Role of Diffusion Weighted Magnetic Resonance Imaging in Evaluation of Hepatocellular Carcinoma Post Chemoembolization

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMMR/2022/v34i1031355

Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/85815

Received 07 February 2022
Accepted 17 April 2022
Published 21 April 2022

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the fifth most common type of cancer in the world and the third most prevalent frequent death reason in patients who suffers from malignant tumors. The apparent diffusion coefficient (ADC) is a method for measurement of the water movement within tissues. Tumors that are viable have a high cellularity, and the cells’ membranes are intact which water molecules' mobility is restricted, leading to a small ADC. But from the other hand, necrosis increases the permeability of the cell of the membrane, permitting water molecules to circulate freely and resulting in an elevation in the ADC’s relative value. This study aimed to describe the use of diffusion weighted MRI to evaluate hepatocellular carcinoma in the setting of trans-arterial chemoembolization (TACE).

Methods: This study was carried out on 30 patient (males: 22, eight females) aged 42 to 75 years with biopsy-proven HCC, candidate for trans arterial hepatic chemoembolization as a plan of treatment. They were referred from Oncology Department to MRI unit in radiology department over a period of 1 year starting in June 2018 to June 2019.

Results: According to diffusion weighted Imaging (DWIs) and ADC values, there were 4 TP (13.33%), 22 TN (73.33%), 2 FP (6.66%) and 2 FN (6.66%) studies. The P-value by Fisher’s Exact was <0.05 denoting agreement between the DWI and ADC findings and the standard of references. Regarding the false negative studies by diffusion MRI, On diffusion images, there was

*Corresponding author
a loss of signal or a mild sustained hyperintensity with a bright ADC map, so it was considered as well ablated lesion. Follow up triphasic CT or dynamic MRI after 3-6 months shows lesion enhancement at arterial phase and wash out at both venous and delayed phases with restricted diffusion. The optimal cutoff value for sensitivity and specificity is 1.06; at this ADC value, the sensitivity is 66.66 percent and the specificity is 91.66 percent.

**Conclusions:** ADC obtained from diffusion-weighted magnetic resonance imaging has developed into a useful biomarker for the response of tumours to therapy. Analysis of the ADC value is helpful in detecting the presence of residuals in non-successfully ablated lesions.

**Keywords:** Magnetic resonance imaging; hepatocellular carcinoma; trans-arterial chemoembolization; apparent diffusion coefficient.

1. **INTRODUCTION**

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer in the known universe and the third most prevalent a frequent reson of death in patients who suffers from malignant tumors [1].

The major risk confounders for HCC are liver cirrhosis and Infection with HBV/HCV. Additionally, additional variables such as non-alcoholic steatohepatitis (NASH) and aflatoxin are significant and prevalent in certain regions of the globe [2].

Recent successful treatments for HCC include liver resection, transplantation, and a variety of local ablative and trans-arterial therapy. Although surgical resection and liver transplantation are excellent curative therapy, only around 20% of patients, most of whom are discovered via routine screening, may benefit from these surgical procedures [3].

Instead of surgery, the disease has been treated using a variety of minimally invasive techniques. These involve transcatheter arterial chemoembolization (TACE) and percutaneous therapy, such as percutaneous microwave coagulation therapy, percutaneous ethanol injection therapy, and radiofrequency ablation therapy [4].

TACE has the greatest effect on the tumour of any chemotherapy agent by injecting chemotherapeutic agents selectively or superselectively into tumour vessels and by embolization of particles, reducing tumoral blood flow as a result, the tumour remains in prolonged contact with the chemotherapeutic agents [5].

TACE efficacy monitoring with imaging is critical for assessing the success of treatment and directing upcoming therapy. Moreover, techniques of imaging and response criteria for imaging have had a limited capacity to produce clinically useful information regarding the magnitude of tumour ablation [6]. Unenhanced CT confirms successful introduction of the chemoembolization mixture into the selected lesions. Assessing contrast enhancement in a tumour with remaining iodized oil on contrast-enhanced computed tomography, on the other hand, can be complicated as a result of the beam's hardening artefacts caused by iodized oil's high resistance to X-ray radiation. The presence of iodized oil has no effect on the MRI signal intensity; As a result, MRI provides a more precise definition of a residual viable tumour [4].

MRI provides anatomical, functional, and molecular parameters for assessing treatment response. T1- and T2-weighted images without contrast reveal changes in morphology, adequacy of fluid in the body, and fibrosis, and dynamic contrast enhanced MRI can deliver information on perfusion. Contrast-enhanced MRI is susceptible to changes associated with treatment with regard to blood volume and vascularity, that is thought to be related to tumour angiogenesis [7,8].

Tumors that have been successfully treated with TACE are typically exchanged with necrosis and a reduction in interstitial tissue fluid as a result of the concurrent arterial occlusion. On dynamic contrast-enhanced MRI enhancement following TACE may represent granulation tissue or residuum of tumour in conjunction with necrotic areas. Distinction is made according to the enhancement phase. Tissue with granulation has a rescheduled rate of while remaining tumoral tissue uptake causes early arterial enhancement, indicating that TACE treatment was unsuccessful [9].

ADC estimated in diffusion-weighted MRI has developed into a favourable and encouraging biomarker of therapeutic response in tumours. ADC is a parameter that quantifies the water
mobility within tissues. Viable tumors have a high degree of cellularity, with intact cell membranes, which limits water molecule motion, as a result of which the ADC is relatively low. On the other hand, necrosis rises the permeability of the membrane, permitting molecules of water to move freely and resulting in a rise in ADC relative to normal [6,10].

This study aimed to describe Diffusion-weighted MRI's role in evaluation of hepatocellular carcinoma post-TACE.

2. PATIENTS AND METHODS

This research was conducted on 30 patients (22 males, 8 females) both with biopsy-proven HCC, candidate for trans arterial hepatic chemoembolization as a plan of treatment. They were referred from Oncology Department to MRI unit in radiology department over a period of 1 year starting in June 2018 to June 2019.

Exclusion criteria were patients who disagreed to join the research, contraindications to MRI as having any metallic implants as pacemaker, aneurysm clips, joint replacement or any other electr., claustrophobic patients, Patients with moderate to end-stage renal disease or a serum creatinine level of greater than 2 mg/dl, Uncooperative cases with ental and behavioral disorders, cases who were medically unstable with low vital signs or having consciousness disorders.

All patients in this study were submitted to the following: full history, laboratory investigations (Serum creatinine with e GFR), radiological examination.

2.1 Patient Preparation

1. No specific preparation of the patients, such as fasting or not drinking, was needed before MRI. 2. The patients were asked about any contraindication as cardiac pacemaker, aneurysm clips, joint replacement or any other electr, claustrophobic patients, Patients with moderate to end-stage renal disease or a serum creatinine level of greater than 2 mg/dl, Uncooperative cases with ental and behavioral disorders, cases who were medically unstable with low vital signs or having consciousness disorders.

All patients in this study were submitted to the following: full history, laboratory investigations (Serum creatinine with e GFR), radiological examination.

2.2 MRI Protocol

a. Pre-contrast standard study include Axial T1WIs (TR=10msec, TE=4.6msec, flip angle=15°), Axial T2 WIs (TR=560msec, TE=26-28msec, flip angle=90°), Axial T2 sat suppression (SPAIR) (TR=560msec, TE=26-28msec, flip angle=90°), these sequences were performed using FOV=315-350 mm, slice thickness=7mm, interval=2mm for all axial pre-contrast sequences.

b. Diffusion weighted Imaging (DWIs): diffusion MR images were conducted prior to the dynamic investigation utilising breath eliciting a single fat-suppressive injection spin echo echoplanar sequence that incorporated diffusion gradients in the direction of phase encoding, and frequency encoding ,section selection, and data recording by EPI read out.

- It is determined by the application of the b values 0, 500 and 1000 sec/mm². - The parameters for acquisition were as follows:(TR/TE: 1700/76msec), matrix 120x95, FOV minimally possible, slice thickness 7mm, Interval 2mm, scan time 3-6min.

c. Study with enhanced dynamic contrast: To circumvent the contrast agent's effect on the value of ADC, a dynamic examination was conducted following the diffusion study, Following a bolus injection of 0.1 mmol/kg body weight of Gd-DTPA, a dynamic study was conducted, the antecubital vein was flushed with 20ml of sterile saline solution. Contrast media and saline solution injections were performed manually, The technique of dynamic imaging was applied in a triphasic fashion; A dynamic series was made up of one sequence of pre-contrast images and four consecutive post-contrast series were performed, which include late arterial, early arterial, and portal. Venous phase imaging having intervals value of 18-21 seconds (17-20 s) for image acquisition while holding one's breath and one second for prior to the start of each phase imaging, followed by a 5-minute delayed phase imaging. All cases were imaged at the end of expiration, to minimise the risk of image misregistration. The acquisition parameters were 3.3–4.5/1.4–1.9; the flip angle was ten; the number of averaged signals was one; the parallel imaging factor was 1.8; the matrix size was 172x135; the field of view was 300–400 mm; and the slice thickness was 2–3 mm.

d. The ADC map and the ADC value are as follows:

- On the workstation, pixel-based ADC maps were generated.

ADC calculations were performed using the three b values (0, 500, and 1000 sec/mm²).
Calculating the ADC value is a fully automated process that can be accessed via the workstation.

2.3 Statistical Analysis

SPSS was used to conduct the statistical analysis v26 (IBM Inc., Chicago, IL, USA). The mean and standard deviation of quantitative variables were calculated (SD) and compared between the two groups utilizing unpaired Student's t-test. The frequency and percentage values for qualitative variables were used (percent) and were examined using the Chi-square test or, when adequate, Fisher's exact test. Statistical significance was defined as a two-tailed P value of 0.05.

3. RESULTS

Demographic data were represented in Table 1.

Virus C was the most common risk factor of the studied patients as it was found in 26 patients (88%), virus B was found in 2 patients (6%) and Bilharziasis was found in only 2 patients (6%) while chronic alcoholic liver disease wasn't found in the patients in this study Fig. 1.

The patients were classified according to Child Pugh score (Table 3) as follow; 20 patients (66.6%) with class A and 10 patients (33.4%) with class B, however, no patients were classified as class C. Tables 2, 3.

The diameter of the analyzed lesions ranged from 1.6 to 8.6 cm with a mean of 5.9 and SD ± 1.83, there is no significant changes of lesions diameter before and after chemoembolization. Maximum, minimum, and mean diameters of the lesions Table 2.

<table>
<thead>
<tr>
<th>Age Subgroup (in years)</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>&lt; 50</td>
<td>4 (13.3%)</td>
<td>2 (6.7%)</td>
<td>6 (20%)</td>
</tr>
<tr>
<td>50 ≤ 65</td>
<td>12 (40%)</td>
<td>4 (13.3%)</td>
<td>16 (53.3%)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>6 (20%)</td>
<td>2 (6.7%)</td>
<td>8 (26.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>22 (73.3%)</td>
<td>8 (26.7%)</td>
<td>30 (100%)</td>
</tr>
</tbody>
</table>

Fig. 1. Risk factors for HCC in the studied 30 patients
Table 2. Classification of the studied 30 patients according to child pugh score

<table>
<thead>
<tr>
<th>Child Pugh score</th>
<th>N. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class A</td>
<td>20 (66.6%)</td>
</tr>
<tr>
<td>Class B</td>
<td>10 (33.4%)</td>
</tr>
<tr>
<td>Diameter of the lesions in cm</td>
<td>5.9 ± 1.83</td>
</tr>
</tbody>
</table>

**Child-Turcotte-Pugh Classification for Severity of Cirrhosis**

<table>
<thead>
<tr>
<th>Clinical and Lab Criteria</th>
<th>Points*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>None</td>
</tr>
<tr>
<td>Ascites</td>
<td>None</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>&gt; 3.5</td>
</tr>
<tr>
<td>Prothrombin time</td>
<td></td>
</tr>
<tr>
<td>Seconds prolonged</td>
<td>&lt;4</td>
</tr>
<tr>
<td>International normalized ratio</td>
<td>&lt;1.7</td>
</tr>
</tbody>
</table>

*Child-Turcotte-Pugh Class obtained by adding score for each parameter (total points)*

- **Class A**: 5 to 6 points (least severe liver disease)
- **Class B**: 7 to 9 points (moderately severe liver disease)
- **Class C**: 10 to 15 points (most severe liver disease)

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Fig. 2. Child Pugh classification and score

After full interpretation of MRI images, the signal intensity of the lesion was analyzed in all sequences as follow:

**Pre contrast T1&T2 MRI studies:**

In the pre-embolization study, the signal intensity on T1&T2WIs was variable; T1WI revealed 18 hypointense, 6 isointense lesions and 6 lesions with heterogenous intensity. T2WI revealed 16 hyperintense, 6 hypointense, 2 isointense lesions and 6 lesions with heterogenous intensity, In the post-embolization studies, the signal intensity on T1&T2WIs was also variable; T1WI revealed 14 hyperintense, 4 hypointense, 4 isointense lesions and 8 lesions with heterogenous intensity. T2WI revealed 4 hyperintense, 12 hypointense, 6 isointense lesions and 8 lesions with heterogenous intensity Fig. 4.

Regarding the false positive studies by dynamic MRI, the lesions in the post-embolization studies had a perilesional early arterial enhancement with wash out on delayed phase becoming iso to hypointense to surrounding liver. So, they were considered as residual tumors. Follow up triphasic CT studies after 3 months were free. Findings were in favor of perilesional arterioporal shunt.

According to DWIs and ADC values, there were 4 TP (13.33%), 22 TN (73.33%), 2 FP (6.66%) and 2 FN (6.66%) studies. The P-value by Fisher’s Exact was <0.05 denoting agreement between the DWI and ADC findings and the standard of references.
Fig. 3. The signal intensity of the 30 analyzed lesions on T1 and T2 WIs in pre-TACE MRI studies

Fig. 4. The signal intensity of the lesions on T1 and T2 WIs in post-TACE MRI studies

Table 3. Total results of the dynamic MRI and the diffusion MRI and ADC map studies of the 30 patients

<table>
<thead>
<tr>
<th>Final result</th>
<th>Study result</th>
<th>-ve</th>
<th>+ve</th>
<th>FEp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TN</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>-ve</td>
<td>24</td>
<td>80%</td>
<td>6</td>
<td>20%</td>
</tr>
<tr>
<td>+ve</td>
<td>18</td>
<td>60%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>FP</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>+ve</td>
<td>12</td>
<td>40%</td>
<td>6</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>TP</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>80%</td>
<td>2</td>
<td>6.66%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20%</td>
<td>2</td>
<td>6.66%</td>
</tr>
</tbody>
</table>

TN: True negative, TP: True positive, FN: False negative, FP: False positive, MRI: magnetic resonance imaging, ADC: apparent diffusion coefficient
Regarding the false positive studies by diffusion MRI, there was sustaining hyperintensity in the diffusion images compared with the signal drop of background liver parenchyma with increasing b factors combined with low or isointensity signal in ADC map. Follow up CT after 3 months was free. Regarding the false negative studies by diffusion MRI, there was lost signal on diffusion images or mild sustained hyperintensity with bright ADC map, so it was considered as well ablated lesion. Follow up triphasic CT or dynamic MRI after 3-6 months shows lesion enhancement at arterial phase and wash out at both venous and delayed phases with restricted diffusion Table 3.

After analysis of the ADC value:

The different ADC values elicited from the corresponding ADC maps were calculated. The difference between ADC variables among the malignant residue and well-ablated groups were statistically significant P value 0.048. The ROC curve obtained by plot at different cut off values is shown in (Fig. 3). The best cut off that maximizes sensitivity and specificity is 1.069, at this ADC value the sensitivity is 66.66% and specificity is 91.66%.

A statistical software showed that the area under the curve is C=0.856 with CI from 0.724 to 0.938. It seems from the ROC that ADC variable is a good indicator to differentiate marginal recurrence of HCC from perilesional pseudo- or benign lesions (Area under the curve: - excellent 0.9-1, good 0.8-0.9, fair 0.7-0.8, poor 0.6-0.7, fail 0.5-0.6) Fig. 5.

4. DISCUSSION

HCC is the majority frequently occurring primary liver cancer. It argues over 500,000 lives each year and is the world’s 3rd most frequent aggravate of cancer death. The majority of cases who are deemed unsuitable for resection/transplantation possess fewer invasiven procedures aiming for local control of the disease [11].

Here is some proof that MRI is more precise in detecting recurrent or residual tumours than other radiological modalities [5].

MRI is used to distinguish zones that have been completely treated from those that contain residual tumour. The characteristics of MRI enhancement are indicative of efficacy of treatment and may indicate the requirement for additional intervention. Enhancement of tumour growth is used as a disease-response biomarker [12]. Techniques for functional MRI, as an example diffusion-weighted imaging (DWI), are
able of identifying changes in a timely manner in the MR signal within tissues within a few weeks of treatment, according to the degree of integrity of the cell membrane [13].

Viable tumour cells’ intact membranes obstruct water diffusion, whereas increased water diffusion is observed in necrotic tumour cells with disrupted cell membranes. The apparent diffusion coefficient is a constant that quantifies the mobility of water (ADC). Thus, tumour necrosis is related to increased in the ADC value, enabling for the distinction of viable and necrotic tumour portions [14].

According to dynamic MRI images after chemoembolization, Youssef et al. [15] stated that 47.5% of cases had no enhancement and 52.5% of cases had persistent enhancement, while Elsaid, et al. [16] stated that 37% of cases had no enhancement and 63% had a residual enhancement for reader 1, while 33.3% of cases had no enhancement and 66.7% had residual enhancement for reader 2, but in this study 60% of cases had no enhancement and 40% had residual enhancement.

In this work out of 30 patients with HCC & who had undergone transarterial chemoembolization, 24 patients revealed no residual tumour tissue. The remaining six cases, on the other hand, demonstrated residual tumour tissue.

DWI had a 66.66% sensitivity, a 91.66% specificity, a 66.66% positive predictive value, and a 91.66% negative predictive value.

Youssef et al. [15] stated higher sensitivity of 100%, 100%, 82%, 76.47% and 83.9% respectively. However, Goshima, et al. [17] stated that diffusion-weighted imaging has a lower sensitivity of (60.7%).

Yu et al. and Ebeed et al. stated that DWI positive predictive value 68% and 70% respectively that almost corresponding with this study. However, Elsaid et al. [16] and Youssef et al. [15] recorded PPV of about 82.76%, 92.86% and 72% respectively which are higher than this study value.

The data on DWI used to assess response can still be extremely heterogeneous, this is most likely due to the fact that the study protocols and MR hardware and software used were different [13].

Yu et al. [18] mentioned that when DWI and Gd-MRI were combined, DWI increased sensitivity compared to Gd-MRI alone (92 vs. 85 percent; p = 0.125), but only in a non-significant way, whereas specificity reduced from 65 to 50%. As a result, DWI appears to be of limited to no value for detecting recurrences in this literature.

In the studies Holtas, et al. [19] and Tung, et al. [20] of sterile liquefactive necrosis and intracavitary microhemorrhage are recognised as the reason for hyperintensity in diffusion-weighted MR images of necrotic malignant lesions.

Thus, we hypothesised in this study that the cases that are false positive are most likely the result of liquefactive necrosis or intralesional haemorrhage (hyperintense on T1WIs), which restricts diffusion.

In Ebeed, et al. [21] study, He utilized b values of 50, 400, and 800 sec/mm2 to prevent the intravoxel perfusion effect associated with low b values (fewer than50) and to maximise lesion characterization accuracy. Elsaid, et al. [16] used b values of 0,300 and 600 s/mm².

Oure result revealed that by using the ROC analysis, the ADC variable is a reasonable predictor of HCC marginal recurrence in comparison to pseudo- or benign perilesional lesions.

Ebeed, et al. [21] mentioned that the variation in the ADC variables seen between malignant residual and well eliminated groups was statistically significant (P = 0.009).

In Youssef, et al. [15] study, The ADC values that were determined revealed a significant difference (P value 0.05) between those obtained in active tumoral regions and those obtained in necrotic regions. The values of area under the curve for ADC was 0.7, and the maximum combined sensitivity and specificity were 79.6% and 69.6%, respectively, at the value of ADC cutoff 1.395 mm²/sec.

In El Said, et al. [16] study it was unable to estimate from the ROC curve an ADC cut off value due to the fact that the insignificant AUC and necrotic tissue may be misidentified as necrotic as a result of well-differentiated HCC.

Comparable to what was observed by Yuan, et al. [22] who indicated that, on occasion, necrotic and viable tumour tissues may be hard to characterise solely based on DW-MRI visual assessment. Nonetheless, We might be able to
compute an average of post-treatment for lesions that responded well and those that did not.

A study of Yu, et al. [18], ADC was used to interpret the marginal recurrence of HCC from pseudo- or benign lesions in the perilesional region. The findings of This study revealed no statistically significant differences; the ADCs differed for non-tumorous lesions and overlapped significantly with the viable tumour portion, making any cutoff points not possible to establish.

In Mannelli, et al. [23] study, The findings indicate that a weaker correlation of ADC with the degree of histopathologic tumour necrosis than contrast-enhanced subtraction MRI. But even so, the diagnosis of complete tumour necrosis showed no difference between the two methods. They believe that DWI can be used in place of contrast-enhanced MRI based on the findings of their study in the management of cases who cannot have gadolinium contrast material administered, like those with renal insufficiency.

Wang, et al. [24] results revealed that while the ADC value may be a useful tool for assessing response of HCC, it may be less sensitive to tumour recurrence than DCE MR imaging. Goshima, et al. [17] asserted that DWI was not a consistent predictor of post treatment local HCC recurrence when compared with gadolinium-enhanced MRI, although he found significant increases in ADC values, it was incapable to ascertain value of ADC cut off as a result of the wide range of calculated ADC values.

Yuan, et al. [22] stated that, DWI is capable of quantifying HCC tumour necrosis, and the value of ADC can be used to distinguish between necrotic and viable tumour tissues. Furthermore, DW-MRI enhanced the detection of liver lesions. As a result, DWI may be a viable option for monitoring HCC patients following chemoembolization in the short term and may be used to direct patient management in order to minimise CT examination-related radiation exposure and the risk of nephropathy caused by contrast material.

From the above findings We recommend DW MRI as a beneficial adjunct method that enhances the sensitivity and specificity of DCE MRI, particularly in cases who are unable to maintain their breath properly, resulting in a degraded DCE MR quality.

Gluskin, et al. [25] according to the authors, the primary advantage of diffusion MRI images is that they improve confidence in the diagnosis of HCC, particularly when it is not possible to administer contrast intravenously or when small lesions adjoining to vessels are present.

5. LIMITATIONS
First, histopathologic correlation was challenging because cases were not candidates for liver transplantation or lesion resection., Second, some inescapable errors including the partial volume effect occur in placement of ROI for ADC measurement value particularly with lesions <2cm in diameter.

6. CONCLUSIONS
ADC obtained from diffusion-weighted MRI has developed into a useful biomarker for tumour response to therapy. Analysis of the ADC value is helpful in detecting the presence of residuals in non-successfully ablated lesions.

The ADC variable was reported to be a reasonable predictor of marginal recurrence of HCC in comparison to benign or pseudo-lesions.

CONSENT
A signed informed consent was acquired from the patient or relatives of the patients.

ETHICAL APPROVAL
The study was done after approval from the Ethical Committee Tanta University Hospitals.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/85815