—Original Article—

Assessing tumor angiogenesis in colorectal cancer by quantitative contrast-enhanced endoscopic ultrasound and molecular and immunohistochemical analysis

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ABSTRACT

Background and Objectives: Data on contrast-enhanced endoscopic ultrasound (CE-EUS) for colorectal cancer (CRC) evaluation are scarce. Therefore, we aimed to assess the vascular perfusion pattern in CRC by quantitative CE-EUS and compare it to immunohistochemical and genetic markers of angiogenesis. **Patients and Methods:** We performed a retrospective analysis of CE-EUS examinations of 42 CRC patients, before any therapy. CE-EUS movies were processed using a dedicated software. Ten parameters were automatically generated from the time-intensity curve (TIC) analysis: peak enhancement (PE), rise time (RT), mean transit time, time to peak (TTP), wash-in area under the curve (WiAUC), wash-in rate (WiR), wash-in perfusion index (WiPI), wash-out AUC (WoAUC), and wash-in and wash-out AUC (WiWoAUC). The expression levels of the vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2 genes were assessed from biopsy samples harvested during colonoscopy. Microvascular density and vascular area were calculated after CD31 and CD105 immunostaining. **Results:** Forty-two CE-EUS video sequences were analyzed. We found positive correlations between the parameters PE, WiAUC, WiR, WiPI, WoAUC, WiWoAUC, and N staging (Spearman $r = 0.437$, $r = 0.336$, $r = 0.462$, $r = 0.437$, $r = 0.358$, and $r = 0.378$, respectively, $P < 0.05$), and also between RT and TTP and CD31 vascular area ($r = 0.415$, and $r = 0.421$, respectively, $P < 0.05$). VEGFR1 and VEGFR2 expression did not correlate with any of the TIC parameters. **Conclusions:** CE-EUS with TIC analysis enables minimally invasive assessment of CRC angiogenesis and may provide information regarding the lymph nodes invasion. However, further studies are needed for defining its role in the evaluation of CRC patients.

Key words: Angiogenesis, colorectal cancer, contrast-enhanced endoscopic ultrasound, microvascular density, vascular endothelial growth factor receptor

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INTRODUCTION

Angiogenesis is a critical process for tumor growth and metastasis, resulting from the interaction of numerous growth factors and signaling molecules which lead to activation of endothelial cells and formation of new vessels.[1] The main role is attributed to the vascular endothelial growth factor (VEGF) family, which includes several cytokines that promote their pro-angiogenic effect by binding to specific tyrosine kinase receptors.[2] VEGF-A, the key factor of the VEGF family, and its receptors VEGFR1 (Flt-1) and VEGFR2 (Flk/KDR) have been shown to be overexpressed in colorectal cancer (CRC) compared to normal tissue. It has also been suggested that their expression was associated with more aggressive tumor phenotypes.[3,4] While the prognostic significance of tumor angiogenesis is still controversial, the biological understanding of this process is important for the development and improvement of novel therapeutic strategies in oncology. Current treatment regimens for advanced CRC already include a combination of cytotoxic and antiangiogenic drugs which have improved survival for these patients.[5]

Conventional means used for assessing angiogenesis include the determination of microvascular density (MVD), usually using panendothelial markers for immunohistochemical staining, such as factor VIII, CD31, and CD34.[6] On the other hand, as a dynamic process, angiogenesis might be better assessed with functional imaging techniques to enable real-time visualization of changes in vascularity and the effects of targeted biological therapies.

Contrast-enhanced ultrasound (CEUS) has been extensively used for imaging of tumor vascularization, initially by amplification of the Doppler signal following intravenous administration of contrast agents. With more recent developments which included second-generation microbubble contrast agents and improved technology with dedicated contrast harmonic imaging mode, CEUS has opened the avenue for the evaluation of tumor angiogenesis by enabling signal detection even from low-velocity flow microvessels.[7] Furthermore, quantitative evaluation of perfusion is possible from CEUS sequences based on time-intensity curves (TICs), which display the variations in time of the average signal intensity inside a region of interest (ROI).[8] Recently, contrast-enhanced harmonic imaging has been added to endoscopic ultrasound (EUS) examinations, enabling better characterization of lesions and differentiation between benign and malignant tumors.[9] Despite increased resolution provided by EUS examinations, data on the use of contrast enhancement for the real-time assessment of angiogenesis in CRC are limited to a couple of studies which included rectal tumors only.[10,11] Their results showed the possibility of using contrast-enhanced endorectal ultrasound for the evaluation of tumor vascularity, and correlations were found between one of the TIC parameters and MVD.[11] In these studies, rigid rectal ultrasound probes were used, whereas a forward-viewing radial echoendoscope has been recently developed, and proved safe in reaching and staging tumors throughout the entire colon.[12]

Therefore, the aim of our proof-of-concept study was to evaluate the perfusion pattern in CRC using contrast-enhanced EUS (CE-EUS) combined with quantitative TIC analysis based on a dedicated software, and to compare the resulting parameters with markers of tumor angiogenesis derived from immunohistochemical (MVD and vascular area) and genetic analysis (VEGFR1 and VEGFR2 gene expression).

PATIENTS AND METHODS

Patients

The study was conducted under approval from the Local Ethical Committee and included 42 consecutive patients (25–80 years old) with a CRC diagnosis. We performed a retrospective analysis of recorded movies of the patients' CE-EUS examinations. We included patients diagnosed with primary rectal tumors referred for local staging by EUS, as well as patients with colonic tumors, with no prior treatment with chemo- and/or radio-therapy, who had undergone CE-EUS examination and for whom biopsies were stored in our bio-bank for molecular analysis. Informed consent was obtained before the investigation. Diagnosis confirmation was obtained from histological examination of endoscopic biopsies.

Contrast-enhanced endoscopic ultrasound

EUS was performed with a radial front-viewing echoendoscope (EG-3670URK, Pentax, Hamburg, Germany) coupled with a Hitachi Preirus US System (Hitachi Medical Corp, Tokyo, Japan) with an incorporated harmonic imaging contrast examination mode. The front-view radial EUS scope enabled examination of more proximal tumors located along the colon. Patients were prepared for examination as

for standard colonoscopies, through oral administration of polyethylene glycol solutions. A water-filling balloon placed over the distal tip of the echoendoscope as well as water instillation were used as needed for clear imaging of the colorectal wall layers. The tumors were described in terms of their echostructure, size, and the extent of invasion into the bowel wall and surrounding structures. Lymph nodes located near the tumor were reported, and those larger than 5 mm in diameter, with hypoechoic appearance, and round shape were suspected of malignancy. Staging was performed according to the American Joint Committee on Cancer classification[13] based on EUS and pathological examination of the surgical specimen whenever intervention was the first-line treatment. For stenosing tumors, EUS examination was conducted from the distal side of the lesion. CE-EUS was performed after the staging procedure by choosing a transverse section which contained a large tumor area, as seen on the B-mode panel while enabling a steady position for the echoendoscope. We used a second generation contrast agent (SonoVue, Bracco, Milan, Italia) including sulfur hexafluoride in phospholipid coating microbubbles which was administered in a single 4.8 mL bolus injection through a 20 G intravenous cannula, followed by a flush of 5 mL 0.9% sodium chloride solution. For all CE-EUS examinations, dynamic wide-band contrast harmonic imaging mode was selected with the mechanical index set-up at 0.2. With the timer starting at the moment of contrast injection, video clips were recorded and stored as audio video interleave files on the system hard drive for later analysis.

Time intensity curve analysis

For the quantitative analysis of tumor perfusion, we used a dedicated off-line software application entitled VueBox™ (Bracco Suisse SA, Switzerland) which enables a standardized quantification across different users and ultrasound systems. The application processes Digital Imaging and Communications in Medicine (DICOM) files after linearization and evaluates tissue perfusion by displaying the mean intensity within a ROI as a function of time. From fitted TIC curves, the program automatically generates several amplitude or temporal parameters relevant to the blood flow and volume of the selected ROI.[14] For our study, the saved video clips were first converted to DICOM files and then transferred from the US system hard drive to an off-line working station. Only the first sixty seconds after bolus injection were analyzed by one of the examiners who were blind to the results of the genetic and immunohistochemistry analyses. With the appropriate settings for our US system available within the application, the ROI was manually drawn to delineate the entire lesion. The motion compensation function was applied to remove any in-plane movement. The TIC parameters included in our analysis were: peak enhancement (PE) representing the maximum intensity in the TIC curve, rise time (RT) calculated from the beginning of enhancement until PE, mean transit time (mTT) defined as the mean time taken by the contrast microbubbles to transit the ROI, time to peak (TTP)-time from zero intensity to maximum intensity, wash-in area under the TIC curve (WiAUC), wash-in rate (WiR), wash-in perfusion index (WiPI = WiAUC/RT), wash-out AUC (WoAUC), and wash-in and wash-out AUC (WiWoAUC) representing the total area under the TIC curve [Figure 1].

Genetic analysis

Total RNA from tumor and normal mucosa samples harvested during colonoscopy was isolated with PureLink® RNA Mini Kit from Ambion (Lyfe Technologies). Samples concentration was measured by spectrophotometry (Eppendorf Biophotometer, Eppendorf, AG, Hamburg, Germany). An Agilent 2010 Bioanalyzer (Agilent Technologies Inc., USA) was used to assess the integrity of isolated RNA.

Two-step real-time quantitative polymerase chain reaction (qRT-PCR) was used to assess the expression level of VEGFR1 and VEGFR2 genes. First, synthesis of complementary DNA (cDNA) from total RNA was performed using High Capacity cDNA Reverse

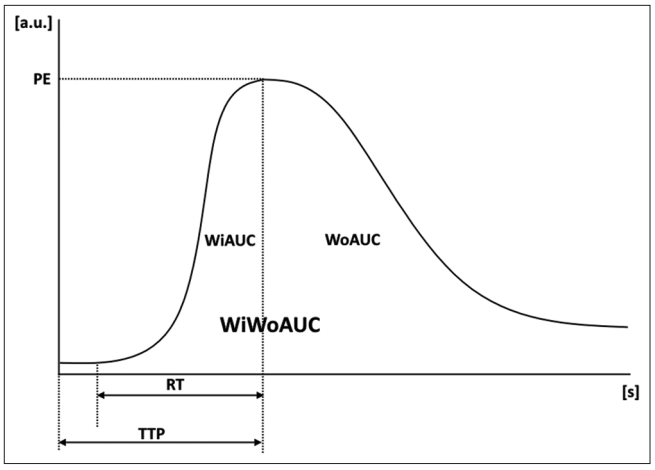

Figure 1. Graphic representation of the time-intensity curve and derived parameters. PE: Peak enhancement, RT: Rise time, TTP: Time to peak enhancement, WiAUC: Wash-in area under the curve, WoAUC: Wash-out area under the curve, WiWoAUC: Wash-in and wash-out area under the curve, a.u.: Arbitrary units, S: Seconds

Transcription Kit (Applied Biosystems, Foster City, CA, USA). In the second step, the PCR products were amplified and quantified using the TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and specific TaqMan Gene Expression Assays for target genes and for endogenous control gene (FLT1/VEGFR1-Hs01052961_m1; KDR/VEGFR2-Hs00911700_m1 and GAPDH-Hs99999905_m1). At least one negative control reaction was performed in each plate. Since the efficiency of the primers and probes used in all the reactions was 100%, $2^{-\Delta\ \Delta Ct}$ method was used for calculating fold changes between paired samples.

Histopathology and immunohistochemical analysis

Pathology diagnosis was based on hematoxylin and eosin staining of formalin-fixed paraffin-embedded biopsy samples obtained during colonoscopy. Immunostaining of the slides was performed with antibodies targeting CD31, a panendothelial marker, and CD105, a marker for the proliferating endothelium, as specified by the manufacturer (Dako, Glostrup, Denmark). The "hot-spot" method was used to measure MVD from three to four random images captured under the ×40 objective from within the areas with the highest vascular density ("hot-spots"). The vascular-related CD31 and CD105 stained endothelia were manually traced on the images using a stylus pen, then these areas were counted and measured as MVD and vascular areas using the image analysis package Image ProPlus AMS7 software (Media Cybernetics, Bethesda, Maryland, USA), as previously described.[15]

Statistical methods

Data were analyzed using GraphPad Prism, version 6.01 for Windows (GraphPad Software, La Jolla California, USA). Besides descriptive statistics, the Spearman's test was performed to look for correlations between CE-EUS parameters, tumor staging, histologic and genetic markers, whereas the nonparametric Kruskal–Wallis test was used for comparisons of multiple groups, with statistical significance defined for a $P < 0.05$. Wilcoxon matched-pairs signed rank tests were performed for statistical analysis of target genes expression in paired samples.

RESULTS

Most of the included patients were found to have advanced tumors (T3-32 cases and T4-2 cases), and 26 of them had nodal involvement. Patients' clinical and pathological data are presented in Table 1. CE-EUS examination showed that 40/42 (95.24%) of the tumors were well vascularized, with either a homogeneous uptake of the contrast agent or inhomogeneous enhancement resulting from stronger peripheral uptake and avascular areas toward the intestinal lumen, with the latter pattern most frequently seen in advanced tumors (T3, T4) [Figure 2]. Only 2/42 (4.76%) of the colorectal tumors were hypoenhancing [Figure 3].

The TIC parameters resulting from the automated analysis are presented in Table 2. When comparing the parameter values to clinical and pathological data, we found a positive correlation between the parameters PE, WiAUC, WiR, WiPI, WoAUC, WiWoAUC, and N staging, with $r = 0.437$, $r = 0.336$, $r = 0.462$, $r = 0.437$, $r = 0.358$, and $r = 0.378$, respectively ($P < 0.05$). The bar graphs represented in Figure 4 show the differences between N stages for the parameters PE, WiR, WiPI and WiWoAUC. No other correlations were found between CE-EUS parameters and T stage or histologic tumor grade [Table 3].

Contrast-enhanced endoscopic ultrasound parameters and vascular endothelial growth factor receptor expression

Gene expression assessed in 42-paired samples revealed that VEGFR1 and VEGFR2 are expressed in both tumor and normal mucosa, with higher mean

Table 1. Patients characteristics (*n*=42)

Clinical/pathological feature	Numeric value
Mean age±SD (range), years	63.87±11.32 (25-80)
Gender (male/female)	36/6
Tumor location	
Descending colon	1
Sigmoid	3
Recto-sigmoid junction	5
Rectum	33
Histology	
Adenocarcinoma	
G1	11
G2	23
G3	4
Undetermined	4
T stage*	
T1	2
T2	6
T3	32
T4	2
N stage*	
N0	16
N1	17
N2	9

*Incomplete EUS TNM staging for four patients with stenosing tumors. SD: Standard deviation, EUS: Endoscopic ultrasound, TNM: Tumor node metastasis

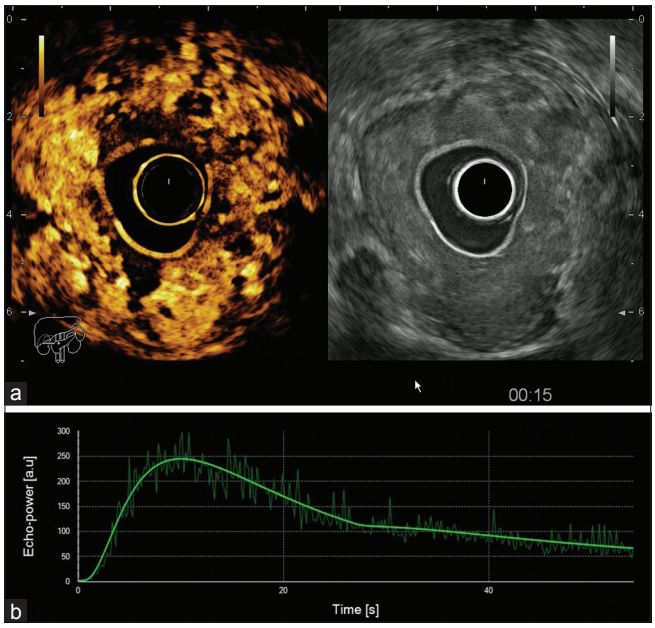

Figure 2. (a) Contrast-enhanced endoscopic ultrasound image from a well vascularized rectal adenocarcinoma with inhomogeneous enhancement of the contrast agent visible in the left panel; (b) the time-intensity curve resulting from off-line analysis of the contrast-enhanced endoscopic ultrasound sequences (PE = 242.78 a.u, WiWoAUC = 5628.25 a.u.). CE-EUS: Contrast-enhanced endoscopic ultrasound, WiWoAUC: Wash-in and wash-out area under the curve, a.u.: Arbitrary units, PE: Peak enhancement

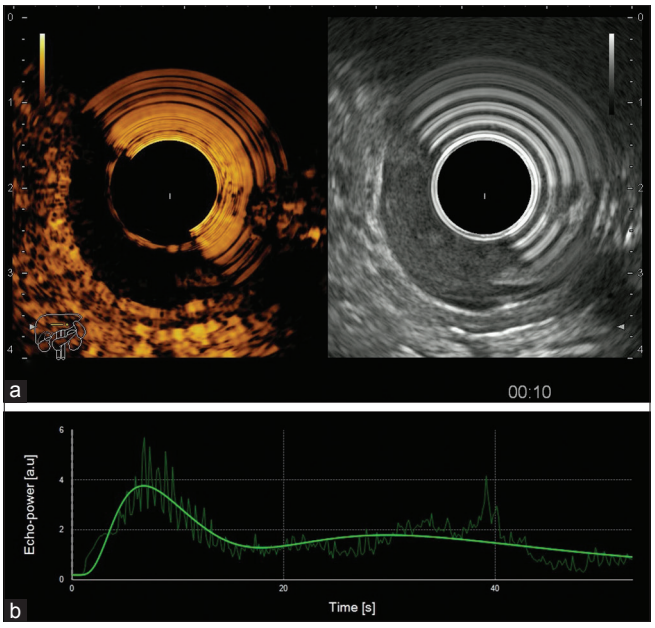

Figure 3. (a) Contrast-enhanced endoscopic ultrasound examination of a sigmoid adenocarcinoma which demonstrated weak uptake of the contrast agent, as shown in the left side image; (b) the resulting time-intensity curve (PE = 3.58 a.u., WiWoAUC = 34.39 a.u.). CE-EUS: Contrast-enhanced endoscopic ultrasound, WiWoAUC: Wash-in and wash-out area under the curve, a.u.: Arbitrary units, PE: Peak enhancement

expression levels in tumor tissue compared to paired noninvaded normal mucosal samples (P = 0.0001, Wilcoxon matched pairs signed rank test). However, the expression of VEGFR1 and VEGFR2 genes was not significantly different in individually paired sample (tumor and normal tissue) (P = 0.8184, Wilcoxon signed rank test). No significant correlation was found between CE-EUS parameters and VEGFR1 and VEGFR2 expression in tumor tissue [Table 3].

Contrast-enhanced endoscopic ultrasound parameters and immunohistochemistry

The mean ± standard deviation MVD count based on pathology slides stained for CD31 was 279.54 ± 102.18 vessels/mm^2 (range 102.66–480.92 vessels/mm^2) while using CD105 staining we found a mean value of 210.89 ± 90.36 vessels/mm^2 (ranging between 41.06 and 374.15 vessels/mm^2). The vascular area reported for CD31 slides was 10.68% ±4.09% (range 3.02–21.41), and 6.12% ±3.65% (ranging between 1.94% and 16.86%) for CD105 staining. We found a positive correlation between the parameters RT, TTP and the CD31 vascular area with r = 0.415 (P = 0.020), and r = 0.421, respectively (P = 0.018). The correlation coefficients between all TIC parameters and MVD and vascular areas are presented in Table 3.

DISCUSSION

CRC represents a significant global health burden as the third most frequent malignancy in males, and second in females. The highest incidence rates are seen in more developed countries, whereas mortality rates have been decreasing in these areas as a consequence of screening programs and improved therapies.[16] Treatment protocols for CRC are the result of a multidisciplinary approach and include the addition of chemo- and radio-therapy to surgical techniques. Recent advancements in understanding tumor biology have translated into the recognition of novel molecular therapeutic targets, such as VEGF and EGFR (epidermal growth factor receptor) pathways. Specific therapies (bevacizumab, regorafenib, aflibercept, cetuximab, panitumumab) have been added as first and second-line treatment options for patients with advanced metastatic CRC, bringing significant benefits in survival rates.[17] As treatment decisions for each patient have become more challenging, novel imaging methods and valid biomarkers need to be developed for accurate pretherapeutic evaluation and follow-up of tumor response to treatment, to optimize outcomes.

CEUS has been used for imaging angiogenesis at many tumor sites, and consequently better characterization

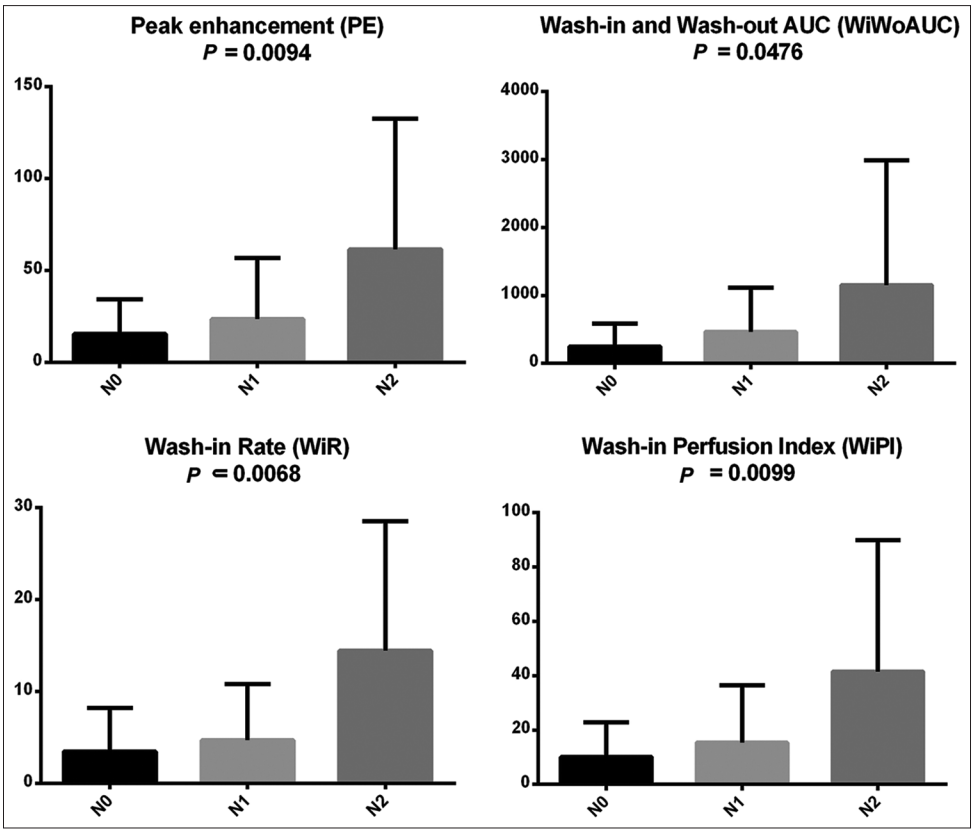

Figure 4. Graphic representation of time-intensity curve derived parameters for different N stages

Table 2. Values of time-intensity curve parameters generated by computed analysis

TIC parameter	25% percentile	Median	75% percentile	Mean value±SD
PE (a.u.)	6.570	15.34	35.95	28.56±43.14
WiAUC (a.u)	27.09	88.90	147.3	158.4±249.6
RT (s)	4.930	7.550	9.747	8.819±6.409
mTT (s)	28.50	42.90	125.4	81.32±80.52
TTP (s)	7.385	10.24	13.71	11.81±7.673
WiR (a.u)	1.450	3.345	7.803	6.315±8.968
WiPI (a.u)	4.120	10.46	23.15	18.97±28.94
WoAUC (a.u)	36.30	201.6	311.4	374.0±730.8
WiWoAUC (a.u)	56.98	298.3	438.6	526.1±987.5

PE: Peak enhancement, WiAUC: Wash-in area under the curve, RT: Rise time, mTT: Mean transit time, TTP: Time to peak, WiR: Wash-in rate, WiPI: Wash-in perfusion index, WoAUC: Wash-out area under the curve, WiWoAUC: Wash-in and wash-out area under the curve, a.u.: Arbitrary units, TIC: Time-intensity curve, SD: Standard deviation

Table 3. Correlation coefficients between contrast-enhanced endoscopic ultrasound parameters, staging, and genetic and histology features

TIC parameter	T	N	G	VEGFR1	VEGFR2	CD31 MVD	CD31 vascular area	CD105 MVD	CD105 vascular area
PE	0.057	0.437*	−0.029	−0.206	−0.189	0.083	−0.153	0.156	0.317
WiAUC	0.092	0.336*	0.035	−0.218	−0.187	0.152	0.020	0.121	0.285
RT	0.159	0.015	0.091	−0.073	−0.046	0.016	0.415*	0.070	0.265
mTT	−0.186	−0.028	0.022	0.189	0.129	−0.138	0.235	−0.028	0.298
TTP	0.211	−0.092	0.173	−0.053	0.004	0.080	0.421*	0.111	0.248
WiR	0.005	0.462*	−0.037	−0.195	−0.199	−0.025	−0.336	0.010	0.205
WiPI	0.052	0.437*	−0.026	−0.203	−0.188	0.081	−0.148	0.141	0.313
WoAUC	0.078	0.358*	−0.013	−0.054	−0.061	0.178	0.044	0.132	0.138
WiWoAUC	0.071	0.378*	−0.016	−0.091	−0.087	0.229	0.066	0.197	0.215

Spearman test *r* values, **P*<0.05. PE: Peak enhancement, WiAUC: Wash-in area under the curve, RT: Rise time, mTT: Mean transit time, TTP: Time to peak, WiR: Wash-in rate, WiPI: Wash-in perfusion index, WoAUC: Wash-out area under the curve, WiWoAUC: Wash-in and -out area under the curve, TIC: Time-intensity curve, VEGFR: Vascular endothelial growth factor receptor, MVD: Microvascular density

of lesions has been obtained based on their perfusion pattern, with the possibility of differentiating benign from malignant tumors.[7] With quantitative evaluation available, CEUS has also been applied to monitoring treatment response for several tumor sites, such as colorectal metastases, hepatocellular carcinoma, gastrointestinal stromal tumors, and renal carcinoma. Studies have shown that it can reveal early changes in vascularity, taking place before tumor shrinkage occurs.[18] Recent results from a multicenter trial which included 539 patients with different solid tumor types undergoing antiangiogenic therapy showed that, among TIC parameters, AUC was a valid criterion that could predict tumor progression at 30 days from baseline.[19] For CRC only a few studies have been conducted to investigate the role of contrast enhancement during ultrasound and initial data resulted from transabdominal examinations.[20-22] Based on quantitative evaluation from CEUS examinations of colorectal tumors, a positive correlation was found between the TIC parameter AUC and MVD ($r = 0.686$, $P = 0.0019$).[21]

Although CE-EUS can overcome some of the disadvantages of transabdominal ultrasound examination of the gastrointestinal tract, such as artifacts induced by luminal gas or abdominal fat, and benefits from increased imaging resolution, experience in CRC has been scarce so far and included mainly rectal cancer cases. One initial study on 227 patients with both benign and malignant rectal lesions that were examined by contrast-enhanced endorectal ultrasound found different patterns of perfusion between rectal adenocarcinomas, adenomas, and inflammatory lesions. Parameters of TIC analysis were also different between benign and malignant lesions and also differed from the normal rectal wall.[10] However, as authors also stated, biases could have resulted from the placement of the ROI which included only a small area within the tumor section. In a different trial 66 rectal cancer patients underwent contrast-enhanced endorectal ultrasound followed by TIC analysis of the recorded movies within a ROI drawn at the most enhanced area of the tumor. Weak associations were found for one of the parameters, enhanced intensity, with MVD ($r = 0.295$, $P = 0.016$), and also with the histological grade ($r = -0.264$, $P = 0.007$).[11]

In both previously published studies a rigid rectal probe was used for ultrasound examination, and therefore only rectal tumors were included. The frontal view radial echoendoscope used in our study enables both endoscopic and ultrasound imaging and this allowed us to include more proximal tumors as well. Although surgery has been traditionally considered to be the mainstay in the treatment of colon cancer patients, more recent studies, have demonstrated the benefits of neoadjuvant chemotherapy in locally advanced colon cancer, which can result in significant tumor volume reduction and even induce a complete pathological response in some cases.[23,24] Moreover, neoadjuvant chemotherapy for locally advanced colon cancer is even considered by current guidelines.[5] Therefore, accurate imaging techniques should be sought to enable a proper selection of patients for neoadjuvant therapy and also to measure tumor response. The forward-viewing radial echoendoscope is able to safely reach colonic tumors and enables more accurate staging compared to computed tomography (CT), as reported by preliminary studies.[12,25]

Regarding the perfusion pattern, our findings are similar to previous reports,[10] showing that most of the tumors (95.24% of cases) were enhanced completely or with inhomogeneous uptake of the contrast resulting from necrotic areas. For TIC analysis, we used a commercially available software which was specifically designed for standardized measurements across different ultrasound platforms, and could, therefore, increase reproducibility of results between different centers.[14] The ROI was manually drawn to include the entire transverse section chosen for CE-EUS examination, which we considered to be more representative of the tumor vascularity, and the feature of automatic in-plane motion compensation was selected for more accurate measurements. No association was found between the calculated TIC parameters and VEGFR1 or VEGFR2 gene expression as determined by qRT-PCR, not even when a subgroup analysis was conducted, including only the cases with tumor overexpression of the two markers (data not shown). A possible explanation could derive from the very complex molecular signature of the angiogenic process, which results from the interaction of numerous other growth factors released by both tumor and host cells.[1] We did find a positive correlation between the parameters RT and TTP and tumor vascularity as assessed by CD31 immunohistochemical staining with the calculation of vascular area ($r = 0.415$ and $r = 0.421$, respectively, $P < 0.05$). Weak positive associations were also observed between PE, WiAUC, mTT, WiPI and CD105 vascular area ($r = 0.317$, $r = 0.285$, $r = 0.298$ and $r = 0.313$, respectively), but statistical significance was not reached, possibly as a

consequence of the small number of included patients. Also, immunohistochemical parameters of vascularity render morphological data from a limited microscopic area which is inevitably bound to sampling errors, whereas imaging methods such as CE-EUS provide functional parameters representative for a wider ROI which might better reflect the dynamics of tumor angiogenesis.

Furthermore, several CE-EUS parameters, including PE, WiAUC, WiR, WiPI, WoAUC, and WiWoAUC positively correlated with the lymph node status, suggesting that highly vascularized tumors are prone to having nodal invasion, as TIC parameters are related to blood volume and flow of the selected ROI. The presence of lymph node metastases in CRC has been previously associated with increased angiogenesis as assessed by CD105 and VEGF overexpression.[26,27] Although tumor staging according to the TNM system provides important prognostic information in CRC, it is not able to predict the individual risk for each patient, as clinical outcomes may differ considerably between tumors of the same pathological stage. Many studies have looked for the possibility of predicting clinical outcomes in CRC based on the intratumoral angiogenesis as assessed by the calculation of MVD, and as results of a meta-analysis point out, it seems that high MVD can predict poor relapse-free survival and overall survival.[6] However, MVD calculation requires an invasive procedure for sampling, using different staining protocols according to each manufacturer's indication. Moreover, the method implies the selection of vascular "hot-spots" from the slides, which may not be representative for the entire tumor, all at a significant workload for the pathologist. Therefore, other markers are needed to supplement information, and whereas several histopathological features that can potentially predict outcomes have been described,[28] functional imaging methods might provide valuable real-time information for clinical decisions making. Imaging of vascularity in CRC has been previously performed with dynamic contrast-enhanced MRI and perfusion CT, with inconclusive results published so far.[29-31] CE-EUS examination, on the other hand, is a safe, nonionizing, less expensive and therefore repeatable imaging technique, which unlike CT and MRI uses contrast agents with strictly intravascular distribution and therefore might represent a better measure for tumor blood flow and volume.[8]

There are some limitations to be mentioned regarding our study. We found that unlike with parenchymal organs, it was difficult to include in the analysis the normal colorectal wall for reference, which was either too thin or not visible in the same ultrasound section, as was the case of circumferential tumors. However, this limitation can be overcome by reproducing the same settings for each investigation and for the same lesion across repeated examinations. Furthermore, our retrospective analysis included a heterogeneous group represented mostly by advanced tumors (T3 and T4), although patients with CRC are more often diagnosed with later-stage disease.[32] Furthermore, it is precisely the patients with locally advanced tumors that are suitable for neoadjuvant therapy, which is the standard of care for rectal cancer and is becoming a feasible strategy for colonic tumors as well. Consequently, these patients need appropriate methods for tumor evaluation, especially in the setting of prospective multicenter trials.[33]

CONCLUSIONS

CE-EUS represents a feasible method for imaging angiogenesis in CRC, enabling real-time assessment of tumor perfusion and also quantitative evaluation based on TIC analysis. As our results and previous studies suggest, novel prognostic factors may derive from CE-EUS examinations which could aid in the better selection of patients that would benefit from more potent first-line therapies. However, data across studies are inconsistent so far, therefore standardization of the methodology is necessary in studies that will follow. Based on the translation of previously reported results on CEUS performed for different tumor locations, this method could also be used for the early prediction of response to targeted antiangiogenic therapies, as changes in vascularity occur earlier than any morphological alterations.

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Conflicts of interest

There are no conflicts of interest.

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