Original Article

Wash-out of Hepatocellular Carcinoma: Quantitative Region of Interest Analysis on CT

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Abstract

Introduction: This study aims to determine if the quantitative method of region-of-interest (ROI) analysis of lesion attenuation on CT may be a useful adjunct to the conventional approach of diagnosis by visual assessment in assessing tracer wash-out in hepatocellular carcinomas. Materials and Methods: From a surgical database of 289 patients from 2 institutions, all patients with complete surgical, pathological and preoperative multiphasic CT scans available for review were selected. For each phase of scanning, HU readings of lesion obtained (Lesion$_{arterial}$, Lesion$_{PV}$ and Lesion$_{equilibrium}$) were analysed using receiver operating curves (ROC) to determine the optimal method and cut-off value for quantitative assessment of tumour wash-out (Lesion$_{arterial - equilibrium}$, Lesion$_{PV - equilibrium}$ or Lesion$_{peak - equilibrium}$). Results: Ninety-four patients with one lesion each met the inclusion criteria. The area under the curve (AUC) values for Lesion$_{arterial - equilibrium}$ (0.941) was higher than the AUC for Lesion$_{pv - equilibrium}$ (0.484) and for Lesion$_{peak - equilibrium}$ (0.667). Based on ROC analysis, a cut-off of 10HU value for Lesion$_{arterial - equilibrium}$ would yield sensitivity and specificity of 91.5% and 80.9%, respectively. ROI analysis detected 9/21 (42.9%) of lesions missed by visual analysis. Combined ROI and visual analysis yields a sensitivity of 82/94 (87.2%) compared to 73/94 (77.7%) for visual analysis alone. Conclusion: Using a cut-off of 10 HU attenuation difference between the arterial and equilibrium phases is a simple and objective method that can be included as an adjunct to visual assessment to improve sensitivity for determining lesion wash-out on CT.

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Key words: Arterial hypervascularity, Region-of-interest analysis, Wash-out

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. It is estimated to cause approximately half a million deaths annually and is an epidemiologically important tumour.[1] HCC arises mainly from patients with pre-existing chronic viral hepatitis and serological markers of chronic viral infection serve both to identify patients at risk as well as contribute supporting evidence for a diagnosis of HCC.

The current diagnosis of HCC is highly dependent on dynamic radiological imaging. Several guidelines exist in this respect. According to the guidelines of the European Association for Study of the Liver (EASL) guidelines, the diagnosis of HCC is established if 2 imaging modalities [ultrasound, computed tomography (CT) or magnetic resonance imaging] show a coincidental nodule with arterial hypervascularisation in a cirrhotic liver regardless of Alpha-fetoprotein (AFP) levels, or if a single modality shows a lesion when the AFP levels are more than 400 ng/mL.[2] In the guidelines of The American Association for Study of Liver Diseases (AASLD), a second modality to confirm the lesion is not necessary if (i) the lesion is greater than 2 cm, (ii) the liver is cirrhotic, and (iii) the lesion shows arterial hypervascularity and washes out in the portal

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venous (PV) or equilibrium phase.[3] A lesion is usually deemed hypervascular when it is hyperdense in relation to the surrounding liver in the hepatic arterial dominant phase and deemed to show wash-out when it becomes hypodense to the surrounding liver in the equilibrium phase.[4,5]

In addition, the AASLD also recommends that if AFP is greater than 200 ng/mL and the mass is greater than 2 cm in diameter with radiological appearance suggestive of HCC (large and/or multifocal disease with arterial hypervascularity) in the presence of cirrhosis, the likelihood of HCC is high and biopsy is not essential. Liver biopsies have attendant risks of hemorrhage and seeding (1% to 3%) and the practice of liver biopsy for lesions with typical radiological appearances is not common in dedicated liver oncology centres.[6]

The above consensus statements are predicated on establishing whether a lesion demonstrates arterial hypervascularisation or wash-out in the PV or equilibrium phase. Hence the presence of wash-out is critical to the diagnosis of HCC. Currently, the diagnosis is established qualitatively by visually comparing density of the lesion with the background liver. This method may sometimes fail if the lesion is perceived to be isodense to the surrounding liver. It is also subjected to intraobserver and interobserver variability. Quantitative analysis of lesion attenuation on CT can be easily performed using region of interest (ROI) analysis, a function that is available in most modern picture archival and communication systems (PACS).

In this study, we sought to determine the optimal method and cutoff value for determining wash-out based upon Hounsfield unit (HU) readings of the lesion. We hypothesise that region of interest (ROI) analysis can improve the conventional method for the diagnosis of HCC wash-out on CT by visual analysis. By applying the optimal cut-off value ($HU_{cut-off}$), we combined ROI analysis with visual analysis to determine if this is true. To the best of our knowledge, there has been no published scientific data on this matter.

Materials and Methods

We conducted a retrospective review of a total of 289 histologically confirmed HCC patients derived from a surgical resection database from 2 institutions (National Cancer Center, Singapore and the Singapore General Hospital) with a common hepatopancreatobiliary surgery program. All cases were resected over a period of 4.3 years (between 7 August 2003 and 17 December 2007). Approval was obtained from the institutional review boards for this study. Surgical, histopathological and radiological records (including preoperative CT scans) available for all cases on the hospital inpatient electronic records system common to both institutions were reviewed. The patients' gender, age,

date of scan, date of surgery, histological grade and size of the lesions were abstracted. One hundred patients with complete records available for review, including surgical notes, pathological grading and preoperative multiphasic CTs (the reports as well as images on the integrated hospital PACS) were included in the study, while those who did not were excluded from the study.

All patients had undergone multiphasic CT scans of the liver (including arterial, PV and equilibrium phases) at one of the 2 study institutions. A total of 7 different helical CT scanners were employed (Asteion, Toshiba, Tokyo, Japan; XPRESS GX, Toshiba, Tokyo, Japan; MX 8000 IDT 16, Philips Medical Systems, Best, The Netherlands; Somatom Sensation Cardiac, Siemens Medical Solutions, Erlangen, Germany; Dual Source Somatom Definition, Siemens Medical Solutions, Erlangen, Germany; Aquillon 64, Toshiba, Tokyo, Japan; Lightspeed VCT 64, GE Medical Systems, Milwaukee, Wisconsin, USA) .

All images were acquired using the following parameters: voltage, 120kV; tube current, 180 to 230 mAs; matrix size, 512 x 512; reconstruction slice thickness, 5 or 7.5 mm (depending on the machine used). Each patient underwent bolus injection of 100 mls of non ionic iodinated contrast (Omnipaque 350, Nycomed Imaging AS, Oslo, Norway) at an injection rate of 3 to 4 mls per second. Bolus tracking over the aorta at the level of the celiac axis (threshold 100 HU) was used to time the arterial phase. The PV and equilibrium phases were scanned at an average delay of 45 seconds and 3 minutes, from the time of injection, respectively.

The CT scans were assessed for adequacy of the arterial phase by looking for well opacified hepatic arteries, faint portal veins, zebra patterns of the spleens and cortical nephrograms. Five patients were excluded due to technically inadequate arterial phase.

Qualitative assessment was performed by a team comprising 2 diagnostic radiologists reading in consensus. A representative CT section containing the lesion was selected. The lesions were observed for their enhancement pattern, relative to the surrounding liver (hypodense, isodense and hyperdense) during each phase of the CT study. Wash-out was considered to be present if the lesion was hypodense to surrounding liver in either the PV or equilibrium phases. Isodensity on PV and equilibrium phase was not considered as wash-out even if the lesion was hyperdense on the arterial phase.

Quantitative assessment was performed separately by one of the two radiologists based on the selected representative section. Sufficient time was allowed to overcome learning effects. ROI analyses of the lesions as well as the surrounding liver were obtained individually for each phase of the scan. A lesion with a diameter smaller than 1 cm (radius <5 mm)

is assumed to be too small to be accurately characterised and would have been excluded. The area of the corresponding circle ROI should be at least 78 mm^2. Hence we excluded one patient where the area of the ROI obtained was less than 78 mm^2. The total study population was thus 94 patients.

For large, heterogeneous lesions, hypodense necrotic areas were avoided. An attempt was made to place the ROI in the area showing maximum density on the arterial phase that was measureable (at least 78 mm^2). The area (mm^2) and mean Hounsfield unit (HU) for each ROI reading was recorded.

For all cases, we recorded the CT attenuation of lesion in the arterial (Lesion$_{arterial}$), PV (Lesion$_{PV}$) and equilibrium (Lesion$_{equilibrium}$) phases, as well as the corresponding CT attenuation of surrounding liver tissue in the respective phases. For all cases, we determined the lesion wash-out based on (i) the difference in CT attenuation of the lesion between the arterial and equilibrium phases (Lesion$_{arterial - equilibrium}$), (ii) the difference in CT attenuation of the lesion between the portal venous and equilibrium phases (Lesion$_{PV - equilibrium}$) and (iii) the difference in the CT attenuation of the lesion between the peak attenuation (arterial or portal venous, whichever was higher) and equilibrium phases (Lesion$_{peak - equilibrium}$).

The Lesion$_{arterial - equilibrium}$, Lesion$_{PV - equilibrium}$ and Lesion$_{peak - equilibrium}$ attenuation differences were tabulated accordingly. Receiver operating characteristic (ROC) analyses were performed to determine the method and HU$_{cut-off}$ that provided optimal sensitivity and specificity. The discriminatory property of each parameter was assessed by calculating the area under the curve and the associated 95% confidence intervals. Data analysis was performed in Stata V 10.0 (Stata Corp, College Station, Tx, USA) and level of significance was set at 5%.

For the method that provided the best results, we further performed a stratified analysis, looking at the results by the subgroups of the various grades of tumour differentiation. We further calculated the number of lesions for which would have been considered to show wash-out by ROI analysis by applying to our series the HU$_{cut-off}$ that provided optimal sensitivity and specificity.

Results

Ninety-four patients with one lesion each were studied. The mean age of the patients was 63.7 years (range, 19 to 84). There were 77 males and 17 females. The mean of the longest diameter of the lesions was 5.6 cm (range, 1.2 to 16.7). The mean measured area of ROI was 142.4 mm^2 (range, 79 to 323). The peak attenuation of the lesions was seen in the arterial phase in 26 cases (27.7%), and in the PV phase in 67 cases (71.3%). There was no change in

HU between the arterial and PV phase in one (1.1%) case.

The ROC curves and their corresponding area under the curve (AUC) for (i) Lesion$_{arterial - equilibrium}$ (ii) Lesion$_{PV - equilibrium}$ and (iii) Lesion$_{peak - equilibrium}$ are shown in Figures 1, 2 and 3, respectively. ROI analysis by the Lesion$_{arterial -}$
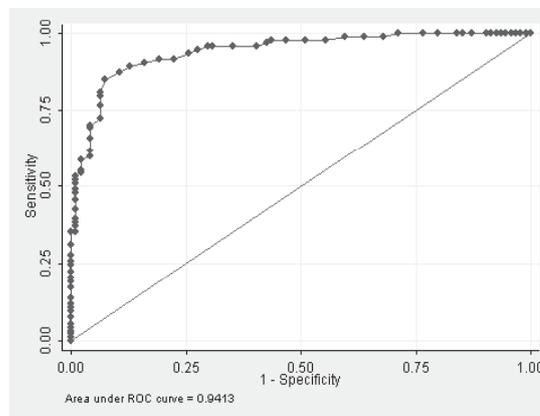
Fig. 1. ROC curve for Lesion$_{arterial - equilibrium}$ (95% CI, 0.909-0.973).
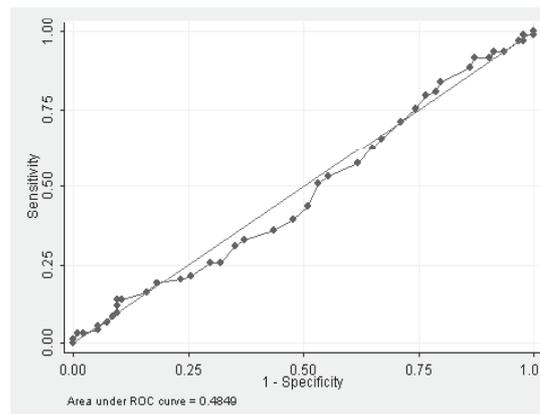
Fig. 2. ROC curve for Lesion$_{PV- equilibrium}$ (95% CI, 0.402-0.568).
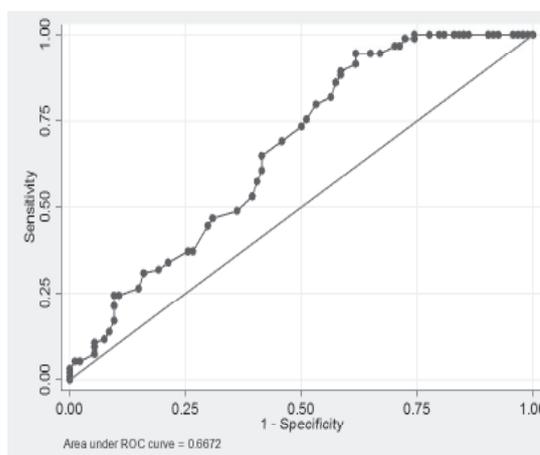
Fig.3. ROC curve for Lesion$_{peak- equilibrium}$ (95% CI, 0.590-0.745).

$_{equilibrium}$ method showed better results (AUC = 0.941) than both Lesion$_{PV-equilibrium}$ (AUC = 0.484) and Lesion$_{peak-equilibrium}$ (AUC= 0.667) methods. The mean attenuation difference for tumour (Lesion$_{arterial-equilibrium}$) was 10.0 HU (95% CI, 6.5 to 13.5) while that for surrounding liver was –20.6 (95% CI, –23.0 to –18.2). This was statistically significant (P <0.0001).

Wash-out was visually appreciated in 73/94 (77.7%) lesions (Table 1). Based on ROC analysis, a HU$_{cut-off}$ of 10 for Lesion$_{arterial-equilibrium}$ yields a sensitivity and specificity of 91.5% and 80.9% respectively. ROI analysis detected 9/21 (42.9%) lesions that would have been missed by visual analysis alone. The mean attenuation difference in these 9 lesions was 7.5 HU (range, –19 to 38). Combined ROI and visual analysis therefore yields detection of 82/94 (87.2%) lesions.

Eighty-two lesions were assigned a histological grade based on the modified Edmundson's criteria. Six lesions were assigned descriptive grades. The remaining 6 lesions had no histological grade assigned. We grouped the graded lesions into well-differentiated (including Edmundson's grade 1, n = 13), moderately differentiated (including Edmundson's grade 2, n = 41) and poorly differentiated HCC (including Edmundson's grades 3 and 4, n = 34). We found the ROC analysis for well-differentiated HCC to be similar to the other histological subtypes (Table 2).

Discussion

HCC is a hypervascular tumour with increased blood supply. Classically the tumour demonstrates hepatic arterial enhancement. During the PV and equilibrium phases, the tumour washes out.[7] Several groups have explored non-invasive diagnosis of HCC by imaging. Two important points have emerged from these consensus criteria by the EASL and AASLD: (i) hypervascularity in the hepatic arterial phase and (ii) wash-out in the PV phases (either early or late).

Conventionally, the wash-out characteristics of HCC are assessed visually in relation to the surrounding non-tumourous liver parenchyma. The phenomenon of "wash-out" in HCC can be appreciated if we consider the fact that the vascular space constitutes a larger fraction of the total tracer distribution volume in the lesion, as compared with the interstitial space. Hence, the overall enhancement pattern of HCC parallels that of the surrounding liver, which is predominantly a "vascular space". HCC enhances in the arterial phase as the vascular spaces are supplied by the hepatic artery. It continues to receive variable blood supply from the portal vein in the PV phase; after which, the contrast returns to the systemic circulation via the veins and is redistributed to the interstitium in the rest of the body.[8] The arterial and venous concentration of contrast decreases due to dilution. As the surrounding liver can be considered as a large vascular space supplied by the hepatic artery and the portal vein, when the contrast concentration in the hepatic artery and portal vein falls in the equilibrium phase, the liver attenuation also decreases. This same phenomenon is observed in the HCC as a large proportion of its volume is made up of vascular spaces hence accounting for the wash-out phenomenon.[9-11]

Therefore, in order for wash-out to be visually perceived, there has to be a larger drop in CT attenuation in the lesions as compared to the surrounding liver between the arterial and the equilibrium phases (Figs. 4 and 5). The liver shows peak enhancement in the PV phase that may mask further increase in attenuation that may take place with the lesions. This explains why, even though the majority (71.3%) of lesions showed peak attenuation in the portal venous phase, they appeared as isodense or hypodense to surrounding liver.

ROI analysis is feasible because, as our study shows, the attenuation of the lesion is greater in the arterial phase than in the equilibrium phase (mean = 10 HU), while that of surrounding liver is greater in the equilibrium phase than in the arterial phase (mean = –20.6 HU). From this study, it appears that calculating the attenuation differences of a lesion between the arterial and equilibrium phases (Lesion$_{arterial-equilibrium}$) yields the best results to tumour establish wash-out. The results of Lesion$_{PV-equilibrium}$ are poorer since both normal liver and the majority of lesions enhanced maximally in the PV phase. Similarly, Lesion$_{arterial-PV}$ would not be meaningful and was not calculated since the majority of the lesions continued to increase in attenuation into the PV phase.

Knowing the optimal HU cut-off for determining lesion

Table 1. Results of Visual Analysis (Hyperdense, Isodense or Hypodense) According to Phase of Scanning

Phase	Hyperdense	Isodense	Hypodense
Arterial	80	14	0
Portal venous	12	28	54
Equilibrium	2	19	73

Table 2. ROC Analyses (Lesion$_{arterial-equilibrium}$) Based on Tumour Grade (differentiation) (n = 88). Tumour Grade was not Available for Data Analysis in 6 Patients

Tumour grade	Number of lesions	AUC	95% CI
Well-differentiated	13	0.932	0.828-1.000
Moderately-differentiated	41	0.946	0.896-0.996
Poorly-differentiated	34	0.952	0.904-1.000

wash-out is useful in clinical practice as CT diagnosis of HCC by visual assessment alone can sometimes be difficult and is subject to both intraobserver and interobserver variability. Based on our findings, a $HU_{cut-off}$ of 10 for $Lesion_{arterial - equilibrium}$ is optimal for determining lesion wash-out.

In our study, using visual analysis alone would have missed 21.3% of lesions (Fig. 6). While our results may be similar to the study by Yan et al[12] (where wash-out was deemed to be present in 72.4% of cases), having a method that can improve on visual analysis alone would be desirable. We were able to achieve this by combining ROI analysis with visual analysis for determining wash-out. In our series, ROI analysis was able to detect 9/21 (42.9%) lesions

that would have been missed by visual analysis alone. However, given the small sample size, this difference was not statistically significant. The combined method would yield a sensitivity of 87.2% of HCC as compared to 77.7% for visual analysis alone.

We acknowledge that there are a few limitations to this study. Firstly, the diagnoses of the cases were already known as these were cases selected from a database of surgically proven HCC. This may have led to bias in the visual analysis of the lesions, increasing its sensitivity. Furthermore, the diagnosis of HCC is also based upon arterial hypervascularity, for which a non-contrast scan is required to make the diagnosis, but was beyond the scope of our study.
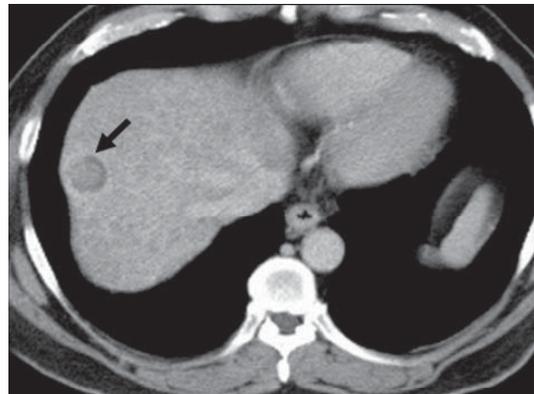
Fig. 4. Wash-out as determined by both ROI and visual analyses. Axial CT image in the (a) arterial and (b) equilibrium phases showing a hypervascular lesion that demonstrates wash-out. The $Lesion_{arterial-equilibrium}$ was 18 HU, which is consistent with wash-out. The lesion is also clearly suspicious for HCC by visual assessment.
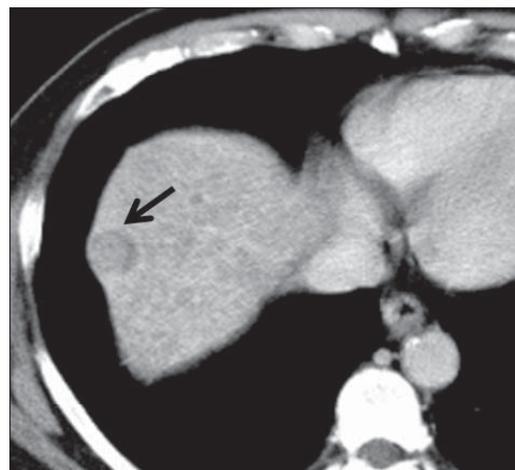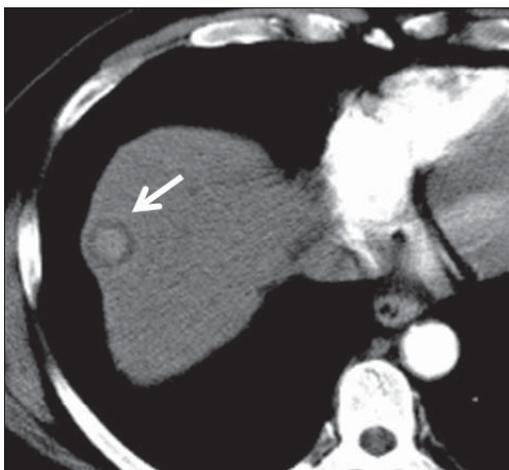
Fig. 5. Wash-out as determined by visual analysis. Axial CT image in the (a) arterial and (b) equilibrium phases showing a hypodense lesion in the latter consistent with wash-out by visual assessment. $Lesion_{arterial-equilibrium}$ was –10 HU, which theoretically means that the lesion did not "wash out" in the equilibrium phase. The perceived wash-out is partly related to the hypodense rim surrounding the lesion and partly to the fact that surrounding liver enhanced to a greater degree than the tumour itself.
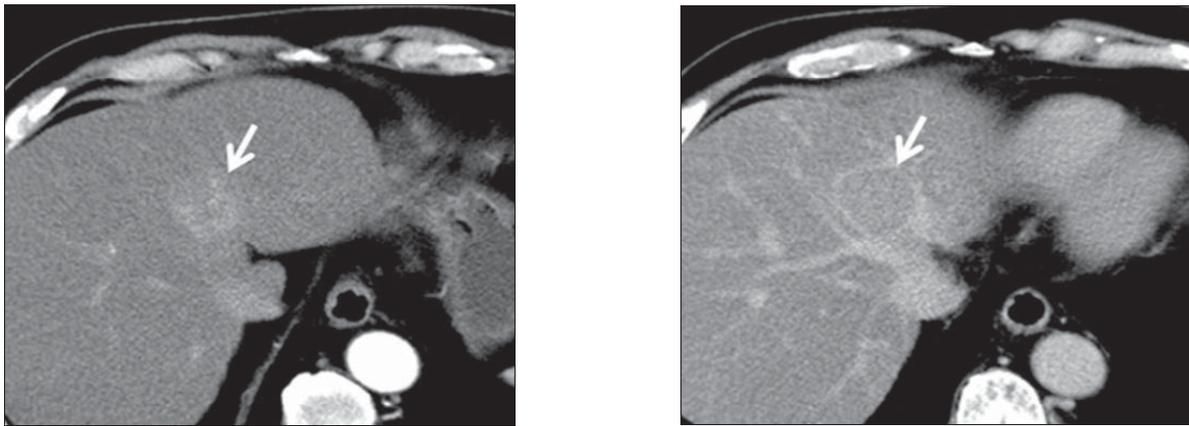
Fig. 6. Wash-out as determined by ROI analysis. Axial CT images in the (a) arterial and (b) equilibrium phases showing a hyperdense lesion (arrows) which becomes isodense, and therefore negative by visual assessment. Lesion$_{arterial - equilibrium}$ was 24 HU, consistent with wash-out by ROI analysis. Presence of a psuedocapsule may have confounded visual analysis.

Secondly, different scanners were included in this study. Although similar imaging protocols were used, slight differences in results would have been present. However, without doing so, we would not have been able to study such a large patient population (with surgicopathological correlation). Although the images were reconstructed to different thicknesses, these should not have affected the analysis of larger lesions. Furthermore, in order for our results to be applied to clinical practice, minor technical differences would invariably be present and therefore should be accepted as confounders.

Thirdly, although we were able to derive specificity values based on ROC analysis, the true specificity of ROI analysis for lesion wash-out needs to be validated by including population that contains both HCC and other hypervascular liver lesions (such as focal nodular hyperplasia or haemangiomas), as this will have important clinical and therapeutic implications.

Fourthly, one foreseeable advantage of ROI analysis is that it may improve characterisation of HCCs smaller than 2 cm in size, since these lesions are more likely to demonstrate atypical patterns of enhancement observed on visual analysis. We were not able to establish this relationship from this study due to the small sample size.

Finally, while we were consistent in using a single reader for the application of ROI analysis, it was not possible to standardise the size and position of the measurements for all cases due to heterogeneity of lesion attenuation. Analysis of necrotic tumours posed the biggest problem, as the exact extent of viable tumour was not always evident. Hence we chose to measure the most highly attenuating region of each lesion. To further reduce inaccuracy of density measurements, lesions smaller than 1 cm have been excluded from the study.

The advantage of our study lies in a large study population that is homogeneous and with histopathological validation. CT evaluation of HCC has conventionally been based on a visual assessment of lesion density in relation to surrounding liver, which is subjective. Direct measurement of lesion attenuation changes between the arterial and equilibrium phases is a simple and objective method that can be included as an adjunct to visual assessment to improve sensitivity for determining lesion wash-out. In addition, ROI analysis is a function that is ubiquitous to most modern PACS software systems, and can be easily applied to routine clinical practice.

Conclusion

In summary, our study has achieved several objectives. Firstly, it shows that Lesion$_{arterial - equilibrium}$ is the method of choice for ROI analysis of lesion wash-out. Secondly, in our series, a HU$_{cut-off}$ of 10 provides optimal sensitivity and specificity. Lastly, addition of the ROI analysis (Lesion$_{arterial - equilibrium}$) to visual analysis can improve the sensitivity of CT in determining lesion wash-out in HCC.

REFERENCES

1. El-Serag HB. Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 2002;35:72-8.

2. Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J Hepatol 2001;35:421-30.

3. Bruix J, Sherman M, Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of Hepatocellular Carcinoma. Hepatology 2005;42:1208-36.

4. Hwang GJ, Kim MJ, Yoo HS, Lee JT. Nodular hepatocellular carcinomas: detection with arterial-, portal-, and delayed-phase images at spiral CT. Radiology 1997;202:383-8.

5. Marrero JA, Hussain HK, Nghiem HV, Umar R, Fontana RJ, Lok AS. Improving the prediction of hepatocellular carcinoma in cirrhotic patients with an arterially-enhancing liver mass. Liver Transpl 2005;11:281-9.

6. Ryder SD. Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults. Gut 2003;52:1–8.

7. Yu SC, Yeung DT, So NM. Imaging features of hepatocellular carcinoma. Clin Radiol 2004;59:145–56.

8. Koh TS, Thng CH, Hartono S, Lee PS, Choo SP, Poon DY, et al. Dynamic contrast-enhanced CT imaging of hepatocellular carcinoma in cirrhosis: feasibility of a prolonged dual-phase imaging protocol with tracer kinetics modeling. Eur Radiol 2009;19:1184-96.

9. Oliver JH 3rd, Baron RL, Federle MP, Rockette HE Jr. Detecting hepatocellular carcinoma: value of unenhanced or arterial phase CT imaging or both used in conjunction with conventional portal venous phase contrast-enhanced CT imaging. AJR Am J Roentgenol 1996;167:71-7.

10. Yan FH, Shen JZ, Li RC, Zeng MS, Wu D, Zhou KR, et al. Enhancement patterns of small hepatocellular carcinoma shown by dynamic MRI and CT. Hepatobiliary Pancreat Dis Int 2002;1:420-4.

11. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. AJR Am J Roentgenol 1980;135:1037-43.

12. Koh TS, Thng CH, Lee PS, Hartono S, Rumpel H, Goh BC, et al. Hepatic metastases: in vivo assessment of perfusion parameters at dynamic contrast-enhanced MR imaging with dual-input two-compartment tracer kinetics model. Radiology 2008;249:307-20.