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FULL PAPER

Early prediction of response of sorafenib on hepatocellular carcinoma by CT perfusion imaging: an animal study

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Objective: This study evaluated the feasibility of CT perfusion parameters for the early efficacy prediction of sorafenib in the treatment of hepatocellular carcinoma (HCC) in rats.

Methods: CT hepatic perfusion measurements were performed in the livers of 40 rats implanted with rat HCC. The rats in the experimental group ($n = 28$) were treated by oral gavage with sorafenib (20 mg per day), whereas the rats in the control group ($n = 12$) were treated by normal saline. Rats were classified into the responder group if the maximum diameter of their tumour had decreased 21 days after treatment, whereas the other rats were classified into the non-responder group. Data were analysed using the Pearson correlation analysis or analysis of variance.

Results: CT perfusion was used to depict haemodynamic changes before and after treatment. The arterial

liver perfusion was significantly decreased in the responder group on Day 11 after treatment with sorafenib (from 71.5 to 53.4 ml min⁻¹ 100 ml⁻¹), whereas no significant changes were observed in the non-responder group ($p = 0.87$). The maximum diameter of the tumour was also significantly decreased in the responder group on Day 21 after treatment ($p = 0.042$), whereas the maximum tumour diameter was significantly increased in the control group ($p = 0.001$).

Conclusion and advances in knowledge: CT perfusion could be used to quantitatively analyse the haemodynamic changes in the treatment of HCC with sorafenib, which indicates that this approach may be developed for the early prediction of treatment efficacy for sorafenib.

Hepatocellular carcinoma (HCC) is ranked as the fifth most common cancer worldwide and the third most common cause of cancer-related deaths.¹ In the past few years, with increasing knowledge of molecular regulatory mechanisms of cancer progression, targeted antineoplastic drugs have been rapidly developed. Sorafenib, a molecular inhibitor of multiple protein kinases,² is the first approved molecular targeted drug for the treatment of HCC. It has been demonstrated that sorafenib can prolong the survival of patients with advanced HCC.³ Although sorafenib has been proven effective in the treatment of HCC, some patients develop adverse reactions or show no treatment effects, and, in some extreme cases, sorafenib has been shown to shorten survival in patients.⁴ If imaging techniques could be used to predict the therapeutic efficacy of sorafenib during the early stage of treatment, unnecessary treatment could be avoided, which would undoubtedly have a significant effect on reducing the physiological distress and financial burden of patients.

The New Response Evaluation Criteria in Solid Tumours⁵ are currently the primary criteria for evaluating therapeutic efficacy in solid tumours. The evaluation criteria for tumour

progression or remission measure the changes in the maximum diameter of a tumour, although this measure is not reliable in clinical practice. For example, tumour necrosis occurs within the mass after treatment, but tumour size is not simultaneously reduced. Under these circumstances, false-positive results may occur in the imaging evaluation, which would hide the true therapeutic efficacy. Perfusion imaging techniques are based on dynamic contrast-enhanced CT scans that can detect the haemodynamic status and functional changes in organs and tissues earlier than the morphological changes; as a result, these techniques can be used for the early detection and diagnosis of tumours.^{6–8} This study sought to investigate whether CT perfusion imaging could be used to depict the effects of sorafenib on the inhibition of tumour angiogenesis in the treatment of HCC using a rat model.

METHODS AND MATERIALS

Experimental design and animal model

The management of experimental animals met the requirements of the Guideline on the Humane Treatment of Laboratory Animals, which was issued by the Ministry of Science and Technology in 2006. The tumour-bearing rat

model was established in two steps. First, rat HCC cells (CBRH-7919) were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Three nude mice (4–6 weeks old) were purchased from the Beijing Laboratory Animal Research Centre. A CBRH-7919 cell suspension (0.10–0.15 ml, cell number: 6×10^6 to 1×10^7) was injected into the hind limbs and subscapularis of the nude mice, and the subcutaneous tissue of these mice at the injection site became relatively hard 7 days after surgery. The lobulated and fixed soft-tissue nodules with poor movement could be felt by palpation, and these nodules were considered as tumour donors when they had grown to approximately 1.5 cm. In the second step, 40 specific pathogen-free standard Wistar male rats (4–6 weeks old) were purchased from the Laboratory Animal Centre at Hebei Medical University, Hebei, China. Dexamethasone was administered intramuscularly at 2.5 mg kg^{-1} to all rats, 3 days before and 7 days after the tumour transplantation. Each rat was anaesthetized with phenobarbital (130 mg kg^{-1}) and ketamine (16 mg kg^{-1}) before surgery. The tumour nodules were excised after the nude mice were anaesthetized and were then cut into small pieces of approximately 3–5 mm in diameter. These tumour pieces were implanted into the rats under CT guidance. Then, the tumour-bearing rats were randomly divided into two groups: an experimental group consisting of 28 rats and a control group consisting of 12 rats. CT perfusion scans were performed to assess intrahepatic tumours on Days 9, 20 and 31 after the tumour cells were transplanted. Treatment with sorafenib was started on Day 10. The end point of the experiment was set as Day 21 after treatment in the experimental group. All rats were euthanized at the end point, and immunohistochemical examinations were performed. In the experimental group, rats with decreased tumour size and without extrahepatic metastases were classified as the responder group. The other treated animals were classified as the non-responder group.

CT scan protocol

A dual-source CT (SOMATOM® Definition Flash, Siemens Healthcare, Forchheim, Germany) was used to perform whole-liver plan CT scans, from the top of the diaphragm to the lower edge of the liver. The parameters were set as follows: tube voltage, 120 kV; tube current, 74 mAs; slice thickness, 3.0 mm; and pitch value, 0.6. A binocular syringe power injector and 24 G catheter were used for contrast medium injection. Diluted iopromide (1 ml) (Ultravist 300 mg I ml^{-1} ; Schering Pharmaceutical Co. Ltd, Guangzhou, China) was injected through the femoral vein (or internal jugular vein) followed by 2 ml of saline, both at an injection rate of 0.5 ml s^{-1} . After 2 s, perfusion scans were performed on whole rat livers, with a tube voltage of 80 kV, tube current of 150 mAs, cycle time of 1 s (scanning time was 1 s, and interval time was 1 s), a total scanning time of 41.2 s, slice thickness of 1.0 mm, slice distance of 1 mm and convolution kernel of B20f. Delayed scanning was performed on the chest and abdomen immediately after the perfusion scans, with the scan parameters the same as those for the plain CT scans. Abdominal compression was used to reduce motion artefacts during scanning.

Application of the molecular targeted drug

The sorafenib solution was prepared for treatment. The sorafenib tablets (200 mg Nexavar®; Bayer, Leverkusen, Germany)

were treated with 500 μl of dimethyl sulfoxide to dissolve the sugar coating of the tablet, and 1 ml of polyoxyethylene castor oil and 1 ml of 95% alcohol were added to emulsify and dissolve the sorafenib tablets. The dissolved sorafenib suspension was mixed with 100 ml of saline. The rats in the experimental group were treated with the sorafenib solution by oral gavage, at 20 mg sorafenib per day, once a day for 21 days. The rats in the control group were treated with the same dose of saline using the same procedure. The basic vital signs of the rats were evaluated during the gavage procedure. A high dose of anaesthetics was given to euthanize the rats after completion of the experiments.

Data analysis

All images were evaluated on a commercial workstation (MMWP; Siemens Healthcare). The tumour size and perfusion parameters of all rats were measured in consensus by two radiologists with 5 years' experience in radiological diagnosis. The maximum diameter of the tumour was obtained from delayed images. The ruler tool was used to measure the maximum diameter of the tumours. The measurement was repeated three times. The mean value was calculated and recorded for further analysis.

The CT perfusion images were analysed using commercial software (Syngo VPCT Body; Siemens Healthcare). The perfusion image analysis was performed in the following steps. First, the dedicated liver mode/algorithm was selected, and the image was visually examined to check the presentation of motion artefacts. If a motion artefact was present, the motion correction was applied. Otherwise, the image analysis proceeded directly to the next step. In the second step, a region of interest (ROI) was drawn on the abdominal aorta and portal vein, with a diameter slightly smaller than the diameter of the vessels. Attention was paid to avoiding the partial volume effect on the edge during measurement. Because the ROI of the spleen can be irregular, this should be determined by outlining the spleen as large as possible and drawing along the edges. The third step was to obtain the time–density curve and the haemodynamic parameters: blood flow (BF) (millilitres per minute per 100 millilitres), blood volume (BV) (millilitres per 100 grams), arterial liver perfusion (ALP) (millilitres per minute per 100 millilitres) and the hepatic perfusion index (HPI) (%). The ROI was placed on the tumour region, which should include the largest section of the tumour, especially in the region with relatively clear enhancement around the edge of the tumour. However, the region connected to the liver was avoided and the measurement of the hepatic tissue did not include the visible blood vessels. To reduce measurement error, we performed measurements of each lesion for three successive slices and used the mean value for further data analysis.

Histological analysis

The hepatic lobe with the tumour was obtained, fixed and embedded in paraffin. Sections were cut to approximately 4 μm in thickness for haematoxylin and eosin staining and the assessment of CD34 and vascular endothelial growth factor (VEGF) expression. The percentage of VEGF-positive tumour cells and the CD34⁺ density were calculated under microscopy. Two highly experienced pathologists with 16 and 10 years' experience performed the immunohistochemical analysis. The assessment criteria for the immunohistochemical analysis of VEGF and CD34 were reported previously.⁹ Briefly, all endothelial cells or endothelial

clusters stained by CD34 antibody were counted as micro-vessels, as long as they were separate micro-vessels and not tumour cells or other connective tissues. The following structures were not counted as micro-vessels: indistinguishable and blurred stained cells, any structure with a thick muscle layer or the luminal area of vessels >8 erythrocytes in diameter. The histological analysis was started with image browse under microscopy with low magnification ($100\times$). Four areas with a high density of tumour blood vessels were selected for micro-vessel density (MVD) analysis under high magnification ($200\times$). The MVD was counted using an eyepiece micrometre counting grid in an area of $90.25\ \mu\text{m}^2$. The mean value of the MVD from four selected areas was recorded for the final data analysis. The cells with positive VEGF expression appeared as brown-yellow in colour and contained dark brown granule-like structures distributed in the cytoplasm. Similar to CD34 stain, in four areas, the percentage of stained tumour in each field of view was recorded, and the mean percentage was recorded for the final analysis.

Statistical analysis

SPSS® v. 13.0 software (SPSS Inc., Chicago, IL) was applied for all data analyses. Quantitative data were expressed as the mean $\pm$ standard deviation (mean $\pm$ SD). A p -value <0.05 was considered statistically significant. Comparisons between the responder, non-responder and control groups were performed using analysis of variance (ANOVA). Comparisons between before and after treatment in the three groups were performed using ANOVA. Pearson correlation analysis was applied for correlation analysis between the MVD and each of the perfusion parameters.

RESULTS

One rat in the experiment group presented skin necrosis on the right hind leg after 1 week of gavage treatment. Therefore, we reduced the dose of sorafenib to $10\ \text{mg kg}^{-1}$ per day and gave this rat local skin care to the right hind leg. However, the

Figure 1. (a, b) CT perfusion imaging of rats with hepatocellular carcinoma [arrowheads on (a)] prior to treatment. (a) Blood flow (BF) image; (b) arterial liver perfusion (ALP) image. (c–h) CT perfusion imaging of the responder group, non-responder group and control group after 21 days of treatment, respectively. (c, e, g) BF images in the three groups, showing an uneven distribution of BF in the tumour tissue. Significantly greater perfusion was observed on the edge of the tumour (arrowheads). Regions with high perfusion were more commonly observed in the control group. (d, f, h) ALP images in the three groups, showing smaller sized tumours in the responder group. In addition, the ALP volume was significantly lower than that observed in the control group. (a–d) Perfusion images from the same rat before and after treatment.

Table 1. CT perfusion measurements in the normal liver and liver tumour before and after treatment

Parameters	Control group (<i>n</i> = 11)		Non-responder group (<i>n</i> = 9)		Responder group (<i>n</i> = 19)	
	Normal liver	Tumour	Normal liver	Tumour	Normal liver	Tumour
Before treatment						
BF	189.5 ± 37.1	94.9 ± 61.5	169.2 ± 41.3	85.7 ± 45.4	195.8 ± 33.6	82.0 ± 27.1
BV	32.3 ± 7.6	12.8 ± 3.4	30.5 ± 5.5	13.3 ± 3.5	37.4 ± 10.2	14.3 ± 2.7
ALP	17.6 ± 3.7	82.8 ± 59.4	12.4 ± 3.6	82.5 ± 41.2	19.4 ± 3.5	77.5 ± 25.1
HPI	9.9 ± 3.1	91.0 ± 9.6	9.1 ± 2.5	94.5 ± 7.6	13.1 ± 5.9	96.5 ± 3.8
11 days after treatment						
BF	165.4 ± 45.3	78.9 ± 26.4	190.6 ± 43.2	86.0 ± 27.4	173.2 ± 48.2	75.2 ± 21.4
BV	27.8 ± 4.3	13.5 ± 2.6	34.8 ± 4.5	13.4 ± 3.9	29.5 ± 9.8	12.2 ± 3.3
ALP	20.1 ± 3.1	74.6 ± 16.8	29.5 ± 10.1	73.5 ± 11.6	16.3 ± 4.7	53.4 ± 23.8
HPI	10.3 ± 3.6	93.7 ± 8.7	14.8 ± 8.5	97.8 ± 2.7	11.5 ± 6.6	91.2 ± 9.4
21 days after treatment						
BF	178.3 ± 55.2	86.9 ± 34.8	204.9 ± 32.4	70.1 ± 26.3	181.7 ± 42.5	50.1 ± 28.9
BV	27.4 ± 6.2	15.6 ± 1.9	33.8 ± 6.9	9.8 ± 3.8	33.3 ± 7.1	6.6 ± 2.9
ALP	23.8 ± 5.7	75.3 ± 13.4	26.2 ± 8.3	61.5 ± 17.2	29.4 ± 6.2	35.2 ± 10.3
HPI	12.5 ± 2.9	96.3 ± 3.7	11.5 ± 4.4	92.2 ± 9.7	14.9 ± 5.1	85.7 ± 13.2

ALP, arterial liver perfusion; BF, blood flow; BV, blood volume; HPI, hepatic perfusion index. The values represent the mean ± standard deviation.

symptoms were not alleviated after 3 days of observation, so we stopped sorafenib treatment. In addition, one rat in the control group died on Day 20, with an autopsy showing multiple intrahepatic, intraperitoneal and pulmonary metastases. At the end point of the study, 39 rats completed all CT perfusion scans, with 19 rats classified in the responder group, 9 rats classified in the non-responder group and 11 rats in the control group.

Arterial liver perfusion and blood volume perfusion images

Figure 1 shows ALP and BV images obtained on Day 21 after treatment. These images revealed areas of abnormal perfusion in the control group. The perfusion level within the tumours of the non-responder group was lower than in the control group, and the images from the responder group showed even lower levels of perfusion.

Comparison of perfusion parameters between groups

The perfusion parameters were not significantly different among the control group, non-responder group and responder group prior to treatment (Table 1). However, the ALP values demonstrated significant differences between the three groups after 11 days of treatment ($p = 0.01$); in particular, the ALP of the responder group decreased from $71.5 \text{ ml min}^{-1} 100 \text{ ml}^{-1}$ before treatment to $53.4 \text{ ml min}^{-1} 100 \text{ ml}^{-1}$ after treatment ($p = 0.001$). No significant changes were observed in the control group and the non-responder group ($p = 0.34$ and 0.87 , respectively). The values for BF, BV and HPI were not significantly

altered in the three groups at 11 days after treatment, whereas after 21 days of treatment, BF and BV were significantly decreased in the responder group when compared with the values obtained prior to treatment ($p = 0.01$ for both parameters). However, this difference was not statistically significant in the control group ($p = 0.47$ and 0.79 , respectively). BF, BV and ALP were also significantly decreased in the non-responder group when compared with the values obtained prior to treatment ($p = 0.02$, 0.01 and 0.01 , respectively).

Figure 2. Hepatocellular carcinoma growth in the three groups before and after treatment. The tumour diameter increased continuously in the control group, while the tumour diameter was decreased in the responder group after 21 days of treatment. d, days.

Figure 3. (a-c) Light microscopy showing irregular tumour vessels with brown staining using the CD34 antibody in the control group, non-responder group and responder group. The number of micro-vessels in the tumour was significantly higher in the control group than in the non-responder and responder groups.

(a)

(b)

(c)

Comparison of maximum tumour diameters

The maximum diameters of tumours were not significantly different between the three groups prior to treatment (Figure 2). However, after 21 days of treatment, the mean maximum diameter of the tumours decreased significantly from 0.87 to 0.59 cm in the responder group ($p = 0.042$), although no significant difference in the maximum tumour diameter was observed in the non-responder group ($p = 0.49$). By contrast, the mean maximum diameter of the tumours increased significantly from 0.75 to 1.53 cm in the control group ($p = 0.001$).

Table 2. Linear correlation analysis between perfusion CT parameters and micro-vessel density (MVD)

Parameters	r	p
BF	0.329	0.53
BV	0.244	0.38
ALP	0.647 ^a	0.01
HPI	0.347	0.59

ALP, arterial liver perfusion; BF, blood flow; BV, blood volume; HPI, hepatic perfusion index.

^aA significant linear correlation was detected between ALP and MVD.

Correlation between arterial liver perfusion and micro-vessel density

Table 2 presents the positive correlation observed between the ALP and MVD ($r = 0.647$). However, BF, BV and HPI were not significantly correlated with MVD. Figures 3 and 4 show that MVD was significantly lower in the responder group than in the control group and the non-responder group after sorafenib treatment ($p < 0.01$).

DISCUSSION

Tumour growth and metastasis is a complex multistep process. In this process, angiogenesis is considered to be an important factor in the tumour ecosystem because it affects the biological behaviour of tumours. It has been shown that sorafenib inhibits serine/threonine-specific protein kinase and their receptors in the tumour cells and blood vessels, thereby inhibiting tumour angiogenesis.² The present study demonstrated that CT perfusion could be used to evaluate the difference in tumour angiogenesis between the responders and non-responders to sorafenib. The CT perfusion parameter ALP indicated significantly reduced arterial perfusion in the lesion area after 11 days of treatment. However, at that time, there was not a statistically significant change in the maximum diameter of the tumour, which suggests that CT perfusion parameters could serve as an earlier predictor of the efficacy of sorafenib treatment.

CD34 staining showed a large amount of nascent tumour blood vessels in the region of the HCC before and after treatment in the control group. However, the MVD significantly decreased in the responder group after treatment. This reduction led to a significant reduction in BF in the responder group. In addition, the CT perfusion parameter ALP was correlated with MVD changes ($r = 0.647$), which reflected the hepatic artery angiogenesis changes in the tumour before and after treatment. Theoretically, the BF and BV measurements reflected the blood supply of the studied tissues. However, the correlation between BF, BV and MVD was relatively weak in this study ($r = 0.329$ and 0.244 , respectively). One possible reason for the weak correlation could be that the HCCs primarily received blood from arteries. ALP might be a more sensitive earlier indicator than BF/HPI for the reduction of artery blood supply in the tumour region.

Sorafenib inhibits receptor tyrosine kinases and serine/threonine kinases in the Raf/ERK/MEK pathways, resulting in an extension

Figure 4. Box plot showing the difference in CD34-stained micro-vessels among the three groups. The number of micro-vessels was significantly reduced in the responder group. FOV, field of view.

of the tumour cell division cycle and the inhibition of cell growth.¹⁰ In this study, the maximum diameter of the tumours was significantly different between the experiment group and the control group after 21 days of treatment. Thus, the growth rate of the tumour either slowed down or the size of the tumour was reduced in the experiment group. After sorafenib treatment, the rats in the non-responder group showed relatively slower tumour growth than the control group, and the tumour size was reduced compared with that measured prior to treatment.

Hepatic perfusion scans using multidetector CT have the advantage of being performed when a subject is breathing under resting conditions. Although even breathing movements cause motion artefacts, they can be corrected by post-processing software, thereby reducing the examination failure rate. In addition, each CT perfusion parameter can be selectively applied in the clinical evaluation of cancer treatment. In this study, the

changes in CT perfusion parameters could predict the effect of sorafenib in the treatment of HCC after 11 days of treatment. Therefore, we believe that this technique demonstrated a relatively high sensitivity. However, the radiation dose associated with this technique remains the primary limitation that prevents the use of successive CT perfusion scans.¹¹ Colour Doppler flow imaging and MRI can also quantitatively represent the haemodynamic changes in tissues and organs. However, the limitation of dynamic contrast-enhanced ultrasonography is the motion artefacts caused by breathing when targeting small-sized tumour lesions at a deep location. In addition, only a single tumour lesion can be focused on for observation during each imaging procedure. The results can also be easily affected by the examiner's experience, and the consistency of results is relatively poor.¹² MR perfusion imaging has gradually developed and improved, although MR perfusion imaging still requires sophisticated software, higher spatial and temporal resolution and an MR machine with a high-gradient field to achieve perfusion imaging of the liver.¹³ CT perfusion imaging of the liver was first successfully applied by Miles et al¹⁴ in 1993. After 20 years, CT perfusion imaging has been well developed and widely applied in clinical studies,^{15–17} and the main priority of future research is the reduction of the perfusion radiation dose.

The present study has limitations. First, the number of rats is small. A study with a large number of rats could improve the reliability of the results. Second, we did not perform imaging studies regarding the effect of sorafenib on survival time, because all rats were euthanized after 21 days of treatment for immunohistochemical analysis.

In summary, CT perfusion imaging could predict the therapeutic effect of sorafenib on the CBRH-7919 rat model at an early stage of tumour treatment. Moreover, a positive correlation was found between ALP and MVD. The rapid identification of subjects who do not respond to treatment will certainly help with designing and modifying the clinical treatment plan.

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