Assessment of Angiogenesis of Hepatocellular Carcinoma using Dynamic Contrast Enhanced MR and Histopathologic Correlation in an Experimental Rat Model

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Directed by Professor Ki Whang Kim

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

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The stepwise course of hepatocellular carcinomas (HCC) develop through a progressive pathway from a benign regenerative nodule to a dysplastic nodule (DN) to small HCC, and finally to the overt HCC. Differential diagnosis remains difficult especially in areas of DN or small well-differentiated HCC. Recently developed dynamic contrast enhanced (DCE) magnetic resonance (MR) imaging is a noninvasive imaging technique that can be used to quantify perfusion changes in the liver. The aim of our study was to assess the perfusion parameters and angiogenesis of HCC using DCE MR and to correlate it with histopathologic findings in an experimental rat model.

In this study, 20 rats were continuously infused with diethylnitrosamine (DEN) for tumor induction, with the use of mini-pumps. After 32 to 36 weeks of DEN treatment, the rats underwent MRI of the liver with a 3-T MR imaging system. Perfusion parametric maps and perfusion parameters such as, time to peak (TTP) and peak enhancement (PE) were obtained by using a commercially available software package (Nordic ICE; NordicNeuroLab, Bergen, Norway). The nodules were corresponded precisely to DCE MR

images.

A total of 13 nodules were found in 12 rats; 5 DNs were identified in 5 rats and 8 HCCs (3 Edmonson grade I, 2 Edmonson grade I-II, 3 Edmonson grade II) were found in 7 rats.

There were no statistical differences in blood perfusion parameters (TTP and PE) between nodules and their surrounding hepatic parenchyma. There were no significant differences in mean value of TTP, in histogram peak height (HPH), or in width of TTP between DN and HCC. However, there were significant differences in mean values of between PE and HPH of PE between DN and HCC. Mean value and HPH of PE showed statistically significant correlation with tumor grade.

In conclusion, there were significant differences in mean value and HPH of PE between DN and HCC. In addition, there were substantial negative correlations between tumor grades and perfusion parameters with mean value and HPH of PE in DN and well-differentiated HCC.

DCE MR imaging can be used in the differential diagnosis and management of liver disease along the multistep process of heparocarcinogenesis.

Key words : hepatocarcinogenesis, dysplastic nodule, hepatocellular carcinoma, dynamic contrast enhanced magnetic resonance

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I. INTRODUCTION

Chronic viral hepatitis B and C, alcohol abuse, and liver steatosis are major risk factors for the development of liver fibrosis, cirrhosis, and subsequent risk of end-stage liver disease, all of which can lead to hepatocellular carcinoma (HCC)[1]. HCC is the most common primary malignant tumor of the liver and the third most common cause of cancer mortality worldwide[2]. The great majority of HCCs develop in patients with chronic liver disease, particularly cirrhosis and chronic viral hepatitis[3-4]. The stepwise carcinogenesis of HCC is well known. An International Working Party of Gastroenterology proposed terminology in which dysplastic features of the hepatic nodule are expressed[3, 5-6]. Many HCC develop through a progressive pathway, from a benign regenerative nodule to a dysplastic nodule (DN)

to a DN with microscopic foci of an HCC, which may enlarge and replace the nodule, giving rise to a small HCC, and finally to the overt HCC[4, 7]. Differential diagnosis remains difficult particularly in the DN or small well-differentiated HCC. HCC is a highly vascularized tumor, and angiogenesis is one of the most important phenomena in the process of stepwise hepatocarcinogenesis[8].

Magnetic resonance imaging (MRI) has an important role in the evaluation of diffuse and focal liver diseases and plays an important role in the differential diagnosis of disease. The diagnostic value of the dynamic enhancement characteristics of the background liver and liver tumor has been well recognized[9]. Recently developed dynamic contrast enhanced (DCE) magnetic resonance (MR) imaging is a noninvasive imaging technique that can be used to derive quantitative parameters that reflect microcirculatory structure and function in imaged tissues[10]. DCE MR imaging has been applied to quantify perfusion changes in the liver parenchyma observed in hepatic fibrosis and cirrhosis, and to quantify the angiogenic activity in malignant focal liver lesions[11].

To our knowledge, there have been very few studies about hepatocellular nodules using DCE MR[7, 12]. The aim of our study was to assess the perfusion parameters and angiogenesis of HCC using DCE MR and correlate it with histopathologic findings in an experimental rat model.

II. MATERIALS AND METHODS

1. Animal Model

The study protocol was approved by the institutional review board for animal research. Six-week-old male Sprague-Dawley rats (N=20) weighing 120–150 g were used for tumor induction. The animals were acclimated for one week and maintained under specific pathogen-free environmental conditions with temperatures between 20°C and 24°C, and with 55% humidity in a 12 h light/dark cycle with free access to clean diet.

For induction of liver tumors, mini-pumps (Alzet model 2002;ALZA Scientific Products, Palo Alto, CA) provided a continuous infusion (0.5 μL/h for 2 weeks, nominal reservoir volume 200 μL) of diethylnitrosamine (DEN; N0756, Sigma Chemical Co., St. Louis, MO) dissolved in dimethyl sulphoxide (DMSO) at a dose level of 45mg/pump. DEN is a carcinogen inducing liver tissue changes of progressive malignancy[13-14]. The pathological aspects of these lesions are comparable to human hepatocarcinogenesis.

The mini-pumps were implanted into the peritoneum of the rats, during a surgical procedure that was performed under ether anesthesia. After 3 weeks, the implanted mini-pumps were removed.

2. MR Imaging

The rats underwent MRI of the liver, 32 to 36 weeks after DEN treatment. The rats were examined with a 3-T MR imaging system (Achieva 3.0T; Philips, Best, The Netherlands). Animal-dedicated radiofrequency (RF) coil were used for RF excitation and signal reception. All rats were anesthetized for MRI with an intraperitoneal administration of 1 mg/kg zoletil 50 (Vibac Laboratories, Carros, France).

For detection of hepatic nodules, conventional liver MR protocols were used as follows: transverse T1-weighted gradient echo (TR/TE=450/11msec; section thickness, 2mm; no intersection gap; matrix, 332×320; field of view, 80×80 mm) and transverse T2-weighted gradient echo sequences (TR/TE=2527/20msec; section thickness, 2mm; no intersection gap; matrix, 332×320; field of view, 80×80 mm; flip angle).

DCE MR was performed using gadolinium diethylene triaminepenta acetate (Gd-DTPA) (Magnevist; Schering AG, Berlin, Germany) through the nodule with fast T1 weighted 3-dimensional imaging technique (THRIVE sequence : T1-weighted High Resolution Isotropic Volume Examination, TR/TE=4.7/2.7msec, flip angle=10), resulting in a temporal resolution of 15 sections / 3.1 seconds. Gd-DTPA was rapidly administered manually by one investigator via the tail vein of the rat at a dose of 0.1mmol Gd/kg. A bolus of contrast material was followed by a 1.0mL bolus of

saline flush. This sequence was applied continuously for 200 measurements. Immediately after the last set of MR imaging, the rats were sacrificed for pathologic examination.

3. Histopathologic Analysis

The livers were removed and then fixed in a 4% paraformaldehyde solution. The whole liver was serially sectioned into 3mm-thick sections corresponding to the transverse plane that aligned closely to the MR imaging. The nodules had corresponded precisely to DCE MRI using the transverse images as a reference. After paraffin embedding, standard hematoxylin-eosin staining was used to assess the nature of nodules. Histological examination was conducted by 2 hepatopathologists and a consensus was achieved. All nodules were defined grossly and microscopically following the criteria and nomenclature of the International Working Party on the Terminology of Nodular Hepatocellular Lesions[5].

4. Data analysis

DCE MR imaging data were transferred from the MR imagers to an independent personal computer for perfusion analysis. Perfusion parametric maps were obtained by using a commercially available software package (Nordic ICE; NordicNeuroLab, Bergen, Norway). Regions of interest (ROIs) were determined for each hepatic

nodule and its surrounding hepatic parenchyma, avoiding the inclusion of imaging artifacts and major vascular structures by one radiologist (J.H.L), who was blinded to the pathologic results.

For semiquantitative assessment of the perfusion parameters of nodules and surrounding hepatic parenchyma, time to peak (TTP) and peak enhancement (PE) were estimated.

5. Statistical analysis

Wilcoxon signed rank tests were used to compare perfusion parameters of nodular lesions (DN and HCC) and surrounding hepatic parenchyma. Statistical differences in perfusion parameters between DN and HCC were analyzed using the Mann-Whitney U test. The correlations between DN and each grade of HCC with perfusion parameters were analyzed using Spearman's coefficient of rank correlation. Significant differences were defined as those with $P < 0.05$. All statistical analyses were performed using several commercially available software packages (MedCalc version 8.2.1.0; MedCalc Software, Mariakerke, Belgium).

III. RESULTS

1. Pathologic findings.

Out of 20 rats with DEN exposure for induction of liver tumors, 2 died

spontaneously before we could complete the study and 2 could not obtain MR imaging due to technical problems. Based on precisely correlating nodular lesions in the resected liver with the location of nodular lesions visualized with MR, a total of 13 nodules were found in 12 of the 16 rats. Among these, 5 DNs were identified in 5 rats, with diameters ranging from 2.6mm to 9.8mm (mean 5.8mm). There were 8 HCCs (three Edmonson grade I, two Edmonson grade I-II, three Edmonson grade II) found in 7 rats, with diameters ranging from 4mm to 17mm (mean 9.5mm).

2. MR and MR perfusion imaging findings.

We found no statistical differences in comparing the blood perfusion parameters (TTP and PE) between nodules and their surrounding hepatic parenchyma (Tables 1 and 2).

Table 1. Comparisons of perfusion parameters between dysplastic nodule and adjacent liver parenchyma

	DN (N=5)	Parenchyma (N=5)	*P* value
Mean TTP	45.2745	136.2550	1.0000
HPH of TTP	39.9813	109.6293	0.1875
Width of TTP	94.9960	210.0363	0.8125
Mean PE	19.4884	100.4900	0.1250
HPH of PE	17.0634	86.5368	0.4375
Width of PE	19.2155	89.7172	0.1875

DN : dysplastic nodule, TTP : time to peak (s)

HPH : histogram peak height, PE = peak enhancement (Hounsfield unit).

Table 2. Comparisons of perfusion parameters between hepatocellular carcinoma and adjacent liver parenchyma

	HCC (N=8)	Parenchyma (N=8)	*P* value
Mean TTP	6.1979	14.9317	0.6406
HPH of TTP	1.3472	1.9244	0.4375
Width of TTP	40.0164	42.5050	0.8438
Mean PE	10.7776	8.7597	0.7422
HPH of PE	10.8081	7.9754	0.5469
Width of PE	11.7619	14.6764	0.1484

HCC : hepatocellular carcinoma, TTP : time to peak (s)

HPH : histogram peak height, PE : peak enhancement (Hounsfield unit).

There were no significant differences in mean value of TTP, histogram peak height (HPH) and width of TTP between DN (Figure 1) and HCC (Figure 2). There were significant differences in the mean values of PE and HPH of PE between DN and HCC (Table 3).

The relationship between perfusion parameters and tumor grade is summarized in Table 4. Mean value and HPH of PE showed substantial correlation with statistical significance.

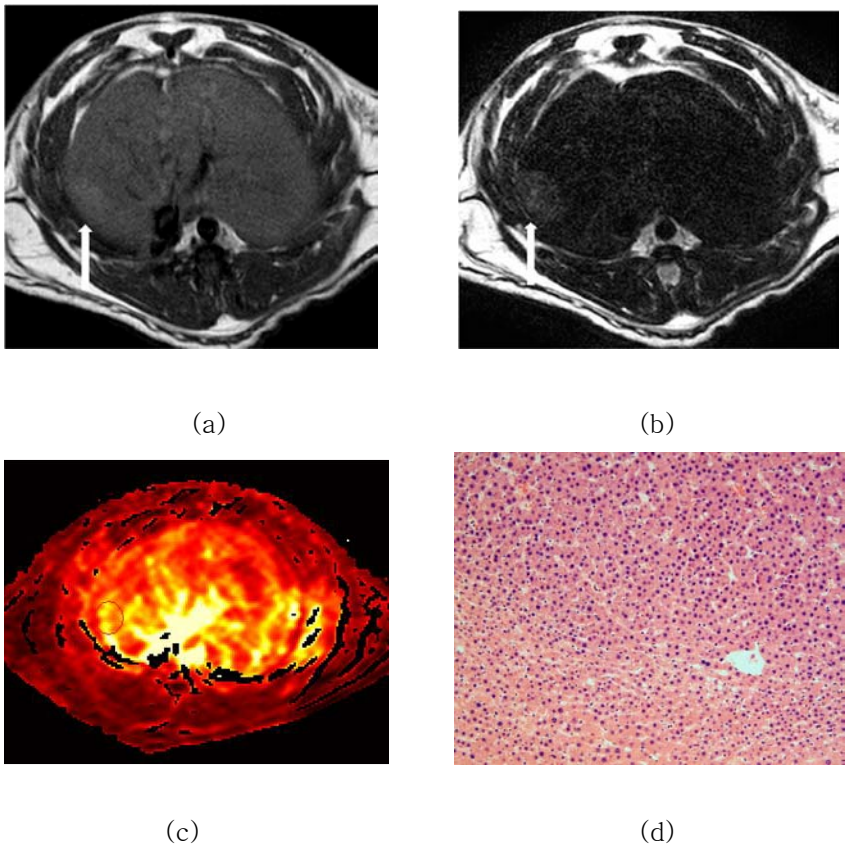
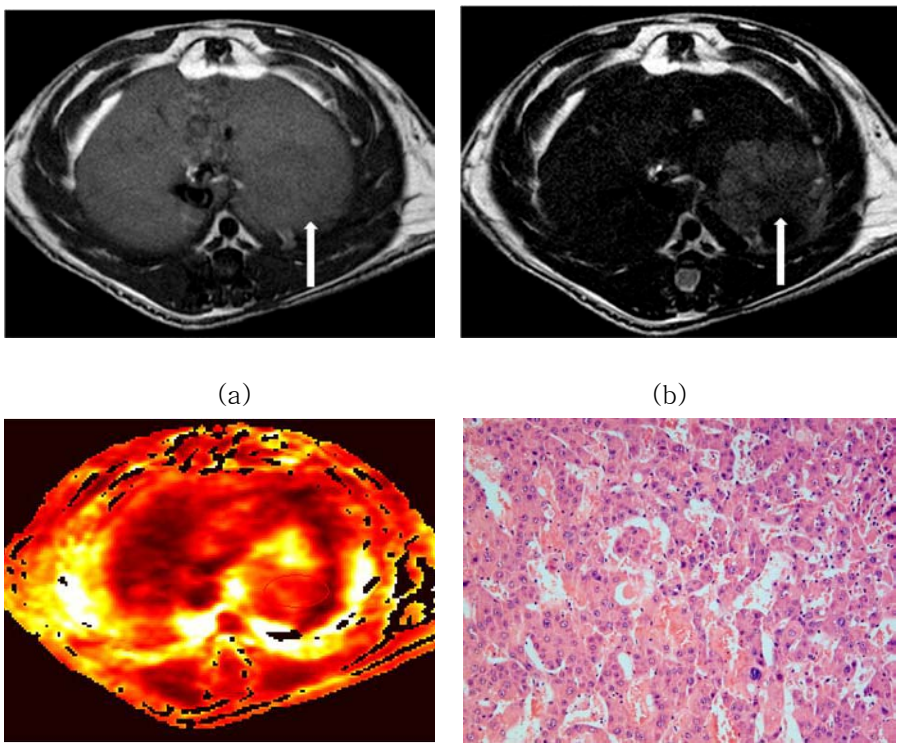


Figure 1. Dysplastic nodule in rat liver.

(a) T1-weighted gradient-echo MR image shows slightly high signal intensity nodule(arrow) in liver. (b) T2-weighted gradient-echo MR image shows slight high signal intensity nodule(arrow) in liver. (c) Peak enhancement map is derived from DCE MR imaging data, and ROI was drawn in the nodule. (d) The nodule was pathologically proved to be a DN (hematoxylin-eosin [H-E] stain, X 100).



(a) (b)

(c) (d)

Figure 2. HCC in rat liver.

(a) T1-weighted gradient-echo MR image shows slightly low signal intensity nodule(arrow) in liver. (b) T2-weighted gradient-echo MR image shows high signal intensity nodule(arrow) in liver. (c) Peak enhancement map is derived from DCE MR imaging data, and ROI was drawn in the nodule. (d) Histologic examination reveals HCC (hematoxylin-eosin [H-E] stain, X 400).

Table 3. Comparisons of perfusion parameters in DN and HCC

	DN (N=5)	HCC (N=8)	*P* value
Mean TTP	136.2550	14.9317	0.0653
HPH of TTP	109.6293	1.9244	0.0932
Width of TTP	210.0363	42.5050	0.5237
Mean PE	100.4900	8.7597	0.0295
HPH of PE	86.5368	7.9754	0.0109
Width of PE	89.7172	14.6764	0.3543

DN = dysplastic nodule, HCC = hepatocellular carcinoma

TTP = time to peak (s), HPH =histogram peak height

PE = peak enhancement (Hounsfield unit).

Table 4. Correlation of tumor grade with perfusion parameters in experimental rat

	Correlation (rho)	*P* value
Mean TTP	-0.518	0.0726
HPH of TTP	-0.495	0.0865
Width of TTP	-0.149	0.6060
Mean PE	-0.607	0.0355
HPH of PE	-0.610	0.0346
Width of PE	-0.338	0.2418

TTP = time to peak (s), PE = peak enhancement (Hounsfield unit)

HPH =histogram peak height.

IV. DISCUSSION

In human, most HCC arise in the background of chronic hepatitis/cirrhosis. HCC usually follows a multistep sequence, and de novo development of HCC may occur, but this appears to be rare[3]. It is now well established that HCCs develop from precancerous (dysplastic) lesions, including dysplastic foci and DN. Morphologically recognizable lesions during hepatocarcinogenesis include dysplastic foci, DN (high grade or low-grade), early HCCs, and progressed HCCs[3]. Computerized tomography (CT), MRI and ultrasound (US) have all been utilized for the evaluation of liver nodules such as HCC. However, the diagnosis of precancerous lesions is still difficult because of the overlapping between DN and RN, DN and early HCC on ultrasound, CT and MRI.

Blood supply to the various nodules in cirrhotic liver is complex. Regarding the blood supply, three types of feeding vessels can be recognized in large liver nodules, as described above: (1) branches of the hepatic artery, (2) branches of the portal vein, and (3) abnormal (nontriadal) arteries[3]. The proportion of blood supply derived from each type of vessel differs in accordance with the presence and grade of malignancy in each particular nodule. Small HCCs are supplied by nontriadal arteries, whereas early HCCs and dysplastic nodules may receive blood supply from both portal tracts and nontriadal arteries. However, the nontriadal arteries of early HCCs are insufficiently developed. The similarities in blood supply of these three types of

nodular lesions result in significant overlap of findings on dynamic imaging[4]. Hayashi et al.[15] shows that, when HCC emerges in a cirrhotic liver, the intranodular normal portal and arterial supply gradually decreases, whereas the abnormal arterial supply provided by nontriadal arteries increases, following the increase in the grade of malignancy of the nodule.

MR imaging has an important role in the evaluation of diffuse and focal liver diseases. Recently developed perfusion imaging of the liver can be described as a quantitative imaging method reflective of hepatic parenchymal and hepatic lesion blood flow[9]. Therefore, this technique may be more valuable for the differential diagnosis of nodules in liver. Perfusion MR imaging has become synonymous with DCE MR imaging. DCE MR imaging has been applied to quantify perfusion changes in the liver parenchyma observed in hepatic fibrosis and cirrhosis, and to quantify the angiogenic activity in malignant focal liver lesions[11]. In liver fibrosis, the decrease in portal blood flow is compensated by an increase in arterial blood flow, resulting in an elevated arterial perfusion fraction(known as the arterial buffer response)[16]. Changes in blood transit time of contrast agents and relative arterial to portal venous blood flow can be estimated using DCE MR imaging[11].

DCE MR imaging methods can be divided into semiquantitative and quantitative techniques[11]. Semiquantitative method can be processed by the analysis of signal intensity(SI) changes in organs or lesions of interest. This can be possible with T1-

weighted images given the T1-shortening effects of gadolinium-based contrast media. Semiquantitative DCE MR imaging methods have the advantage of being easy to implement, without the need for pharmacokinetic model, conversion to tracer concentration, or input functions[11]. Limitations are related to the variability of MR SI and numerous sources of bias, such as scanning protocols, amplifier gain, injection technique, and cardiac output, which could potentially limit the reproducibility of semiquantitative parameters[11].

In our study, there were no perfusion parameters(TTP and PE) differences between nodule(DN or HCC) and their surrounding hepatic parenchyma. Tajima et al[17] point out that the degeneration or disappearance of preexisting hepatic arteries causes a decline in intranodular arterial blood flow in DNs or well-differentiated HCCs. Insufficient growth of neovascularized arteries coupled with the disappearance of preexisting hepatic arteries results in hypovascularity compared with the surrounding hepatic parenchyma. As a result of a marked increase in neovascularized arteries, neovascular blood flow becomes dominant. The portal blood supply decreases with advancement of the tumor and eventually, the tumor is fed mainly by arterial flow. If preexisting hepatic arteries and portal veins have not yet decreased, DNs would have the same blood supply as the surrounding cirrhotic liver. In addition, it is well known that some well-differentiated HCCs are hypovascular. Highly differentiated tumors may have nearly normal vascular

architecture, while highly anaplastic tumors tend to demonstrate extremely disorganized and heterogeneous networks of vascular spaces without recognizably mature elements[18] .

In the present study for the comparison of DN and HCC, DCE MR parameters (Mean value and HPH of PE) showed statistical significance. Xu and colleagues[7] reported that the PE of HCCs was significantly higher than cirrhotic liver, but PE of DNs was lower than cirrhotic liver. PE in our study correlates with grade of tumors (DN and HCC) with statistical significance. There was a significant negative correlation between mean value and HPH of PE and grade of tumor (DN and HCC). These results suggest that mean value of PE may decrease with multistep process of hepatocarcinogenesis from DN to well-differentiated HCC. All of the HCC in our study was Edmonson grade I or II, and we think that HCC in our study showed decreased PE because of decreased intranodular arterial blood flow, insufficient growth of neovascularized arteries, and disappearance of preexisting hepatic arteries in well-differentiated HCC. Moderately and poorly differentiated HCCs shows increased intranodular arterial blood supply and markedly decreased portal venous supply[4]. Xu et al[7] reported that the PE of HCCs was significantly higher than cirrhotic liver, moderate or poorly differentiated HCC may show increased PE in DCE MR imaging. Histogram analysis was performed by assessing the peak height of the histogram distribution of perfusion parameters in the tumor. This approach

was chosen because the resulting height was directly determined on the basis of the underlying heterogeneity of blood supply. We think that histogram analysis of the perfusion parameters can be a useful diagnostic tool to predict heterogeneity of blood supply in hepatocellular nodules.

There are several limitations to our study. Firstly, the number of lesions is small and there might be selection bias. And all of the HCC in our study was well-differentiated HCC with Edmonson grade I or II. However, despite the small sample size, there was statistical significance between DN and HCC in some of the perfusion parameters. Additional studies with larger numbers of patients would be important. Secondly, we used semiquantitative parameters for evaluation of blood supply in hepatic nodules. The drawbacks are that these semiquantitative parameters did not accurately reflect contrast medium concentration in the tissue of interest and were subject to variations in MR scanner settings. However, semiquantitative curve analyses can be extremely valuable, particularly in their application to tumor characterization. The slope percentage showed an almost significant correlation with the microvascular density (MVD) which is related with degree of vascularization and perfusion of tumor[19]. Despite its simplicity, the semiquantitative method used in our study has achieved satisfactory results. Another limitation is that we could not correlate hepatic tumor with MVD or vascular endothelial growth factor (VEGF) for quantification of angiogenesis. MVD is currently considered the gold standard for

histological assessment of the degree of angiogenesis within a tumor. VEGF is the only growth factor proven to be specific and critical for blood vessel formation[20]. We had failed to standardize the level of VEGF staining in rats. But, the results of correlation studies on dynamic MRI and the VEGF expression are conflicting, and it is likely that VEGF expression may not fully describe the functional angiogenetic status of the tumor[21-22].

Using DCE MR imaging, we will be able to understand hemodynamic changes in tumors and many other pathologic diseases, including multistep hepatocarcinogenesis. DCE MR imaging can be performed repeatedly and thus presents a major advantage in the evaluation of dynamic changes in tumor angiogenesis and may allow for the early detection of HCC. In the future, DCE MR imaging will become increasingly important in diagnosis and management of liver disease, with a standardized imaging protocol and methods of image analysis.

V. CONCLUSION

After the study on the DCE MR of HCC in an experimental rat model the conclusions are as follows.

1. There were significant differences in mean value and HPH of PE between DN and HCC.
2. There were substantial negative correlation between tumor grades and perfusion

parameters with mean value and HPH of PE in DN and well-differentiated HCC. But, further evaluation with a large number of the cases with histopathologic correlation is needed.

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ABSTRACT(IN KOREAN)

실험용 백서의 간세포암모델에서 자기공명 관류영상을 이용한 신생혈관생성의 정량화 및 병리소견과의 비교.

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이 제 희

다수의 간세포암은 양성 재생성 결절에서 이형성 결절과 소간세포암을 거쳐 진행성 간세포암으로 발전하는 다단계발암과정으로 발암한다. 이형성 결절과 고분화 간세포암의 감별진단은 현재까지도 특히 어려운 상태로 남아있다. 최근에 개발된 자기공명 관류영상은 비침습적인 영상법으로, 간에서의 혈류변화를 정량화 할 수 있다. 본 연구의 목적은 실험용 백서의 간세포암모델에서 자기공명 관류영상을 이용한 신생혈관생성 및 혈류인자의 정량화 및 병리소견과의 비교 분석이다.

총 20마리의 백서가 간암 유도에 사용되었고, diethylnitrosamine (DEN)을 미니펌프를 이용하여 지속적으로 투입하였다. 백서는 DEN 처리후 32주에서 36주 사이에 3.0 테슬라 자기공명영상 시스템으로 자기공명 관류영상을 시행하였다. 혈류

지도와 time to peak(TTP)나 peak enhancement(PE)와 같은 혈류 인자 값을 상용 소프트웨어(Nordic ICE)를 이용하여 얻었다. 각각의 결절들은 자기공명 관류영상과 정확하게 상응하였다.

총 13개의 결절이 12마리의 백서에서 발견되었다. 이 중에서 5마리의 백서에서 5개의 이형성 결절과 7마리의 백서에서 8개의 간세포암(3개의 Edmonson 등급 I, 2개의 Edmonson 등급 I- I I, 3개의 Edmonson 등급 I I)이 발견되었다.

결절과 주변 간조직에서 얻은 혈류인자값(TTP와 PE)에 통계학적인 차이는 없었다. 이행성 결절과 간세포암 사이에 평균 TTP값, 히스토그램 최빈(histogram peak height, HPH) TTP값, TTP값의 폭에 통계학적인 차이는 없었다. 이행성 결절과 간세포암 사이에 평균 PE값, 히스토그램 최빈 PE값에 통계학적으로 유의한 차이를 보였다. 평균 PE값과 히스토그램 최빈 PE값은 종양의 등급에 따는 통계학적으로 유의한 연관성을 보였다.

결론적으로, 자기공명 관류영상은 다단계 간세포발암의 과정에서 감별진단 및 간질환의 관리에 사용될 수 있다.

핵심되는 말 : 간세포발암, 이형성 결절, 간세포암, 자기공명 관류영상