Implications of inflammation and remodeling of the enteric glial cells in colorectal adenocarcinoma

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Abstract

Aim: The aim of our study was to assess glial fibrillary acidic protein (GFAP) glial cell phenotype in the enteric nervous system (ENS) in colorectal adenocarcinoma of different tumor grading and, also, to establish correlations between these changes and the tumor proliferative activity and the tumor-infiltrating leukocytes.

Patients, Materials and Methods: We ran an observational, prospective study on a group of 52 patients diagnosed with colorectal adenocarcinoma. They were surgically treated in the 1st Surgery Clinic of the Emergency County Hospital of Craiova, Romania. From the surgically resected pieces, after pathological confirmation and tumor grading, 3-μm thick seriate sections were cut and processed for immunohistochemistry for detecting GFAP, S100, CD45 and Ki-67.

Results: Evaluation of GFAP glial cell type in the ENS of colorectal cancer with different stages of differentiation showed that the density of these nervous elements is higher in well-differentiated (G1) colorectal tumors compared to moderately differentiated (G2) and poorly differentiated (G3) colorectal tumors. For well-differentiated colorectal adenocarcinoma, we did not find any correlations between GFAP glial cell type in the ENS and the tumor proliferative activity or with tumor-infiltrating leukocytes. In what the moderately and poorly differentiated adenocarcinoma are concerned, we found a high inverse variation between GFAP glial cell type in the ENS and the proliferative activity, on one hand, and, between GFAP glial cell type in the ENS and the tumor-infiltrating leukocytes, on the other hand.

Conclusions: The decrease in the density of GFAP glial cell type in the ENS with tumor grading of colorectal cancer and the inverse variation with the tumor proliferative activity and with the tumor-infiltrating leukocytes might serve as putative prognostic factors in colorectal cancer.

Keywords: colorectal adenocarcinoma, GFAP glial cell type in the ENS, tumor-infiltrating leukocytes.

Introduction

Although, in the last decades, important progress was made in what the screening for colorectal neoplasm or the medical and surgical therapeutic methods are concerned, in 2012, in Europe, about 1000 patients were diagnosed each day with colorectal neoplasm, this type of neoplasm being the third most frequent neoplasm in all the 27 countries of the European Union, representing 13% of all cancers [1]. Worldwide, colorectal cancer was on the third place after the lung (1.61 million cases) and breast (1.38 million cases), being responsible of 9.7% of the total cancer burden [1.23 million cases of colorectal cancer (CRC)] [2].

The exact way of colorectal neoplastic transformation and also the tumor progression and metastasis are not yet fully understood, so, in consequence, many studies are being performed in order to identify new therapeutic strategies.

Gliadin enteric cells, considered for a long period of time as mere supportive and accompanying cells for the neurons, are the main component of the enteric nervous system [3]. However, in the last decades, the role of gliadin enteric cells has been reconsidered, both in what the disease and health status are concerned, as many studies showed that this type of cells is an important binding element between the intestinal epithelium’s cells, the immune system’s cells, the enteric neurons and the entero-endocrine cells [4]. Both gliadin enteric cells, involved in maintaining the integrity of the intestinal barrier having an antiproliferative role [5], and also the immune system’s cells may play an important role in colorectal neoplasm’s pathogenesis and evolution [6, 7].

The aim of our study was the assessment of gliadin fibrillary acidic protein (GFAP) glial cell type in the enteric nervous system (ENS) in colorectal adenocarcinoma’s different tumor grading and, also, establishing correlations.
between these changes and the tumor proliferative activity and the tumor leukocyte infiltrate.

Patients, Materials and Methods

Patients and specimens

We ran an observational, prospective study on a group of 52 patients diagnosed with colorectal adenocarcinoma in the 1st Medical Clinic, Department of Gastroenterology, Emergency County Hospital of Craiova, Romania, between 2015 and 2016. They were surgically treated in the 1st Surgery Clinic of the same Hospital.

According to the Declaration of Helsinki of the Human Rights (1964) and all current national and international legislation, the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania, approved our study which included only adults who had been informed regarding to the objectives of the study and had also given written informed consent for the procedures and tissue collection.

All histological and immunohistochemical analyses were performed in the Research Center for Microscopic Morphology and Immunology, affiliated to the University of Medicine and Pharmacy of Craiova.

Tissue processing and immunohistochemistry

The slides were examined by optical microscopy and classified in line with the pTNM staging, in accordance with the criteria established by the World Health Organization (WHO) for colon and rectum, as well (G1), moderated (G2) and poorly differentiated (G3) tumors.

After re-confirming the pathology and tumor grading, we cut from each block seriate sections with a thickness of 3 μm each, deparaffinized them in xylene, then we rehydrated them in graded alcohol series, and then we performed immunohistochemical stainings using anti-S100, anti-GFAP, anti-CD45, and anti-Ki-67 antibodies. Firstly, by using microwaving in citrate buffer pH 6 for 20 minutes, we processed the sections for antigen retrieval, then in order to block the activity of endogenous peroxidase, the sections were incubated for 30 minutes with DAB or with Hematoxylin in order to obtaining pure spectral signatures of the respective staining (see further below). Also, negative controls were obtained by omitting the primary antibodies in the immunodetection sequences.

Image processing

For capturing and quantifying the targets’ immunohistochemical expression, we used a motorized microscope Nikon Eclipse 90i (Elta 90 Medical Research, Bucharest, Romania) equipped with a multispectral camera Nuance FX and the Nuance unmixing software (Perkin Elmer, Hopkinton, MA, USA), in order to acquire light microscopy images as spectral cube data. A separating spectral library was also created from individual slides that had been stained with either Hematoxylin or DAB (as described above), and subsequently has been utilized to unmix and separate DAB/Hematoxylin signals in order to characterize the immunohistochemical expression patterns of interest (Figure 1). On the resulting monochromatic images, by using the Image-Pro Plus AMS 7 image analysis software (Media Cybernetics, Bethesda, MD, USA), on 10 random images that were taken with a 20× objective, the unmixed DAB signal was quantified in terms of area and integrated optical density (IOD = average intensity/density of each area of interest). Only the tumor epithelia was consider in this analysis, by manually defining epithelia as regions of interest utilizing a user-defined lasso tool in the image analysis software. Moreover, for each patient and for each histopathological grade, the resulting data were averaged and finally compared.

The nervous tissue was firstly quantified by using S100 protein and then the expression of GFAP was quantified.

All Hematoxylin–Eosin slides were entirely scanned on the motorized Nikon 90i microscope at 4× magnification, in order to report nerve density to total tissue area.

After this, image files were loaded in Image-Pro Plus, and by using a microscopic stage micrometer, the pixel-size calibration for 1 mm was made, then, images were segmented and an RGB (red, green, blue) profile was further created for automatically selecting the tissue and measuring its area.

Statistical analysis

The data were analyzed using Student’s t-test, ANOVA (analysis of variance) with Bonferroni’s post-hoc correction and Pearson’s correlation coefficient.

The data were reported as mean ± standard deviation (SD). In all cases, p<0.05 was used to indicate statistical significance. Moreover, p-values <0.05, <0.01 and <0.001 representing significant differences were signalized with *, **, and ***.

Results

Relationship between clinico-pathological parameters of the patients, suffering from colorectal adenocarcinoma, included in the study and GFAP, common leukocyte antigen (CLA) and Ki-67 immunohistochemical expression features are globally presented in Table 1.
Implications of inflammation and remodeling of the enteric glial cells in colorectal adenocarcinoma

**GFAP glial cell type in the ENS in different tumor grading**

The evaluation of GFAP glial cell type in the ENS in colorectal cancer's different stages of differentiation showed that the density of these nervous elements was higher in well differentiated (G1) colorectal tumors with a percentage area for this type of cells of 0.004197±0.001632%, while, in moderately differentiated (G2) colorectal tumors a percentage area of 0.003741±0.001632% was recorded and a significant decrease of this parameter to 0.00123±0.000812% was observed in poorly differentiated (G3) colorectal tumors (Figure 1A).

Comparing the average nervous density of GFAP glial cell type in the ENS in colorectal cancer’s different stages of differentiation by using ANOVA test followed by Bonferroni’s post-hoc test, we noticed a statistically significant difference between the density of GFAP enteric glial cell type in the well and poorly differentiated tumors \(p=0.007\) and, also, between the density of GFAP enteric glial cell type in the moderately and poorly differentiated tumors \(p=0.023\) while, in what the density of this type of cells was concerned, between well differentiated and moderately differentiated tumors statistically significant differences were not observed. It has to be mentioned that GFAP enteric glial cell type represented about 2.52% of the total nervous tissue area, calculated using S100 immunolabelling, this type of cells being expressed especially in the Auerbach plexus (Figure 2), while in the Meissner plexus no signal was recorded for this type of cells.

**Assessment of the proliferative activity**

Colorectal cancer’s proliferation activity for the patients included in our study was evaluated by using Ki-67 monoclonal antibody. In what patients diagnosed with well differentiated (G1) colorectal adenocarcinoma were concerned, the proliferative tumor activity was of 44.2435±29.2526% (Figure 1B). Comparing the average of the proliferative tumor activity in different colorectal cancer’s differentiation stages by using ANOVA test followed by Bonferroni’s post-hoc test, we noticed a statistically significant difference between the proliferative activity of well differentiated and moderately differentiated tumors \(p=0.023\) and, also, between the proliferative activity of well differentiated and poorly differentiated tumors \(p=0.008\), while between the proliferative activity of the moderately and poorly differentiated tumors no statistically significant differences were recorded.

**Table 1 – Relationship between clinico-pathological parameters of the patients, suffering from colorectal adenocarcinoma, included in the study and GFAP, CLA and Ki-67 immunohistochemical expression (Student’s t-test)**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of cases</th>
<th>GFAP [%/mm² (Mean±SD) P-value</th>
<th>Area of CLA [μm² (Mean±SD) P-value</th>
<th>IOD of CLA Mean±SD P-value</th>
<th>Ki-67 [% of cells (Mean±SD) P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis [years]</td>
<td>&lt;65 17</td>
<td>0.00206812±0.0002126</td>
<td>6452.7618±2168.097</td>
<td>.036*</td>
<td>795519.08±283709.27</td>
<td>.021*</td>
</tr>
<tr>
<td></td>
<td>≥65 35</td>
<td>0.00369529±0.000217</td>
<td>3950.7955±2755.226</td>
<td>.489</td>
<td>603126.68±236820.22</td>
<td>.446</td>
</tr>
<tr>
<td>Gender</td>
<td>Male 30</td>
<td>0.00350512±0.0002098</td>
<td>4944.8447±2943.3263</td>
<td>.302</td>
<td>570477.11±253870.97</td>
<td>.164</td>
</tr>
<tr>
<td></td>
<td>Female 22</td>
<td>0.00370796±0.00017794</td>
<td>4730.0189±2326.7155</td>
<td>.302</td>
<td>497365.42±494295.16</td>
<td>.302</td>
</tr>
<tr>
<td>Site of primary tumor</td>
<td>Distal 41</td>
<td>0.00315407±0.00020077</td>
<td>5106.9236±2680.2706</td>
<td>.297</td>
<td>616831.46±328992.89</td>
<td>.213</td>
</tr>
<tr>
<td></td>
<td>Proximal 11</td>
<td>0.00246816±0.00022579</td>
<td>3685.9371±3720.3834</td>
<td>.297</td>
<td>477365.42±494295.16</td>
<td>.213</td>
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<td>Gross appearance</td>
<td>Exophytic 23</td>
<td>0.00272584±0.00014519</td>
<td>4043.1132±3001.6188</td>
<td>.302</td>
<td>493589.06±357717.35</td>
<td>.164</td>
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<tr>
<td></td>
<td>Infiltrative 29</td>
<td>0.00322120±0.00022555</td>
<td>5334.3317±2676.9976</td>
<td>.302</td>
<td>648567.05±39551.09</td>
<td>.239</td>
</tr>
<tr>
<td>Size [cm]</td>
<td>&lt;5 19</td>
<td>0.00282145±0.000303</td>
<td>5605.54±2431.452</td>
<td>.371</td>
<td>694667.68±315354.2</td>
<td>.239</td>
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<tr>
<td></td>
<td>≥5 33</td>
<td>0.00314994±0.0001548</td>
<td>4623.28±2934.59</td>
<td>.239</td>
<td>557803.73±358665</td>
<td>.239</td>
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<td>Depth of invasion</td>
<td>T1–2 15</td>
<td>0.003549±0.0001889</td>
<td>1650.33±546.601</td>
<td>.298</td>
<td>203437.28±74834.3</td>
<td>.022**</td>
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<tr>
<td></td>
<td>T3–4 37</td>
<td>0.00294±0.000206</td>
<td>5669.47±2521.63</td>
<td>.298</td>
<td>203437.28±74834.3</td>
<td>.022**</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>N0–1 43</td>
<td>0.00363699±0.0002147</td>
<td>3604.2528±1941.0852</td>
<td>.022*</td>
<td>430134.03±224281.22</td>
<td>.003**</td>
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<td></td>
<td>N2 9</td>
<td>0.00189427±0.0000987</td>
<td>7503.2710±2417.8182</td>
<td>.022*</td>
<td>930455.09±30575.39</td>
<td>.003**</td>
</tr>
</tbody>
</table>

GFAP: Glial fibrillary acidic protein; CLA: Common leukocyte antigen; SD: Standard deviation; IOD: Integrated optical density. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\).
Assessment of the inflammation in colorectal cancer

The tumor-infiltrating leukocytes of the patients included in our study was immunohistochemically evaluated by using the anti-CD45 monoclonal antibody, also known as the common leukocyte antigen (CLA), calculating both the area and the IOD of this antibody’s signal in all the three tumor differentiation degrees (Figures 3–5). We noticed a gradually increase of both the area and IOD (Figure 1, C and D) from the well-differentiated colorectal adenocarcinoma (2104.296±826.0828 μm² for area and 259863.0316±106263.5552 for IOD) to the moderately differentiated colorectal adenocarcinoma (4450.312±1328 μm² for area and 523583.331±151044.9749 for IOD) to poorly differentiated colorectal adenocarcinoma (7693.812±1840.834 μm² for area and 946815.1146±236017.7378 for IOD). By comparing the average of the tumor-infiltrating leukocytes in different colorectal cancer differentiation degrees by using ANOVA test followed by Bonferroni’s post-hoc test, we observed that there was a statistically significant difference between the leukocyte infiltrate from the well differentiated and moderately differentiated tumors \( (p=0.025 \text{ for area; } p=0.048 \text{ for IOD}) \), between the leukocyte infiltrate from the moderately and poorly differentiated tumors \( (p=0.002 \text{ for area; } p=0.001 \text{ for IOD}) \) but also between the leukocyte infiltrate from well differentiated and poorly differentiated tumors \( (p=0.000 \text{ both for the area and for IOD}). \)

Correlations between GFAP glial cell type in the ENS, proliferative activity and tumor-infiltrating leukocytes in different tumor grading

We next followed, on one hand, correlations between GFAP glial cell type in the ENS and the proliferative activity in every differentiation stage of colorectal adenocarcinoma and, on the other hand, between GFAP glial cell type in the ENS and tumor-infiltrating leukocytes. For well-differentiated (G1) colorectal adenocarcinoma, we did not find correlations between GFAP glial cell type in the ENS neither with tumor proliferative activity nor with tumor-infiltrating leukocytes. In what the moderately adenocarcinoma was concerned, we found a high inverse variation between GFAP glial cell type in the ENS and the proliferative activity \( (r=-0.780) \), on one hand, and, between GFAP glial cell type in the ENS and the tumor-infiltrating leukocytes \( (r=-0.779) \), on the other hand. In what poorly differentiated tumors were concerned, a moderate inverse variation between GFAP glial cell type in the ENS and tumor proliferative activity \( (r=-0.469) \) was observed together with a high inverse variation between GFAP glial cell type in the ENS and tumor-infiltrating leukocytes \( (r=-0.764) \). Regarding the global correlation between the GFAP glial cell type in the ENS of the entire group of patients included in the study and the tumor-infiltrating leukocytes, a high global inverse variation was noticed \( (r=-0.739). \)

Figure 1 – (A) Immunohistochemical expression of GFAP-glial cell type in the ENS in different tumor grading. (B) Assessment of the proliferative activity of colorectal neoplastic cells in different tumor grading. (C) Assessment of the inflammation with the area of CLA in colorectal neoplastic tissue. (D) Assessment of the inflammation with the IOD of CLA in colorectal neoplastic tissue. GFAP: Glial fibrillary acidic protein; ENS: Enteric nervous system; CLA: Common leukocyte antigen; IOD: Integrated optical density.
Implications of inflammation and remodeling of the enteric glial cells in colorectal adenocarcinoma

Figure 2 – Example of spectral unmixing for the series of slides that highlight GFAP-glial cell type in the ENS in colorectal cancer (A) immunostained for GFAP with DAB and counterstained with Hematoxylin. (B) Pure DAB and Hematoxylin signals are showed either overlapping or (C and D) individually. The big square represents an enlarged representation of the small square from the same slide. Scale bar represents 20 μm.

Figure 3 – Assessment of the inflammation in G1 tumor grading. Example of spectral unmixing for the series of slides (A) immunostained for CLA with DAB and counterstained with Hematoxylin. (B) Pure DAB and Hematoxylin signals are showed either overlapping or (C and D) individually. A small number of inflammatory cells can be observed in the epithelium areas. Scale bar represents 20 μm.
Figure 4 – Assessment of the inflammation in G2 tumor grading. Example of spectral unmixing for the series of slides (A) immunostained for CLA with DAB and counterstained with Hematoxylin. (B) Pure DAB and Hematoxylin signals are showed either overlapping or (C and D) individually. In G2, an increasing number of inflammatory cells was observed in comparison with G1 epithelium areas. Scale bar represents 20 μm.

Figure 5 – Assessment of the inflammation in G3 tumor grading. Example of spectral unmixing for the series of slides (A) immunostained for CLA with DAB and counterstained with Hematoxylin. (B) Pure DAB and Hematoxylin signals are showed either overlapping or (C and D) individually. There are more inflammatory cells in G3 than in G1 and G2 epithelium areas. Scale bar represents 20 μm.
**Discussion**

Understanding the interaction of the additional factors from the tumor microenvironment, such as the nervous cells or inflammatory cells, with colorectal neoplasm tumor cells may bring into light many mechanisms of the colorectal neoplasm’s pathogenesis.

In the present study, we observed for the first time in the literature that GFAP enteric glial cells type density in the ENS in colorectal adenocarcinoma decrease with the tumor grading and we have also noticed an inverse variation with different negative prognostic factors, such as tumor proliferation stage and inflammatory tumor infiltrate, for this type of neoplasm. On the other hand, we previously reported a decrease of the percentage area of Auerbach myenteric plexus and also of the Meissner submucosal plexus with tumor grading in colorectal adenocarcinoma [9].

Moreover, in previous studies we observed an increase in the number of some receptors, such as beta 2 adrenergic receptors, together with tumor grading, highlighting the neoplastic interrelationships that take place at the colorectal level [10, 11].

Currently, the glial enteric cells, known for a long period of time as only having supportive role for the enteric neurons, are well known for their role in maintaining the homeostasis of the enteric neurons, in providing support and stability for the enteric nervous system from the intestinal wall, in the enteric neurotransmission, in regulation of the intestinal epithelial barrier functions, and they may, also, be an important source of the substrate of the enzymes involved in neurotransmitters’ synthesis [3, 12–17]. Savidge et al. showed that glial enteric cells by glial-derived S-nitrosogluthathione (GSNO) play an important role in maintaining the intestinal barrier’s integrity [18]. Thus, both in vitro and in vivo GSNO was directly correlated with a positive adjustment of perijunctional F-actin, tight-junction-associated proteins zonula occludens-1 and occludin, with an important role in maintaining the integrity of the intestinal barrier, and genetic deletion of the glial enteric cells in transgenic mice causes alterations of the intestinal barrier, while, administration of GSNO inhibits the increasing intestinal permeability and inflammation [18]. By synthesizing transforming growth factor-β1 (TGF-β1), the glial enteric cells inhibit the proliferation of the intestinal epithelium cells both in vitro and in vivo, while their ablation stimulates cell proliferation by increasing thymidine incorporation in epithelial cell [5]. This has also been highlighted in neoplastic cells cultures HT-29, Caco-2 and T84 in colorectal adenocarcinoma [5]. Another glial mediator with antiproliferative role is 15-deoxy-Δ12,14-prostaglandin J2 (15dPGJ2), derived from prostaglandin D2 [19]. Pro-epidermal growth factor (proEGF), also synthesized in the glial enteric cells, was proved to be involved in the intestinal epithelial wound repair process [20]. All the studies mentioned above suggest that glial enteric cells by the soluble factors that they synthesize play an important role in maintaining the intestinal barrier and in controlling cell proliferation, so, impairing their integrity in colorectal adenocarcinoma, as reported in our study, would be a favorable element for further tumor cell proliferation and metastasis.

In contrast to the findings from our study, which suggest that the density of GFAP enteric glial cells type in the ENS presented a reverse variation with the tumor grading, a recent study published by Valès et al. showed that glial enteric cells modulate the function of the colon cancer stem cells (CSCs) and they are associated with tumorigenesis and also promote it [21]. According to this study, both in vitro and in vivo, colonic tumor cells activate the glial enteric cells, which develop pro-tumorigenic abilities, and these activated glial enteric cells cause via prostaglandin E2 (PGE2)-dependent pathways and increase in size of tumorspheres [21].

It is well known that many inflammatory cells and some factors (cytokines) secreted and released by them have, on one hand, an antitumor role, and, on the other hand, may be involved in the initiation, progression and tumor metastasis [22–27]. As other malignant tumors, colorectal cancer presents many types of inflammatory cells such as: neutrophils, natural killer cells, mast cells, dendritic cells and tumor-associated macrophages, but, paradoxically there are also myeloid-derived suppressor cells whose main role is to suppress T-cell responses, thus favoring carcinogenesis [22, 28]. Some cytokines and chemokines are involved in colorectal cancer by mutation and epigenic changes, resistance to cell death, growth and tumor proliferation, angiogenesis and metastasis [29, 30].

Because of this, at the moment, it is well known the role of non-steroidal anti-inflammatory drugs in colorectal cancer’s prevention and, also, as an adjuvant treatment in this pathology [31]. In what the relation between the immune system and the glial enteric cells is concerned, little is known so far, particularly with regard to this interaction in patients with colorectal cancer. To this extent, our study was just an observational one highlighting for the first time in the literature that there is a reverse variation between the density of GFAP glial cells type in the ENS and the leukocyte tumor infiltrate. But, we can speculate that this relation is due, on one hand, to the fact that the density of the glial enteric cells decreases with the tumor grading, thus allowing the immune system’s cells to exert their protumoral effects, as we have mentioned before. On the other hand, the decrease in the density of the glial enteric cells with the tumor grading and with the increase in the leukocyte tumor infiltrate may be explained also by inhibiting the ability of glial enteric cells to act as antigen-presenting cells [19, 32], thus allowing tumor cells to escape from the immune response.

**Conclusions**

The decrease in the density of GFAP glial cell type in the ENS with tumor grading of colorectal cancer and the reverse variation with the tumor proliferative activity and with the leukocyte tumor infiltrate might serve as a prognostic factor in colorectal cancer. However, evidence for such a role of GFAP glial cell type remains indirect and needs further confirmation.

**Conflict of interests**

The authors declare that they have no conflict of interests.

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