performed at a magnification of $\times 200$ and calculated automatically according to the algorithms described previously by measuring the nuclear integrated optical density (IOD), representing the cytometrical equivalent of DNA content (10).

The procedure was performed for all nuclei, and the overall mean represents the DNA content or DNA index (DI). The mean IOD of human lymphocytes (control cells) was used as the diploid standard (2c) and reference for DI calculation for targeted cells. DNA histograms were generated and a tumor was classified as diploid when the DI ranged from 0.9 to 1.1 and the relevant DNA histogram revealed only one peak at 2c, and aneuploid when either of these two criteria was absent.

Table I. *Clinical and laboratory characteristics of patients.*

Parameter	Frequency	Percentage
Gender		
Male	55	55
Female	45	45
Performance status before therapy		
70	13	13.0
80	42	42.0
90	22	22.0
100	23	23.0
Performance status on therapy		
Improvement	18	18.0
Stable	37	37.0
Worsening	45	45.0
Symptoms		
Fever	27	27
Weight loss	55	55
Pain	20	20
Metastasis		
Lymph nodes	52	52.0
Liver	65	65.0
Lung	25	25.0
Peritoneal	50	50.0
Pelvic	36	36.0
Local recurrence	35	35.0
Bone	8	8.0
Skin	0	0
Adrenals	2	2.0
Other examined parameters		
Use of epoetin	25	25.0
Blood transfusion	9	9.0
Hypoalbuminemia	15	15.0
Response		
CR	5	5.0
PR	19	19.0
SD	36	36.0
PD	40	40.0

CR: Complete response, PR: partial response, SD: stable disease, PD: progressive disease.

Stem cell measurements. The immunohistochemical score (IHS) for immunostaining of CD133, as a marker of stem cells, was calculated for each case. The scoring system used was similar to previously published methods (4). CD133 immunostaining was measured in the cytoplasm of isolated cancerous cells and in luminal secretions of malignant gland and towards the luminal surface of malignant glandular structures. The extent of positively stained epithelial cells was estimated and classified on a four-point scale as follows: no staining=0%, 1=1-10%, 2=11-25%, 3=26-50%, and 4=51-100%.

Statistical analysis. Summary statistics of continuous variables were based on the mean followed by the corresponding 95% confidence interval, or standard deviation and range. In the case of severely dispersed variables values were transformed into the natural logarithm; in such cases the reported statistics were based on the geometric mean. Categorical variables are summarized by absolute and relative frequencies.

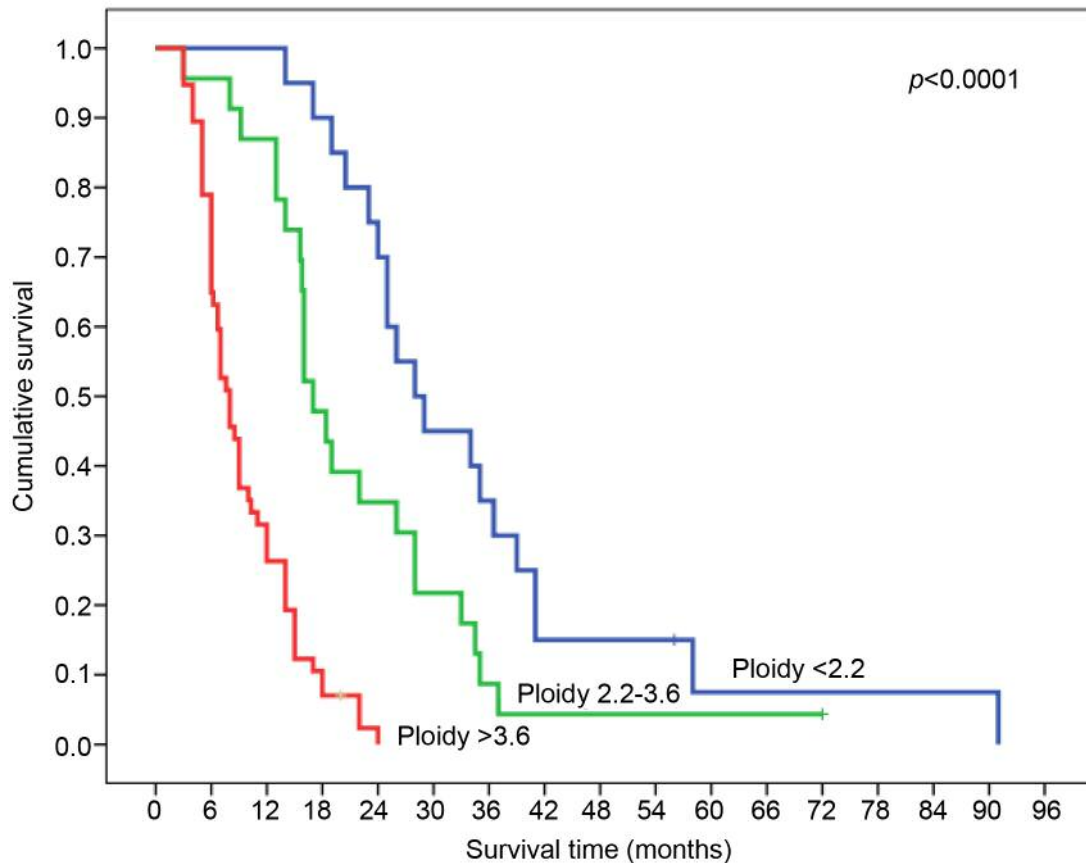


Figure 1. Survival according to ploidy.

One-way analysis of variance (ANOVA) or Chi-squared statistic were applied in order to investigate the association of patient responses with clinical characteristics.

Time-to-event analysis with regards to overall survival was investigated by log-rank statistics and illustrated by Kaplan–Meier curves. Variables with level of significance of $\alpha=1\%$ at the univariate analysis as well as demographics and stem cells were inserted into the Cox proportional hazard regression model in order to identify potential prognostic factors for survival.

All tests were two-sided and the acceptable level of statistical significance was set at 5% and were performed with SPSS 12.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

From the total of 100 patients with histologically-confirmed CRC initially participating in the study, all were included in the statistical analysis. A number of clinical parameters and their relationship with survival and overall response were studied. Descriptive statistics are summarized in Tables I and II.

Patients. Patient characteristics are presented in Tables I and II.

Differences in clinical parameters between treatment groups.

Univariate analysis of response. A univariate analysis comparing response to therapy with regards to the clinical parameters under study was carried out. The differences in clinical parameters between response groups were studied with the use of bivariate tests and statistically significant associations between response and clinical parameters were identified. Responding groups (Responders, complete and partial response to therapy: 24.5% of patients; non-responders, progressive disease: 40.8% of patients) had significant differences with regards to several parameters. Responders in comparison to non-responders had low ploidy score ($p < 0.0001$), improved performance status by therapy ($p < 0.0001$), low CEA ($p < 0.001$) and CA 19.9 values ($p < 0.0001$), and had rarely presented anemia ($p < 0.007$), anorexia ($p < 0.0001$), weight loss ($p < 0.016$), mucositis ($p < 0.044$), or fatigue ($p < 0.001$) during therapy.

Univariate analysis of survival. The mean follow-up was 73 months. A univariate analysis was applied to compare survival with regards to the remaining clinical parameters. The

Table II. Clinical and laboratory characteristics of patients (n=100).

Examined parameters	Geometric mean±SD (range)	95% CI
Age, years	58.55±8.77 (38.00-78.00)	
BSA, m ²	1.79±0.13 (1.40-2.10)	
CRP, mg/l	0.81 (0.00-35.00)	0.47-1.22
CEA, ng/ml	29.67 (1.00-6140.00)	20.51-42.74
CA19-9, U/ml	84.35 (1.00-4194.00)	60.93-116.63
Ploidy score	3.01±0.86 (1.65-4.27)	2.84-3.18
Stem cells, %	19.09±18.09 (0.00-83.00)	15.50-22.68

CI: Confidence interval, SD: standard deviation, BSA: body surface area, CRP: C-reactive protein, CEA: carcinoembryonic antigen, CA19-9: cancer antigen 19-9.

differences in clinical parameters were improvement of performance status ($p<0.0001$), responders ($p<0.0001$); low ploidy score ($p<0.0001$) (Figure 1), CEA ($p<0.0001$) and CA 19.9 ($p<0.0001$) and absence of weight loss ($p<0.0001$), neurological toxicity ($p<0.004$), thrombocytopenia ($p<0.016$), anorexia ($p<0.0001$), weight loss ($p<0.0001$), mucositis ($p<0.04$), and fatigue ($p<0.007$).

Univariate analysis of ploidy. A univariate analysis of ploidy with regards to the remaining clinical parameters presented significant correlations between ploidy and the examined parameters, namely a low ploidy score was associated with improvement of performance status ($p<0.001$), and response to therapy ($p<0.001$).

Univariate analysis of stem cell percentage. A univariate analysis comparing stem cell percentage with regards to the remaining clinical parameters did not find significant correlations between stem cell frequency and improvement of performance status ($p=0.928$), response ($p=0.750$), gender ($p=0.561$), ploidy ($p=0.939$) nor the remaining parameters examined.

Multivariate analysis. A multivariate Cox proportional odds regression model was implemented for the study of the parallel effect of parameters on survival. The best model was selected with the use of automated techniques. In the multivariate analysis, independent unfavorable prognostic factors were increased ploidy score ($p<0.0001$), existence of bone metastases ($p=0.044$), use of epoetin ($p=0.014$), and existence of side-effects such as anorexia ($p=0.033$), mucositis ($p=0.002$), and weight loss ($p=0.001$) (Table III).

Discussion

In the present study, we tried to evaluate the prognostic influence of stem cells in relation with clinical and laboratory parameters in a multivariate process.

Table III. Multivariate Cox proportional odds regression of survival.

Factor	B	HR	95.0% CI		p-Value
			Lower	Upper	
Bone metastasis	0.846	2.331	1.022	5.314	0.044
Epoetin	0.756	2.129	1.167	3.886	0.014
Ploidy	10.757	5.797	3.740	8.988	<0.0001
CA19-9	0.254	1.289	1.122	1.482	<0.0002
Anorexia	0.160	1.174	1.013	1.360	0.033
Mucositis	0.202	1.223	1.074	1.393	0.002
Weight	0.894	2.446	1.472	4.065	0.001

CA19-9: Cancer antigen 19-9, CI: confidence interval, HR: hazard ratio.

Cancer stem cells are distinct within tumor for their specific characteristics that give them the dynamic of tumor genesis, metastasis, recurrence and resistance to chemotherapy. CD133, a pentaspan transmembrane protein, is the most widely used stem cell marker for the identification of tumor-initiating cells in patients with CRC. The regulation of CD133 expression is controlled by many extracellular and intracellular signs. It has been revealed that hypoxia, mitochondrial dysfunction or the depletion of the mitochondrial DNA reversibly up-regulate the expression of CD133 (11). CD133 immunostaining was measured in the cytoplasm of isolated cancerous cells and in the malignant glands' luminal secretions and towards the luminal surface of malignant glandular structures (12).

In the present study, no significant correlations were found between stem cell percentage and improvement of performance status or response. Despite the negative statistical result for this sample of patients, the existence of CD133 cells in the transition zone of neoplastic and normal tissue indicates the need for further analysis of a larger population after the identification of additional markers such as CD44 may reveal any existing correlation between colon cancer stem cells and survival or treatment response (1-4, 13, 14).

DNA ploidy is a well-established prognostic and predictive factor (5-7). In the present study, a low ploidy score was associated with improved performance status and response to treatment in metastatic patients with CRC.

Furthermore, this analysis confirms the predictive and prognostic importance of laboratory parameters such as CEA and CA 19.9, as well as clinical factors, such as the improved performance status and the absence of weight loss, anaemia, thrombocytopenia, mucositis and fatigue (5-7, 15-18).

In the multivariate analysis, the independently unfavorable prognostic factors were increased ploidy score, existence of bone metastases, the use of epoetin and the existence of treatment-related side-effects such as anorexia, mucositis and weight loss. Patients with ploidy scores 2.2-3.6 had 2.94-fold

and those with >3.6 had 4.98-fold higher probability of death, respectively, as compared to those patients with a ploidy score <2.2.

In conclusion, this study represents a comprehensive analysis of molecular, clinical and laboratory parameters with possible predictive or prognostic role in patients with metastatic CRC receiving palliative chemotherapy. Our study highlights the predictive and prognostic role of well-established factors such as DNA ploidy, cancer markers such as CEA and CA 19.9, and good performance status. Further analysis should be conducted in order to identify the prognostic and predictive significance of CSC in patients with metastatic CRC. Randomized controlled population studies in a prospective setting should be performed in order to evaluate the best model of markers to allow a personalized therapeutic process.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

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