INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy worldwide, accounting for over 9% of all cancer cases [1, 2]. Europe has one of the highest incidences, with the fastest increase being documented in the Eastern Europe countries [3]. It has been estimated that about 30% of the patients will develop distant metastases [4], and more than 50% of the patients with stage III disease will develop local recurrence and/or metastases [4, 5].

The existence of drug-resistant colon cancer stem cells (CSCs) is thought to be one of the most important reasons behind treatment failure in CRC, these cells ultimately being the likely cause of metastasis and recurrences [6-9]. The strategies for colon CSCs identification and isolation are still a matter of debate [10-12]. During the last decade, several molecules have been proposed as colon CSC surface markers including CD133 and CD166 [12-14].

Prominin-1 (PROM1, CD133) is a 120-kDa transmembrane glycoprotein [15], preferentially localized in plasma membrane protrusions and microvilli [16], that might play a role in cell polarity and migration via cell-cell and cell-matrix interactions...
In addition, several studies have evidenced that CD133 expression may serve as an independent marker for prognosis and chemoresistance in CRC [7, 18-21]. However, other authors have failed to establish a correlation between CD133 expression and tumour progression or survival [14, 22], making thus questionable its predictive value in CRC.

Activated Leukocyte Cell Adhesion Molecule (ALCAM, CD166) is a 110-kDa transmembrane glycoprotein that belongs to the immunoglobulin superfamily of cell adhesion molecules [23], which mediates intercellular adhesion [24, 25]. Over the past decade, alterations in expression of CD166 have been reported to correlate with an aggressive disease phenotype in a variety of cancers [26-30]. However, inconsistent data also exist regarding the prognostic significance of CD166 expression in CRC [19, 31-34].

In the present work, we aimed to investigate the expression patterns of CD133 and CD166 in colon carcinoma, both individually and as colocalization ratios, in order to assess their significance as prognostic markers.

**METHODS**

**Patients and specimens**

This retrospective study was based on a consecutive series of 45 colon adenocarcinoma cases treated by surgical resection in the Craiova Emergency Clinical Hospital between 2011-2012. The available clinicopathological information included gender, age, tumour size, depth of invasion, lymph node metastasis, distant metastasis, tumour stage, grading, and lymph node status. Due to the short period of the study, only two deaths were recorded among these patients, following cardiovascular complications. The tumours were graded as well, moderately and poorly differentiated, according to the WHO grading criteria [35]. Staging was based on pathological findings according to the AJCC cancer staging manual (2010) [36]. For statistical purposes, stages I and II were grouped as early forms, while stages III and IV as advanced stages. In order to compare the immunoreactivity of the markers, we have also chosen from our pathology archive 15 cases of low grade dysplasia (LGD), 10 cases of high grade dysplasia (HGD), and 10 tissue blocks of normal colon mucosa. The study was carried out with full local ethical committee approval and a written informed consent obtained from all the patients.

**Enzymatic immunohistochemistry**

Hematoxylin and eosin (H&E)-stained slides were reviewed to confirm the diagnosis. From the corresponding blocks, sections were incubated with the corresponding primary antibodies (Table I) at 4°C, overnight, followed by signal amplification using the Histofine Simple Stain MAX Peroxidase Mouse and Rabbit detection systems (Nichirei Biosciences, Tokyo, Japan) according to the manufacturer’s protocols. Detection was performed with 3,3’-Diaminobenzidine (DAB) chromogen substrate solution (Vector Laboratories, Peterborough, United Kingdom). The slides were counterstained with Mayer hematoxylin (Sigma-Aldrich, Munich, Germany).

CD133 and CD166 expression was evaluated by two independent pathologists, utilizing a blind methodology, without knowledge of clinical and pathological information. The sections were scanned at high magnification to assess the positivity of staining in tumour cells. Intraluminal CD133 expression patterns were not taken into account. This data was used for phenotypic characterization and descriptive localization of the signal in the tissues, and was followed by immunofluorescent semiquantitative analysis (see below).

**Multiple fluorescence immunohistochemistry**

For triple stainings (CD166, CD133, and Ki-67), the sections were first incubated with the mouse anti-human Ki-67 (1:100), amplified with a human-adsorbed peroxidase polymer-based system (Nichirei Biosciences) for 30 minutes, and visualized with Alexa Fluor 488 labeled tyramide (Invitrogen, Bucharest, Romania), with a precipitation time of 1.5 minutes. The anti-Ki-67 primary antibody was eluted from the slides using an acidic SDS-glycine solution [37]. The next two primary antibodies were added together on the sections, followed next day by a mixture of goat anti-mouse Alexa Fluor 546 (Invitrogen) and goat anti-rabbit Alexa Fluor 610 (Invitrogen) antibodies (1:300) for 90 minutes at room temperature. In all cases, the slides were counterstained with DAPI (Invitrogen), incubated for 30 seconds in a 0.3% alcoholic solution of Sudan Black (for quenching lipid autofluorescence), and finally coverslipped.

Negative controls were first obtained by omitting the primary antibodies. Moreover, in order to ascertain the specificity of the primary antibodies, species and subclass-specific isotype

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<td>PROM1 (CD133)</td>
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* Antigen retrieval utilized: microwaving in 0.1 M Citrate pH=6
antibodies were utilized matching the concentrations of the respective primary antibodies (Table I and Supplementary Fig. 1, http://www.jgld.ro/2014/2/supplementary-Fig.1.jpg).

**Image processing and colocalization analysis**

Fluorescent images were grabbed under a 40× objective utilizing a Nikon Eclipse 90i motorized microscope equipped with a 1.4-megapixel monochrome Rolera-XR cooled CCD camera (Q-Imaging, Surrey, BC Canada), together with the Image ProPlus AMS analysis software (Media Cybernetics, Bethesda, MD, USA). As signal areas and intensities were highly variable in the tissues, pictures for image analysis were collected from the areas with the highest densities of CD166, CD133 and respectively Ki-67 signals. Images were obtained by sequentially scanning each channel with specific pairs of highly selective custom-made filters in order to eliminate the cross-talk of the fluorophores and to ensure a reliable quantification (Chroma Technology Corp., Bellows Falls, USA). All images were originally stored in Image ProPlus’s proprietary format, and then they were subjected to a blind deconvolution algorithm based on a multi-pass adaptive point spread function subtraction of diffracted light (AutoDeblur, Image ProPlus AMS package). Before the actual semiquantitative analysis, stromal areas and intraluminal areas were deleted utilizing manual selection, thus only epithelia being further investigated here for further sorting out highly variable stromal and unspecific intraluminal signals. CD166 and CD133 immunolabelling areas were first semi-quantitatively reported as square microns per 40× objective area, utilizing a fix RGB-profile threshold for each of the two signals and for all slides analyzed. Two-signal colocalization studies further utilized deconvolved images, with the results being reported as Pearson’s correlation coefficients of the intensity distribution between the two channels [38].

In addition, on the triple immunolabeled CD166-CD133-Ki-67 (DAPI) slides, after evaluating the areas and the overlapping coefficients of CD166 and CD133, we intended to evaluate if these data would show any connection with the percentage of proliferating cells out of the total DAPI-stained nuclei for normal, dysplastic and respectively tumour epithelium (defined herein as proliferating index).

**Statistical analysis**

As the study followed-up the patients over a time interval of only 2 years, and with only 2 patients being deceased during this time, no significant survival analysis could be followed for this type of cancer. As only a few of the metastasis tissue blocks were readily available for further investigation, this study focused on the evaluation of primary tumours and not any corresponding metastases.

Both signal areas (expressed as square microns) and CD133-CD166 correlation coefficients were averaged for each case in part and then for all cases from each particular pathological status (or control tissue samples). 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.

**RESULTS**

**Clinicopathological data**

We examined retrospectively a total of 45 colon cancer patients, 33 males and 12 females, with ages ranging from 49 to 81 years (mean 69.73 years). Topographically, in 16 cases the tumours were located on the right colon and in 29 cases on the left colon. According to the AJCC classification, 19 cases were in stage I, 9 in stage II and 17 in stage III. Histopathologically, 14 adenocarcinomas were classified as well differentiated, 17 as moderately differentiated, 6 as poorly differentiated, and 8 as mucinous carcinomas (Table II). Investigating the proliferative index, we noticed a clear-cut increase from control areas (2.32%±0.80), LGD (4.80%±1.66), to HGD (8.89%±5.17), and adenocarcinoma (13.35%±6.75) [F(1,128) = 64.81, p < 0.001] (Fig. 1, A).

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**CD133 expression patterns**

A weak CD133 expression in the residual normal mucosa was noted in the cytoplasm (as a strong dotty-like or with a more uniform pattern) of enterocytes from both the surface and intestinal crypt epithelium. The reactivity was also present along the luminal border of the enterocytes that covered the intestinal crypts (Fig. 2, A-B). Also, the expression was seen with variable intensities at the level of endothelial cells, nerves and ganglia of myenteric and submucosa plexuses, in the associated inflammatory infiltrate, and in the smooth muscle cells from within the tumours and around them (Fig. 2, C-F).
Fig. 2. Immunoexpression patterns of CD133 in normal colon tissue. Expression pattern cytoplasmic, along the luminal border of the enterocytes that cover the intestinal crypts (A-B). Signal seen with variable intensities at the level of endothelial cells, nerves and ganglia of colon plexuses, in the associated lymphocytic inflammatory infiltrate, and in the smooth muscle cells (C-F). Scale bars represent 100 µm.

We noticed a few scattered cells being positive for CD133 in the tumour stroma, most likely being hematopoietic stem cells, endothelial or mesenchymal progenitor cells. In addition, we observed a gradual increase in the CD133 expression from non-neoplastic mucosa, to LGD and towards HGD (Fig. 3, A-D).

In tumour specimens, CD133 staining was detected both in the cytoplasm and on the apical plasma membrane of cells from within tumour gland-like entities (Fig. 4, A-B). When we examined the whole tumours, we noticed a heterogeneous staining pattern, with tumour areas showing different staining intensities alternating with negative areas. The percentage of stained tumour epithelia ranged from 0.4% to 36.8% of the 40× objective area (or between 169.8 µm² and 15623.6 µm²). In addition, adenocarcinomas with well and moderate differentiation had a tendency for higher CD133 levels compared to poorly differentiated tumours and mucinous adenocarcinomas (Fig. 1, B; 4, C-F).

Overall, average CD133 expression area was significantly higher in dysplasia (11802.46 ± 6538.75 µm²) as compared to control (7076.48 ± 3721.93 µm²) (p=0.0069) and tumour areas (8193.62± 4776.23 µm²) (p=0.0185) (Fig. 1, C). Only for well and moderate differentiated tumours (8755.08 ± 4868.091 µm²), CD133 expression was higher than in control tissues (7076.48 ± 3721.93 µm²) (p=0.03034) (Fig. 1, B). Regarding the proliferating index, we showed a moderate indirect correlation with CD133 expression areas for normal mucosa (r=-0.409). CD133 expression area was higher in tumours without metastases (p=0.0014); and regarding the tumour stage, we found that it showed larger expression areas for early stages...
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Fig. 3. Qualitative assessment of CD133 expression in the progression of colon adenocarcinomas. CD133 (green) expression gradually increase from non-neoplastic colon mucosa (crypt base- A and body- B of Lieberkuhn gland), to low-grade dysplasia (C) and towards high-grade dysplasia (D). Scale bars represent 50 µm.

Fig. 4. Immunoexpression patterns of CD133 in tumour specimens. Reactivity present in cytoplasm and at the apical plasma membrane of epithelial tumour gland-like entities, and sometimes at the level of intraluminal cellular debris (A-B). CD133 reactivity more obvious in the well and moderate differentiated tumors (C-D), when compared with mucinous adenocarcinomas (E-F). Scale bars represent 100 µm.

(9490.44 ±4853.18 µm²) when compared to advanced stages (6133.98 ± 3951.196 µm²) (p=0.010).

**CD166 expression patterns**

In normal mucosa, CD166 expression was prevalent as a membranous staining along the luminal and lateral borders of the cells forming the base of the crypts (Fig. 5, A-B). Also, a weak granular cytoplasmic staining with a predominant supranuclear and apical topography was observed in enterocytes from both the surface and intestinal crypts epithelium. In addition, CD166 staining was noticed in the ganglia of the submucosal and myenteric plexuses and nerves, and in some inflammatory or stromal cells (most likely fibroblasts) (Fig. 5, C-D). A stronger reaction than in the adjacent normal mucosa was seen in the dysplastic lesions (Fig. 3, A-D), but with lower expression areas than in tumour specimens. The percentage of CD166 staining areas in tumour epithelium ranged from 0.89% to 28.86% of the 40× objective area (or between 377.8 µm² and 12252.65 µm²).

Tumour reactivity for the anti-CD166 antibodies showed both cytoplasmic and membranous patterns, with a more pronounced membranous expression (Fig. 5, E-F). Overall, tumours showed a heterogeneous staining pattern inside the cancerous lesions. Compared to CD133, CD166 tumour reactivity areas seemed to be lower, although the differences were not always significant (Fig. 1, B-C). In relation to the tumour grade, we noticed a slight and non-significant reduction in CD166 staining for poorly differentiated tumours.
and mucinous adenocarcinomas (Fig. 5, G-H). The reactivity was focal and restricted to abortive glands, exhibiting a prevalent membranous pattern.

Overall, the only significant difference for CD166 expression was between dysplasia (6035.60 ±5892.99 µm²) and normal tissue (3059.73 ±3421.65 µm²) (p=0.0436) (Fig 1, C). Again, only for well and moderately differentiated tumours (6191.31 ±3178.78 µm²), CD166 expression was higher than in control tissue (3059.73 ±3421.65 µm²) (p=0.00324). As for the proliferating indexes, we found a moderate indirect correlation with CD166 expression areas for confirmed adenocarcinoma (r=-0.428).

There was no difference for CD166 expression in tumours with or without lymph node metastases (p>0.05). For the tumour stage, we found that only for poorly differentiated tumours, CD166 expression was higher in advanced stages (5581.73 ±3275.67 µm²) compared to early stages (2466.30 ± 1742.11 µm²) (p=0.039). Its expression area was higher in non-recidivated tumours (6399.44 ±3413.76 µm²) compared to recidivated tumours (3887.18 ±1887.83 µm²) (p=0.0084).

Overall, CD133 expression correlated with that of CD166, both in normal mucosa and in preneoplastic and neoplastic lesions (r=0.306) (Fig. 1, B-C). A higher correlation was noticed only for the dysplastic lesions (r=0.52).

Colocalization of CD133 and CD166

Colocalization areas for CD133/CD166 were obvious especially at the cell membrane level along the entire spectrum of lesions (Fig. 6, A-D). For dysplastic lesions, particularly in high grade lesions (HGLs) we also noticed signals colocalization at the level of apical cytoplasm areas (Fig. 6, A). For the tumour specimens, colocalizations were also observed in the cytoplasm of tumour cells (Fig. 6, E-F).

Overall, CD166-CD133 colocalization coefficients were higher for HGD (0.82±0.041) compared to all tumours accumulated together (0.68±0.148) (p=0.033) (Fig. 1, D). Due to the high heterogeneity between cases, there was no difference between the correlation degrees, for normal and tumour tissue samples (p>0.05).

Colocalization coefficients had higher values in early stage tumours (0.77±0.088), compared to advanced tumours (0.63±0.154) (p=0.0014). Well and moderate differentiated tumours with recurrences (0.72±0.133) had higher colocalization coefficients compared to non-recurrent tumour cases (0.64±0.123) (p=0.043).

Overall, there was a weak indirect correlation between the proliferative index and the CD133-CD166 colocalization coefficients (r=-0.382), and a strong indirect correlation with the CD133-CD166 colocalization coefficients for LGD (r=-0.863) (Fig. 7, A- B).

DISCUSSION

One of the reasons behind treatment failure in colorectal cancer is the existence of drug-resistant colon CSCs [6-9]. During the last decade, many CSC surface markers were proposed, including CD133 and CD166 [12-14]. Inconsistent data exist regarding the prognostic significance of these markers’ expression in CRC [14, 18, 22, 32-34]. Thus, we aimed to investigate the immunoexpression patterns of CD133 and CD166 in colon carcinoma, both individually and in combination, in order to assess their significance as prognostic markers.

CD133 and CD166 expression patterns

CD133 expression in the non-neoplastic colon mucosa was weak compared with dysplastic and neoplastic tissues, and was mostly present in the cells from the base of the crypts, similar to the results of previous studies [6, 14, 18]. The most common expression pattern found was a cytoplasmic dot-like staining, with a less spread membranous-like pattern. 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 [14, 33, 34, 39].

In addition, for both markers, we noticed a higher expression in the associated dysplastic lesions compared to their reactivity.
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in the non-neoplastic mucosa, suggesting a possible involvement of these proteins in colon carcinogenesis. In this line, for mouse colon carcinogenesis, Arena et al [40] observed a significantly increased expression of CD133 in preneoplastic lesions, which tended to decrease with tumour development. Regarding the tumorigenic potential of CD166, it was hypothesized [34] that the upregulation of CD166 might be an early event in malignant cell transformation in colon carcinogenesis, because it was found in all adenomas of the colon.

In our study, both CD133 and CD166 were expressed to different extents in all primary cancer tissue samples. 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% [6, 14, 18, 19, 21, 22, 41], while for CD166, CRC reactivity varied between 34 and 76% [14, 33, 34]. The prevalent tumour 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 [6, 14, 21, 22, 33, 42].

Most studies reported that CD133 expression is mainly detected on the cell membranes, at the luminal surface of the CRC carcinomatous cells [21, 22, 43]. Some authors proposed that apical/endoluminal membranous CD133 staining is a characteristic of well oriented, polarized and differentiated cells, while cytoplasmic staining may be indicative of putative CSCs [44, 45]. These large discrepancies have been attributed mainly to methodological variations and different scoring systems [18, 46, 47]. It seems that only membranous CD133 overexpression correlates with patient survival times, recurrence-free survival times and chemoresistance [21, 48]. However, it was speculated that the shift of CD133 from cytoplasmic to a membranous localization underlies the transition of epithelial cells towards a more invasive phenotype [48].

Regarding the staining patterns of CD166 in CRC, most studies showed a predominant membranous expression for CD166, with the cytoplasmic staining pattern not being present in the absence of the membrane-like staining [33]. However, Weichert et al [34] and Levin et al [39] observed cytoplasmic staining without a membranous expression, and this alteration supports a potential functional role of this molecule in tumorigenesis in either cell adhesion or signaling pathways. In addition, Weichert et al [34] found that patients with strong membranous staining had significantly shorter survival times.

Fig. 6. Colocalization of CD133 (green) and CD166 (red) in normal and tumour samples. Along the entire spectrum of lesions, colocalization areas more obvious at the cells membrane level (arrows in series A, D). In high grade dysplastic lesions (arrow in series A) and in tumour specimens (arrow in series E-F), colocalization areas were also observed in the cytoplasm. Enlarged insets represent areas increase CD133-CD166 colocalization. Scale bars represent 50 µm.
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. 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 the identification of low-, intermediate-, and high-risk cases of CRC [32].

**CD133 and CD166 colocalization**

The present study performed for the first time an integrated assessment of CD133 and CD166 expression areas in colon cancer in association with the proliferative index, based on triple immunostainings for CD166, CD133, and Ki-67 in FFPE tissue. The prognostic value of CD166/CD44 combined positivity did not contribute to independent prognostic information in a multivariate analysis performed by Lugli et al, revealing no added benefit for the risk stratification in patients with CRC [14].

Other authors observed that only CD133+/CD44+ CRC cells displayed tumorigenic potential, suggesting that the use of both markers could identify colorectal CSCs much more accurately [49, 50]. However, Muraro et al [51] found that the expression of CD133 or the coexpression of CD166/CD44 or CD24/CD44 did not appear to identify reliably CSC populations in established CRC cell lines.

In our study, the colocalization areas for CD133/CD166 were obvious especially at the level of cell membranes along the entire spectrum of lesions. In high grade dysplasia, we also noticed colocalizations at the level of apical cytoplasm areas while in the tumour specimens colocalizations were also observed in the cytoplasm of carcinomatous cells. Statistically, the CD133-CD166 colocalization coefficients were higher for HGD compared to all tumours pulled together. Also, we noticed higher values in early stage tumours compared to advanced tumours, while well and moderately differentiated tumours with recurrences had higher colocalization coefficients compared to non-recurrent tumour cases.

**Clinicopathological correlation of CD133 and CD166 expression**

Regarding the tumour grading, we noticed a reduced CD133 and CD166 staining in poorly differentiated and mucinous adenocarcinomas compared to well and moderately differentiated tumours, and even to dysplastic lesions. Literature data regarding CD133 are contradictory, with some authors finding a higher expression in well and moderately differentiated tumours compared to poorly differentiated and mucinous tumours [19, 22, 41, 52], while other groups did not find any significant associations of CD133 with tumour differentiation [21, 53-55]. While Weichert et al [34] failed to show a significant correlation between CD166 expression and tumour grade in colon carcinomas, Tachezy et al [33] found a significant inverse correlation of its expression with tumour cell differentiation.

Regarding the tumour stage, we found that CD133 expression was higher in early stages compared to advanced stages for the entire tumour spectrum, while for CD166 expression we found a higher reactivity only in poorly differentiated tumours and in advanced stages. We noticed that
while for CD166 expression there was no difference according to lymph node status, for CD133 the reactivity was higher in tumours without metastases, even though we could not rule out that our study was limited by the relatively low number of patients enrolled in this ongoing project.

Chen et al [48] established through meta-analysis that CD133 overexpression correlates with the T3-4 tumour status, with the N positive status, and with vascular invasiveness. These findings come to certify the more invasive ability of the CD133+ colon cancer cells compared to the negative ones, established by in vitro [56] and in vivo [57] studies. In the most comprehensive study regarding CD166 expression in CRC, Lugli et al [14] established a significant association between the loss of CD166 expression and the larger tumour sizes, nodal metastasis and infiltrating tumour border configuration. However, other authors showed no association of CD166 expression with clinical and histopathological parameters, such as age, gender, TNM classification, resection margin status [32, 33].

All these data suggest the possibility of individualized adjuvant therapy and post-treatment surveillance aimed at identifying colon cancer patients with the highest likelihood of disease recurrence or progression based on CD133/CD166 markers' expression within the primary tumour [58].

CONCLUSIONS

Overall, our results suggest that the colocalization of CD133 and CD166 is an early event in colon tumorigenesis, with the highest colocalization coefficients being recorded in patients with HGD. 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 observed in well-differentiated and early stage tumours.

Authors' contribution. CM and DP wrote the manuscript, performed pathological examination, immunohistochemistry, statistical analysis. IC, AB and TC were responsible with patient follow-up, and proofread the manuscript. AS was involved in writing, patient follow-up, statistical analysis.

Conflicts of interest. No conflict to declare.

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REFERENCES


