

Contrast-Enhanced Ultrasonographic Studies on Pancreatic Carcinoma with Special Reference to Staining and Muscular Arterial Vessels

HIDEYA SUGA*^{§§}, YOSHINOBU OKABE*, OSAMU TSURUTA*, YOSHIKI NAITO**[§],
HISAFUMI KINOSHITA[†], ATSUSHI TOYONAGA[‡], NAOFUMI ONO[§],
KAZUHIKO OHO^{§§}, MASAMICHI KOJIRO^{§§}
AND MICHIO SATA*

*Division of Gastroenterology Department of Medicine, **Department of Pathology and

[†]Department of Surgery, Kurume University School of Medicine, Kurume, 830-0011,

[‡]Yasumoto Hospital, Fukuoka 830-0112, [§]Eguchi Hospital, Saga 830-0032,

^{§§}Yanagawa Hospital, Fukuoka 832-0077, Japan

Received 13 September 2013, accepted 3 December 2013

J-STAGE advance publication 17 February 2014

Edited by MINORU YAGI

Summary: Comparative study of contrast-enhanced ultrasonography (CE-US) and histopathology of surgically resected specimens in 13 patients with pancreatic carcinoma. A time intensity curve was used to determine the percentage brightness increase in cancerous and normal regions and the patients were divided into two groups, hyperperfusion, with a percentage brightness increase over 80% (n=6) and hypoperfusion, with an increase of less than 80% (n=7) on CE-US. The hyperperfusion group included well-differentiated tubular adenocarcinoma, adenosquamous cell carcinoma and acinar cell carcinoma, while all 7 patients in the hypoperfusion group had moderately differentiated tubular adenocarcinoma. Immunological staining (α -SMA and anti-CD34) of the resected specimens showed significantly higher microartery count (MAC) in the hyperperfusion group ($p < 0.005$) than in the hypoperfusion group or normal pancreas. In the normal pancreas, the mean vessel diameter was significantly higher (over 100 μm) than in the hyperperfusion group (30 μm ; $p < 0.005$).

It was concluded that a muscular arterial vessel density of less than 30 μm is an important factor in determining staining degree and carcinoma progression by CE-US in pancreatic carcinoma.

Key words pancreatic carcinoma, Contrast-Enhanced Ultrasonography, microartery count, muscular arterial vessels, Levovist

INTRODUCTION

With advances in diagnostic imaging techniques and their widespread application, the ability to diagnose pancreatic cancer has improved significantly. Among these techniques, external ultrasonography, a simple and non-invasive method of examination, plays an important role in the diagnosis of pancreatic carcinoma.

Since Levovist (Schering AG, Berlin, Germany), an ultrasonic contrast agent that can be used intravenously, became available in September 1999, it has been possible to evaluate in detail the hemodynamics of lesions non-invasively by using images [1,2].

Using Levovist with the coded harmonic angio mode developed by General Electric Health Care (Milwaukee, Wisconsin, USA) provides high sensitivity that enables detailed examination of the hemody-

Correspondence to: Hideya Suga, MD. Division of Gastroenterology Department of Medicine, Kurume University School of Medicine Address; 67 Asahimachi, Kurume, 830-0011, Japan. Tel: +81-942-31-7561 Fax: +81-942-34-9413 E-mail: suga516@med.kurume-u.ac.jp

Abbreviation: CE-US, contrast enhanced ultrasonography; MAC, microartery count; MVD, micro vessel density; TIC, time intensity curve; TI, tumor intensity; VEGF, vascular endothelial growth factor; PI, pancreas intensity.

namics of the pancreas as well as precise evaluation of the staining degree and the extent of pancreatic carcinoma.

It has been reported that since tumor vascularity is an important factor for determining cancer progression [3], detailed evaluation of blood flow with contrast enhanced ultrasonography (CE-US) can have an impact on chemotherapy for pancreatic carcinoma and can be a prognostic predictor [4].

While other diagnostic imaging techniques have shown poor blood flow in pancreatic carcinoma [5], few studies have examined intratumoral microvessels and contrast enhanced staining. There have been no comparative studies of US and histology to examine the effect of histological factors on staining enhancement of CE-US.

The aim of the study was to clarify the relationship between intratumoral muscular arterial vessels and staining degree in CE-US.

SUBJECTS AND METHODS

Subjects

CE-US was performed on 84 consecutive patients with pancreatic carcinoma from September 2002 through June 2006 in Kurume University hospital. Surgical treatment was indicated and successfully performed in 13 cases, in which normal and cancerous regions of the pancreas were simultaneously delineated by CE-US and surgical enucleation was possible. They were 8 males and 5 females with a mean age of 64.8 ± 7.5 years. These 13 patients with surgical speci-

mens were enrolled in the study.

The tumor was located in the pancreas head, body or tail of the pancreas in 5, 7 and 1 patient, respectively. Tumor size averaged 24.4 ± 12.5 mm in diameter. There were 2 well-differentiated adenocarcinomas, 8 moderately differentiated adenocarcinomas, 2 adenocarcinomas, and 1 acinar cell carcinoma (Table 1). The study was performed according to the guidelines of the Helsinki Declaration. Informed consent was obtained from all patients.

CE-US examination

The coded harmonic angio mode of a GE LOGIQ 7 (General Electric Health Care, Milwaukee, Wisconsin, USA) ultrasonic scanner was used, transmitting at 2.2 MHz and receiving at 4.4 MHz. Acoustic power was at the default setting with a mechanical index of 0.6-1.2. The focus point was just below the bottom of the lesion. The US contrast agent Levovist, which is composed of 99.9% galactose and 0.1% palamic acid, was used in all patients. Levovist at 300 mg/ml (2.5 g) was administered intravenously at 1 ml/second, and after 10 to 15 seconds the patient was made to hold his or her breath during observation of the lesional area with a continuous transmission (7Hz). When the lesion and peripheral blood vessels were delineated, the transmission frequency was changed to 2 Hz and after observation of blood vessels intermittent transmission was performed for 2 to 4 seconds to obtain a perfusion image.

US Image Analysis

Following the completion of examination, using low data stored hard disk of the ultrasound observation device, regions of interest were established in cancerous and normal regions of the pancreas and time-

TABLE 1.

Characteristics of patients with pancreatic carcinoma (n=13)

Sex (Male/ Female)	8/5
Age (years)	64.8 ± 7.5
Tumor location	
head	5
body	7
tail	1
Tumor size (mm)	24.4 ± 12.5
Histological type	
Well differentiated adenocarcinoma	2
Moderately differentiated adenocarcinoma	8
Adeno-squamous cell carcinoma	2
Acinar cell carcinoma	1

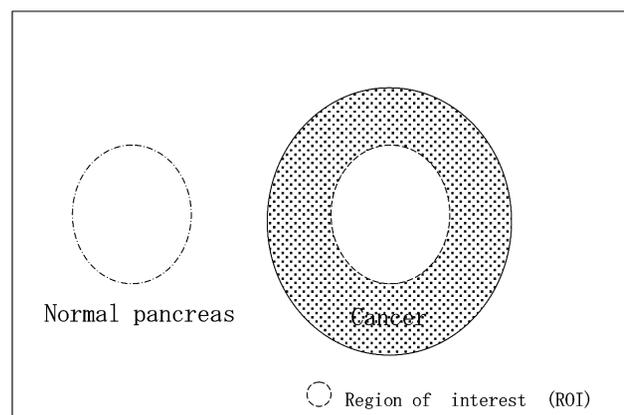


Fig. 1-a. Regions of interest (ROI) were set equally in normal parts and transverse sections of the tumors.

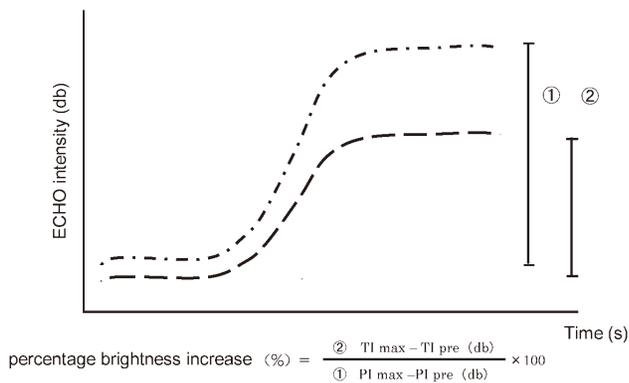


Fig. 1-b. Percentage brightness increase was differentiated into maximum intensity and baseline intensity using the TIC, and PI and TI were obtained. Percentage brightness increase (%) = TI max - TI base/PI max -PI base × 100. TIC,time intensity curve. PI, pancreas intensity. TI, tumor intensity.

intensity curves (TIC) were generated (Fig. 1). The percentage increase in brightness due to the contrast medium at maximum intensity and baseline intensity were quantified using the TIC, and pancreas intensity (PI) for normal tissue and tumor intensity (TI) for cancerous tissue were obtained. Quantitative assessment was performed using the formula; percentage brightness increase (%) = TI max - TI base/PI max -PI base ×100. Cases with a percentage brightness increase over 80% were allocated to the hyperperfusion group and those with a percentage brightness increase of less than 80% to the hypoperfusion group. The ultrasonic examinations were performed by two CE-US specialists.

Pathological examination

Resected specimens were examined by the same two pathologists. To determine the number of muscular arterial vessels, transverse sections of the largest part of the tumor were selected and stained with hematoxylin and eosin and double immunostained with α-SMA and anti-CD34. Under 200x magnification, the mean number of muscular arterial vessels in 10 fields was calculated and the mean microartery count (MAC) was obtained. The muscular arterial vessels were then classified by vessel diameter (>30 μm, 30>60 μm, 60 ≥ 100 μm and ≥ 100 μm), and the MACs were obtained for each classification.

Statistical analysis

Histological evaluation was performed using the Japan Pancreas Society classification [6]. Data are ex-

pressed as mean ± SD. 1) Percentage contrast increase and tissue images, 2) percentage contrast increase and MAC, and 3) percentage contrast increase and MAC by vessel diameter were examined. A P value of 0.05 was considered significant. All the analysis was done using JMP (SAS Institute, Cary, North Carolina, USA).

RESULTS

1) Percentage contrast increase and tissue images

Among the 6 cases in the hyperperfusion group, there were 2 well-differentiated tubular adenocarcinomas, 1 moderately differentiated tubular adenocarcinoma, 2 adenosquamous carcinomas and 1 acinar cell carcinoma (Fig. 2). All 7 cases in the hypoperfusion group were moderately differentiated tubular adenocarcinoma (Fig. 3).

2) Percentage contrast increase and MAC

Muscular arterial vessel density evaluated by MAC was 9.2±5.4 (mean ± SD; P<0.05) in the hyperperfusion group, which was significantly higher than in

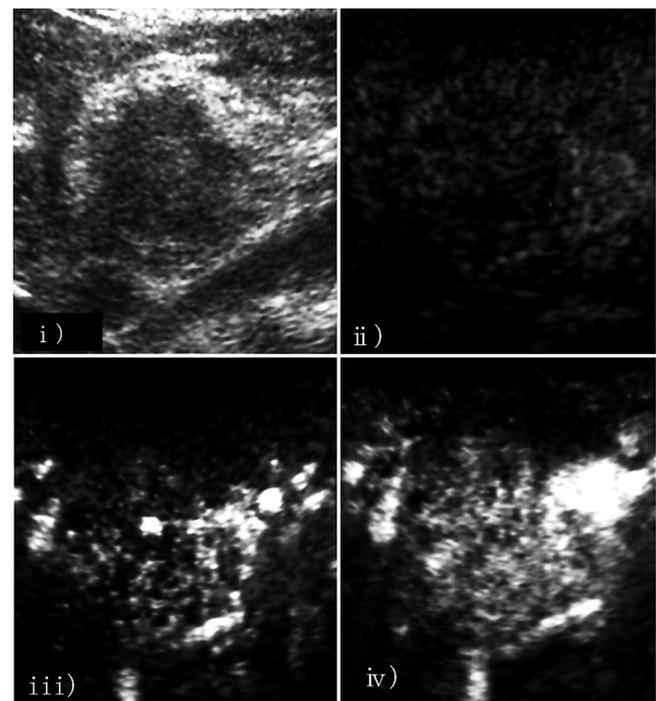


Fig. 2-a. i) Percentage brightness increase was differentiated into i) Pancreas head carcinoma by B-mode (20×30mm). ii) No Levovist flow seen in a tumor 21 sec after injection. iii) Inflow of Levovist is seen in a tumor 37 sec after injection. iv) The same staining as normal pancreas was observed 47 sec after injection. CE-US, contrast-enhanced ultrasonography.

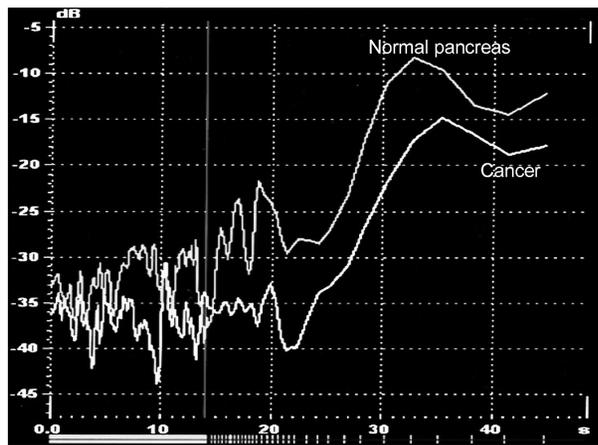


Fig. 2-b. Percentage brightness increase was 81.5%.

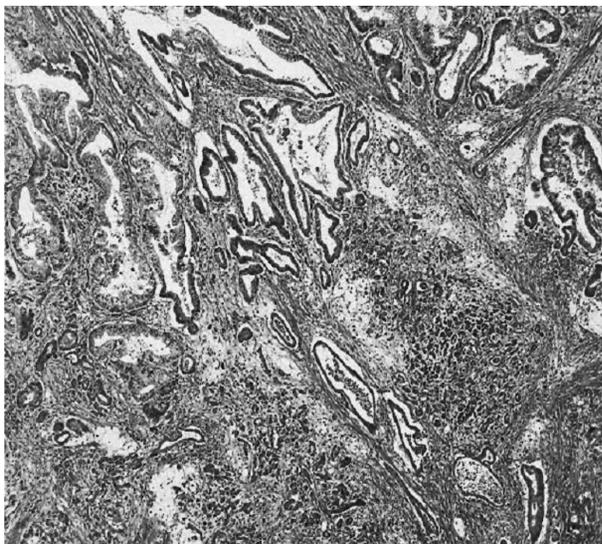


Fig. 2-c. HE staining. Tubular adenocarcinoma, well differentiated type.

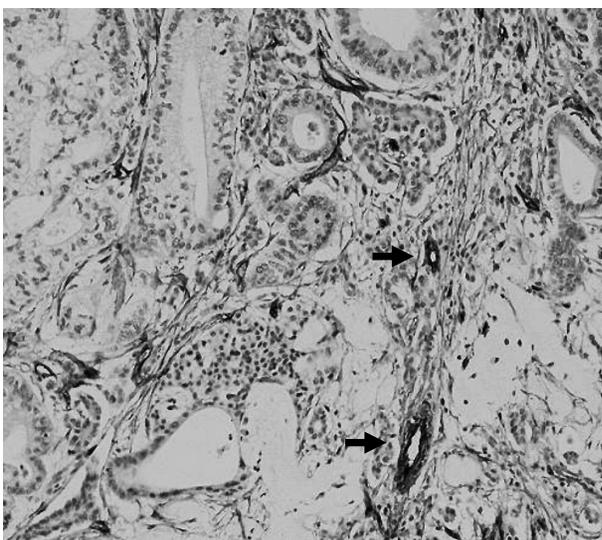


Fig. 2-d. Double immunostaining with α -SMA and anti-CD34. Numerous muscular arterial vessels were observed in interstitial tissue (arrows).

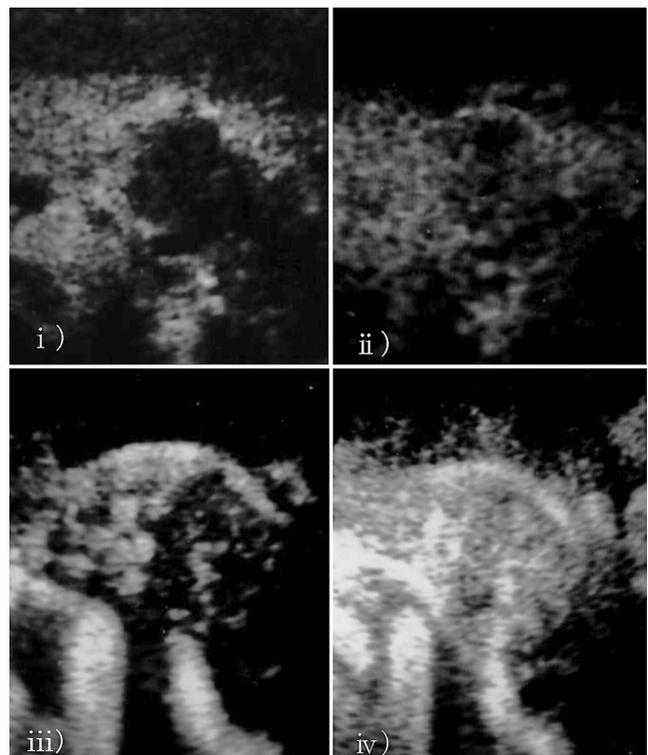


Fig. 3-a. i) Pancreas body carcinoma by THI (15 \times W17mm). ii) Before Levovist infusion (0sec). iii) Inflow of Levovist is seen in a tumor 37sec after injection. iv) The weaker staining than normal pancreas was observed 31sec after injection. THI, Tissue Harmonic Imaging

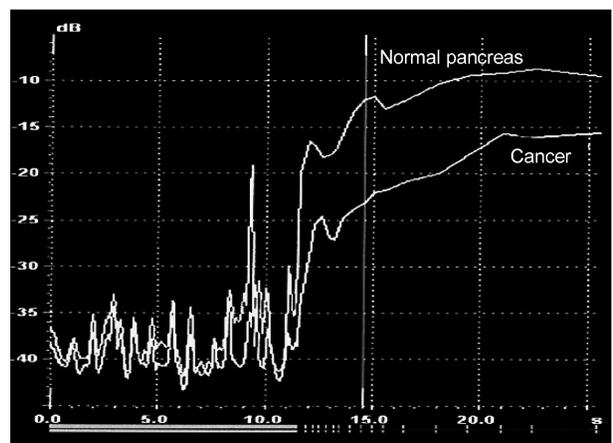


Fig. 3-b. Percentage brightness increase was 74.0%.

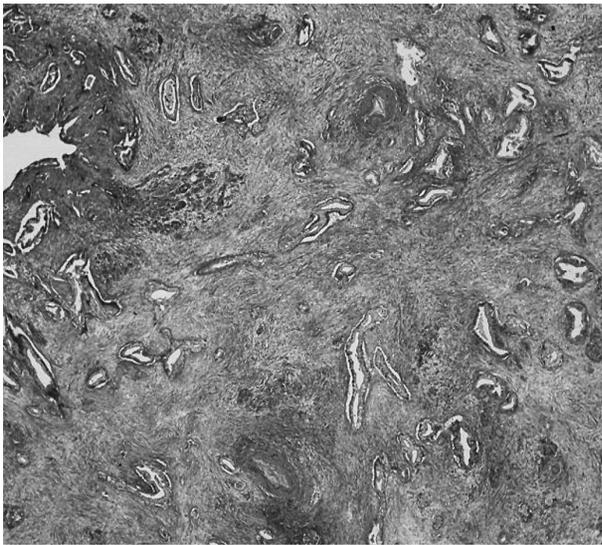


Fig. 3-c. HE staining. Tubular adenocarcinoma, moderately differentiated type.

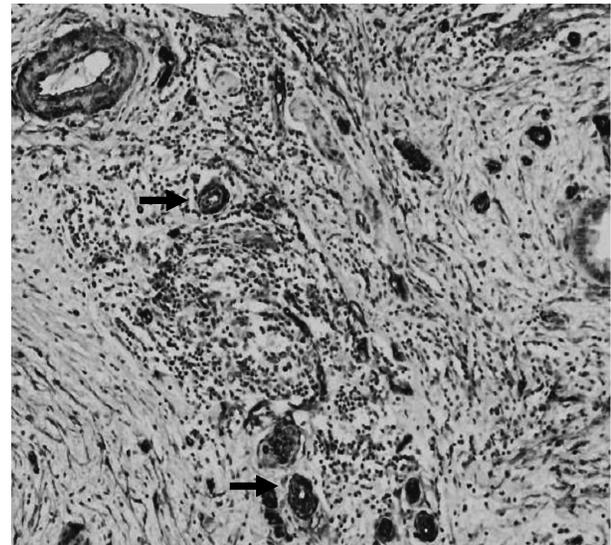


Fig. 3-d. Double immunostaining with α -SMA and anti-CD34. Numerous muscular arterial vessels were observed in the inside of a tumor. (arrows)

TABLE 2.
Comparison of MAC evaluated by CE-US

	Pancreatic cancer		Normal pancreas (n=13)
	hyperperfusion group (n=6)	hypoperfusion group (n=7)	
MAC(total)	9.2±5.4 ^{a, b}	5.5±3.4	4.9±2.1
30µm>	7.2±5.0 ^{a, b}	3.9±3.2	2.2±2.2
30µm≤60µm	0.9±1.0	0.7±0.8	0.9±0.9
60µm≤100µm	0.6±0.7	0.5±0.7	0.9±1.3
100µm≤	0.6±1.0	0.4±0.7	1.2±0.6 ^{b, c}

^a p<0.05 versus normal pancreas

^b p<0.05 versus hypoperfusion group

^c p<0.05 versus hyperperfusion group

MAC, micrartery count

CE-US, contrast-enhanced ultrasonography

TABLE 3.
Comparison of MAC in tubular adenocarcinoma

	Histological type	
	Well differentiated (n=2)	Moderately differentiated(n=8)
30µm>	9.0±5.5*	3.2±2.9
30µm≤60µm	0.8±0.3	0.7±0.8
60µm≤100µm	0.3±0.7	0.3±0.7
100µm≤	1.2±1.5	0.5±0.7

* p<0.05 versus Moderately differentiated adenocarcinoma

MAC, microartery count

TABLE 4.
Comparison of cancer histology with CE-US findings

CE-US findings	hyperperfusion group (n=6)	hypoperfusion group (n=7)
Histology		
Medullary type	1	0
Intermediate type	3	4
Scirrhou type	2	3

CE-US, contrast-enhanced ultrasonography.

the hypoperfusion group (5.3 ± 3.4) or normal tissue (4.9 ± 2.1). There was no significant difference in MAC between the hypoperfusion group and the normal pancreas.

3) Percentage contrast increase and MAC by vessel diameter

MAC with a diameter of less than $30 \mu\text{m}$ was 7.2 ± 5.0 in the hyperperfusion group ($P < 0.05$), which was significantly higher than in the hypoperfusion group (2.2 ± 2.2) and normal pancreas (4.9 ± 2.1). There was no significant difference between the MAC values in the 3 groups for vessels with a diameter of 30 to $60 \mu\text{m}$ and 60 to $100 \mu\text{m}$.

For vessels with a diameter greater than $100 \mu\text{m}$, MAC was 1.2 ± 0.6 ($P < 0.05$) in normal pancreas, which was significantly higher than the 0.6 ± 1.0 in the hyperperfusion group and 0.4 ± 0.7 in the hypoperfusion group (Table 2).

In tubular adenocarcinoma the MAC for vessels with a diameter less than $30 \mu\text{m}$ was 9.5 ± 5.5 ($P < 0.05$) in well-differentiated adenocarcinoma, which was significantly higher than the 3.2 ± 2.9 found in moderately differentiated adenocarcinoma (Table 3).

No significant difference was observed in the amount of interstitial tissue in cancerous lesions between the hyperperfusion and hypoperfusion groups (Table 4).

DISCUSSION

Imaging diagnostic methods show hypovascular tumors in pancreatic carcinoma and until now, the blood flow in tumors could not be delineated. Folkman proposed the idea that in solid tumors, the supply of oxygen and nutrients by diffusion limits the size of the tumor to approximately 250 to $300 \mu\text{m}$ [7]. His assumption was that unless angiogenesis occurred, a tumor could become no larger than 1 to 2 mm , and that vascularization was essential for tumor metastasis and enlarge-

ment. Following publication of these proposals, studies were undertaken to examine microvessel density (MVD), vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP) and other angiogenesis factors, and there have also been reports of how their expression and mechanisms are related to angiogenesis [8-10]. MVD in particular is an indicator of the degree of angiogenesis and its use as a prognostic factor is anticipated [11].

Many studies on the different prognoses of hyper vascular tumors by means of MVD were reported concerning breast cancer [12], colon cancer [13] and gastric cancer [14]. Benckert et al. examined the correlation between MVD and VEGF and prognosis in pancreatic cancer, and reported that multivariable analysis showed high MVD to be an independent prognostic factor [15]. D'Onofrio et al. examined the relation between CE-US and angiogenesis and found a correlation between the contrast effect of CE-US and CD34-positive blood vessels pathologically examined in 42 pancreatic tumor patients [16]. There have, however, been only a few reports on the correlation between CE-US and MVD in pancreatic carcinoma, and a review of Pub Med and Japan Medical Abstracts Society found no reports on the direct correlation between CE-US and pathological findings. Until the advent of Levovist, US-angiography (US-A) was performed using carbon dioxide microbubbles injected into the hepatic artery as a contrast medium.

In pancreatic tumors, Koito et al. reported that US-A was superior to contrast CT and angiography for differentiating between mass forming pancreatitis and pancreatic ductal carcinoma [17]. However, angiographic examination is essential but highly invasive. On the other hand, Levovist enables intravenous evaluation of blood flow. Compared to other contrast agents, Levovist has not been reported to cause allergic reactions or renal disorders and seems to be an extremely safe contrast agent [18]. Furthermore, unlike the contrast medium used by CT, it is able to show a pure

vessel image.

Although pancreatic carcinoma is thought to be a hypovascular tumor, it should have some blood flow for growth. With US-A, the cancerous part of the pancreas is delineated as a poorly stained region [17]. Therefore, it was judged as hypovascular tumor. With CE-US, pancreatic tumors are shown distinctly and both sensitivity and specificity are high [19,20], because blood-flow evaluation is attained. There have also been reports of the usefulness of the staining patterns for tumor differentiation and for determining the surgical indication [1,21]. In previous reports, however, the presence or absence of blood flow was determined visually and staining patterns were analyzed on that basis rather than by evaluating the degree of staining by intensity. We analyzed the TIC obtained by CE-US and compared the percentage brightness increase of the cancerous and normal parts of the pancreas. The results suggested that percentage brightness increase might be correlated with histological type in pancreatic carcinoma.

Differences in staining in US-A and CE-US are believed to be due to differences in bubble size (CO₂ > 30 μm, Levovist approximately 1.3 μm). This study showed that since there were many muscular arterial vessels with a diameter of less than 30 μm in cancerous parts, CO₂ bubbles could not pass through these blood vessels and they would therefore have been delineated as poorly stained areas. With Levovist, however, the vessels were delineated as stained areas. Therefore, the percentage of muscular arterial vessels less than 30 μm is the factor that determines the staining intensity in pancreatic cancer. As a result, with CE-US, quantitative evaluation of staining becomes an indirect evaluation of microvessel density, which could be a useful prognostic predictor for pancreatic carcinoma.

In conclusion, the CE-US density of muscular arterial vessels less than 30 μm is an important factor in determining staining degree and carcinoma progression in pancreatic carcinoma.

REFERENCES

1. Kitano M, Kudo M, Maekawa K, Suetomi Y, Sakamoto H et al. Dynamic imaging of pancreatic disease by contrast enhanced coded phase inversion harmonic ultrasonography. *Gut* 2004; 53:854-859.
2. Sofuni A, Iijima H, Moriyasu F, Nakayama M, Nakamura K et al. Differential diagnosis of pancreatic tumor using ultrasound contrast imaging. *J Gastroenterol* 2005; 40:518-525.
3. Klagsbrum M, Folkman J: Angiogenesis. In: peptide growth factor and their receptors II. *Handbook of Experimental Pharmacology*. Springer-Verlag, Berlin 1990; 95:549-665.

4. Masaki T, Ohkawa S, Amano A, Ueno M, Miyakawa K et al. Noninvasive assessment of tumor vascularity by contrast-enhanced ultrasonography and the progressive of patients with nonresectable pancreatic carcinoma. *Am Cancer Society* 2005; 103:1026-1035.
5. Ichikawa T, Haradome H, Hachiya J, Nitatori T, Ohyomo K et al. Pancreatic ductal adenocarcinoma: preoperative assessment with helical CT versus dynamic MR imaging. *Radiology* 1997; 202:655-662.
6. Japan Pancreas Society. *Classification of Pancreatic Cancer* 2nd English edition. Tokyo: Kanehara & CO., Ltd., 2003.
7. Folkman J: Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; 285:1182-1186.
8. Poon RT, Fan ST, Wong J: Clinical significance of angiogenesis in gastrointestinal cancers: a target for novel prognostic and therapeutic approaches. *Ann Surg* 2003; 238:9-28.
9. Kutami R, Nakashima Y, Nakashima O, Siota K, and Kojiro M. Pathomorphologic study on the mechanism of fatty change in small hepatocellular carcinoma of humans. *J Hepatol* 2000; 33:282-289.
10. Vizo B, Navarino A, Giacobino A, Cristiano C, Parti A et al. Pilot study to relate outcome in pancreatic carcinoma and angiogenic plasma factors /circulating mature / progenitor endothelial cells: Preliminary result. *Cancer Sci* 2010; 101:2448-2454.
11. Fujimoto K, Hosotani R, Wada M, Lee JU, Koshiha T et al. Expression of two angiogenic factors, vascular endothelial growth factor in human pancreatic cancer, and its relationships to angiogenesis. *Eur J cancer* 1998; 34:1439-1447.
12. Kim SW, Park SS, Ahn SJ, Chung KW, Moon WK et al. Identification of angiogenesis in primary breast carcinoma according to the image analysis. *Breast Cancer Res Treat* 2002; 74:121-129.
13. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, and Ellis LM. Expression of endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; 55:3964-3968.
14. Tanigawa N, Amaya H, Matsumura M, Shimomatsuya T, and Horiuchi T. Extent of tumor vascularization correlation with prognosis and hematogenous metastasis in gastric carcinoma. *Cancer Res* 1996; 56:2671-2679.
15. Benckert C, Thelen A, Cramer T, Weichert W, Gaebelein G et al. Impact of microvessel density on lymph node metastasis and survival after curative resection on pancreatic cancer. *Surg Today* 2012; 42:169-176.
16. D'Onofrio M, Mallago R, Zamboni G, Vasori S, Falconi M et al. Contrast-enhanced ultrasonography better identifies pancreatic tumor vascularization than helical CT. *Pancreatol* 2005; 5:398-402.
17. Koito K, Namieno T, Nagakawa T, and Morita K. Inflammatory pancreatic mass: differentiation from ductal carcinoma with contrast-enhanced sonography using carbon dioxide microbubbles. *AJR* 1997; 169:1263-1267.
18. Otis S, Rush M, Boyajian R. Contrast-enhanced transcranial imaging: result of an American two phase study. *Stroke* 1995; 26: 203-209.
19. Rickes S, Unkrodt K, Neye H, Ocran KW, and Wermke W. Differentiation of Pancreatic Tumor by Conventional

- Ultrasound, Unenhanced and Echo-enhanced Power Doppler Sonography. *Scand J Gastroenterol* 2002; 37:1313-1320.
20. Oshikawa O, Tanaka S, Ioka T, Nakaizumi A, Hamada Y et al. Dynamic Sonography of Pancreatic Tumors: Comparison with Dynamic CT. *AJR* 2002; 178:1133-1137.
21. Faccioli N, D'Onofrio M, Malogo R, Zamboni G, Falconi M, et al. Resectable pancreatic carcinoma; depiction of tumoral margin at contrast enhanced ultrasonography. *Pancreas* 2008; 37:265-268.