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Ultrasonographic characterisation of an experimental model of liver metastases from colon carcinoma in rats

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Abstract
Objective: To determine the ultrasonographic characteristics of liver metastases induced in a rat model; to evaluate the usefulness of ultrasonography in the noninvasive evaluation of tumor infiltration.
Material and methods: We seeded the livers of 46 WAG/RijCrl rats with CC-531 syngeneic colorectal carcinoma cells by intrasplenic inoculation. At 21, 28, 35, 42, 70 days after tumor seeding, we performed a series of ultrasonographic examinations to evaluate tumor induction in different groups of animals: 37 rats were studied with a 10 MHz linear probe and 9 were studied with a 6–18 MHz multifrequency probe. The following signs were considered indicative of tumor development: intrahepatic nodules, sinuate liver borders, lobe enlargement, and extrahepatic masses. Ultrasonographic findings were verified at autopsy. We determined the number of implants, size (less than 3 mm, between 3 and 7 mm, or greater than 7 mm), and lobe location for each technique.
Results: Compared to the autopsy results, ultrasonography detected 64%of the animals with disease. All the extrahepatic masses were correctly diagnosed. Metastases were identified in 90%of the rats with lesions greater than 7 mm, in 75%of those with implants between 3 and 7 mm, and in 25%of those with lesions less than 3 mm. In the group in which we used the 6–18 MHz probe, we detected 50%of the lesions less than 3 mm.
Conclusion: Ultrasonography was useful for monitoring the experimental model and enabled the noninvasive oncologic evaluation of the rat liver with reasonable sensitivity.

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PALABRAS CLAVE
Neoplasia;
Neoplasia del hígado, experimental;
Neoplasia, experimental;
Ecografía;
Experimentación animal

Caracterización ecográfica de un modelo experimental de metástasis hepáticas de carcinoma de colon

Resumen
Objetivo: Determinar las características ultrasonográficas de la patología metastásica hepática inducida en un modelo múrido, para evaluar la utilidad de la ecografía en la valoración no invasiva de la infiltración tumoral.
Material y métodos: El hígado de 46 ratas WAG/RijCrl fue diseminado con células singénicas de carcinoma colorrectal CC-531 mediante inoculación intraesplénica. En los días 21, 28, 35, 42 y 70 posteriores a la siembra tumoral, se realizaron series de ecografías para valorar inducción tumoral en diferentes grupos de animales; 37 ratas se estudiaron mediante sonda lineal de 10 Mhz y 9 con sonda multifrecuencia de 6–18 Mhz. Como signos ecográficos de desarrollo tumoral se consideró la detección de nódulos intrahepáticos, festoneado del contorno hepático, megalias de lóbulos y masas extrahepáticas. Los hallazgos ecográficos se verificaron tras necropsia, y en ambas técnicas se determinó el número de implantes, tamaño (< 3, entre 3–7 y > 7 mm) y localización lobular.
Resultados: Comparado con los resultados de la necropsia, en ecografía se detectaron lesiones en el 64%de los animales con patología. Todas las masas extrahepáticas fueron correctamente diagnosticadas. Se identificaron metástasis en el 90%de ratas afectadas por lesiones > 7 mm, en el 75%de animales con implantes de 3–7 mm y en el 25%con lesiones < 3 mm. Con la sonda de 6–18 Mhz se detectaron el 50%de lesiones < de 3 mm.
Conclusión: La ecografía resultó útil en la monitorización del modelo experimental y posibilitó, con razonable sensibilidad, la valoración oncológica no invasiva del hígado múrido.

Introduction

Colorectal neoplasia is one of the most common tumour processes in the Western world; as the third leading cause of neoplastic disease, it is responsible for approximately 10%of cancer deaths.[1]

Almost half of the patients suffering from colorectal neoplasia will develop hepatic metastases and, in about 15-25%of these, it will already be present at the time of diagnosis.[2,3] The treatment of choice for metastatic disease is surgery, which shows the best long term survival with a five-year survival rate of around 24-38%,[4] which can reach 58%for certain patients.[5] However, only 8-27%of cases will be candidates for surgery.[6]

For patients not suitable for surgical therapy, alternative local therapies have been developed, such as ethanolisation, radiofrequency thermal ablation or transarterial chemoembolisation. However, these procedures are relatively inefficient, and there is therefore a requirement for the development of new therapies that, prior to clinical application, show adequate local antitumour effects and minimal systemic toxicity. Experimental cancer models are useful for estimating the *in vivo* effectiveness of the new therapies. Classically, development of the experimental model itself requires a strict follow-up and control of tumour progression that usually involves culling animals of different series and at different time intervals. Alternatively, diagnostic imaging methods that are able to non-invasively detect and monitor the progression or regression of an experimental neoplastic process could be employed.

The use of ultrasonography in the experimental cancer field has not been evaluated in depth. In this study, the ultrasonographic characteristics of hepatic metastases of colon adenocarcinoma induced in the murine liver were studied with the objective of evaluating the use of ultrasound for non-invasive assessment and monitoring of an experimental cancer model.

Materials and methods

The experimental study was performed on 46 WAG/RijCrl rats (17 male, 29 female) weighing 170–286 g. All procedures conformed to the regulations protecting animals used for experiments and other scientific purposes (Royal Decree 1201/2005, October 10).

The rats were kept in an animal facility under standard conditions: 22ºC average temperature, 55%relative humidity, 12-hour periods of alternating light and darkness, and standard *ad libitum* feed and water diet.

To induce hepatic metastases, the animals were inoculated with syngeneic cells of colon adenocarcinoma

Table 1 Echographic-pathological correlation

Experiment (weeks after tumour inoculation)	Echography Metastases (number)/ size/ location/ semiology	Necropsy Metastases (number)/ size/ location
ECO-I (3)		
1	No	Yes (1) < 3 ML
2	No	Yes (3) < 3 RL + ML
3	No EM	No EM
4	No EM	No EM
5	No	Yes (1) < 3 CL
6	No	Yes (1) < 3 ML
ECO-II (4)		
7	No	Yes (3) < 3 CL
8	No EM	Yes (7) 3-7 CL + LL + EM
9	No	No
10	No	No
11	No	Yes (4) < 3 ML
12	No	Yes (4) < 3 CL
13	Yes (1) < 3 CL s	Yes (3) < 3 CL
14	Yes (1) < 3 ML s	Yes (1) < 3 ML
15	No	Yes (1) < 3 CL
16	Yes (2) 3-7 CL n + le	Yes (9) 3-7 CL + RL
17	No	Yes (5) < 3 CL + ML
18	Yes (1) < 3 CL s	Yes (10) < 3 CL
19	Yes (1) < 3 CL n	Yes (10) < 3 CL
ECO-III (5)		
20	No	No
21	No	No
22	Yes (5) > 7 LL le	Yes (5) > 7 LL
23	Yes (tumour) > 7 CL le	Yes (tumour) > 7 panlobular
24	Yes (tumour) > 7 CL le	Yes (tumour) > 7 panlobular
25	Yes (tumour) > 7 CL le	Yes (tumour) > 7 CL + LL
26	No	Yes (tumour) > 7 CL + RL
27	Yes (2) 3-7 CL n	Yes (6) 3-7 CL + RL + LL
28	No	No
29	No	No
ECO-IV (6)		
30	Yes (tumour) > 7 panlobular le	Yes (tumour) > 7 panlobular
31	No	Yes (1) 3-7 CL
32	Yes (1) 3-7 ML s	Yes (9) 3-7 CL + ML
33	Yes (2) 3-7 LL s	Yes (tumour) 3-7 LL
34	No	Yes (3) < 3 CL + LL
35	Yes (1) > 7 CL le	Yes (tumour) > 7 CL + ML
36	No	Yes (4) < 3 CL + ML
37	Yes (1) 3-7 CL le	Yes (1) 3-7 CL
38	Yes (1) 3-7 CL le	Yes (1) 3-7 CL
39	No	Yes (4) < 3 CL + ML
40	No	No
41	No EM	No EM
42	No EM	No EM
ECO-V (10)		
43	Yes (2) > 7 CL + LL le	Yes (7) > 7 CL + LL
44	Yes (tumour) > 7 panlobular + EM le	Yes (tumour) > 7 panlobular + EM
45	No EM	No EM
46	Yes (tumour) > 7 panlobular + EM le	Yes (tumour) > 7 panlobular + EM

— In the metastases section, a reference is made to their presence or absence and, in parenthesis, to the number of implants found. The term "tumour" is used to denote very profuse infiltrations in which it is not possible to determine the exact number of independent metastasis.
— Ultrasound semiology of tumour development. s: scalloped contour; le: lobe enlargement: n: intrahepatic nodes; tumour: extensive neoplastic infiltration, non-individualised confluent metastases.
— Hepatic location of the metastases. CL: caudate lobe; RL: right lateral lobe; LL: left lateral lobe; ML: medial lobe; EM: extrahepatic tumour implants; panlobular; tumour implants in all hepatic lobes.

(cellular line: CC-531) on day zero of the experiment using the splenic reservoir technique, which can induce metastases in 60-70% of the animals. The inoculation procedure required exposing the spleen through a 3 cm long subxiphoid midline laparotomy, which allowed a direct intra-splenic injection of 250,000 tumour cells suspended in 0.5 ml of Hank's solution using a 27 G needle. Five minutes after the injection, a splenectomy was performed to avoid developing a primary tumour and, finally, the laparotomy was sutured. During the tumour inoculation process, the animals were kept under inhalation anaesthesia with halothane.

The ultrasound was performed using two different clinical systems: one "Logiq 7" model from General Electric Healthcare equipped with a 10-MHz linear probe, and a "MyLab60 XVision" echogram from Esaote equipped with a multifrequency linear probe of 6–18 MHz. Thirty-seven rats were evaluated using the 10-MHz system and nine rats using the multifrequency system. The 46 animals were studied in 5 experimental groups: ECO-I (n = 6), ECO-II (n = 13), ECO-III (n = 10), ECO-IV (n = 13) and ECO-V (n = 4), which were evaluated at 21, 28, 35, 42, and 70 days from the splenic injection of tumour cells. The animals identified as 11 to 19 were studied using the multifrequency probe of 6–18 MHz (table 1). During the ultrasound procedures, intraperitoneal anaesthesia was used with an initial injection of diazepam (5 mg/kg) and an injection of Ketamine (100 mg/kg) 10 minutes later. Before ultrasonography, the top halves of the rats' abdomens were shaved using a standard shaving cream.

The liver of an adult rat is segmented with a smooth contour, distinct edges and a maximum anteroposterior size of 20 mm. Anatomically, several hepatic lobes have been defined: medial right and left, lateral right and left, and caudate. In the ultrasound explorations, the sensitivity of the technique for the diagnosis of hepatic metastatic infiltration and the ultrasound characterisation of the metastases was assessed. In accordance to the criteria used in human pathology,[7] the following findings were considered signs of neoplastic development:

— Intrahepatic nodes of different and distinguishable echogenicity from the surrounding parenchyma.
— Scalloping of the hepatic contour caused by focal bulges on the organ surface.
— Enlargement of the hepatic lobes characterised by disorder of the ultrasound pattern and the convexity of the edges.
— Solid masses located extrahepatically.

Following the ultrasonographic studies, all animals were sacrificed by deep inhalation anaesthesia with ether and cervical dislocation. The livers were extracted and, after dissecting the different lobes, visual analysis was performed to determine the tumour infiltration characteristics. To compare the ultrasound results with the necropsy results, the number of tumour implants, as well as their size and location, was established for both techniques. The replacement of hepatic tissue by profuse neoplastic infiltration is described as tumour involvement and when all lobes are involved, it is called "panlobular". The neoplastic infiltration is classified as nodes < 3 mm, nodes 3–7 mm, nodes > 7 mm.

Samples were also taken from 10 specimens for histological analysis. These samples were placed in 10% formalin and subsequently stained with hematoxylin-eosin for optical microscopy. Microscopy was used to confirm the neoplastic nature of lesions found using ultrasonography and visual analysis.

Qualitative variables (e.g., location, size, and detection) were measured by the rate of occurrence as a percentage of the necropsy findings, which was considered the gold standard.

Results

All experiments showed hepatic neoplastic development in the necropsy studies of 74% of the inoculated rats. The use of ultrasound enabled the detection of neoplastic lesions in 64% of the animals with pathology (table 1).

In the comparative study between the imaging and necropsy findings in relation to the lobular location of the metastases, ultrasound found metastases in 55% of the animals with neoplastic involvement of the caudate lobe. Tumour infiltrations were detected in the left, right and medial lobes in 50, 33 and 33% of the animals. All of the extrahepatic masses were correctly identified, and were not associated with hepatic tumour development in 5 animals.

Using ultrasound, neoplastic lesions were detected in 90% of the rats affected with metastasis > 7mm, in 75% of the animals with implants from 3–7mm and in 25% of the rats with lesions < 3mm. However, it is important to note that the lesions < 3mm were revealed in the studies performed with a multifrequency probe (6–18 MHz); an experiment that, when evaluated independently, showed a 50% of detection rate for these minimal lesions. The size of the extrahepatic masses varied between 5 and 17 mm.

When comparing the ultrasound criteria of tumour development with the necropsy findings, it was possible to correlate the ultrasound signals with the different morphological manifestations of the profuse neoplastic infiltrations. Intrahepatic, rounded and hyperechoic nodes were distinguishable from the surrounding hepatic tissue and, in a later anatomopathological study, were proven to be small metastases surrounded by healthy lobular tissue (fig. 1). The ultrasound findings that related to the scalloping or bulging of the hepatic contour corresponded to small subcapsular metastatic implants (fig. 2). In our study, the enlargement of the lobes was characterised by diffuse bulging of contours, loss of distinct boundaries and a heterogeneous ultrasound pattern, where hyperechoic foci were distributed throughout the hepatic parenchyma. These findings were shown to be the manifestation of profuse tumour infiltrations (fig. 3). Finally, histological studies proved that the solid and lobular masses located extrahepatically were manifestations of tumour implants in the omentum or peritoneum.

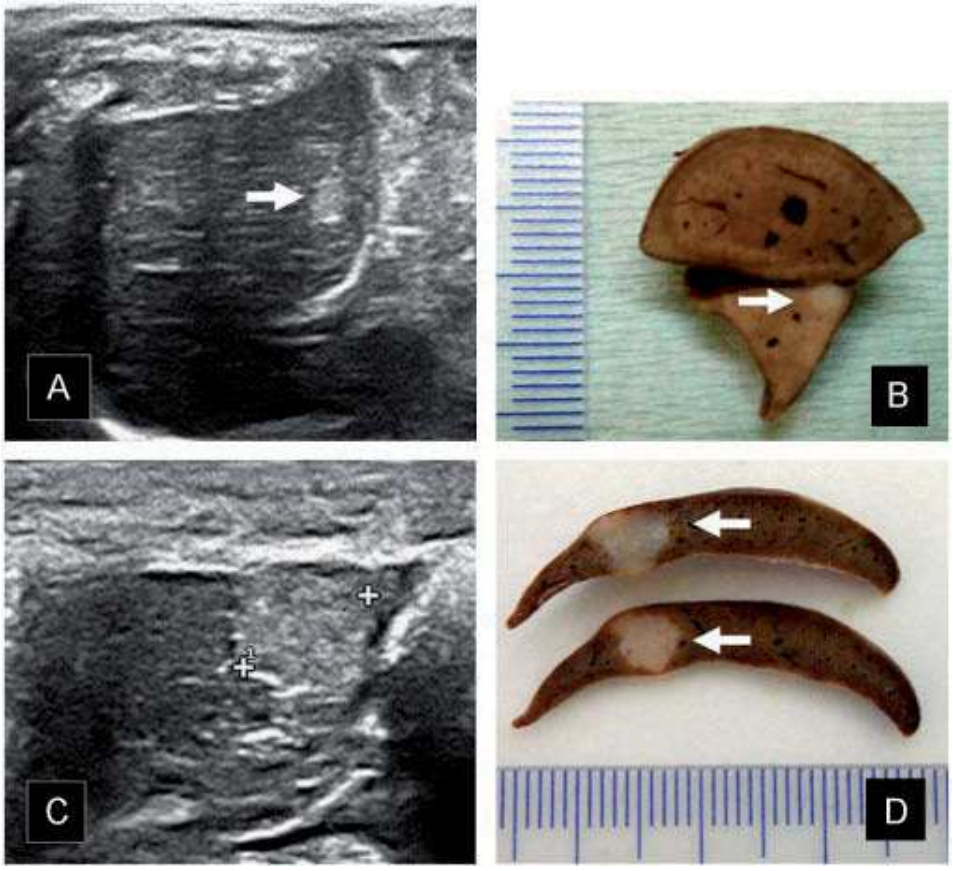

Figure 1 Intrahepatic nodes. A) In the ultrasound with an 18-MHz probe, a 2.5 mm hyperechoic metastasis is identified in the medial caudate lobe (arrow). B) After hepatic extraction and fixation in formalin, the histological incision shows a tumour node of pearly appearance. C) Hepatic ultrasound where a 5 mm diameter hyperechoic mass is observed. It reaches the organ surface and slightly deforms it. D) Histological sections corresponding to the lesion previously described.

Discussion

The oncological studies with animals mimic the human neoplastic process. The artificial model of metastasis induction without primary neoplasia is based on the intravascular injection of a large quantity of anaplastic tumour cells, which enables the study of the late phases of the metastatic process. In our study, the onset of hepatic lesions was triggered by an intrasplenic tumour injection, which is useful for studying metastatic hepatic pathology.[8]

In experimental metastatic systems, the metastatic yield has to be controlled. The histological estimate of the size and number of metastases is a procedure that evaluates the overall quantity of neoplastic tissue and its distribution in the colonised organ parenchyma.[9] It is an ideal system for hepatic pathology since the tumour invasion in the liver is irregularly distributed among the lobes and is frequently located deeply intraparenchymally.[9,10] This study has calculated, via ultrasound, the yield of hepatic metastases in a rat model.

In clinical terms, a series of ultrasonographic signs were established whose identification is correlated with the presence of hepatic tumour pathology.[7] In general, the neoplastic nodes are recognisable since they show different acoustic impedance with respect to the surrounding tissue[11] and, in this context, it is noteworthy that even though the sonographic appearance of the cell line is nonspecific, highly vascularised lesions[12,13] and digestive tube metastasis[14-16] are usually hyperechoic.

With the observation of the different ultrasonographic signals, it is possible to correctly detect pathology in 64%of the animals with malignant lesions. The extrahepatic masses, which occurred during the splenic injection process and are possibly a result of inadvertent extravasation of the neoplastic cells, are simple to diagnose and their presence was correctly determined in all cases. This is most likely due to the notable differences in acoustic impedance that exist between the tumour tissue and the hyperechoic peritoneal fat that envelops these implants. The identification of intrahepatic lesions is more difficult. In previously published works, experimental models of hepatic lesions were studied, such as hepatoma, metastatic murine melanoma or human colon and breast cancer metastasis.[17-19] In these models, the tumour lesions invariably had a hypoechoic appearance and were distinct from the adjacent parenchyma. However, the

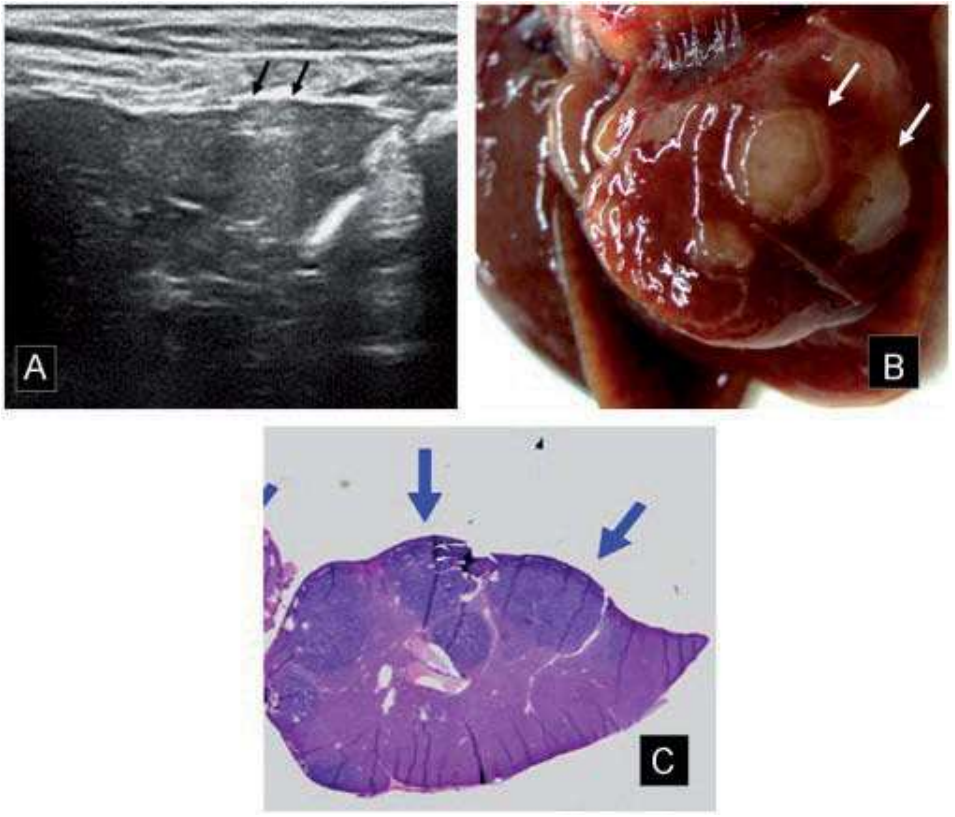

Figure 2 Subcapsular implants, scalloped contour. A) In the ultrasound, 2 hyperechoic lesions bulge the surface of the left caudate lobe (arrows) in an extension of 3mm. B) Necropsy, detail of the caudate lobe. The presence of subcapsular implants detected in the ultrasound is proven (arrows) and a higher number of smaller metastases are also observed. C) Histological section, after fixation in formalin and staining. The neoplastic lesions, of bluish colour (arrows), cause the scalloped surface of the hepatic lobe.

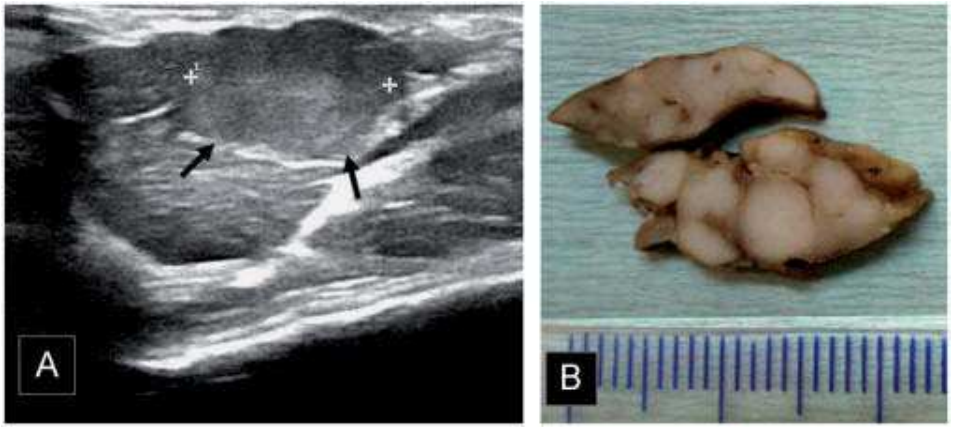

Figure 3 Lobe enlargement. A) Mass of 15 mm that extensively infiltrates the caudate lobe (arrows), which has a swollen appearance. The echogenicity of the mass is similar to that of the liver; however, the deformation of lobular anatomy enables its identification. B) Necropsy, histological cuts after fixing in formalin. Extensive confluent neoplastic metastatic involvement, caudate lobe tumour.

echogenicity of the observed lesions in our model are different; the colon metastases appear as hyperechoic nodes.

One of the essential factors for detecting lesions in their early stages is the acoustic potency of the echographic probes used. In experimental studies of murine hepatoma using 10-MHz probes, the detection limit of neoplastic lesions was established as a 5-mm tumour.[18] In other studies with 15-MHz probes, the limit dropped to 3.7 mm[19] and, with echographic biomicroscopy techniques[20] using 40-MHz probes with an

effective focal depth of 6 mm, it was possible to detect lesions < 0.3 mm in diameter.[17] In our model, it was possible to identify 90 %of the lesions > 7 mm and only 25 %of lesion < 3 mm. However, when considered in isolation, it was possible to identify 50 %of the lesions < 3 mm with a multifrequency linear probe of 6–18 MHz. It is evident that a higher sonic frequency enables the detection of lesions in earlier stages. However, when choosing ultrasound equipment, the type of animal studied needs to be taken into consideration. To evaluate the entire rat liver, it is necessary to reach an exploration depth of up to 25 mm, a requirement that limits the use of high frequency probes. Therefore, when selecting ultrasound equipment for experimental use, an appropriate balance between spatial resolution and depth of ultrasound penetration has to be found.

This study has limitations, above all derived from the small number of explorations that could be done with the higher frequency probe. However, in view of the scarce literature related to the technical use of non-invasive monitoring in the experimental cancer field, we believe it is interesting to present these preliminary results as they can provide a basis for more exhaustive research and that provide data of greater statistical relevance.

In conclusion, it is our experience that it is possible to perform echographic studies of the whole rat liver with an acceptable sensitivity for neoplastic detection, especially when using a relatively high (18 MHz) multifrequency probe. Based on these considerations, ultrasound has to ability to non-invasively monitor the natural progression of tumor progression and evaluate the efficiency of potential anti-neoplastic therapies.

Conflict of Interests

The authors have no conflicts of interest to declare.

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J.J. Echevarria: responsible for the conception, design, data collection and writing.

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All authors have read and approved the final version of this article.

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