PhD THESIS

NOVEL IMAGING METHODS FOR THE ASSESSMENT OF TUMOUR ANGIOGENESIS IN COLORECTAL CANCER PATIENTS

-ABSTRACT-

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Key words
Colorectal cancer (CRC), angiogenesis, confocal laser endomicroscopy (CLE), immunoendoscopy, transrectal endoscopic ultrasound (EUS), contrast enhanced endoscopic ultrasound (CE-EUS)
INTRODUCTION

Colorectal cancer represents an important cause of morbidity and mortality, with 1.200.000 new cases being diagnosed worldwide each year (1). Its incidence is characterized by a high interregional variability under the influence of different environmental risk factors and genetic predisposition (2). Although national data are limited, a constant increase in the number of diagnosed cases has been noted, CRC being currently the most frequent digestive cancer, and consequently there has been an increase in the mortality rate (3,4).

The care plan of CRC patients requires a multidisciplinary approach. The development of novel biological therapies directed against the vascular endothelial growth factor (bevacizumab) or endothelial growth factor receptor (cetuximab, panitumumab) represents an important step in oncology and has brought the possibility of increased survival for patients (5). These agents are already part of the treatment protocols in metastatic CRC combined with cytotoxic medication. Choosing the right therapeutic strategy for each patient represents a complex decision and it is necessary to identify predictive biomarkers in order to progress towards individualised therapies.

Angiogenesis represents a critical process for tumour growth and metastasis, resulting from the complex interaction of numerous growth factors and molecules (6). Considering the controversial prognostic role of angiogenesis, the importance of understanding this process arises mainly from the development and improvement of novel therapeutic strategies in oncology. Currently used techniques for assessing angiogenesis include genetic and immunohistochemical studies of angiogenic factors (7). These methods, however, are based on tissue sampling and are inevitably prone to errors, while fully understanding the biological mechanisms involved in angiogenesis requires both morphological and functional studies.

Confocal laser endomicroscopy represents a novel endoscopic technique that enables microscopic analysis of the GI mucosa by using topically or systemically administered contrast agents (8). Its clinical applications are currently growing and have gone beyond the description of mucosal morphology to functional and molecular imaging, revealing new opportunities for clinical and basic science research.

Contrast enhanced ultrasound has been intensively used for characterising and detecting lesions by assessing their microvascular enhancement pattern. Consequently CE-US has been
used for monitoring the response to anti-angiogenic therapy, enabling early recognition of changes in tumour vascularity (9). Contrast enhancement has also been used with endoscopic ultrasound, as a high-resolution imaging technique, mainly for assessing pancreatic masses (10). Despite its increased resolution and recent technology advancements, the use of CE-EUS for the evaluation of CRC has not been previously reported.

Therefore, in this study two minimally invasive high-resolution imaging techniques (CLE and CE-EUS) were used in the evaluation of colorectal cancer patients aiming to improve diagnosis and to find means of assessing tumour angiogenesis.

PERSONAL CONTRIBUTIONS

MATERIAL AND METHODS

The study took place in the Gastroenterology Clinic and the Research Centre of Gastroenterology and Hepatology of the University of Medicine and Pharmacy of Craiova between January 2010 and June 2012, with a prospective nature and included several groups of patients as follows:

- Group I included 119 patients with CRC for a study of the main demographic and clinical characteristics.
- Group II consisted of 32 patients with malignant and benign colorectal lesions: 16 cases of CRC, 6 patients with adenoma and 10 patients with colitis. All patients underwent CLE examination and images were digitally stored for later analysis based on texture features, looking for a method to objectively classify colorectal lesions.
- Group III was subject of an ex vivo CLE examination and included paired biopsies of normal and malignant mucosa that were obtained from 6 patients with CRC, during colonoscopy. The biopsies were specifically stained by using fluorescently labelled anti-CD31 antibodies.
• Group IV included 18 patients with CRC that were examined by CE-EUS for evaluation of tumour vascularity. Video sequences were stored for later analysis of time-intensity curve parameters.

RESULTS AND DISCUSSIONS

During the considered time period 119 patients were diagnosed with colorectal cancer in the Endoscopy Unit of the Gastroenterology Clinic of the University of Medicine and Pharmacy of Craiova, representing 10.5% of all colonoscopies performed.

The mean age ± SD of the patients was 67.10 ± 10.23 years, with limits ranging between 34 and 90 years old, most of them (92.4%) being diagnosed over the age of 50. There was a predominance of male patients (67%).

More than 50% of the tumors were located in the rectum and sigmoid, the exact distribution being as follows: 5 cases located in the cecum (4.2%), 14 in the ascending colon (11.76%), 11 in the hepatic flexure (9.24%), 6 cases in the transverse colon (5.04%), 3 in the splenic flexure (2.52%), in the descending colon 12 cases (10.08%), 30 in the sigmoid (25.21%) and 38 in the rectum (31.93%).

Histopathology examination revealed that 70.6% of cases were represented by moderately differentiated adenocarcinoma.

The role of confocal laser endomicroscopy in the diagnosis and angiogenesis assessment of colorectal cancer patients

In vivo study - Texture analysis based on CLE images

In this study 256 images recorded during CLE examinations were analyzed as follows: 80 images from CRC examinations, 70 from normal mucosa, 30 CLE images of colorectal polyps and 76 images from colitis patients.

Analysis was performed using the free, public domain image processing tool ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and the GLCM texture analyzer plug-
in (Cabrera, USA). Based on grey level co-occurrence matrices five texture parameters (contrast, entropy, correlation, angular second moment - ASM and inverse difference moment - IDM) were calculated for two scales (1 and 5 pixels) and two orientations (0° and 90°), resulting in 20 variables for each image. Statistically significant differences between the mean values of all parameters were found by the ANOVA test (< 0.001). A multiple comparisons test was performed after the one-way ANOVA which identified the different group means. The parameters that could differentiate between CRC images and normal mucosa, colorectal polyps and colitis were ASM for 1 pixel scale and 0° orientation and IDM for 5 pixels and 0°, and 5 pixels and 90°, respectively (Table 1).

**Table 1** - Mean values ± SD (95% CI) of the best GLCM classifiers found by texture analysis of CLE images

<table>
<thead>
<tr>
<th></th>
<th>ASM 1 pix; 0°</th>
<th>IDM 5 pix; 0°</th>
<th>IDM 5 pix; 90°</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRC</td>
<td>0.0077 ± 0.0132</td>
<td>0.1436 ± 0.0418</td>
<td>0.1404 ±0.0423</td>
</tr>
<tr>
<td></td>
<td>(0.0048 - 0.0106)</td>
<td>(0.1345 - 0.1527)</td>
<td>(0.1310 - 0.1498)</td>
</tr>
<tr>
<td>Normal</td>
<td>0.0031 ± 0.0062</td>
<td>0.1220 ± 0.0446</td>
<td>0.1087 ± 0.0386</td>
</tr>
<tr>
<td></td>
<td>(0.0011 - 0.0051)</td>
<td>(0.1116 - 0.1324)</td>
<td>(0.0995 - 0.1179)</td>
</tr>
<tr>
<td>Polip</td>
<td>0.0005 ± 0.0004</td>
<td>0.0882 ± 0.0251</td>
<td>0.0835 ± 0.0247</td>
</tr>
<tr>
<td></td>
<td>(0.0004 - 0.0007)</td>
<td>(0.0788 - 0.0975)</td>
<td>(0.0743 - 0.0927)</td>
</tr>
<tr>
<td>IBD</td>
<td>0.0012 ± 0.0025</td>
<td>0.1205 ± 0.0312</td>
<td>0.1158 ± 0.0299</td>
</tr>
<tr>
<td></td>
<td>(0.0007 - 0.0018)</td>
<td>(0.1134 - 0.1276)</td>
<td>(0.1090 - 0.1227)</td>
</tr>
<tr>
<td>ANOVA</td>
<td><em>p &lt; 0.0001</em></td>
<td><em>p &lt; 0.0001</em></td>
<td><em>p &lt; 0.0001</em></td>
</tr>
</tbody>
</table>

ASM - angular second moment; IDM - inverse difference moment.

ROC analysing was performed to test the performance of these parameters in discriminating between CRC and normal mucosa. An area under the curve of up to 0.750 (95% CI, 0.673 - 0.817, *p*=0.0001) was found for IDM (5 pixels, 90°), with a cut-off value of 0.136 that could differentiate CRC from normal mucosa with 52.50% sensitivity and 88.57% specificity (PPV 84%, NPV 62%).

The best classifier in discriminating between malignant and benign tissue (normal mucosa, adenoma, colitis) was ASM (1 pixel; 0°) with an area under the ROC curve of 0.783 and a cut-off value of 0.0009 that enabled 81.2% sensitivity and 70.5% specificity (PPV 55.6%, NPV 89.2%).

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Texture analysis has been used for interpreting and classifying medical images since 1973, with initial studies including radiology and ultrasound images. In the last years many published papers have reported the use of texture analysis with CT and MRI sequences, for a variety of benign and malignant pathologies, including CRC (11).

This was the first attempt to use texture analysis for classifying colorectal lesions based on CLE images. A pilot ex vivo study showed the feasibility of such a quantitative analysis for Barrett esophagus, by using images from microendoscopy examination of fresh tissue resected during endoscopy (12).

Both ASM and IDM represent measures of image homogeneity, with low values indicating inhomogeneous pictures, and were found able to classify colorectal lesions. Thus, ASM and IDM parameters showed descending values for normal mucosa, colitis and adenoma, but higher values for CRC. Similar findings were reported by a recent study that applied texture analysis to magnifying colonoscopy images (13). The same parameters that characterize homogeneity had increased values in the case of type V_N colonic polyps of the Kudo and Tsuruta classification, associated with massive submucosal invasive carcinoma. One possible explanation could be that the loss of normal crypt architecture that is replaced by malignant cells results in a more simplified, amorphous structure.

Texture analysis offers information beyond that available to the examiner’s visual inspection, without the need of acquiring additional images. In the future, determining the best combination between GLCM parameters could enhance the diagnosis yield of CLE examinations and offer support for clinical decisions.

Ex vivo study - A feasibility study for the use of confocal laser endomicroscopy for the morphometric evaluation of microvessels in colorectal cancer using targeted anti-CD31 antibodies

This study included paired biopsy samples of normal and neoplastic mucosa from 6 patients with advanced CRC that were specifically stained, using fluorescently labeled antibodies directed against CD31 and examined by CLE. Five images were analyzed for each tissue sample.

CLE detected a specific fluorescent signal in all examined biopsy samples, with differences noted between normal and tumor vessels. In normal biopsies the vessels formed a regular network with a honeycomb pattern and showed regular distribution of diameters. On the
other hand, the vessels in tumor samples appeared dilated and irregular in shape, with variable diameters along their length. The vascular pattern is used in classifying colorectal lesions also during \textit{in vivo} CLE examinations, following intravenous administration of fluorescein, an unspecific contrast agent that tends to diffuse into the interstitial space (14). In this study the use of a specific endothelial staining enabled in addition a morphometric analysis by displaying entire vascular segments.

The average vessel diameter in tumor samples was $13.5 \pm 0.5 \ \mu m$ (95% CI 12.6 - 14.5), significantly higher than the vessel diameter in normal colorectal mucosa ($8.5 \pm 0.4 \ \mu m$, 95% CI 7.6 - 9.3, $p<0.0001$). Vascular density was $242.4 \pm 11.0$ vessels/mm$^2$ in tumor tissue samples (95% CI 219 - 266 vase/mm$^2$) and $188.7 \pm 12.8$ vessels/mm$^2$ in the normal tissue (95% CI 162 - 215, $p=0.0027$). The diameter distribution showed more vessels of larger diameters in tumor samples compared to normal tissue.

The CLE results were validated by a conventional immunohistochemical technique which revealed a positive signal for CD31 in both control and malignant samples. There was a similar trend for increased MVD and vascular area in the samples of colorectal cancer, in agreement with previously reported results (15). In the normal mucosa, the average MVD was $211.2 \pm 42.9$ while in the malignant tissue it was $351.3 \pm 39.6$/mm$^2$ ($p=0.1072$). The vascular area was almost 3 times higher for CRC ($8.5 \pm 2.1\%$) compared to the normal samples ($2.9 \pm 0.5\%$), $p=0.0536$. Although the differences are clear, statistical significance was not reached, probably because of the small number of samples.

Recent studies have reported specific imaging by CLE of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), two already established therapeutic targets for advanced CRC (16-18). However, this seems to be the first study to use fluorescently labeled antibodies directed against an endothelial marker (CD31) for CLE examination. This method generates similar results as traditional immunohistochemistry, but on fresh non-fixed tissue. Thus, any processing artifact of the biopsies is avoided and imaging of the tissue is performed within a short time after biopsy collection, which considerably speeds up the diagnosis process (19). These results can be translated into a new technique, \textit{immunoendoscopy}, pending on the discovery of novel targeted contrast agents to be used in clinical trials.
This study originally included 20 patients with CRC that were prospectively examined by CE-EUS, of which 18 were subject of a quantitative analysis of the recorded video sequences. Loco-regional staging assessed either by EUS or after surgery where possible showed that most patients had advanced tumors, 15 having T3 and T4 stages, 9 of which were the patients with lymph nodes metastases. The histopathological exam classified most tumors as moderately differentiated adenocarcinomas.

Most CRC examined by CE-EUS were well vascularized with uptake of the contrast agent in the arterial phase in 15 cases. The enhancement was either equally distributed through the tumor or inhomogeneous with stronger peripheral uptake and avascular areas towards the intestinal lumen, probably representing tumor necrosis areas. In 3 patients the tumors were hypoenhancing.

Time-intensity curve analysis applied to the films recorded during CE-EUS examinations enabled a quantitative assessment of tumor perfusion. Average values ± SD of the resulting parameters are presented in Table 2.

Table 2 - Parameters resulting from time-intensity curve analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean value ± SD</th>
<th>Min - Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT (s)</td>
<td>10.08 ± 3.85</td>
<td>4.17 - 16.67</td>
</tr>
<tr>
<td>TTP (s)</td>
<td>24.03 ± 10.94</td>
<td>9.33 - 56.17</td>
</tr>
<tr>
<td>Imax (a.u.)</td>
<td>41.43 ± 19.24</td>
<td>10.1 - 80.1</td>
</tr>
<tr>
<td>AUC (a.u.*s)</td>
<td>5477.45 ± 2922.68</td>
<td>830.1 - 11735</td>
</tr>
</tbody>
</table>

*a.u. - arbitrary units; AT - arrival time; TTP - time to peak; Imax - peak intensity; AUC - area under the curve*

An inverse correlation was noted between the presence of positive lymph nodes and Imax and AUC, but without reaching statistical significance (Spearman $r = -0.439, p = 0.0683$). Both parameters showed decreased values in the presence of lymph node involvement compared to N0 stages (Imax $32.37 \pm 16.86$ a.u. vs. $50.59 \pm 17.82$ a.u, $p = 0.0415$ and AUC $4023 \pm 2243$ a.u.*s vs. $6932 \pm 2891$ a.u.*s, $p = 0.0298$). No other correlation was found between TIC parameters and the considered histopathology factors.
The development of contrast agents for ultrasound examination (CEUS) has enabled real-time assessment of intratumoral perfusion and also a quantitative analysis of the vascularization (20). By using this method CEUS examination demonstrated the early treatment effect of anti-angiogenic agents in primitive hepatocellular carcinoma, renal carcinoma and liver metastases from CRC (21). A recent study found increasing values for the parameter AUC in T2, T3 and T4 stages by using transabdominal CEUS (22). The same parameter, AUC was significantly higher in colorectal adenocarcinomas compared to adenomas and positively correlated with MVD assessed by immunohistochemistry (23).

The use of transabdominal ultrasound in the examination of the GI tract is limited by artifacts induced by intraluminal gas, peristalsis and abdominal fat. Some of these shortcomings can be overcome by CE-EUS, benefiting from the advantages of a high resolution imaging technique.

Although EUS has been used to evaluate rectal tumors since 1985 and current international guidelines recommend its use for pre-therapeutic staging of patients with rectal cancer (24), the role of contrast examination during EUS has not been so far assessed for colorectal tumors (25).

This study showed the feasibility of CE-EUS in CRC patients. The use of a radial echoendoscope with frontal view enabled examinations further in the sigmoid and ascending colon. There were however some technical difficulties during the study. The normal colorectal wall could not be included as control in the analysis due to the circumferential spread of some of the tumours and its small size. In this context a quantitative analysis is all the more necessary to get a full picture of the vascular pattern. This was possible by offline analysis of stored video sequences based on time-intensity curve parameters. Although the study group was relatively small and conclusions cannot be drawn with high confidence, results show that Imax and AUC have a potential as prognosis factors.

Further prospective studies on larger groups are necessary for defining the role of CE-EUS for CRC and improving the examination technique.
CONCLUSIONS

- Colorectal cancer is the most frequent digestive cancer, diagnosed in 10.5% of all colonoscopies performed during the considered time period.
- Confocal laser endomicroscopy is a novel endoscopic technique that enables both a histologic diagnosis in real-time, based on the microvascular pattern and changes in crypt architecture, and an objective analysis of the recorded images.
- Texture analysis of CLE images identified the best classifiers of colorectal lesions: angular second moment and inverse difference moment. These parameters were able to differentiate between CRC and control groups with good sensitivity and specificity and an area under the ROC curve up to 0.783.
- Further studies to confirm the results and include these texture parameters in automatic models of classification will reduce intra- and interobserver variability and enhance the diagnosis yield of CLE examinations.
- From a technical point of view immunoendoscopy is feasible and in the future it could be used for individualized therapies in oncology. By using fluorescently labelled antibodies directed against an endothelial marker (CD31) selective imaging of vessels in tumor and normal colorectal mucosa was possible with CLE, similar to conventional immunohistochemistry, but on fresh, non-fixed tissue.
- The microvascular pattern of CRC samples during CLE examination was irregular, with dilated vessels, clearly different from the normal vascular network.
- Morphometric analysis of CLE sections with specific staining for CD31 demonstrated significant differences between normal and malignant vessels, with both diameter and vascular density showing increased values in CRC samples.
- Endoscopic ultrasound proved to be feasible for the assessment of intratumoral perfusion using contrast-enhanced, low mechanical index examination. In most cases colorectal tumours were enhanced completely or with the presence of avascular areas after contrast administration.
Computer aided analysis of the video sequences enabled in addition a quantitative evaluation of tumor vascularity based on time-intensity curve derived parameters. Imax and AUC had different values between N0 stage and cases with lymph node involvement, suggesting a possible prognostic role for these parameters. Further studies on larger groups of patients are necessary to improve the examination technique and define its role in the evaluation of CRC.

On the long term these novel endoscopic techniques could allow a real-time histologic diagnosis using CLE validated by objective means of classification and consequently allow rapid access to the appropriate treatment of CRC patients, while CE-EUS could offer a close monitoring of specific anti-angiogenic therapies through a quantitative study of vascularity.
SELECTED REFERENCES


