Hyperenhanced Rim Surrounding Liver Metastatic Tumors in the Postvascular Phase of Sonazoid-Enhanced Ultrasonography: A Histological Indication of the Presence of Kupffer Cells

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Key Words
Liver cancer · Sonazoid · Contrast-enhanced ultrasonography · Kupffer cell · Programmed cell death-1 antibody · CD8-positive T cell

Abstract
Aim: A hyperenhanced rim (termed ‘HER’) in the postvascular phase is detected in some cases of liver metastasis by Sonazoid-enhanced ultrasonography (US). Here, the association of the HER with histological features was investigated to clarify the cause of this characteristic imaging pattern.
Subjects and Methods: A total of 13 hepatic nodules obtained from 11 patients with metastatic liver cancer who underwent Sonazoid-enhanced US followed by surgical resection were analyzed. The distribution density of CD68-positive cells in the tumor rim and the nontumor area was calculated and compared between the HER-positive and HER-negative groups. The relation between the pathological features of the tumor rim and the rate of necrosis within the tumor was also investigated.
Results: In the HER-positive group (n = 8), the distribution density of CD68-positive cells was 2.9 ± 0.9, which was significantly higher than that (1.0 ± 0.3) in the HER-negative group (p < 0.05). Inflammatory cell infiltrates, including CD8-positive lymphocytes, were detected in all the HER-positive cases in the area surrounding the tumor, while fibrosis was observed in all the HER-negative cases. The necrotic area within the tumor was significantly larger in the HER-negative group.
Conclusion: The HER-positive sign in liver metastases could reflect an increase in Kupffer cells in the tumor rim. The presence of the HER was associated with inflammatory cell infiltrates including CD8-positive lymphocytes surrounding the metastatic liver tumor.

Introduction
Until recently, contrast-enhanced ultrasonography (US) was primarily used for the evaluation of the hemodynamics of liver masses [1–6]. The development of
contrast-enhanced US using Sonazoid enabled not only the observation of the hemodynamic state, but also detailed evaluation of liver masses by generating functional images to visualize the distribution of Kupffer cells [7–14]. Images obtained during the vascular phase with excellent temporal resolution [15] and postvascular images [16] obtained during the postvascular phase are both clinically useful. Metastatic liver tumors that are poorly visualized by conventional B-mode US can often be detected as a clear defect by Sonazoid-enhanced US [13, 17]. Increasing evidence suggests that the ability of Sonazoid-enhanced US to detect liver metastases is superior to that of computed tomography [3, 13, 18]. Qualitative diagnosis, including determination of the degree of differentiation of newly developed, small, well-differentiated hepatocellular carcinomas (HCCs) [5, 15, 19–23], has been achieved by evaluating the decreased echogenicity associated with a reduced distribution of Kupffer cells [24–26]. However, the evaluation of Kupffer images in the postvascular phase has focused only on the inside of the lesion, whereas altered imaging patterns in the area surrounding the lesion have not attracted much attention.

In some cases of liver metastases, a 2- to 3-mm hyper-enhanced band-like area is observed in the tumor periphery during the postvascular phase of Sonazoid-enhanced US. This imaging pattern was tentatively termed ‘hyper-enhanced rim’ (HER) in the postvascular phase. In the present study, the histopathological features of resected liver metastases were compared by dividing lesions into two groups, a HER-positive and a HER-negative group, to investigate the causes of this characteristic imaging pattern.

<table>
<thead>
<tr>
<th>HER in the postvascular phase</th>
<th>positive group (n = 8)</th>
<th>negative group (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness of HER, mm</td>
<td>2.4±0.4 (2–3)</td>
<td>–</td>
</tr>
<tr>
<td>Age, years</td>
<td>67±12 (41–81)</td>
<td>62±8 (50–73)</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>6:2</td>
<td>4:1</td>
</tr>
<tr>
<td>Primary site, n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Duodenal papilla</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tumor diameter, cm</td>
<td>1.9±0.8 (1.1–3.7)</td>
<td>1.9±0.5 (1.1–2.5)</td>
</tr>
<tr>
<td>Time after intravenous injection of Sonazoid, min</td>
<td>51±34 (20–110)</td>
<td>58±33 (40–110)</td>
</tr>
<tr>
<td>Interval from contrast-enhanced US to surgery, days</td>
<td>35±12 (15–42)</td>
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</tr>
</tbody>
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### Subjects and Methods

The study included 13 hepatic nodules [mean diameter, 1.8 ± 0.7 cm (range, 1.1–3.7)] obtained from 11 patients with liver metastases (7 men and 4 women with a mean age of 65 ± 11 years) who underwent contrast-enhanced US followed by surgical resection between September 2011 and August 2013. The primary sites were the colon in 9 patients, the duodenal papilla in 1 patient, and the breast in 1 patient.

The 13 nodules were divided into 8 HER-positive and 5 HER-negative nodules. There were no significant differences in age, gender, primary site, tumor diameter, time after intravenous injection of Sonazoid, and interval from contrast-enhanced US to surgery between the two groups (table 1). Contrast-enhanced US was performed by observing images in the postvascular phase at 30–60 min after intravenous injection of 1 ml of Sonazoid with a mechanical index of 0.20–0.22. The US equipment and probes used were AplioXG (Toshiba) with PVT-374BT or PVT-375BT, and Aloka-Preirus (Hitachi) with EUP-C715 or EUP-L52.

For histological evaluation, formalin-fixed, paraffin-embedded blocks of resected specimens of liver metastases were cut into 3 serial 3-μm sections at the maximum tumor diameter. The sections were subjected to immunohistochemical staining for macrophages, hematoxylin and eosin (HE) staining, or Masson’s trichrome staining.

The primary antibody used for macrophage staining was anti-CD68 (KP1; Dako, Glostrup, Denmark), a macrophage marker. Diaminobenzidine was applied as a chromogen for immunohistochemical staining. The stained slides were observed under a light microscope at low magnification, and 3 fields with adequate staining were randomly selected from an area within 2 mm from the tumor margin and photographed using a ×40 objective lens. Similarly, 3 fields were randomly selected from a nontumor area located at least 5 mm from the tumor margins and photographed as described above. The number of CD68-positive cells was determined from printed photographs. Results were expressed as the number of CD68-positive cells per each unit area (0.35 × 0.26 × 3 mm²), and the distribution density of CD68-positive cells in the tumor rim relative to that in the surrounding nontumor liver parenchyma was calculated.

### Table 1. Comparison of clinical and radiological characteristics between HER-positive and HER-negative groups

<table>
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<tr>
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<th>HER in the postvascular phase</th>
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<td>Thickness of HER, mm</td>
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Inflammatory cell infiltrates in the tumor rim and the morphological features of the tumor were evaluated using HE-stained slides. The inflammatory cell infiltrates were defined as positive if the density of inflammatory cells was higher than that of Glisson’s capsule in a nontumor area located at least 5 mm from the tumor. The necrotic area within the tumor was also measured. Fibrosis and capsule formation in the tumor rim were evaluated by Masson’s trichrome staining. Capsule formation was defined as positive when a band-like fibrosis was detected in a circumferential pattern surrounding the tumor.

The Student t and χ² tests were used to evaluate the statistical significance of differences.

### Results

The mean thickness of high-echo bands (fig. 1) in the HER-positive nodules was 2.4 ± 0.4 (range, 2–3) mm.

In the HER-positive group (n = 8), the number of CD68-positive cells per unit area in zones within 2 mm from the tumor margin was 247 ± 86 and the distribution density relative to that in the nontumor areas (84 ± 20) was 2.9 ± 0.9 (range, 1.6–4.2). In the HER-negative group, the number of CD68-positive cells was 85 ± 34 and the distribution density relative to that in the nontumor areas (85 ± 35) was 1.0 ± 0.3 (range, 0.6–1.3; table 2).

The number of CD68-positive cells in the tumor rim and the relative distribution density were significantly higher in the HER-positive group (n = 8) than in the HER-negative group (p < 0.05 both).

In the histological analysis of the tumor rim, inflammatory cell infiltrates were significantly more common in the HER-positive group (8/8) than in the HER-negative group (2/5; p < 0.05; table 2). Fibrosis in the tumor rim was more common in the HER-negative (8/8) than in the HER-positive group (4/8; p = 0.057). An established capsule was more common in the HER-negative than in the HER-positive group. There were no significant differences in the compression of the hepatic cord and the presence of peliosis in the tumor rim between the two groups.

### Table 2. Comparison of histopathological findings between HER-positive and HER-negative groups in resected specimens

<table>
<thead>
<tr>
<th>At tumor margin</th>
<th>HER in the postvascular phase</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive group (n = 8)</td>
<td>negative group (n = 5)</td>
</tr>
<tr>
<td>Density of CD68-positive cells</td>
<td>247 ± 86 (99–388)</td>
<td>85 ± 34 (40–136)</td>
</tr>
<tr>
<td>Ratio of distribution density of CD68-positive cells</td>
<td>2.9 ± 0.9 (1.6–4.2)</td>
<td>1.0 ± 0.3 (0.6–1.3)</td>
</tr>
<tr>
<td>Inflammatory cell infiltrate</td>
<td>8 (100%)</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>4 (50%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Fibrous capsule</td>
<td>1 (13%)</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>Compression of hepatic cord</td>
<td>4 (50%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Peliosis</td>
<td>5 (63%)</td>
<td>2 (40%)</td>
</tr>
</tbody>
</table>

### Fig. 1. HER in the postvascular phase in a metastatic liver tumor. A band-like area of 2–3 mm (arrowheads) showing hyperenhancement relative to the surrounding parenchyma in the postvascular phase of Sonazoid-enhanced US is observed in a circumferential pattern in the tumor rim of a metastatic liver cancer of 2.5 cm in size, detected as a defect in the S5 (a). A band-like area (arrowheads) of similar thickness is observed in the periphery of a small nodule of 8 mm in size found in the vicinity (b). These findings were termed ‘hyperenhanced rim (HER)’ in the postvascular phase image.
(For legend see next page.)
A necrotic area within the tumor was detected at a higher rate in the HER-negative group than in the HER-positive group (p < 0.05; table 2; fig. 2, 3).

Discussion

Contrast-enhanced US using Sonazoid is widely used in Japan, and qualitative diagnosis by contrast-enhanced US is included in the diagnostic criteria for hepatic lesions proposed by the Japan Society of Ultrasonics in Medicine (JSUM) in 2012 [16]. The JSUM guidelines for contrast-enhanced US were published by Kumada et al. [15] in 2014. The World Federation for Ultrasound in Medicine and Biology (WFUMB) guidelines for contrast-enhanced US were published in 2013 [9, 10].

In these criteria, early enhancement confined to the tumor margin (ring enhancement) is described as one of the characteristic findings of liver metastases [7, 15, 16]. This imaging pattern is thought to result from the enhancement of viable cell components in the tumor margin and of surrounding noncancerous tissues associated with hyperplasia of the fibrous stroma, inflammation, and angiogenesis [7, 15, 27], and may disappear in the portal phase. These findings appear to be similar to those associated with the HER in the present study, since they both show ring enhancement in the tumor rim; however, they are present only in the early arterial phase of vascular phases up to 120 s. This indicates that they differ from the HER findings, which were confined to the postvascular phase, suggesting that the two imaging patterns are caused by different processes.

On the other hand, the characteristic features of liver metastases in the postvascular phase have been clearly defined [1, 15, 16]. Unlike the slightly decreased enhancement found in small, well-differentiated HCCs associated with a decreased density of Kupffer cells within the tumor [5], observation in the postvascular phase with high spatial resolution is useful for detecting liver metastases. Tumors within the liver parenchyma showing a wedge-shaped enhancement in the vascular phase due to tumor infiltration to the peripheral portal branches and Glisson’s capsule [28] can be detected by this method. In our institution, contrast-enhanced US is performed as a screening test to detect liver metastases by searching for a defective area at 30–60 min (average of 50 min) after intravenous injection of Sonazoid.

The detection of malignant tumors in the postvascular phase is generally based on findings in the defective area, such as changes in the echogenicity within the tumor, rather than on changes in the tumor rim. In this regard, the HER described in the present study could be a novel imaging finding of contrast-enhanced US. Since postvascular images reflect the distribution of Kupffer cells, the HER could be an area containing a high density of Kupffer cells. To test this hypothesis and to investigate the types of liver metastases associated with a high density of Kupffer cells in the tumor rim, we compared histopathological findings between the HER-positive and HER-negative groups.

Our results showed that the number of CD68-positive cells in the tumor rim (within 2 mm from the tumor margin) and the distribution density relative to that in the nontumor areas were significantly higher in the HER-positive than in the HER-negative group. CD68 immunostaining is used for the detection of cells of the histiocytic system, including monocytes and macrophages with phagocytic activity such as Kupffer cells. In the present study, CD68 staining was faint and macroscopic detection of an area of high CD68 staining corresponding to the HER was not possible. However, the number of CD68-positive cells counted under the microscope was approximately three times higher in the tumor rim than in the nontumor areas. Although these results were obtained by counting the number of cells in photographic images of 3 randomly selected fields from the tumor rim and nontumor areas acquired under a microscope with a ×40 objective lens, the difference in the distribution density of CD68-positive cells may reflect the HER pattern. HER positivity could indicate the accumulation of macrophages with phagocytic activity in a circumferential pattern in an area within 2 mm from the tumor margin.

Neutrophil infiltration occurs frequently in the portal region in cases of liver metastasis [29]; however, the path-

Fig. 2. A HER-positive metastatic liver tumor originating from the rectum in a female patient in her 70s. At 90 min after intravenous injection of Sonazoid (in the postvascular phase), a 1.9-cm defect with a clear HER (arrowheads) is detected in the S5 (a). A high-frequency probe enables clear detection of the rim (arrowheads), and its maximum thickness was determined to be 3 mm (b). In a fixed resected specimen (c), the central part of the tumor shows a yellowish-white appearance with a necrotic area occupying 30% of the tumor, as measured under a loupe lens (d). Slight fibrosis in addition to inflammatory cell infiltrates is detected at the tumor rim by Masson’s trichrome staining (e). The distribution density of CD68-positive cells in the tumor rim (f) relative to that in the nontumor area was 3.1 (g) as measured by anti-CD68 immunostaining. HE staining of the tumor periphery and the surrounding liver (h). CD8-positive cells are observed in the surrounding liver (i) and in the tumor (j).
Fig. 3. HER-negative metastatic liver cancer (a case associated with the presence of a HER-positive nodule) originating from the rectum in a male patient in his 60s. At 60 min after intravenous injection of Sonazoid (in the postvascular phase), a clear 2.2-cm defect is detected in the S5 (a). There is no high-echo band in the tumor rim (a). In this case, a HER-positive nodule of 0.8 cm in size was detected in the vicinity (b). In a fixed resected specimen, most of the tumor has a yellowish-white appearance, while the adjacent HER-positive nodule looks whitish (c). Under a loupe lens, necrosis is detected in 95% of the tumor (d). Masson’s trichrome staining shows a thick fibrous capsule surrounding the whole tumor and the tumor rim (arrows; e). CD68 staining showed that the distribution density of CD68-positive cells was 0.6 in the tumor rim of the HER-negative nodule (f) and 1.6 in that of the HER-positive nodule (g). CD68-positive cells were rare in the nontumor area (h).
logical findings associated with macrophage accumula-
tion in the tumor periphery have not been reported.

Functional imaging modalities targeting the phagocyt-
ic activity of reticuloendothelial cells such as Kupffer cells
include phytate colloid scintigraphy and superparamag-
netic iron oxide MRI. However, the former has an ex-
tremely low resolution capability and a limited capacity
for detecting alterations of 2–3 mm in size. Although su-
perparamagnetic iron oxide MRI is superior to unen-
hanced MRI for the detection of liver metastases [30], an
imaging feature corresponding to the HER sign has not
been reported to date. In this respect, Kupffer cell accu-
mulation is only detectable by imaging modalities based
on Sonazoid-enhanced US with a high spatial resolution.

One of the mechanisms by which macrophages accu-
mulate in the periphery of liver metastases could be re-
lated to an increased density of Kupffer cells attached to
the sinusoidal wall in the tumor rim associated with com-
pression by tumor growth. However, pathological exam-
inations revealed no correlation between the compres-
sion of the hepatic cord in the tumor periphery and the
HER area. In addition, there was no correlation between
the presence of peliosis, dilated sinusoids, and the HER
sign. These results suggest that the HER-positive sign
may reflect another process in addition to the distribution
density of attached Kupffer cells. There was a correlation
between inflammatory cell infiltrates in the tumor rim
and the HER sign. Inflamatory cell infiltrates including
CD8-positive cells were found in some HER-positive cas-
es, which we tested in only a few cases.

If CD8-positive T cell (cytotoxic T cell) infiltrates are
consistently associated with HER-positive tumors and
not associated with HER-negative tumors, HER positiv-
ity could be used as an imaging biomarker for the predic-
tion of anti-programmed cell death-1 (PD-1) antibody
treatment response, since PD-1 response correlated with
PD-L1 expression, which also correlated with the pres-
ence of CD8-positive lymphocytes [31, 32].

Fibrosis was detected in the tumor rim in all HER-
negative cases, and some of these cases showed a com-
plete capsule. In addition, there was no correlation be-	ween the proportion of necrotic areas within the tumor
and the HER sign. The proportion of necrotic areas tended
to be higher in the HER-negative group than in the
HER-positive group, and some cases in the HER-negative
group showed complete necrosis within the tumor (fig. 4).

These results led us to hypothesize that migrating mac-
rophages [26] accumulate in the tumor periphery and
mediate the phagocytosis of substances eluted from the
tumor. This could be one of the mechanisms by which
macrophages surround metastatic tumors. As fibrosis, in
addition to the accumulation of inflammatory and/or cy-
totoxic lymphocytes [29], occurs in the tumor periphery,
complete encapsulation of the tumor could decrease the
number of macrophages that come into contact with tu-
mor substances and thereby accumulate in the tumor rim.

Since macrophage accumulation is thought to be
casted by foreign body (tumor cell) phagocytosis, their
localization to the invasive front of the cancer growth area
indicates that macrophages may inhibit the progression

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**Fig. 4.** Comparison of histological findings between HER-positive and HER-negative nodules.

**HER (+) nodule**
- CD68-positive cell: increase
- Inflammatory cell: increase
- Fibrosis: increase
- Ratio of necrotic area: high

**HER (−) nodule**
- CD68-positive cell: equivocal
- Inflammatory cell: increase

**HER (−) nodule**

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of cancer itself. Also, CD8-positive cells may have been activated by tumor-associated antigen through antigen-presenting cells.

Nevertheless, this phenomenon has not been reported previously, even in pathological studies, and can be observed only by Sonazoid-enhanced US. Therefore, further studies using Sonazoid-enhanced US are required to determine whether the prognosis of liver metastasis differs between HER-positive and HER-negative patients, and whether the HER sign is specific to liver metastases and is not observed in other types of hepatic nodules, including HCCs. Also, further study of the immunohistochemistry of CD8-positive cells and PD-L1 in our series may open a new door to immunotherapy of liver cancers through PD-1 monoclonal antibody treatment for liver cancer.

**References**


**Conclusion**

A HER sign in the postvascular phase of Sonazoid-enhanced US detected in the tumor rim of liver metastases could reflect an increase in cells of the histiocytic system including Kupffer cells. The presence of the HER sign was also associated with inflammatory cell infiltrates and CD8-positive lymphocytes, suggesting that HER positivity could be a predictive imaging biomarker of the response to PD-1 monoclonal antibody treatment for liver cancer.

**Disclosure Statement**

All authors declare that they have no conflicts of interest to disclose.

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