Elastography and Contrast-enhanced Ultrasonography in the Early Detection of Hepatocellular Carcinoma in an Experimental Model of Nonalcoholic Steatohepatitis

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Background/objective: The early detection of focal hepatic lesions using ultrasound scanning is challenging, and this challenge becomes even greater in the presence of diffuse parenchymal disease. This study aimed to evaluate the diagnostic performance of elastography and contrast-enhanced ultrasonography (CEUS) in the early detection of hepatocellular lesions in an experimental rat model of nonalcoholic steatohepatitis (NASH). **Methods:** B-mode and Doppler ultrasonography was performed weekly in 30 rats divided into a NASH group ($n = 20$) and a group without liver disease ($n = 10$). The animals underwent elastography and CEUS and were then euthanized. Liver nodules were assessed by histopathology. **Results:** Doppler mapping results of lesions with vascularization were considered indicative of malignancy, with a sensitivity of 29% before and 71% after contrast injection. The specificity was 71% before and 96% after CEUS. Elastograms of positive lesions showed areas of high stiffness, which were indicative of malignancy. This malignancy was confirmed by the histologic evaluation, with a sensitivity of 90% and a specificity of 60%. After CEUS analysis, 4 nodules were identified that were not observed on B-mode ultrasonography. Early wash-in was significantly associated with malignancy (sensitivity of 88% and specificity of 67%). **Conclusions:** Both techniques allow for the correct diagnosis of well-differentiated to moderately differentiated hepatocellular carcinomas with good accuracy in an experimental rat model of NASH. (J CLIN EXP HEPATOL 2013;3:96–101)

Hepatocellular carcinoma (HCC) is the fifth most common cancer in humans and is a recognized complication of advanced nonalcoholic steatohepatitis (NASH).¹

The early detection of focal hepatic lesions by routine clinical examination and ultrasound (US) scanning is challenging, and this challenge becomes even greater in the presence of diffuse parenchymal disease. Despite these challenges, US is still the first choice for screening these lesions.²

Two new technologies, elastography and contrast-enhanced ultrasonography (CEUS), can improve diagnostic results. Elastography has emerged as a new method to

evaluate the stiffness of focal lesions and promises to differentiate lesions based on malignancy.^{3,4} In addition, real-time CEUS with microbubbles has been widely used as a noninvasive modality for the detection and characterization of focal liver lesions in clinical practice.^{5,6}

CEUS has improved the assessment of liver parenchyma relative to the use of conventional gray-scale US and exhibits high accuracy for differentiating malignant focal liver lesions from benign lesions.^{7,8,9} Focal liver lesions in individuals with diffuse liver diseases, such as liver cirrhosis or steatosis, are very common in clinical practice. Nevertheless, the presence of diffuse liver disease increases the difficulty of characterizing these focal lesions.¹⁰

Few studies using CEUS have assessed patients with liver steatosis and cirrhosis.^{11,12} In addition, in animal models, there are few studies on NASH^{13,14} that progresses to steatosis, steatohepatitis and HCC because of the complexity of the model and the relatively long time (40–72 weeks) required to reliably produce HCC. Recently, our group developed an animal model that replicates many features of NASH, including steatohepatitis with ballooning, fibrosis, cirrhosis and HCC.¹⁵

This study aimed to evaluate the diagnostic performance of elastography and CEUS in the early detection

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Abbreviations: HCC: hepatocellular carcinoma; NASH: nonalcoholic steatohepatitis; US: ultrasound; CEUS: contrast-enhanced ultrasonography

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of malignant hepatic nodules in an experimental rat model of NASH that progresses to HCC.¹⁵

MATERIALS AND METHODS

Ethics

All procedures for animal experimentation were in accordance with the Helsinki Declaration of 1975 (NIH Publication no. 85-23, revised 1996) and the Guidelines of Animal Experimentation of the University of Sao Paulo School of Medicine. The present study was reviewed and approved by the Ethics Committee of the School of Medicine of the University of São Paulo (process number 358/10).

Animals

Two groups were compared: control and NASH animals.

The control group included 10 normal adult male Sprague-Dawley rats weighing 250–300 g. The animals were provided a standard diet and water *ad libitum*. Elastography and CEUS were performed on these rats to determine the normal pattern of liver parenchyma and establish the appropriate contrast dose.

In the NASH group, 20 adult male Sprague-Dawley rats received unrestricted amounts of a specially manufactured food (a choline-deficient and high-trans-fat diet) and drinking water containing diethylnitrosamine (135 mg/L) to induce the development of NASH and HCC, as described elsewhere.¹⁵

B-mode and Doppler ultrasonography was performed weekly in all rats until nodules were detected. B-mode ultrasonography was performed to detect abnormalities. In this rat model, nodules were detected after 16 weeks, as described in the literature.¹⁵ When a nodule was detected, it was measured and analyzed by elastography and CEUS. Doppler mapping of the lesions was classified as negative for malignancy when there was no inner vascularization or when vessels were detected only around the nodule.

All data were recorded. All animals received humane care and were housed in an environment with controlled temperature, humidity and ventilation. The study protocols complied with the institution's guidelines. All animals were euthanized after the last examination, and liver tissue samples were obtained for histological analysis. We compared the elastography and CEUS results with the histological findings.

Ultrasonography

The ultrasonography equipment used was a model Aplio XG system (Toshiba, Tokyo, Japan) with a 12 MHz linear transducer. The exams were performed under anesthesia with ketamine hydrochloride (0.2 mg/kg intraperitoneal). The same conditions of luminosity, contrast, intensity and gain of the US system were used for all examinations. All sonographic examinations were performed by a single professional with more than 10 years' experience.

Elastography

Qualitative real-time US elastography was performed with the same equipment used to perform conventional scanning. Sonoelastography was performed with a freehand scanning technique, in which a series of five mechanical compressions was applied. The equipment's software uses a complex algorithm that is able to process all of the data on a lesion in a short time, allowing for the measurement of the degree of tissue distortion. After the data were acquired using the equipment, the curve that best represented the maximal compression was chosen, and the information was provided as an image. The hard and soft areas (ie, areas of high and low elasticity, respectively) were differentiated in a color elastogram. In general, increasing tissue hardness was represented as a change from red to yellow, green and then blue (maximum hardness). These colors represented the relative hardness of the tissues in the elastogram. Elastography images included the target lesion (suspected nodule) and the surrounding tissue because the elasticity values were displayed relative to the average strain inside the region of interest. In our work, the nodules imaged by qualitative elastography were classified as negative for malignancy (soft nodule) or positive for malignancy (hard nodule) based on comparison with the surrounding liver parenchyma.

Contrast-enhanced Ultrasonography

The contrast agent used in the CEUS study was Definity (Lantheus), which was diluted 1:5 in saline solution. This contrast agent was composed of sulfur hexafluoride with a liposome shell. The internal jugular vein was catheterized to inject the contrast agent (0.1 mL of diluted contrast agent). A total of 1 mL of saline solution was sequentially injected.

The type of enhancement during the arterial phase (which occurred 2–5 s after injection) and during the portal and late phases (after 5 s) was evaluated. Focal liver lesions were classified according to the type of enhancement and wash-out. A lesion was classified as malignant when it had early wash-in or no wash-in during the arterial phase and wash-out during the portal or late phase. A lesion was classified as benign when it did not exhibit wash-out.

Histological Analysis

After the animals underwent elastography and CEUS, they were euthanatized, and samples of the liver tissue were assessed by histopathology. Because each liver could have multiple nodules, needle puncture was used to mark the regions of interest identified by the two imaging methods.

Liver tissue was assessed grossly. Samples were fixed in 10% formaldehyde and processed for hematoxylin-eosin and Sirius red staining for histological analysis. Variables were blindly scored by an experienced hepatopathologist

using a modified scoring system¹⁶ adapted from the NASH activity score: macro/microsteatosis (0–3), hepatocyte ballooning (0–2), lobular inflammatory changes (0–3), and fibrosis (0–4).

HCC was classified according to Edmondson–Steiner grading,¹⁷ as recently reassessed by other authors.^{4,18} Dysplastic nodules were classified according the International Consensus Group for Hepatocellular Neoplasia.¹⁹

Statistical Analysis

All data were expressed as the mean and standard deviation or the median. Categorical data were expressed as the number or proportion. All *P* values were two tailed, and $P < 0.05$ was considered statistically significant. To identify potential predictors of malignancy and the diagnostic performance of the imaging methods, the sensitivity, specificity and positive and negative predictive values were calculated.

RESULTS

In the control group, liver samples were obtained from all rats, and as expected, there were no focal lesions.

In the NASH group, four rats died during the anesthesia procedure, and thus, not all assessments could be performed for these rats. In total, 30 liver nodules detected on B-mode ultrasonography from 16 rats were studied. Conventional ultrasonography revealed diffuse increased echogenicity of the liver (relative to the kidney) and heterogenous texture in all rats. The mean nodule size was 0.58 cm (range, 0.33–2.19). Most nodules were localized

in the right lobes of the liver. The locations of the nodules were as follows: five nodules in the left lateral hepatic lobe, six nodules in the left medial hepatic lobe, nine nodules in the right medial hepatic lobe and 10 nodules in the right lateral hepatic lobe.

Doppler mapping results of lesions with vascularization were considered indicative of malignancy, with a sensitivity of 29% before CEUS and 71% after contrast injection. The specificity was 71% before and 96% after CEUS. The accuracy of the Doppler study results was 50% before CEUS and 80% after contrast injection.

Elastograms of the nodules showed positive lesions as areas of maximum hardness (blue areas), which were considered indicative of malignancy and confirmed by histology (Figure 1), with a sensitivity of 90% and a specificity of 60%. The positive and negative predictive values were 0.75 and 0.82, respectively. The four false-negative nodules included three areas that measured less than 0.5 cm in length (classified as HCC on histology) and one that had a cystic appearance because of a necrotic center and it was classified as a mixed tumor (hepato-cholangiocarcinoma). The false-positive nodules were slightly hypoechoic and heterogenous (Table 1) and they were classified as dysplastic nodules based on histology. Results of CEUS study of the two false-positive nodules presented early wash-in in the arterial phase and wash-out in the late vascular phase.

After CEUS analysis, 4 additional nodules were identified (in 3 different rats), which were confirmed with histology. However, only the 30 nodules previously detected on

Figure 1 Elastogram showing a positive lesion (white arrow) with an area of high stiffness and the corresponding image taken in B-mode, which suggests malignancy.

Table 1 Ultrasonography results (elastography and CEUS) compared with the results of histological analysis.

Nodules	Elastography	Wash-in	Wash-out	Histology	Tumor histology grade
1	+	+	+	+	HCC III
2	+	–	+	+	HCC III
3	–	–	–	–	not a tumor
4	–	–	–	–	not a tumor
5	+	+	+	+	HCC II
6	+	+	+	+	HCC III
7	+	+	–	+	HCC III
8	+	–	+	+	HCC II
9	+	+	+	+	HCC III
10	–	–	+	–	not a tumor
11	+	+	+	+	HCC II
12	+	+	+	+	HCC III
13	+	+	+	+	HCC III
14	+	+	+	+	HCC II
15	+	+	+	+	HCC III
16	+	+	+	+	HCC III
17	+	+	+	–	not a tumor
18	+	+	+	–	not a tumor
19	–	–	–	–	not a tumor
20	+	N	N	+	HCC II
21	+	N	N	+	HCC II
22	–	N	N	+	HCC III
23	–	N	N	+	HCC III
24	–	–	+	–	not a tumor
25	–	–	–	–	not a tumor
26	+	+	+	+	HCC III
27	–	–	–	+	HCC II
28	+	+	–	+	HCC III
29	–	+	–	+	HCC II
30	+	+	+	+	HCC III

(+) Positive for malignancy; (–) negative for malignancy; (N) not performed.

B-mode ultrasonography were studied with contrast sonography. In the CEUS analysis, wash-in during the arterial phase was significantly associated with malignancy, with a sensitivity of 88% and a specificity of 67%. In the late vascular phase, the wash-out pattern was significantly associated with malignancy (Figure 2), with a sensitivity of 78% and a specificity of 50%. When defining nodules as malignant when they exhibited both wash-in and wash-out, we found a sensitivity of 70% and a specificity of 67%.

Steatosis and grossly cirrhotic with nodular surface was observed in all of the animals. Microscopically, cirrhosis

Figure 2 CEUS image in the late phase showing a nodule characterized by marked wash-out, indicating malignancy (black arrow).

was found in 75% (12/16) of the animals. Steatohepatitis was found in all cases, with a predominance of macro- and microvesicular steatosis. In addition, pericellular fibrosis was present in all animals. According to Edmondson–Steiner grading, most tumors were solid (Figure 3), well differentiated or moderately differentiated and contained mitotic and apoptotic bodies (22/30; 73.4%). According to the histological analysis, 26.6% (8/30) of the nodules were negative for malignancy (3/8 were dysplastic and 5/8 were regenerative nodules).

The accuracy was 80% for elastography and 70% for CEUS.

DISCUSSION

Small animal oncology models have become essential tools for studying the etiology and development of human tumor diseases in the laboratory setting. Because the lesions

Figure 3 A well-differentiated HCC nodule with a pseudocapsule of collagen (arrow). Edmondson–Steiner grade I (40×).

in these models mimic human tumors, the use of these models has been helpful for the development of new methods of diagnosis and effective therapies and the assessment of the efficacy of treatment strategies. The model used in our research is described in the literature¹⁵ and proposed the development of histological NASH with cirrhosis and hepatocellular carcinoma, resulting from a high trans-fat diet combined with exposure to a known genotoxic agent. This mechanism involves exacerbation of oxidative injury which likely contribute to the development of HCC.^{1,20}

HCC is a slow-growing tumor, and the prognosis largely depends on the evolutionary stage at which the disease is detected.²⁰ Like all diffuse parenchymal diseases, advanced NASH makes the diagnosis of focal lesions difficult when using B-mode US.² The gold standard for the diagnosis of malignancy traditionally requires cytologic or histologic confirmation. However, given the potential complications of liver biopsies in patients with decompensated chronic hepatic disease, noninvasive criteria have been proposed for the diagnosis of HCC.

The diagnostic algorithm largely depends on the size of the nodules, and the literature indicates that small nodules (<3 cm) detected on US in individuals with advanced diffuse hepatic disease represent the most challenging category for the noninvasive diagnosis of HCC.²¹ Currently, the proposed criteria used to differentiate benign and malignant focal liver lesions are based on the pattern of contrast enhancement during the arterial, portal and late phases.^{7,8,9} Nodule detection was performed using B-mode US in our study, but elastography and CEUS were used to confirm the presence of nodules and identify malignant lesions. In our study, three false-negative nodules in the elastography study were very small nodules (less than 0.5 cm in length); two of them were not evaluated by CEUS, and one was also a false negative in the CEUS analysis. The other false-negative nodule had a cystic appearance and was a false negative in both studies. Intranodular arterial blood flow tended to increase as the course of carcinogenesis, but small nodules are usually hypovascular because of the low number of arteries in the tumor, and the number of arteries increases as the size of the tumor increases.²⁰ The hypovascular nature of small nodules makes them difficult to diagnose with Doppler and contrast imaging techniques.²¹ This difficulty in diagnosis of arterial blood flow also occurred with a tumor with a cystic appearance because of the necrotic center. Pathological changes are generally correlated with changes in tissue stiffness. The echogenicity and mechanical attributes of tissue assessed by sonoelastography are generally uncorrelated.³ In many cases, despite the difference in stiffness or mobility, the small size of a pathological lesion (as in these cases) or its location deep in the body makes detection difficult. Moreover, lesions may or may not possess echogenic attributes that make them detectable by US.²²

The two false-positive nodules (Table 1, nodules 17 and 18) in the elastography analysis were also false positives in the CEUS study. These nodules were slightly hypoechogenic and heterogenous and were classified as dysplastic nodules based on histology. Such nodules can be considered potentially premalignant.²³ In clinical practice, a dysplastic nodule is an important finding and should be followed up.

Malignant tumors are stiffer than benign tumors and normal soft tissue; when mechanical compression is applied, malignant tumors deform less than benign tumors, which in turn deform less than the normal surrounding tissue.²² In our study, nodule stiffness was characterized through comparison with surrounding tissue (with NASH and cirrhosis). Qualitative elastography exhibited good sensitivity and specificity in this experimental model. There are a few recent reports in the literature on the application of this type of elastography in liver analysis,^{21,24} but none of these studies was based on a NASH model like this. In our research, considering the animal's size and the ease of compressing the liver, the results obtained using qualitative real-time US elastography were very promising. These successful results may not be the same in humans or other large animals. In humans and large animals, the literature shows good results using technologies other than transient elastography, such as acoustic radiation force impulse and Fibroscan analysis, to assess diffuse liver disease.⁴ However, sonoelastography provided promising perspectives for the assessment of the malignancy of focal hepatic lesions in our sample. The development and validation of these non-invasive diagnostic modalities in an animal model could redefine pathophysiological animal experiments. These new techniques could play an important role in the follow-up of patients with NASH.

Early wash-in in the arterial phase and wash-out in the late vascular phase were strongly suggestive of malignancy, as shown in many studies.^{25,26} Our results showed an accuracy of 70% using CEUS to differentiate benign and malignant nodules. We had false negatives and false positives in our results. These vascular patterns have two explanations: vascular alterations were promoted by the experimental model used in this study, and the false-negative nodules were well-differentiated HCC or dysplastic nodules.

In conclusion, elastography and CEUS yielded diagnoses of well-differentiated to moderately differentiated HCC with good accuracy in this experimental rat model of NASH. Further studies are needed to evaluate the effectiveness of this method in the diagnosis of HCC in human NASH patients.

CONFLICTS OF INTEREST

All authors have none to declare.

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