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Original Article

Application value of ultrasound contrast in gastric cancer examinations and its correlation with microvessel density

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Abstract: Objective: The aim of this study was to examine the application value of ultrasound contrast in gastric cancer examinations and its correlation with microvessel density. Methods: A prospective observational study of 127 gastric cancer patients, diagnosed by gastroscopic pathology biopsy, was conducted. A total of 64 patients received surgical treatment. Ultrasound contrast performance was analyzed and ultrasound contrast parameters of cancerous tissues and adjacent normal tissues were compared, including enhanced time (ET), time to peak (TTP), and wash in rates (WIR). Differences in ultrasound contrast parameters among different tumor staging and differentiation of gastric cancer tissues was analyzed. Immunohistochemical staining was adopted to calculate the microvessel density of cancerous tissues and adjacent normal tissues in patients receiving surgical treatment for gastric cancer, examining the correlation between ultrasound contrast parameters and microvessel density. Results: ET and TTP of cancerous tissues were shorter than those of adjacent normal tissues, but the WIR was higher than that of adjacent normal tissues. Differences were statistically significant ($P<0.05$). Differences of ET, TTP, and WIR in different tumor stages and differentiation of gastric cancer were statistically significant (all $P<0.05$). Later tumor staging signaled lower differentiation was. Shorter ET and TTP indicated larger WIR. Microvessel densities of the cancerous tissues in patients that received surgical treatment for gastric cancer were (85.47±34.25)/200 hpf, significantly higher than in adjacent normal tissues (20.85±4.76)/200 hpf. Differences were statistically significant ($P<0.05$). Pearson's correlation analysis showed a negative correlation between ET and microvessel density ($r=-0.684$, $P=0.019$), a negative correlation between TTP and microvessel density ($r=-0.584$, $P=0.027$), and a positive correlation between WIR and microvessel density ($r=0.712$, $P=0.012$). Conclusion: Ultrasound contrast may be directly applied to assess lesion blood perfusion in gastric cancer examinations, correlating with microvessel density.

Keywords: Gastric cancer, ultrasound contrast, microvessel density, correlation

Introduction

Gastric cancer is one of the most common malignant tumors. It is characterized by rising incidence and mortality rates, which are related to the low precision treatment. Accurate assessment and prognosis are necessary to guide treatment. As a special pathological feature of cancer-specific lesions, neovascularization provides the basic condition for the survival and transfer of the tumor. Analysis of the neovascularization of patients with gastric cancer can reflect the progress and severity of the disease [1]. Microvessel density is closely related to the activity and intensity of neovascularization and has been regarded as one of the most accurate criteria for evaluating neovascularization capability [2]. However, the tissue to calculate microvessel density by immunohistochemical staining is from surgeries or biopsies. They can cause trauma and cannot be reused. It cannot be widely used to detect gastric cancer in the early stages or to dynamically evaluate the status of gastric cancer [3]. In addition, the observation of neovascularization under 200 hpf does not guarantee 100% scientific results [4]. In recent years, domestic and foreign studies have pointed out that the level of lesion blood perfusion in gastric cancer is closely related to microvessel density [5, 6]. As

a result, ultrasound contrast was developed to show lesion blood perfusion condition in real-time and in a clear manner. In recent years, ultrasound contrast has been shown to be an effective method in examining liver cancer, kidney cancer, and pancreatic cancer [7]. However, there still exists controversy about its application value in gastric cancer and reports on its relevance to microvessel density are rare. The objective of this study was to investigate the value of ultrasound contrast in gastric cancer examinations, assessing its correlation with microvessel density.

Materials and methods

Clinical data

A total of 127 gastric cancer patients, diagnosed by gastroscopic pathology biopsy and admitted to the First Affiliated Hospital of Xi'an Medical University, from January 2016 to January 2017, were selected for a prospective observational study. There were 85 males and 42 females, with an average age of (57.9±12.4) years old, ranging from 28 to 83. Regarding lesion sites, 64 were on gastric antrum, 44 on gastric cardia, and 19 on the gastric body. Patients were divided into the following groups based on their clinical and pathological features: 61 cases at stage I-II and 66 cases at stage III-IV; 67 cases of high-moderate differentiation and 60 cases of low differentiation-undifferentiation; 16 cases of distant metastasis and 111 cases of no distant metastasis. Of these, 64 patients underwent surgeries, including 43 males and 21 females, with an average age of (42.5±10.7) years old, ranging from 28 to 65. Regarding lesion sites, 31 were on gastric antrum, 25 on gastric cardia bottom, and 8 on the gastric body.

Inclusion criteria: 1) Patients over the age of 18; 2) Diagnosed with gastric cancer by gastric biopsy, which could be surgically removed; and 3) Patients providing informed consent.

Exclusion criteria: 1) Patients that received non-steroidal anti-inflammatory drugs, radiotherapy, chemotherapy, or immunotherapy before the surgery; 2) Patients with severe liver and kidney dysfunction; 3) Patients that refused to undergo ultrasound contrast or gastroscopy; and 4) Patients allergic to contrast agents.

Ultrasound contrast examination

GE Voluson 730 color Doppler ultrasound diagnostic apparatus was adopted for the exam and 4C probe was used with the frequency of 7.5-10 MHz. The patients had no food intake for more than 8 hours before the examination. After drinking enough water, the patient lied on his back to undergo the abdominal ultrasound examination. All lesion parts were scanned successively, including gastric bottom, gastric body, gastric antrum, and pylorus, locating the cancer site. Changes of different layers of the gastric wall were observed, such as the extent and degree of cancerous infiltration and its impact on the surrounding organs. The number of cancerous lesions was recorded and the best section was selected. Micro-bubble suspension of ultrasound contrast agent Sonazoid and physiological saline was injected into the median cubital vein and then 5 mL physiological saline was injected. Next, the contrast mode examination was started and the patients were asked to hold their breath. The injection conditions of ultrasound contrast agent Sonazoid, as well as the ultrasound echo intensity changes in the cancerous area and surrounding visceral organs, were observed. If the image was not clear or ultrasonic contrast agent Sonazoid faded fast, the ultrasound contrast agent Sonazoid could be injected again after 15 minutes. Contrast observation time was between 5-8 minutes. See **Figure 1**. TIC software was used to analyze the images and generate TIC curves. Ultrasound contrast parameters of cancerous tissues and adjacent normal tissues were calculated, including enhanced time (ET), time to peak (TTP), and wash in rates (WIR).

Detection of microvessel density

Immunohistochemical staining was used to slice and anti-CD34 was used stain the cancerous tissues and adjacent normal tissues in patients receiving surgical treatment for gastric cancer. Microvessel density was calculated. Agents included Zhongshan Jinqiao ZLI-9032 concentrated DAB kit, universal ultrasensitive S-P kit, and monoclonal antibody of rat anti-human primitive hematopoietic cells of Zhongshan Jinqiao (CD34). According to the Weider vascular counting method, after CD34 immunohistochemical staining, five regions

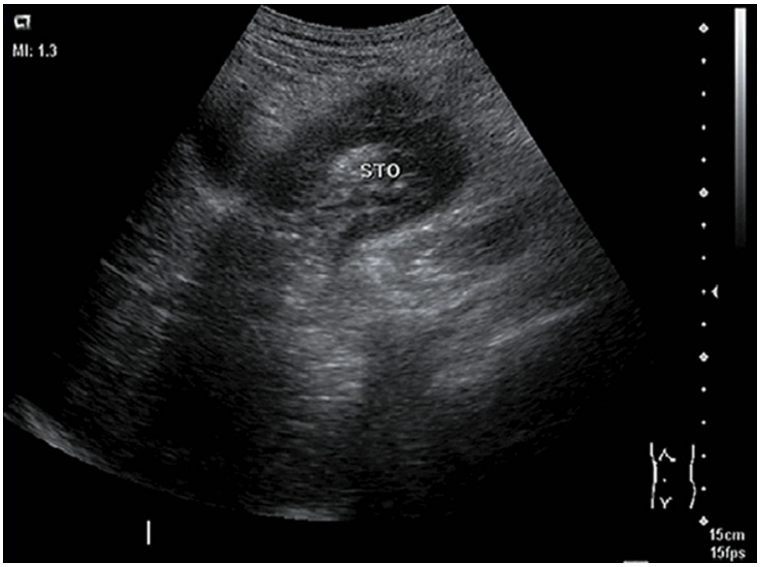

Figure 1. Ultrasound contrast image of gastric cancer. It shows the thickening of the mucosa, continuously disruption of the submucosa, and smooth and complete serosal coat (STO: gastric cavity).

with the highest vascular density were chosen under 100 hpf microscope. The number of microvessels in each region was counted under 200 hpf microscope and the average value was taken as the microvessel density value. If isolated brown yellow vascular endothelial cells or cell clusters were found, they were counted as one microvessel density.

Developmental stages of gastric cancer and the differentiation grading approach

According to tumor node metastasis (TNM) staging, gastric cancer can be divided into four stages. Stage I refers to superficial gastric cancer without lymph node metastasis or gastric cancer in which invaded tumors account for less than a half of the subregion although they have reached muscular layer. Stage II refers to superficial gastric cancer, T2 and T3 cancers with lymph node metastasis in the first stop and T3 cancer without lymph node metastasis. Stage III includes tumors of all sizes with lymph node metastasis in the second stop or those with only lymph node metastasis in the first stop or even no lymph node metastasis but whose tumor size is larger than one area. Stage IV refers to gastric cancer with lymph node metastasis in the third stop or distant metastasis, regardless of tumor size [8].

Tumor grading is based on the differentiation of tumor cells in the pathological section of gastric cancer tissues. High differentiation means that the tumor cells are well differentiated. Moderate differentiation means that the tumor cells are moderately differentiated. Low differentiation means that the tumor cells are poorly differentiated. Undifferentiation refers to the extremely low differentiation of tumor cells.

Outcome measures

Differences in ultrasound contrast parameters, including ET, TTP, and WIR, between cancerous tissues and adjacent normal tissues, were compared. Differences in ultrasound contrast parameters in different tumor staging and differentiation in cancerous tissues, as well as correlation between ultrasound contrast parameters and microvessel density, were analyzed.

Data processing

SPSS18.0 was adopted to process experiment data. Measurement data that conformed to normal distribution laws and met homogeneity of variance are expressed as mean ± standard deviation. Paired (cancerous tissue or adjacent cancerous tissues) or independent sample (other comparison) t-test was adopted for comparisons between the two groups. Correlation between ET, TTP, WIR, and microvessel density was analyzed using Pearson's correlation analysis. $P<0.05$ indicates statistical significance.

Results

Ultrasound contrast performance of gastric cancers

Ultrasound contrast performance of adjacent normal tissues was that the ultrasound contrast agent rapidly entered the artery and the inner, middle, and outer gastric walls showed high enhancement, low enhancement, and high enhancement. The enhancement type was in accord with CT images, showing high-low-high enhancement structure. The conventional ultrasonographic manifestation of cancerous tissues is uneven thickness of the gastric wall or lump-shaped gastric wall, with the ultrasound contrast showing homogeneous or

Table 1. Comparison of ultrasound contrast parameters between cancerous tissues and adjacent normal tissues

Group	Case	ET (s)	TTP (s)	WIR
Cancerous tissues	64	24.85±7.54	10.28±6.02	7.56±2.51
Adjacent normal tissues	64	29.93±6.71	14.62±5.84	5.02±1.58
T		4.817	3.127	2.504
P		<0.001	0.006	0.016

Note: ET, enhanced time; TTP, time to peak; WIR, wash in rate.

Table 2. Comparison of ET, TTP, and WIR of cancerous tissues in different tumor staging

Group	Case	ET (s)	TTP (s)	WIR
Stage I-II	61	26.27±7.91	12.06±6.33	6.57±2.36
Stage III-IV	66	21.95±6.23	8.54±5.21	8.91±2.74
t		5.024	3.542	2.681
P		<0.001	0.004	0.014

Note: ET, enhanced time; TTP, time to peak; WIR, wash in rate.

Table 3. Analysis of differences in ultrasound contrast parameters of cancerous tissues in different tumor grading

Group	Case	ET (s)	TTP (s)	WIR
High-moderate differentiation	67	26.43±7.89	12.25±6.58	6.47±2.41
Low differentiation-undifferentiation	60	20.41±5.96	8.14±4.99	9.15±2.87
T		5.745	3.714	2.847
P		<0.001	0.002	0.012

Note: ET, enhanced time; TTP, time to peak; WIR, wash in rate.

Table 4. Comparison of microvessel density in cancerous tissues and adjacent normal tissues (/200 hpf)

Group	Case	Microvessel density (/200 hpf)
Cancerous tissues	64	85.47±34.25
Adjacent normal tissues	64	20.85±4.76
t		18.429
P		<0.001

uneven rapid high enhancement of local artery while it rapidly withdrew from the venous phase. Compared with the ultrasound contrast performance of adjacent normal tissues, the cancerous tissues showed a "rapid in and out" change, as shown in **Figure 1**.

Comparison of ultrasound contrast parameters between cancerous tissues and adjacent normal tissues

Cancerous tissues had shorter ET and TTP than adjacent normal tissues. Its WIR was larger than that in adjacent normal tissues. Differences were statistically significant (all P< 0.05). See **Table 1**.

Analysis of differences in ultrasound contrast parameters of cancerous tissues in different tumor staging and differentiation

Differences of ET, TTP, and WIR of gastric cacer tissues in different tumor stages and grades were statistically significant (P<0.05). The later the tumor staging was, the lower the differentiation was. The shorter the ET and TTP were, the larger the WIR was. See **Tables 2** and **3**.

Comparison of microvessel density in cancerous tissues and adjacent normal tissues

The microvessel density of cancerous tissue in patients that received surgcal treatment on gastric cancer was (85.47±34.25)/200 hpf, significantly higher than that in adjacent normal tissues (20.85±4.76)/200 hpf. Differences were statistically significant (P<0.05). See **Table 4**.

Correlation analysis between ultrasound contrast parameters of cancerous tissues and microvessel density

Pearson's correlation analysis showed a correlation between ultrasound contrast parameters of cancerous tissues and microvessel density. There was a negative correlation between ET and microvessel density (r=-0.684, P=0.019), a negative correlation between TTP and microvessel density (r=-0.584, P=0.027), and a positive correlation between WIR and microvessel density (r=0.712, P=0.012).

Discussion

Neovascularization can be found in any stage of gastric cancer progression. It offers a mor-

phological basis for tumor growth and metastasis, such as providing access for cancer cells to infiltrate and metastasize and giving cancer cell nutritional support. This helps them to develop biological malignant behavior. It is generally accepted by clinicians that microvessel density is an important and independent index in evaluating the activity and intensity of neovascularization in gastric cancer. It is closely related to the biological characteristics of the tumor [9]. For patients with gastric cancer, greater microvessel density suggests that tumors are more likely to grow faster with adequate nutrition and more mature angiogenesis makes it easier for cancer cells to enter peripheral circulation. Result of this might be their distant metastasis. Finally, patients will have a bad prognosis [10, 11]. As tumor neovascular endothelial cells markers, CD34 molecules have the highest antigen specificity [12]. Based on the above viewpoints, this study calculated microvessel densities using immunohistochemical staining. Results showed that the microvessel density in cancerous tissues in patients that received the surgical treatment for gastric cancer was (85.47±34.25)/200 hpf, higher than the (20.85±4.76)/200 hpf in adjacent normal tissues. Differences were statistically significant, consistent with the research findings of Zou et al. [13]. At present, immunohistochemical staining is mainly used in splicing and anti-CD34 staining of tumor tissues after biopsies or surgical resections. Calculating the microvessel with reference to Weider vascular counting method to density had certain limitations, including obvious trauma, nonrepeatability, cumbersome operation, and a long time, making it difficult to dynamically observe the microvascular circulation [14]. To further accurately and conveniently analyze the neovascularization of gastric cancer, this study used ultrasound contrast to display the lesion blood perfusion in a real-time and dynamic manner. Next, the neovascularization ability was evaluated. In addition, there was a great deal of neovascularization and abundant perfusion in gastric cancer lesions. In analyzing the fluidity of ultrasound contrast agent in ultrasound contrast, particularly its inflow and outflow rates, the lesion area can be displayed very clearly and neovascularization can be accessed accordingly [15]. Plus, this approach can be duplicated with ease.

According to the 127 gastric cancer cases in this study, the local artery showed homogeneous or uneven rapid high enhancement while the vein showed rapid withdrawal. Compared with the ultrasound contrast performance of adjacent normal tissues, cancerous tissues showed a “rapid in and out” change. Consistent with previous studies, it is suggested that ultrasound contrast could be used directly to assess the lesion blood perfusion mainly because Sonazoid, a blood pool agent, would not enter the extravascular space and could accurately reflect blood flow information [16-18]. Compared with the color Doppler ultrasound, the ultrasound contrast agent in ultrasound contrast operates with erythrocytes to enhance tissue echogenicity and shows as enhanced ultrasound contrast, thus quantitatively measuring ET, TTP, and WIR [19]. Chen et al. found, in the selection of gastric cancer ultrasound contrast parameters, that ET and TTP of gastric cancerous tissue are significantly extended more than those in adjacent normal tissues, while WIR is significantly increased compared with adjacent normal tissues [20]. In reference to the findings of Chen et al., this study selected ET, TTP, and WIR as ultrasound contrast parameters [20]. Results showed that the ET and TTP in cancerous tissues were shorter than those in the adjacent normal tissues while the WIR was larger than in adjacent normal tissues. Differences were statistically significant, in accord with results reported by Jia et al. that ET and TTP were shortened and WIR was increased in gastric cancerous tissues [21]. However, there are few reports about differences of ultrasound contrast parameters in gastric cancer with different TNM staging and differentiation. Precise preoperative evaluation of staging and differentiation of gastric cancer can guide treatment and accurately excise the tissue. Zhang et al. analyzed 47 cases of gastric cancer ultrasound contrast performance and found that the pathological types, TNM staging, and differentiation correlated with the ultrasound contrast performance. In this study, the differences of ET, TTP, and WIR of gastric cancer tissues in different tumor staging and differentiation were statistically significant [22]. A more advanced stage and higher differentiation of cancer meant shorter ET and TTP in gastric cancer tissues and higher WIR, consistent with previous studies [23, 24]. It is suggested that ultrasound contrast parameters are closely correlated to pathological features. Ultrasound contrast parameters are especially significant when patients are in serious condi-

tion and the prognosis is poor. This may be related to the large number of microbubbles and neovascularization of cancerous tissues in the early period of ultrasound contrast perfusion, which shorten the contrast enhancement time in the artery and time required to reach peak. Therefore, differences of ultrasound contrast parameters and performance between cancer tissues and adjacent normal tissues are significant.

In this study, anti-CD34 staining was conducted in cancerous tissues and adjacent normal tissues in 64 patients that received surgical treatment for gastric cancer. Microvessel densities were calculated. Since the microvessel density in cancerous tissues was significantly higher than that in adjacent normal tissues, analysis of the correlation between ultrasound contrast parameters of cancerous tissues and microvessel density showed that ET and TTP were negatively correlated with the microvessel density, while WIR was positively correlated with microvessel density. Zhang et al. analyzed the correlation between the ultrasound contrast parameters of cancerous tissues and microvessel density in 84 cases of gastric cancer patients. They found that blood flow signal differentiation, ultrasound echo intensity, and microvessel density were positively correlated [25]. The number of microbubbles in the ultrasound contrast agent Sonazoid was positively correlated with the number of neovascularization in cancerous tissues. Neovascularization often leads to significant changes in ET. TTP is an important indicator of the microbubble flow and velocity of ultrasound contrast agent Sonazoid in cancerous tissues. Levels of blood perfusion in cancerous tissues is also closely related to the Sonazoid microbubble flow and velocity. Therefore, TTP could reflect the blood perfusion level of cancerous tissues. As shown in **Table 4**, the microvessel density of cancerous tissues was (85.47±34.25)/200 hpf in patients that received surgical treatment for gastric cancer, much higher than that in adjacent normal tissues (20.85±4.76)/200 hpf, indicating abundant blood supply of cancerous tissues. Flow quantity and rates of ultrasound contrast agent Sonazoid were high and WIR showed a clear increasing trend, while ET and TTP were significantly shortened. In this regard, ET, TTP, and WIR in cancerous tissues of gastric cancer patients can be used to assess neovascularization ability. Domestic and foreign research has pointed out that ultrasound contrast parameters of other malignant tumors were highly correlated with microvessel density, in accord with present results [26, 27].

In summary, ultrasound contrast can directly reflect the lesion blood perfusion condition during gastric cancer examinations. Plus, it is highly correlated with microvessel density and can reflect neovascularization. It deserves further study and can be applied to prognosis and treatment guidance. However, as few samples are available, it is hard to analyze how ultrasound contrast can offer guidance in the treatment of gastric cancer and its correlation with cell invasive ability. Sample sizes need to be increased in future studies. At the same time, only enhancement characteristics of one region could be observed by each contrast agent injection. Ultrasound contrast parameters vary in accordance with contrast agent dosage and gastric filling degrees, making it difficult to observe the lesion from a holistic view. Thus, there are some blind spots. It is necessary to combine enhanced CT and MRI examinations, improving diagnosis of gastric cancer.

Disclosure of conflict of interest

None.

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