The aspects of angiogenesis in anal canal carcinomas compared with that in colorectal carcinomas

SIMONA GURZU1), J. JUNG1), L. AZAMFIREI2), T. MEZEI1), ANCA MARIA CÎMPEAN3)

1)Department of Pathology
2)Department of Intensive Care
University of Medicine and Pharmacy of Targu Mures
3)Department of Histology, “Victor Babes” University of Medicine and Pharmacy, Timisoara

Abstract
Aim. To compare the angiogenesis in anal canal carcinomas (ACC) with that in colorectal carcinomas (CRC). Methods. A number of 507 CRC, surgical specimens, were analyzed, 12 cases (1.97%) being ACC. In 20 cases from left and right colon (CRC) and in the 12 ACC we analyzed the immunohistochemical parameters related to angiogenesis, utilizing the following LabVision antibodies: CD 31, CD105 (endoglin) and VEGF1. Morphometrical analysis and positive cell counting were performed in the tumoral and peritumoral tissue. Immunoperoxidase method was used. Results. The average age was 63.17 ± 10.87 years in CRC, respectively 57.9 ± 10.05 years in the ACC (p<0.0001). Compared with CRC the ACC occur more frequently at the females (58%). Angiogenesis was expressed in the majority of cases. In CRC, the microvascular density (MVD) was higher than that from ACC. The ratio CD31/CD105 was 1 in ACC and 3 in CRC. VEGF was positive in 25% of ACC and 80% of CRC. In CRC were more mature vessels, marked only with CD31 than immature vessels or endothelial isolated cells marked with both CD31 and CD105. In ACC prevailed the neoformed vessels marked with both CD31 and CD105. Conclusions. The performed assessments have showed a higher incidence of ACC at females and at younger ages. The angiogenesis in ACC was not so high like in CRC and the immature neoformed vessels was more frequently. In ACC, the antiangiogenic treatment that regards the VEGF inhibition seems to be not as efficient as in CRC. The radiotherapy could stop the angiogenesis and could inhibit the vessels' maturation.

Keywords: angiogenesis, anal canal carcinoma, immunohistochemistry.

Introduction
The angiogenesis of CRC is much studied and more treatments are based on the angiogenic type of tumor [1–3]. The angiogenic feature of ACC is revealed by few papers and is insufficiently demonstrated. However, 4% of carcinomas of the digestive tract are represented by ACC [4].

This is not a small percent because the incidence of CRC is increased and also the ACC incidence. It is very difficult to identify, based at the immunohistochemical (IHC) aspects, the cases in which the angiogenesis inhibition could prolonged life.

The angiogenesis could be IHC evidenced with more antibodies. However, a specifically antibody for the endothelial intratumoral cells has not been discovered yet. It is known that the CD31, a platelet endothelial cell adhesion molecule, also marks the mature and neoformed vessels. More studies revealed that the endoglin (CD105) marks just the proliferating endothelium, respectively the neoformed vessels and could be a specifically antibody for neoformed vessels [5]. However, this aspect is very controversial in the literature.

VEGF (Vascular Endothelial Growth Factor) is another angiogenic factor, supposed to be a prognostic marker for CRC [6]. Mastocites, tumoral cells and other cells in hypoxic conditions produce it.

Material and methods
Tissue preparation
Twelve ACC surgical specimens and 20 specimens from left and right colon (CRC), diagnosed in the Department of Pathology of Emergency Hospital Targu Mures, Romania, were used for IHC staining.

From the 20 cases (CRC), 12 were non-mucinous adenocarcinomas (five well-differentiated, three moderately and four poorly differentiated) and six cases were mucinous adenocarcinomas.

The other two cases were malignant polyps. Twelve cases were in pT3, six in pT2 and two in pT4, according to pTNM staging. Five cases were presented lymph node involvement.

Eight ACC cases were non-mucinous adenocarcinomas (NMA), two cases mucinous adenocarcinomas (MA), the other two being squamous cell carcinomas (SCC).

From the NMA, three cases were well-differentiated, other three cases intermediary differentiated and two cases were poorly differentiated.
According to pTNM staging, two ACC cases were in pT2N0 stage, six in pT3N0, two in pT3N1, one in pT3N2 and one in pT4N0 stage.

Primary antibodies

We used the following antibodies, provided by LabVision: CD31 clone OAJC (JC/70A), CD105 clone SN6H and VEGF1.

Immunohistochemical staining

We used the immunoperoxidase method, in formalin-fixed, paraffin-embedded sections.

Sections were deparaffinized, were incubated at 100°C in citrate solution, pH 6 (CD31) or in EDTA, pH 9 (CD105, VEGF) and were washed with distillated water before the hydrogen peroxide incubation.

After this, all sections were washed with Tris Buffered Saline (TBS) and were incubated with primary antibodies for 60 minutes, then were washed with TBS and were covered by Streptavidin Peroxidase Solution for 5 minutes.

After this, they were washed with TBS and were covered with Biotinylated Goat Anti-Polyvalent Solution for other 5 minutes.

The development was performed with substrate-chromogen solution (DAB) for 3–5 minutes. The nuclei were colored with Mayer’s Hematoxylin.

Morphometrical analysis

To determine the microvascular density (MVD) we used the pictures by “hot-spot” regions, at 200 and 400 high power fields.

The pictures were realized with Nikon 800E microscope and were made in the intra- and peritumoral tissue (five pictures for each area).

The count was made using NIH’s ImageJ program-Trial Version. We batch-measured the positive area versus total tissue area ratio, counting the vessels with and without lumen. We eliminated the ulcerated regions and also the regions rich in lymphocytes.

Statistical analysis

For statistical analysis, we used the Statistical Program Graph Pad In Stat 3-Trial Version. First, we collected the data with the program Microsoft Excel.

We used the t-test, chi square test and the contingency tables, Fischer’s test, One way Anova test, determining the values of p and chi. We considered the significant association when p<0.05, with 95% confidence interval.

Results

Aspects of classical clinico-pathological parameters

From CRC (507 cases) the average age was 63.17 ± 10.87 years, with median by 65 years, minimum 24 and maximum 91 years.

The average age in ACC was 57.9 ± 10.05 years. The median age was 57 years, with minimum 43 and maximum 75 years.

Fifty percents of ACC were diagnosed between 51 and 60 years old. Compared with CRC, the ACC were diagnosed at younger ages (p<0.0001).

The CRC was diagnosed more frequently at men (57% in our cases) but the ACC was diagnosed at females, in 58% of cases (p = 0.004).

Regarding the gross features, all ACC were ulcerated tumors, 58% being polypoid-ulcerated and 42% ulcero-infiltrated tumors. In the other colon segments (507 cases), 36% of cases were polypoid-ulcerated tumors, 33% ulcero-infiltrated, 21% polypoid and 10% infiltrated tumors.

Sixty-six percents of ACC were NMA, 17% MA and the other 17% were SCC. In CRC, 68% of tumors were NMA, 31% MA and 1% SCC. The SCC were more frequent in anal canal (p<0.0001).

Compared with CRC, the ACC presented a few malignant polyps (24%, respectively 8%). The difference was statistically significant (p = 0.0033).

The majority of carcinomas were diagnosed in the pT3N0 stage, not being difference between the two groups (Tables 1 and 2).

Table 1 – Distribution of cases according to pTNM staging (pT)

<table>
<thead>
<tr>
<th>pT</th>
<th>CRC</th>
<th>ACC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT0 + pT1</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>pT2</td>
<td>19%</td>
<td>17%</td>
</tr>
<tr>
<td>pT3</td>
<td>70.14%</td>
<td>75%</td>
</tr>
<tr>
<td>pT4</td>
<td>9.02%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Table 2 – Distribution of cases according to pTNM staging (pN)

<table>
<thead>
<tr>
<th>pN</th>
<th>CRC</th>
<th>ACC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pN0</td>
<td>61%</td>
<td>67%</td>
</tr>
<tr>
<td>pN1</td>
<td>28%</td>
<td>25%</td>
</tr>
<tr>
<td>pN2 + pN3</td>
<td>11%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Aspects of angiogenesis

In both CRC and ACC CD31 immunostain was correlated with CD105 expression (p<0.0001). In ACC both antibodies were correlated (p = 0.0002) with size of tumor (Table 3). The size of tumor was not correlated with pTNM staging (p = 0.60).

Table 3 – The correlation between angiogenesis and size of ACC

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>Area CD31</th>
<th>Area CD105</th>
</tr>
</thead>
<tbody>
<tr>
<td>under 10 mm</td>
<td>4 ± 1.25%</td>
<td>2 ± 0.22%</td>
</tr>
<tr>
<td>10–20 mm</td>
<td>6 ± 2.12%</td>
<td>8 ± 2.67%</td>
</tr>
<tr>
<td>21–33 mm</td>
<td>12 ± 4.34%</td>
<td>11 ± 3.56%</td>
</tr>
</tbody>
</table>

In ACC, both CD31 and CD105 immunostains were not correlated with depth of invasion, lymph node involvement or grade of differentiation. The intensity of CD31 MVD seemed to increase with age but not statistically significant (p = 0.25).

VEGF was expressed in 25% of ACC and did not correlated with CD105 (p = 0.81), CD31 (p = 0.08) or other prognostic factors.

In CRC, CD105 was correlated with CD31 immunostain at 400× (p = 0.0004) but not at 200× magnification (p = 0.39).
In CRC we not observed a statistical difference between intratumoral (13.23 ± 3.98%) and peritumoral CD105 MVD average area (12.50 ± 4.43%) neither peritumoral (34.05 ± 8.52%) and intratumoral CD31 MVD average area (29.65 ± 9.78%).

In addition, in ACC we not observed a significant difference between CD31 or CD105 regarding the intratumoral or peritumoral area.

The CD31 and CD105 immunostains, in CRC, were not correlated with grade of differentiation. Only CD31 was reversely correlated with level of penetration (p = 0.05), being higher in pT2 (39 ± 5.41%) than pT3 or pT4 (30 ± 3.70%, respectively 15.5 ± 1.23%). CD31 was also reversely correlated (p = 0.05) with lymph node involvement (37.9 ± 7.44% in pN0, 29.2 ± 4.23% in pN1, and 11 ± 2.52% in pN2).

In CRC VEGF was expressed in 80% of cases. A reverse correlation between VEGF and CD31 was observed (p = 0.004). VEGF was not correlated, in CRC, with CD105 (p = 0.56) or other prognostic factors.

**Discussions**

Although the ACC appear more rarely than CRC, it seems that they have a particular behavior. Our data about the classical clinico-pathological factors are, partially, in concordance with the literature. ACC appear around the age of 55, more frequently at females [7–9].

Grossly, in the majority of cases, these tumors are ulcerated. Regarding the microscopic features, many papers reveal the predominance of SCC and basaloid carcinomas [7, 10].

In our cases, we found the predominance of adenocarcinomas. The ACC is recognized usually in late stages. In opposition with our conclusions, several publications say that the lymph nodes are involved when diagnosis is made [8, 10].

According to the literature, in ACC the CD31 is not correlated with the histologic type, lymph node involvement, patient’s age or neoplastic relapse. Significant correlation was found between CD31 score in ACC and parietal invasion [11, 12].

Many papers revealed that in CRC the CD31 is reverse correlated with survival and direct correlated with patient’s age or neoplastic relapse, lymph node status or parietal invasion, remaining statistically significant factor for survival [11, 13, 14].

Other papers reveal that CD31 could predict the recurrence but only endoglin counts are correlated significantly with liver metastasis [6, 15].

Many studies reveal that the antiangiogenic treatment prolonged the life in CRC with metastasis [2, 3, 16], but we observe a higher MVD in non-metastatic CRC. In most studies CD105 was correlated with CD31 in CRC but we did not found a paper about CD105 correlations in ACC.

VEGF is correlated with parietal invasion and CD31 or CD105 in CRC [6, 15]. For CRC, based at angiogenesis, more treatments are used, with Bevacizumab, Cetuximab or other angiogenesis inhibitors. These treatments regard the VEGF inhibition. For ACC, before surgical intervention, the radiotherapy is used in many cases. In our study, all cases with ACC were analyzed after radiotherapy and CRC cases were analyzed after surgical intervention.

Comparing our results regarding angiogenesis in the two groups, we observed significant differences. In ACC CD31 was correlated only with tumor size but in CRC it was reversely correlated with depth of invasion, lymph node involvement and VEGF.

CD105 was correlated with tumor size in ACC but we did not found a correlation between CD105 and other tumor factors in CRC. The IHC aspects of CD31 and CD105 are shown in the Figures 1 and 2.

The most interesting aspect was represented by the intensity of MVD and by the type of vessels. Therefore, in CRC, the MVD was higher than that from ACC (Table 4).

<table>
<thead>
<tr>
<th>Colon segment</th>
<th>CD31 average area</th>
<th>CD105 average area</th>
<th>VEGF-positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>7.37 ± 3.54%</td>
<td>7.37 ± 4.12%</td>
<td>25%</td>
</tr>
<tr>
<td>CRC</td>
<td>33.55 ± 7.43%</td>
<td>12.70 ± 3.54%</td>
<td>80%</td>
</tr>
</tbody>
</table>

Figure 1 – MVD marked with CD31 in one adenocarcinoma (A) and one squamous cell carcinoma (B)

The ratio CD31/CD105 average area was approximately 1 in ACC and 3 in CRC. VEGF was positive in 25% of ACC and 80% of CRC.
We observed that in CRC were more mature vessels, marked only with CD31 than immature vessels or endothelial isolated cells marked with both CD31 and CD105. In ACC prevailed the neofomed vessels marked with both CD31 and CD105.

Because the treatment was different in the two groups and because in one case with ACC all IHC reactions were negative, we believe that the radiotherapy could stop the angiogenesis and could inhibit the vessels’ maturation. Because the IHC reactions are negative influenced by radiotherapy we supposed that this is the reason why so few articles regard the angiogenic feature of ACC.

Acknowledgements
This work was supported by the Romanian National University Research Council (CNCSIS), Ministry of Education and Research, project frame “Angiogenesis in colorectal carcinomas and their correlations with other prognostic and predictive factors”, Trd Project, no. 550/2007.

References

Conclusions
The ACC seems to have particular behavior compared with CRC. The angiogenesis in ACC are not so high like in CRC and the immature neoformed vessels are more frequently. The percent of VEGF positive cases with ACC is smaller compared with CRC. In ACC, the antiangiogenic treatment that regards the VEGF inhibition seems to be not as efficient as in CRC. The radiotherapy could stop the angiogenesis and could inhibit the vessels’ maturation.

Figure 2 – MVD marked with CD105 in one adenocarcinoma (A) and one squamous cell carcinoma (B)
Corresponding author
Simona Gurzu, Assistant Professor, MD, PhD candidate, Department of Pathology, University of Medicine and Pharmacy, 38 Gheorghe Marinescu Street, 540 136 Târgu Mureș, Romania; Phone +40745–673 550, Fax +40265–210 933, E-mail: simonagurzu@yahoo.com

Received: September 3rd, 2007

Accepted: November 15th, 2007