Contrast-enhanced power Doppler endosonography and pathological assessment of vascularization in advanced gastric carcinomas – a feasibility study

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Abstract

Aim. Besides representing angiogenesis markers, microvascular density (MVD) and vascular endothelial growth factor (VEGF) are two important tools for the assessment of prognosis in patients with gastric cancer. The aim of our study was to assess the Doppler parameters (resistivity and pulsatility indexes) and vascularity index (VI) calculated by contrast-enhanced power Doppler endoscopic ultrasound (CEPD-EUS) in correlation with the expression of intra-tumoral MVD and VEGF in patients with gastric cancer. Material and methods. The study included 20 consecutive patients with advanced gastric carcinoma, but without distant metastasis at initial assessment. All the patients were assessed by contrast-enhanced power Doppler endoscopic ultrasound (EUS) combined with pulsed Doppler examinations in the late venous phase. The vascularity index (VI) was calculated before and after injection of second generation microbubble contrast specific agent (SonoVue 2.4 mL), used as a Doppler signal enhancer. Moreover, pulsed Doppler parameters (resistivity and pulsatility indexes) were further calculated. The correlation between power Doppler parameters and pathological/molecular parameters (MVD assessed through immunohistochemistry with CD31 and CD34, as well as VEGF assessed through real-time PCR) was assessed. Kaplan-Meier survival analysis was used for the assessment of prognosis. Results. Significantly statistical correlations were found between post-contrast VI and CD34 (p=0.0226), VEGF (p=0.0231), VEGF-A (p=0.0464) and VEGF-B (p=0.0022) while pre-contrast VI was correlated only with CD34 expression. Pulsatility index and resistivity index were not correlated with MVD or VEGF expression. Survival analysis demonstrated that VEGF-A is an accurate parameter for survival rate (p=0.045), as compared to VEGF (p=0.085) and VEGF-B (p=0.230). We did not find any correlation between the survival rate and ultrasound parameters (RI, PI, pre-contrast VI or post-contrast VI). Conclusion. Assessment of tumor vascularity using contrast-enhanced EUS, including analysis of spectral Doppler parameters is possible and feasible in gastric cancer patients. A correlation between measured EUS vascularity and pathological parameters of angiogenesis (MVD and VEGF expression) was found.

Keywords: gastric cancer, power Doppler, endosonography, vascular endothelial growth factor, microvessel density

Introduction

Angiogenesis is a complex process, very important in tumor growth and metastasis. The vessels inside the tumors have an incomplete endothelium, a greater permeability, a chaotic distribution and an altered function as compared with normal endothelium [1,2]. Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis and tumor growth being up-regulated through increase of tumor cell production. Microvessel density (MVD), a pathological parameter, represents a conventional method of assessment of angiogenesis in tumors. There are various immunohistochemical markers used for quantification of MVD: anti-factor VIII related antigen polyclonal antibody, CD31, CD34, and recently CD105 [3-6].
Besides representing angiogenesis markers, MVD and VEGF are two important tools for assessment of prognosis in patients with gastric cancer [7-11]. New methods were developed in the past years for the assessment of angiogenesis by non-invasive imaging methods: transabdominal ultrasound, CT scan or magnetic resonance imaging (MRI). However, all these methods have severe methodological flaws, including the lack of real-time characteristics and low resolution.

Doppler parameters such as resistivity index (RI) and pulsatility index (PI) could represent important markers for the assessment of vascularity and angiogenesis [12]. Thus, resistivity index (RI) measured by pulsed Doppler was correlated with MVD in breast and ovarian cancer [13-15], although there has been a lack of data regarding Doppler parameters and gastric cancer. Vascularity index (VI) can be also calculated by color Doppler ultrasound in cancer patients, being usually correlated with MVD and survival [16]. The role of endoscopic ultrasound (EUS) in the assessment of angiogenesis of gastrointestinal tumors is not yet established. In the past years specific microbubble ultrasound contrast agents were developed and the role of contrast-enhanced ultrasound increased, with focal liver masses being the most studied.

The aim of our study was to assess the vascularization of advanced gastric adenocarcinomas using two different methods: imaging (contrast-enhanced power Doppler endoscopic ultrasound) and pathological parameters (MVD and VEGF expression). Consequently, obtaining a correlation between these parameters represented a secondary aim. Power Doppler EUS parameters studied were pulsatility and resistivity indexes (PI, RI), as well as vascularity index (VI). Microvascular density was assessed by conventional immunohistochemistry (CD31 and CD34), being correlated with the expression of VEGFA and VEGFB genes. The study was consequently designed to establish the feasibility of power Doppler EUS (Doppler parameters and vascularity index) for the assessment of vascularization in gastric adenocarcinoma, in correlation with immunohistochemical (MVD) and molecular (VEGF) markers.

Materials and methods

The EUS procedure is routinely performed in Endoscopy Units. All patients who underwent this procedure signed an informed consent for EUS procedures according to the Ethical Committee of the University of Medicine and Pharmacy, Craiova. Our patients routinely undergo contrast-enhanced US examinations, because the second generation microbubble US contrast agent (Sonovue; Bracco Diagnostics, Inc, Bracco, Italy) is approved for clinical use in Romania and other European Union countries.

We prospectively assessed angiogenesis in 20 consecutive patients with advanced gastric carcinoma, but without distant metastasis at the initial assessment. Cancer diagnosis was established by upper gastrointestinal endoscopy completed with conventional pathology exam of biopsy samples. At least 8 samples from the tumor were taken in all patients. All the patients were assessed by contrast-enhanced power Doppler endoscopic ultrasound (EUS) combined with pulsed Doppler examinations in the late venous phase. The tumor stage was established by using imaging methods: transabdominal ultrasound as initial examination, CT scan for M and N stage and EUS for T stage and N stage. In operable patients, the stage was established by surgery and complete pathology exam of the resection piece.

An initial scan was performed by power Doppler EUS, followed by a bolus intravenous injection of 2.4 mL of Sonovue. After approximately 20 seconds the power Doppler gain was reduced because of power Doppler noise (such as color blooming and flash artifacts). The power Doppler gain was set-up at the highest level possible in order to avoid artifacts. The recorder started at 60 seconds after bolus injection, after the stabilization of images. Three movies were recorded in every patient before and after contrast enhancement. Each recorded movie of contrast-enhanced power Doppler EUS was subjected to a computer-enhanced dynamic analysis using a public domain Java-based image processing tool (Image J) [17] with a special plug-in developed by the IT Department of the University of Medicine and Pharmacy Craiova, Romania. The plug-in was used to compute and dynamically analyze the percentage of color pixels from each frame of the movie; every movie had at least 10 seconds and started after the stabilization of the power Doppler signal and disappearance of blooming artifacts, in the late venous phase. All the movies with artifacts were excluded. Vascularity index (VI) was calculated as a percent of color pixels in every frame of the movies and a mean was obtained (fig 1). The vascularity index was calculated before and after injection of a second generation microbubble contrast specific agent (Sonovue 2.4 mL), used as a Doppler signal enhancer. Moreover, pulsed Doppler parameters (resistivity and pulsatility indexes) were further calculated. Three values of PI and RI were obtained by interrogating several intratumoral vessels with arterial-type signals and the average value was used for analysis.

MVD was assessed by immunohistochemistry using CD31 and CD34 markers (fig 2a, b). Hot spots were found by using low-power magnification. After that, a
high-power magnification (200X) was used for counting the vessels in three different fields and an average was calculated. CD31 clone JC 70A (DakoCytomation, Denmark), diluted 1:20 in PBS, was used after 20 minutes pre-treatment of tissues with heat induced epitope retrieval (MW) in DakoCytomation target retrieval solution High pH and 30 minutes incubation at room temperature with primary antibody, with visualization by Dako EnVision+/HRP. Negative control was DakoCytomation Mouse IgG, diluted in the same concentration as primary antibody. CD34 clone QBEnd 10 (DakoCytomation, Denmark), diluted 1:50 in PBS, was used after 20 minutes pre-treatment of tissues with heat induced epitope retrieval (MW) in DakoCytomation target retrieval solution High pH and 30 minutes incubation at room temperature with primary antibody, with visualization by Dako EnVision+/HRP. Negative control was DakoCytomation Mouse IgG, diluted in the same concentration as primary antibody.

The expression of VEGF was considered positive in those samples where the marker was evident in at least 5% of tumoral cells (fig 2c). VEGF clone VG1 (DakoCytomation, Denmark), diluted 1:50 in PBS, was used after 20 minutes pre-treatment of tissues with heat induced epitope retrieval (MW) in DakoCytomation target retrieval solution pH 9 and 30 minutes incubation at room temperature with primary antibody, with visualization by Dako LSAB+/HRP. Negative control was DakoCytomation Mouse IgG, diluted in the same concentration as primary antibody.

Molecular expression of VEGFA and VEGFB genes was also calculated. Both tumor and corresponding peritumoral mucosa were biopsied during endoscopy for all the patients. The samples were collected in RNA later solution (Ambion) and kept at -80°C until RNA isolation. Total RNA was isolated using SV Total RNA Isolation System (Promega) and stored at -80°C until reverse-transcribed. Electrophoresis on denaturing agarose gel was carried out for the assessment of the quality of RNA. The concentration and purity of RNA were measured with a spectrophotometer (Eppendorf Biophotometer). Two steps quantitative Real-Time PCR was performed. In the first step the reverse-transcription of 1μg RNA into complementary single stranded DNA (cDNA) was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In the second step, quantitative PCR was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems) and specific TaqMan® Gene Expression Assays (Applied Biosystems) for target genes and endogenous control gene (VEGFA, VEGFB). The amplifications were carried out in triplicate, in 20 μl volumes, on a Mastercycler® ep replex (Eppendorf). The cycling parameters were: hold 50°C for 2-minutes, hold 95°C for 10 minutes, followed by 50 cycles of PCR at 95°C for 15 seconds and 60°C for 1 minute. Fold changes between tumor and peritumoral
mucosa were calculated according to the Pfaffl method. The differences between the paired samples were considered relevant when the mRNA level varied more than 1.8 folds between the tumor and peritumoral mucosa.

Finally, the t-test and Pearson correlation (r) were used for comparing continuous data, while the chi-square test was used for categorical data. Survival was calculated using the Kaplan-Meier method correlated with demographic, clinical and EUS parameters. Comparisons between groups were performed using the log-rank test. A correlation between contrast-enhanced EUS parameters and clinico-pathological parameters was further established.

**Results**

A total of 20 patients were included in the study (14 men and 6 women), with a mean age of 60 years (range 30 – 73 years, stdev +/- 12.10). The tumors were located in the gastro-esophageal junction/proximal stomach (n=7), stomach body (n=9), antrum (3) and one tumor in resected stomach. Pathological type of the cancer was: adenocarcinoma (17 pts) and signet-ring carcinoma (3 pts). Carcinomas were mainly of intestinal type (14 pts) but there were 6 patients with diffuse type. Concerning the grade of differentiation the tumors were classified as follows: well differentiated (n=2), moderately differentiated (n=8) and poorly differentiated (n=10). The local stage of the disease, evaluated by EUS or surgery (for operated patients) reveals that the majority of patients had locally advanced disease: T1 – 0 patients, T2 – 3 patients, T3 – 14 patients and T4 – 3 patients., N0 – 1 patient, N1 – 9 patients, N2 – 7 patients and N3 – 3 patients. The TNM stage was IIA – 2 patients, IIB – 9 patients, IIIA – 3 patients, IIIB – 4 patients, IIIc – 1 patient and IV – 1 patient, according to the AJCC classification [18]. One patient had M1 stage because of the presence of mediastinal lymph node metastasis, with positive cytology after EUS-guided FNA.

Median values for PI and RI were 1.7 (range 0.25 – 3.5, stdev=0.79) and 0.74 (range 0.4 – 1.3, stdev=0.21), respectively. The median pre-contrast VI was 6.77% (range values between 0.44 – 19.16% with stdev=5.15), while post-contrast VI was 18.31% (range values between 8.34 – 32.11% with stdev=7.35). VEGF was positive in 12 patients (60%). Median values of CD31 and CD34 were 6.87 (stdv 3.12) and 10.62 (stdv 3.02).

No correlation was found between RI and immunohistochemistry parameters, although a higher correlation coefficient was found between RI and pre-contrast VI, but without reaching statistical significance (r=0.54, p=0.2096). There was a correlation between PI and RI (r=0.55, p=NS) and a correlation between PI and pre-contrast VI (r=0.54, p=0.0887), again without reaching statistical significance. Pre-contrast VI was significantly correlated with CD34 expression (r=0.47, p=0.0439), but there was no correlation with CD31 or VEGF, VEGF-A and VEGF-B. However, post-contrast VI was significantly correlated with CD 34 (r=0.54, p=0.0226), VEGF (r=0.51, p=0.0231), VEGF-A (r=0.52, p=0.0464) and VEGF-B (r=0.50, p=0.0022) (table I).

The mortality rate after a follow-up of 730 days was 60%. Based on univariate analysis using Kaplan-Meier survival curves, we proved that the survival rate was influenced by the stage of the disease (fig 3). According to TNM classification, the most important parameter for prognosis was N stage (fig 4) as compared with T stage, but we should consider that in our set of patients only one had distant metastasis (M1) and this parameter was not eligible for the assessment in the analysis. The survival rate was significantly influenced by VEGF expression (both by molecular and immunohistochemical assess-

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ment). However, VEGF-A (fig 5) seems to be a more accurate parameter for survival analysis (p=0.045), as compared to VEGF (p=0.085) and VEGF-B (p=0.230). We did not find any correlation between the survival rate and ultrasound parameters (RI, PI, pre-contrast VI or post-contrast VI).

Discussion

Doppler ultrasound parameters were mainly assessed previously for breast and ovarian cancer. Thus, a correlation of Doppler spectral parameters with tumor angiogenesis and histological growth pattern was established in breast masses. The RI and PI were higher in malignant tumors as compared with those in benign lesions [19]. Although color Doppler mapping has been shown to be useful in distinguishing benign from malignant breast lesions, the intensity of signal and velocity of flow had no correlation with the extent of angiogenesis in breast cancer. The presence of high-flow tumor signal in early breast carcinoma was significantly associated with the presence of axillary lymph node metastases [20]. In a different study analyzing breast cancer patients, a significant correlation was found between power Doppler sonographic measurement of tumor vascularity and MVD detected by immunohistochemical analysis [21]. Low resistance to blood flow as determined by RI or PI measurements based on pulsed Doppler may be positively correlated with the MVD in malignant ovarian tumors [22]. Moreover, vascular pattern and vascular density assessed with power Doppler can better differentiate benign and malignant cervical lymph nodes [23]. These parameters can be also used for the assessment of response after chemotherapy [24].

For gastric cancer patients, the usefulness of contrast-enhanced endoscopic ultrasound (EUS) was not established until now, to the best of our knowledge. Only a single study was previously published, including a limited number of patients with gastric tumors assessed by transabdominal ultrasound [25]. Our data revealed that RI and PI are correlated with pre-contrast VI, but not with postcontrast VI. After contrast-enhancement, power Doppler analysis is frequently associated with flash and overpainting artifacts, inducing errors that might appear in positioning the cursor precisely inside the vessels. Intratumoral vessels are also very small with slow flow, being extremely difficult to differentiate between Doppler signals and background disturbances [26]. We thus consider that is preferable to quantify the spectral Doppler parameters before contrast enhancement.

Different studies on breast cancer demonstrated that a high RI (> 0.7) is well correlated with malignancy [27]. Only 55% of the patients included in our study had a RI
over 0.7, the median value of RI being 0.74. Thus, in gastric cancer, the value of RI does not seem to be so important for the characterization of the lesion. Also, overlapping values of RI and PI should preclude any firm conclusions and establishment of distinct cut-offs for prognosis assessment. However, post-contrast VI seems to be extremely important in the assessment of vascularisation as compared with pre-contrast VI, due to the better correlation with the MVD (assessed by CD31 and CD34) and VEGF expression. In the current study the survival rate was influenced only by high VEGF expression and advanced tumor stage. Survival rate was not influenced by the MVD or Doppler parameters. Thus, even though EUS Doppler parameters and especially post-contrast VI are useful in the assessment of angiogenesis and are correlated with immunohistochemical and molecular parameters, their role in the evaluation of the extension of the disease and the prognostic value is still unclear and should be assessed by larger studies.

The role of VEGF expression in prognosis is still controversial, with the majority of authors considering VEGF as a useful tool for prognosis assessment. In the current study immunohistochemical VEGF expression in tumor samples had a good correlation with overall survival, although the molecular assessment of VEGF-A and VEGF-B seems to be more accurate for prognosis assessment. Thus, the survival rate was significantly influenced by VEGF-A (p=0.045). Although the data presented support the relationship between VEGF-A and survival, there are however controversies in the literature regarding the involvement of VEGF in prognosis, with recent articles that do not support VEGF-A as a prognostic marker [28].

Our study has several limitations. The number of patients included in the analyses was rather small; consequently certain statistical parameters did not reach statistical significance. The study should be replicated in larger patient subgroups, thus proving the value of certain EUS parameters (especially post-contrast VI) for the evaluation of prognosis, beside the TNM stage and VEGF values. Although Doppler ultrasound parameters were found to be closely correlated, they were determined by using contrast-enhanced power Doppler EUS, which is certainly associated with known artifacts. Nevertheless, the introduction of low mechanical index contrast-enhanced EUS techniques might eliminate such artifacts, and it should be the preferred method for the real-time evaluation of angiogenesis during the follow-up of inoperable cancer patients.

**Conclusion**

Assessment of tumor vascularity using contrast-enhanced EUS, including analysis of spectral Doppler parameters is possible and feasible in gastric cancer patients. This concept was certainly demonstrated by the significant correlation between VI (determined by contrast-enhanced power Doppler EUS) and MVD (determined through immunohistochemistry using CD31 or CD34) or VEGF expression (VEGF immunohistochemistry, VEGF-A or VEGF-B gene expression). The role of pre-contrast Doppler parameters (RI and IP) in the assessment of prognosis is still unclear, with larger studies undertaken which should be designed to establish the true role of this method. Only two parameters influenced the overall survival: VEGF expression and stage of the disease. Moreover, VEGF-A gene expression seems to be more accurate than immunohistochemical VEGF expression for prognosis assessment.

**Conflict of interest:** none

**References**


