University of Medicine and Pharmacy of Craiova

Doctoral School

PhD THESIS

TRANSLATIONAL ASSESSMENT OF PUTATIVE CANCER STEM CELLS EXPRESSION IN COLORECTAL CANCER PATIENTS

-REZUMAT-

Scientific coordinator,
Prof.Univ.Dr.Adrian Săftoiu

PhD student,
Irina Florina Cherciu

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TABLE OF CONTENTS

KEY WORDS

Colorectal cancer(CRC), cancer stem cells (CSC), confocal laser endomicroscopy(CLE), imunoendomicroscopy, biomarkers CD133, CD166, CD44, molecular biology, quantitative real-time PCR(qRT-PCR), imunohistochemistry (IHC).

INTRODUCTION

Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide, in Europe approximately 447000 patients being diagnosed in 2012, this representing 13% of all cancers from this region. High variations of CRC incidence are observed along Europe, in Romania representing the second leading cause of mortality and morbidity, in both genders(1) The continuous increase of CRC prevalence, especially among developing countries, is associated with two main aspects: the existing phenomenon of population ageing and the unhealthy westernized lifestyle including a diet with low intake of fresh fruits and vegetables, rich in processed food, fats, red meat and the lack of physical activity(2).

CRC was and continues to be intensely studied. With all the improvements in the oncologic therapeutic protocols registered during the last two decades, the problem of treatment failure and tumor recurrence remains a keystone. Multitudinous therapeutic protocols have been dedicated to CRC and several research studies have determined conventional prognostic factors, without yielding a complete cure in all cases.CRC survival at 5 years remains around 50%(2,3), hence the **aim** of our study: improvement of survival rates among CRC patients.

Actually, two possible models of CRC carcinogenesis are described: stochastic model(stating that any cell has an equivalent potential of cancer initiation and promotion) and the newly concept of cancer stem cells(CSC). This second model brings additional explanation stating that intratumoral heterogeneity can result from a functional diversity among cells in various states of differentiation. Accordingly, the tumors are organized in a definite hierarchical degree and only CR-CSCs are the cancer progenitors, requiring the accumulation of genetic/epigenetic alterations and acquiring subsequently the potential of tumor initiation, tumor growth and therapy resistance (4, 5). In this context, the need of identifying these cells strikes.

CSC identification and localization, through specific markers, represents difficult processes, similar with understanding signaling pathways and other regulatory mechanisms related to CRC. Isolation of CSC from CRC can be achieved by targeting and selecting subpopulation of tumor cells, based on the expression of one or multiple cell surface markers associated with cancer self-renewal, as literature is proposing: CD133, CD166, CD44, CD24, beta1 integrin-CD29, Lgr5, EpCAM (ESA), ALDH-1, Msi-1, DCAMLK1 or EphB

receptors(5,6). The identification of reliable markers targeting specifically the CSCs fraction is an imperative pathway to be followed. It might represent the desired progress in the development of new molecular targeted drugs, appropriate patients selection and stratification, early evaluation of treatment effects and individualized therapy. It remains a controversial topic due to the lack of general accepted markers of CSC.

In consequence, the study of CRC is a challenge requiring translational research and the use of state of art techniques as confocal laser endomicroscopy (CLE) in tandem with molecular biology and imunohistochemistry(IHC) for assessing the expression characteristics of CSC.

CLE is an innovative endoscopic imaging method which enablesreal time histological examination of the colorectal mucosa following the systemic or topic administration of contrast agents. Except describing the mucosal morphological aspects, immunoendoscopy(CLE association with fluorescently labeled antibodies and peptides which directly bind to their targets) allows the functional assessment up to molecular level.

Already proved to be feasible in fundamental research, CLE made the first steps towards the technical translation into the clinical practice (7,8), outlining the framework for early diagnosis and for the innovation of targeted CSC therapies. Although the research regarding CSC has amplified during the last years, their complex biology, an undeniable identification and isolation strategy remains a topic of continuous debate (9).

In this context, we associated a modern imagistic method with already established techniques in order to reach the following **objectives**:1) Identification, assessment and optimization of the most relevant CSC biomarkers from CRC; 2) Assessing the feasibility of CLE use for CRC evaluation and validating the results through IHC and molecular biology techniques.

PERSONAL CONTRIBUTIONS

MATERIAL AND METHODS

The study took place in the Research Center of Gastroenterology and Hepatology, UMF Craiova and in the Clinic of Gastroenterology, Emergency Clinical County Hospital, Craiova, Romania and it included prospectively, during the period October 2012-September 2015, several group of patients, as it follows:

Group I: enrolled 126 patients with CRC in a study regarding the demographic, clinic and anatomopathology peculiarities.The presumptive diagnosis of CRC was suspected in the presence of digestive signs and symptoms as: abdominal pain, diarrhea, constipation or change in bowel habits, hematochezia, rectorragia, tenesmus, anemic syndrome, unexplained weight loss, weakness or fatigue, anemia.CRC diagnosis was confirmed after colonoscopy and biopsy with histological confirmation, the staging being determined through computer tomography or/and endoscopic ultrasonography. Before the minimal invasive explorations and tissue sampling, all patients included in the study signed an informed consent, subsequent to the explanation of the procedures and clarifying possible uncertainties. All the procedures took place in accordance to the Declaration of Helsinki of Ethical Principles for Medical Research Involving Human Subjects, as stated on the World Medical Association website. A data base was created in order to register the general identification data, medical, personal and family history, clinical and paraclinical results, information regarding the administered treatment and survival.

Statistical analysis

The measured parameters for the subjects included in this group were stocked, analyzed and graphically represented utilizing Microsoft Office Excel® was used for stocking and analyzing the registered data. Descriptive statistics of quantitative variables involved several parameters: average, standard deviation, minimum, maximum. Qualitative variables were expressed as percentage.

Group II: involved 13 patients diagnosed with advanced CRC. Minimum two paired biopsies of normal and tumor tissue, with an average diameter of approximately 3mm, were subject of an ex vivo CLE examination after being harvest during routine colonoscopy and specifically stained with fluorescently marked anti-CD133, anti-Cd166 and anti-CD44 antibodies. The CLE evaluation was performed using a dedicated CLE system which integrates a miniature confocal microscope into the distal tip of a conventional flexible endoscope (EC-3870 CIFK, Pentax, Tokyo, Japan). The CLE images presenting the highest fluorescence signal and a clear pattern of the tissue architecture where selected, stored and analyzed offline by using the ImageJ software.

Statistical analysis

The results of stem cell counting were exported in Excel programme, being expressed as average± standard deviation. The Pearson correlation coefficient(R) was applied in order to assess the linear ratio between the paired biopsies. All statistical tests had a signification level settled for 5% ($p<0,05$).

Group III: included 45 patients with colon cancer. An imunnohistochemical study regarding the expression of CD133 and CD166 biomarkers was performed. The available clinicopathological information included gender, age, tumor size, depth of invasion, lymph node metastasis, distant metastasis, tumor stage, grading, and lymph node status. The histology study implied classical staining methods as Hematoxylin and eosin (HE). CD133 and CD166 expression was evaluated by two independent pathologists, utilizing a blind methodology, without knowledge of clinical and pathological information. The phenotypic characterization and descriptive localization of the signal in the tissues was followed by immunofluorescent semiquantitative analysis. The working method followed a standard algorithm with several adjustments according to the antibodies used for immunoblotting: PROM1 (CD133) MAB10525, ALCAM (CD166) HPA010926, Ki-67 M7240, ab91353, ab27478.

Statistical analysis

Differences between groups were investigated utilizing the student's t test and ANOVA analysis, while correlations were reported using the Pearson's statistics. Statistical significance was deemed for $p < 0.05$.

Group IV: involved 60 patients diagnosed with CRC. Tumor and peritumor biopsies were harvested during colonoscopy for all the patients. A two step Real-Time qRT-PCR method was used for measuringCD133, CD166 gene activation level in 51 paired biopsies and CD44 gene activation level in 19 paired biopsies of normal and tumor tissue for the assessment. The complementary DNA (cDNA) synthesized by reverse-transcription was quantified using TaqMan technology.

Statistical analysis

Comparative expression of targeted genes in paired tumor and peritumor mucosa was assessed by Wilcoxon matched-pairs signed rank test. Data are presented as relative mRNA expression of target gene to GAPDH. Student Ttest or paired T test was used in order to determine the signification degree of the differences registered in the gene activation level. Results were considered statistical significant for $p<0.0001$.

RESULTS AND DISCUSSIONS

The study regarding demographic, clinical and pathology included 126 CRC patients with an average age of 61,65 ± 13,25 and the age limits between 24 to 83 years old. CRC incidence appeared to be higher among patients over 50 years old, 85,66% of the diagnosed patients being framed the age groups above this limit. The gender distribution revealed that 61% of the patients from the study group were men. The environmental origin was in most of the cases the urban area. The histology exam reported 75,8% of the tumors as moderately differentiated adenocarcinoma. 60% of the tumor masses were located along the rectum and sigmoid. Considering the short period of the study, survival was calculated only for 3 years after CRC diagnosis (70,37%).

The role of confocal laser endomicroscopy in assessing the cancer stem cells from colorectal cancer

In order to assess the CSC expression through the CLE technique, we proposed three biomarkers: CD133, CD166 and CD44.

Regarding the CLE evaluation of the CD 133 biomarker, only 10 pictures were suitable selection and analyses. The cell count revealed 16,3±3,87 cells/slide for the tumor tissue and 37,45±24,36 cells/slide for the normal tissue ($p<0,01$). Biopsies marked with anti-CD166 antibody showed no fluorescent signal, the images being impossible to be evaluate through CLE, similar with the observations previously published (10).

A number of 537 slides were analyzed, stacked and the most 65 appropriate were selected for cell counting using Image J. The distinct fluorescent signal was analyzed with the CLE scope in all biopsies both in normal and in tumor tissue samples. Standard biopsy sections from normal tissue revealed a well defined layout of the mucosal structures, with normal size and regular arrangement of crypts and a reduced number of CSCs. In tumor samples these characteristics were lost, the architecture had an irregular arrangement with destroyed crypts and unrecognizable structures while the presence of CSCs was increased.

CLE evaluation of the selected images revealed 34, 92±16 cells/slide for the tumor tissue and 22, 77±11, 65 cells/slide for the normal tissue ($p=0,037$). Regarding the correlation between CLE cell count and tumor T or N staging, the highest number of patients was found in T3, N0 or N1 stage category. CD44 expression was higher in tumor versus normal tissue and statistical relevant in stage T3/4: $p= 0,028$, $r= 0,622$ while for N0: $p=0, 1$, $r= 0, 92$ and for N1: $p= 0, 1$, $r= 0, 8$. The average cell count in tumor tissue, according to tumor grading, was as it follows: for G1 grade 42,8±18,8 cells/slide, for G2 34,7±13 cells/slide and for G3 19±9,89 cells/slide. The average cell count from normal mucosa, reported to the grading of adjacent tumor, was for as it follows: for G1 grade 24,2±14,55 cells/slide, for G2 24,6±10.76 cells/slide and for G3 13,5±0,7 cells/slide. The registered differences were not statistical relevant.

To our knowledge, this is the first report on CSCs imaging obtained with CLE by utilizing fluorescently labeled antibodies targeting several cancer stem cell markers, previous studies focusing on the assessment of angiogenesis in CRC (11, 12, 13). We selected as

molecular target a cell surface biomarker, labeling normal and tumor cancer stem cells in order to obtain a distinctive evaluation of the expression patterns. We can assert the feasibility of CLE for the assessment of colorectal tumors, although supplementary studies might be necessary for optimizing the CLE technique. A large spectrum of CSC biomarkers, well established protocols for the immunomarking and larger number of patients from the study group represent several directions to be followed.

Although the results were not statistical relevant in all cases (probably due to the reduced number of patients included in the study groups), and only a partial correlation with tumor staging and grading was observed, several positive aspects regarding CLE should be taken into consideration. Using fresh tissue avoids the appearance of artifacts due to processing but the main advantage is represented by real time imaging of CSCs performed within a short time after specimen harvest which might considerably accelerate the diagnosis procedures.

Even more, in vivo CLE promises results obtained in real time, facilitating the process of patient selection for individualized therapy and also the chance of assessing the treatment effects and patients evolution (14, 15).

Imunohistochemistry assessment of putative cancer stem cells from colorectal cancer

In our study, the triple immunofluorescence CD133/CD166/Ki67 assessment conducted to the observation that both CD133 and CD166 were expressed to different extents in all normal and primary cancer tissue samples. Also, we observed a gradual increase in the CD133 expression from non-neoplastic mucosa, to low grade dysplasia and towards high grade dysplasia.

Regarding **CD166 expression** in normal mucosa, we also noticed a prevalent membranous expression along the luminal and lateral borders of the cells from the base of the crypts, suggesting that, as a cell adhesion molecule, CD166 may play an important role in maintaining the integrity of the stem cell niche, or in directing the cells from the base of the crypts (17, 19, 20).

Tumor reactivity for the anti-CD166 antibodies showed both cytoplasmic and membranous patterns, with a more pronounced membranous expression. Overall, tumors

showed a heterogeneous staining pattern inside the cancerous lesions. The percentage of CD166 staining areas in tumor epithelium ranged from 0.89% to 28.86% of the 40× objective area (or between 377.8 μm2 and 12252.65 μm2).

As for the proliferating indexes, we found a moderate indirect correlation with CD166 expression areas for confirmed adenocarcinoma (r=-0.428).

For the tumor stage, we found that only for poor differentiated tumors, CD166 expression was higher in advanced stages (5581.73 ±3275.67 μm2) compared to early stages (2466.30 ± 1742.11 μm2) (p=0.039).

Colocalization areas for CD133/CD166 were obvious especially at the cells membrane level along the entire spectrum of lesions. Colocalization coefficients had higher values in early stage tumors (0.77±0.088), compared to advanced tumors (0.63±0.154) (p=0.0014).

Regarding the immunohistochemical expression rates of both markers in CRC, literature data indicate very wide variations. Thus, the rate of CD133-positive staining was reported to be between 15.3-91.4% (16, 17, 18, 21, 22), while for CD166, CRC reactivity varied between 34 - 76% (17, 19, 20). The prevalent tumor staining pattern for CD133 in our study was cytoplasmic, while for CD166 it was mainly membranous-like, which is in accordance with previously reported results (16, 19, 21, 22, 23).

Overall, in our study, CD133 expression correlated with that of CD166 in the entire spectrum of lesions, but with higher correlation levels being noticed only for the dysplastic lesions. (r=0.306). A higher correlation was noticed only for the dysplastic lesions (r=0.52). In other studies, CD133 expression correlated with that of CD166, while both did not correlate with CD44, hence the combined analysis of all three markers might be superior in identification of low-, intermediate-, and high-risk cases of CRC (24).

The implications of molecular biology in assessing the gene expression of cancer stem cells from colorectal cancer

Currently, qRT-PCR is considered the gold-standard technique for measuring gene expression, enabling the detection of differences of only a few copies of mRNA per cell. The

analysis of the gene expression using Real-Time PCR provides the opportunity to quantify the different expression levels of a given gene in a patient population.

Three specific CSC genes were targeted in our study: PROM1/CD133, AlCAM/CD166 and CD44, being already established by several other studies involving IHC or molecular biology techniques, that the combined use of biomarkers is efficient for identifying CSC and tumors with negative prognosis. Similar to the literature, the qRT-PCR analysis of our studies paired biopsies detected in different extents all the three genes, in both normal mucosa and in the tumor tissue.(24, 25, 26)

The expression of total CD44 was assessed in 19-paired samples of CRC tumors and adjacent tissue. qRT-PCR analysis revealed that CD44 is expressed in both tumor and peritumor mucosa. Total CD44 expression was higher in tumor tissue compared with paired non-invaded peritumor samples in 15cases (78.95%) and in the remaining 4 cases (21.05%) the difference between paired samples was biologically insignificant(Figure 1).

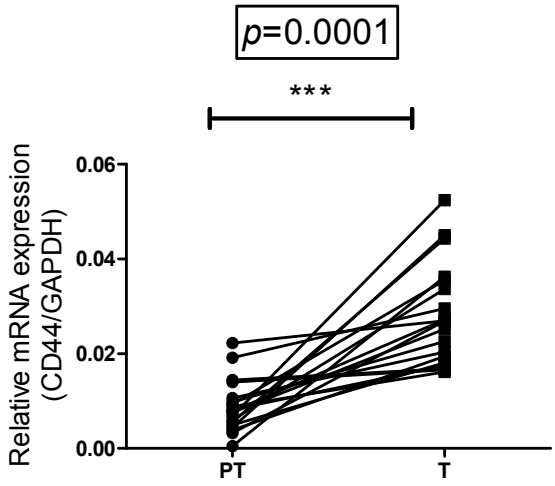

Figure 1. Comparative expression of CD44 mRNA in paired tumor and peritumor mucosa (n=51). Data are presented as relative mRNA expression of target gene to GAPDH. Wilcoxon matched-pairs signed rank test, $p<0.0001$.

The expression of total PROM1 was assessed in 51-paired samples of CRC tumors and adjacent tissue. Total PROM1 was higher expressed in tumor tissue compared with paired non-invaded peritumor samples in 8 cases (15.69%); in 24 cases (47.06%) the expression was higher in non-malignant peritumor tissue and in the remaining 19 cases (37.25%) the difference between paired samples was biologically insignificant(Figure 2).

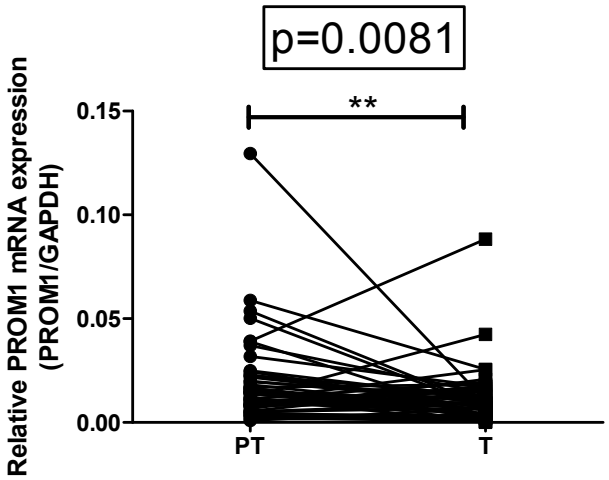

Figure2. Comparative expression of PROM1 mRNA in paired tumor and peritumor mucosa (n=51). Data are presented as relative mRNA expression of target gene to GAPDH. Wilcoxon matched-pairs signed rank test, p<0.0001.

The expression of total ALCAM was assessed in 51-paired samples of CRC tumors and adjacent tissue. The total ALCAM expression was increased in tumor tissue compared with paired non-invaded peritumor samples in 7cases (13.73%); in 7 cases (13.73%) the expression was higher in non-malignant peritumor tissue and in the remaining 37 cases (72.55%) the difference between paired samples was biologically insignificant(Figure 3).

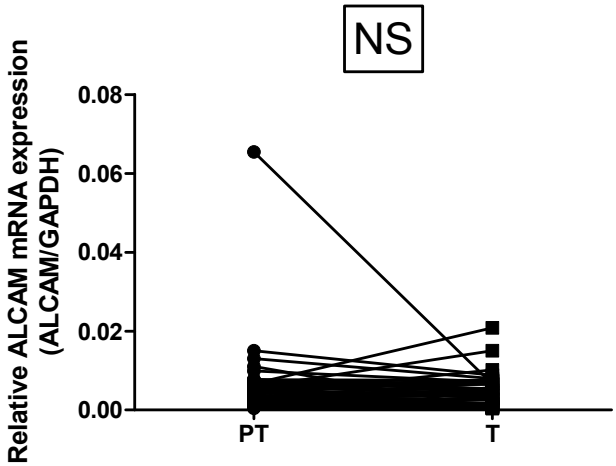

Figure 3. Comparative expression of ALCAM mRNA in paired tumor and peritumor mucosa (n=51). Data are presented as relative mRNA expression of target gene to GAPDH. Wilcoxon matched-pairs signed rank test, p<0.0001.

In our study, the CD44 expression was higher in tumor tissue, alike previous published data(27),being in the same time in consensus with the pronounced CD166 expression in the neoplastic tissue. On the other side, CD133 had a higher activation level in the normal mucosa, opposite to some of the results mentioned in the literature. Therefore, while the CD133 expression is described as heterogeneous in different type of tissues without statistical relevance (28),other studies claim an elevated expression level of CD133 in tumor tissue (29, 30). Regardless the inconstant expression level of CD 133 in different type of tissues, in our study the qRT-PCR method conducted to statistical significant results, attaining a high potential for assessing the CSC expression.

Conclusions:

- Colorectal cancer (CRC) represents the second leading cause of mortality and morbidity in Romania, in both genders, the demographic, clinical and pathology data from of our study being consistent with the literature results.
- CRC carcinogenesis is described by two models: stochastic and the cancer stem cells (CSC) hypothesis. The assessment of CSC through modern imagistic techniques, represented the main research direction in our study.
- Confocal laser endomicroscopy (CLE) is a „state of the art'' endoscopic technique, allowing the scan of a tissue up to molecular level, facilitating the real time histology diagnosis along with an offline computer analysis of the acquired CLE images.
- Imunoendoscopy represents the association between CLE and the use of biomarkers ment to fluorescently stain specific targets. In our study, out of the three markers, only CD44 proved to be feasible in the imunomarking process and in the endomicroscopy assessment of CSC. CD133 imunomarking requires an adjustment of the staining protocol, the results of our study being partially satisfactory. CD166 was inefficient in the process of identifying CSC through CLE.
- The outcomes of our investigation prove the utility of CLE technique, setting a solid background for further research. The minimal time spent between the collecting the biopsies and obtaining the endomicroscopy images, justifies CLE for the transfer into the clinical practice.

- The results of our immunohistochemistry study suggest that the colocalization of CD133 and CD166 is an early event in colon tumorigenesis. Also the co-expression of these two markers could be useful in the prognostic and therapeutic stratification of patients with colon cancer, considering the higher colocalization coefficients noticed in well-differentiated and early stage tumors.
- The molecular biology assessment of the CSC biomarkers, similar to endomicroscopy and imunohistochemistry evaluations, leaded to the observation that CSC occurred in both normal and tumor tissue but in different proportions (CD133 gene was prevalent in normaltissue while CD44 and CD166 geneshad a higher activation level in tumor tissue).
- The combination of CLE with imunohistochemestry and molecular biology is feasible for assessing the expression patterns of CSC markers in colorectal cancer.
- Further extended studies are required for the identification of specific CSC biomarkers for CRC.CSC carry an important role in early diagnosis, prognosis and in the development of individualized therapies, targeting precisely these subgroup of cells which is not targeted momentary by any drug.

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