Hepatic Local Micro-environmental Immune Status in Hepatocellular Carcinoma and Cirrhotic Tissues

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in the world, especially in Guangxi, China. The causes and mechanism of its tumourigenesis and development have not been completely clarified. Some studies revealed that the hepatic local cellular immune function was one of the factors. In the present study, the local micro-environmental immune status was explored by investigating the number, distribution and function of CD_3, CD_{57}, CD_{20}, CD_{68}, and granzyme B (GrB) positive cells in 60 patients with HCC and 62 patients with liver cirrhosis (LC) and its relationship with the prognosis of the patients. The results showed that the number of T and B lymphocytes and natural killer (NK) cells in the liver of HCC patients was significantly higher than that in the LC and normal controls; while the number of macrophages (Mφ) was significantly lower. The number of Mφ in the tissues decreased successively with the decrease of HCC differentiation; GrB-expressing cells in the liver predominantly consisted of CD_{57} positive cells. The number of NK cells, B lymphocytes and GrB-expressing cells in the cancerous tissues of stage I and II was significantly higher than that of stages III and IV. The number of T lymphocytes, NK cells, Mφ and GrB-expressing lymphocytes in HCC cases without metastasis in 15 months was significantly higher than in the metastatic counterparts. The number of T and B lymphocytes, NK cells, and GrB-expressing cells decreased in patients with the progression of the HCC. These results suggest that the number of T and B lymphocytes, NK cells, Mφ and GrB-positive lymphocytes might be important markers in the estimation of hepatic local immune status and be useful factors for predicting the prognosis of HCC patients.

Estatus Inmune Microambiental Local Hepático en el Carcinoma Hepatocelular y los Tejidos Cirróticos

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RESUMEN

El carcinoma hepatocelular (CHC) es uno de los tumores malignos más comunes en el mundo, especialmente en Guangxi, China. Las causas y el mecanismo de su desarrollo y génesis tumoral no han sido completamente aclarados. Algunos estudios revelaron que la función inmune celular local hepática era uno de los factores. En este estudio se exploró el estatus inmune microambiental local, mediante la investigación del número, distribución y función de CD_3, CD_{57}, CD_{20}, CD_{68}, y células positivas para la granzima B (GrB) en 60 pacientes con CHC y 62 pacientes con cirrosis hepática (CH) y su relación con la prognosis de los pacientes. Los resultados mostraron que el número de linfocitos T y B y las células asesinas naturales (NK) en el hígado de los pacientes con HCH, era significativamente mayor que en los controles normales y los pacientes con CH, mientras que el número de macrófagos (Mφ) fue significativamente menor. El número de Mφ en los tejidos disminuyó sucesivamente a la par con la disminución de la diferenciación de CHC; las células expresoras de GrB en el hígado consistían predominantemente en células positivas CD_{57}. El número de células NK, linfocitos B y células expresoras de GrB en los tejidos cancerosos de etapas I y II, fue significativamente más alto que el de las etapas III y IV. El número de linfocitos T, células NK, Mφ y linfocitos expresores de GrB en los casos de CHC sin metástasis en 15 meses, fue significativamente mayor que en las con-

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trapartidas metastáticas. El número de linfocitos T y B, células NK, y células expresoras de GrB, disminuyó en los pacientes a medida que progresaba el CHC. Estos resultados sugieren que el número de linfocitos T y B, células NK, Mφ y linfocitos GrB-positivos podrían ser marcadores importantes en la valoración del estatus inmune local hepático y los factores útiles a la hora de predecir la prognosis de los pacientes de CHC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in the world, especially in Guangxi, China. The causes and the mechanism of its carcinogenesis and development have not been clarified. Some studies revealed that the immune function of the host was a contributing factor and suggested that the immune system is important in the control of the development of HCC (1). The presence of lymphocyte infiltration in human HCC is also evident (2). However, since it is not easy to get the samples, the local immune status of the HCC patients is poorly characterized (3). To the best of the authors' knowledge, no similar studies on the relationship between the immune status and the HCC patients' prognosis have been documented in the literature. The present study explored the hepatic local micro-environmental immune status by immunohistochemically investigating the number, the distribution and some of the functions of hepatic local immunocytes in HCC, paraneoplastic, liver cirrhosis (LC) and normal liver tissues by using monoclonal antibodies of CD_3, CD_{57}, CD_{20}, CD_{68}, and granzyme (GrB). Furthermore, the results were compared with the histologic subtypes and clinical parameters such as clinical stage and metastasis to evaluate the clinical outcome.

SUBJECTS AND METHODS

This retrospective study included 60 cases of HCC, 62 cases of LC and 23 normal controls utilizing tissues surrounding liver cavernous haemangioma. The age of HCC patients ranged from 23 to 73 years, with a median age of 47 years. Among the 60 patients, 57 were males and three were female; 48 cases had cirrhosis and 12 did not; 38 cases were alpha-fetoprotein (AFP) positive. The paraneoplastic tissues were taken at least 2 cm from the cancerous node. All cases were initial hepatectomies in order to avoid the secondary changes of healing post biopsy. In the group of LC patients, 47 were males and 15 were females; the age range was 15 to 74 years, with a median of 43 years. In the group of normal controls, 10 were males and 13 were females; the age range was 26 to 67 years, with a median of 45 years. All cases were randomly chosen from the hepatectomies performed over a one to two-year span in the First Affiliated Hospital, Guangxi Medical University, China, between May 2002 and January 2003.

The histopathologic diagnoses were made according to the World Health Organization (WHO) international histologic classification of HCC. There were 3 cases in grade 1, 38 in grade 2 and 19 in grade 3. According to the TNM classification, their clinical stages were stage I, 3 cases; stage II, 28 cases; stage III, 11 cases and stage IV, 18 cases. Clinical in-formation was obtained from the records. Among 60 HCC patients, 25 cases had metastases within 15 months. Six pa-tients were lost to follow-up. Histopathological diagnosis and classification were made by the same pathologist.

Specimens were routinely fixed in 10% buffered formalin and embedded in paraffin. For the immunohisto-chemical study, the conventional avidin–biotin complex method was employed as described elsewhere using the following monoclonal antibodies: anti-CD_3, anti-CD_{57}, anti-CD_{68}, anti-CD_{20} (Maxim Corp, Fuzhou, China) and anti-GrB (Zhongshan Corp; Beijing, China) (4). Sections of normal lymph node tissues were used as positive controls for CD_3, CD_{57}, CD_{20} and GrB and normal unaffected tissues in the liver were used as positive controls for CD_{68} respectively. PBS replaced specimens were used as negative controls.

The positive cells were recognized and counted in 10 randomly selected high-power fields (HPF 400x) by using a light optic microscope. The number of the positive cells was expressed as mean ± standard deviation (mean ± SD). Mann-Whitney/Wilcoxon test and Pearson correlation analysis were employed to analyze the results of the experiment by utilizing Statistical Package for the Social Sciences 12.0 software for windows. A value of $p < 0.05$ was regarded as statistically significant.

RESULTS

CD_3 positive cells were oval or circular and exhibited a different invasive pattern by which single cells or small clusters of cells were scattered throughout the liver tissues. They were distributed in liver sinusoids in normal hepatic tissue, with a few cells in the portal area. In LC tissues, CD_3 positive cells were gathered in fibrous tissues, besides being distributed partly in sinusoids. In HCC, most of the CD_3 positive cells were found in the surrounding matrix of cancerous and paraneoplastic tissues. Some were distributed in sinusoids of paraneoplastic tissues and blood space in HCC. The number of CD_3^+ cells from highest to lowest was: paraneoplastic, HCC, LC and normal control liver tissues ($p < 0.05$) (Table 1).

The CD_{57} positive NK cells were buff, light brown, brown-yellow or dark brown in cell cytoplasm and cyto-membrane with round or oval shape. They were sprinkled within the liver sinusoids, blood space in HCC and the surrounding matrix without any concentrating trend. Hepatic

Table 1: The number of different cells in various tissues (±s, *n*/HP)

Tissues	*n*	CD$_3$	CD$_{57}$	CD$_{20}$	CD$_{68}$	GrB
HCC	60	60.5 ± 18.9	6.8 ± 5.1	40.3 ± 29.9	24.6 ± 13.4	5.5 ± 4.6
G I	3	65.9 ± 19.7	3.7 ± 1.2	36.9 ± 5.7	43.5 ± 5.1	3.8 ± 4.1
G II	38	59.9 ± 17.6	7.4 ± 2.4	41.8 ± 31.1	25.4 ± 11.8	5.9 ± 4.8
G III	19	61.0 ± 19.1	6.2 ± 2.9	39.0 ± 29.9	20.1 ± 14.6	4.8 ± 4.3
Paraneoplastic	60	80.2 ± 23.5	6.2 ± 4.1	28.0 ± 27.2	43.5 ± 12.9	6.7 ± 3.9
LC	62	53.0 ± 18.7	4.2 ± 2.9	21.5 ± 18.2	41.0 ± 13.5	3.5 ± 2.3
Normal	23	45.4 ± 11.7	4.8 ± 2.3	8.1 ± 5.9	40.3 ± 8.9	3.6 ± 2.4

NK cells were located in the liver sinusoids adherent to the endothelium. The number of CD$_{57}^+$ cells from highest to lowest was: HCC, paraneoplastic, normal control liver and LC tissues. The number of CD$_{57}^+$ cells in HCC was significantly higher than that in LC ($p < 0.05$) and normal liver tissues ($p < 0.05$). The number of CD$_{57}^+$ cells in paraneoplastic tissues was significantly higher than that in LC tissues ($p < 0.05$). There were no differences between paraneoplastic and normal liver tissues, paraneoplastic and HCC tissues (Table 1).

B-cells, as identified by CD$_{20}$ staining, were round or elliptic and could be detected in each section in various degrees. They also exhibited a different invasive pattern in which single cells or small clusters of cells were scattered throughout the liver tissues. In normal hepatic tissues, they were distributed in the portal area; CD$_{20}^+$ cells were present focally in fibrous tissues and in portal area in LC, cancerous and paraneoplastic tissues. The number of CD$_{20}^+$ cells from highest to lowest was: HCC, paraneoplastic, LC and normal control liver tissues. There was no significant difference between the number of CD$_{20}^+$ cells in paraneoplastic and LC tissues while there were significant differences between the other groups ($p < 0.01$) (Table 1).

The CD$_{68}^+$ Mφ were distributed in the blood space of HCC, the sinusoids of paraneoplastic tissues and the normal liver tissues. Most of the CD$_{68}^+$ Mφ in normal liver, LC, the well-differentiated HCC and paraneoplastic tissues had a stellate or spindle shape and were partly adherent to the sinusoidal endothelial cells. Other Mφ showed an oval shape and contained an abundance of cytoplasm. The location and the numbers of Mφ were variable in different patients. The numbers of Mφ with a stellate or spindle shape decreased with the decrease of the HCC differentiation while those with an oval shape and containing an abundance of cytoplasm increased. In some instances, no Mφ could be found in some parts of the poorly-differentiated HCC. The number of Mφ in HCC was significantly lower than that in para-neoplastic, LC and normal liver tissues ($p < 0.01$) but there was no difference among paraneoplastic and LC and normal liver tissues.

The number of GrB$^+$ cells in tissues from highest to lowest was: paraneoplastic, HCC, normal control liver and LC tissues. There was no difference in the number of the GrB$^+$ cells between LC and normal tissues while significant differences occurred between other groups ($p < 0.05$) (Table 1).

The number of Mφ in G1 was significantly higher than in G2 ($p < 0.05$) and G3 ($p < 0.05$). There was no difference between the number of Mφ cells in paraneoplastic tissues and the histological grades. The number of CD$_3^+$, CD$_{57}^+$, CD$_{20}^+$ and GrB$^+$ cells had no relationship with the histological grades either in HCC or paraneoplastic tissues ($p > 0.05$) (Table 1).

The number of CD$_3^+$ cells in the cases of stages I and II was lower than that of III and IV both in HCC and paraneoplastic tissues. This was not statistically significant ($p > 0.05$). The number of CD$_{57}^+$ cells in the cases of stages I and II was higher than that of stage III and IV ($p < 0.05$). There was no relationship between the number of CD$_{57}^+$ cells in paraneoplastic tissues and the clinical TNM stage. The number of CD$_{20}^+$ cells in the cases of stages I and II was significantly higher than that of stage III and IV both in HCC and paraneoplastic tissues ($p < 0.05$). There was no relationship between the number of Mφ in HCC, para-neoplastic tissues and the clinical TNM stage. The number of GrB$^+$ cells in the cases of stages I and II was significantly higher in HCC tissues than that of stages III and IV ($p < 0.05$), but there were no significant differences in paraneoplastic tissues ($p > 0.05$) (Table 2).

As shown in Table 2, the number of CD$_3^+$ cells in the cases with metastasis within 15 months was significantly lower than those without metastasis in HCC tissues ($p < 0.05$). The number of NK-cells in HCC and paraneoplastic tissues in the cases with metastasis within 15 months were both significantly lower than those without metastasis ($p < 0.01$). The number of CD$_{20}^+$ cells in HCC and paraneoplastic tissues in the cases with metastasis within 15 months were both lower than that without metastasis but without significant difference ($p > 0.05$). The number of Mφ in HCC in the cases with metastasis within 15 months was significantly lower than in those without metastasis ($p < 0.05$). The number of Mφ in paraneoplastic tissues in the cases with metastasis within 15 months was higher than that without metastasis but without significance ($p > 0.05$). The number of GrB$^+$ cells in the cases with metastasis within 15 months was significantly lower than those without metastasis in HCC tissues ($p < 0.01$) (Table 2).

Table 2:   The relationship between the number of various positive cells and clinical TNM stages and metastasis

Clinical	n	CD_3^+ in HCC	CD_3^+ in paraneo-plastic	CD_{57}^+ in HCC	CD_{57}^+ paraneo-plastic	CD_{20}^+ in HCC	CD_{20}^+ in paraneo-plastic	CD_{20}^+ in HCC	CD_{20}^+ in paraneo-plastic	GrB^+ in HCC	GrB^+ in paraneo-plastic
TNM stages I, II	31	58.6 ± 15.4	76.2 ± 24.2	8.3 ± 5.3	6.2 ± 4.1	49.1 ± 29.8	35.3 ± 30.6	24.7 ± 14.0	40.5 ± 6.9	6.8 ± 5.3	6.0 ± 3.7
TNM stages III, IV	29	62.5 ± 22.2	84.5 ± 22.3	5.3 ± 4.5	6.3 ± 4.2	31.0 ± 27.5	20.2 ± 20.8	24.5 ± 12.8	46.8 ± 16.6	4.1 ± 3.2	7.4 ± 4.1
With metastasis	25	53.1 ± 16.0	75.7 ± 25.6	2.9 ± 3.1	4.2 ± 2.8	31.4 ± 26.2	22.9 ± 23.4	20.9 ± 11.3	46.6 ± 18.1	2.5 ± 1.6	5.4 ± 4.0
Without metastasis	29	65.5 ± 21.1	81.4 ± 23.2	9.4 ± 4.5	7.5 ± 4.5	46.2 ± 32.4	29.9 ± 30.6	30.1 ± 14.4	41.1 ± 5.3	7.0 ± 4.3	7.4 ± 3.6

The scatter diagram showed that there was a linear trend between the number of Mφ and NK cells in HCC. In further linear correlation analysis, there was a linear positive correlation between the number of Mφ and NK-cells (r = 0.344, p < 0.05). There was a linear trend between the number of CD_{57}^+ and GrB^+, $CD3^+$ and GrB^+ cells in HCC and paraneoplastic tissues. They all had linear positive correlations (Table 3).

Table 3: The relationship between the numbers of various positive cells

Tissues	CD_3 and GrB		CD_{57} and GrB		CD_{57} and CD_{68}	
	r	P	R	P	R	P
HCC	0.318	< 0.05	0.673	< 0.01	0.344	< 0.01
paraneoplastic	0.312	< 0.05	0.508	< 0.01	-0.091	0.489
LC	0.14	0.279	0.492	< 0.01	-0.015	0.907
Normal	-0.093	0.673	0.742	< 0.01	0.013	0.952

DISCUSSION

T cells, B cells, NK cells, and Mφ are the main components of the immune system in the body. T cells play an important role in leading cell immune function, specifically killing tumour cells (5) and stimulating a specific and durable anti-tumour response (3). B cells produce the antibodies leading to the immune effect of body fluid (6). CD_{68} is expressed in the Mφ in all of the tissues. Kupffer cells (KC) are the Mφ residing in the sinusoids of the liver and play important roles in maintaining homeostasis and in the host anti-tumour defense mechanism (7). They are also involved in the pathogenesis of liver diseases such as viral hepatitis, alcoholic liver injury, chemically mediated liver injury, liver fibrosis and hepatocyte regeneration (8–9). CD_{57} is a glycoprotein expressed in the leucocytes and neural endocrine cells. Anti-CD_{57} antibody was first used to identify the NK cells in the subgroup of lymphocytes. Hepatic NK cells, named Pit cells, were first described in 1976 by Wisse *et al*. The name Pit cell was introduced because of the characteristic cytoplasmic granules which in Dutch are called Pits, resembling the pits in a grape. It has been proven that hepatic NK cells are more

cytotoxic and have stronger tumour killing ability than blood and spleen NK cells (10). They have been demonstrated to attack tumour cells or virus-infected cells by the following: 1) identifying and adhering to the target cells; 2) activating cells and conducting 'lethal hit' including to deliver killing cytokines by the perforin/granzyme pathway or inducing the target cells with *Fas* expression on the surface to allow apoptosis by *Fas ligand (FasL)* (10–15). The present results indicate that GrB-expressing lymphocytes had positive correlation with CD_3 and CD_{57} positive cells in HCC, paraneoplastic, LC and normal liver tissues. However, it needs further investigation to confirm that GrB-expressing cells are T lymphocytes or NK cells.

Some experiments have shown that the number of NK cells in the HCC tissues from patients with metastases increased but their activity decreased (16). Similar results are reported herein. The mechanism of the increase of NK cells in HCC has not been clarified. It is known that the major histocompatibility complex class I (MHC-I) on tumour cells negatively regulates NK cell-mediated cytolysis (10). The HCC cells with defect of MHC-I are identified by the NK cells and these cells will accumulate in the areas and this causes the increase of NK cells in HCC. Meanwhile, it may play important non-specific anti-tumour function. Further study is required on how to enhance the activities and functions of NK cells in HCC.

The quantities of T lymphocytes and NK cells in HCC were obviously more than those in LC and normal liver tissues. The quantities of GrB^+ cells in paraneoplastic tissues and HCC were higher than in normal liver tissues and LC, which proved that in the tumour tissues, the activated CTL and NK cells obviously increase and may be related to the stimulus of the tumour antigen (17). GrB-expressing lymphocytes in paraneoplastic tissues were higher than those in the HCC tissues. This may be because tumour necrosis influenced the expression of GrB. When B cells are stimulated by antigen or mitogen in HCC tissues, fission and hyperplasia occur and hence, the number of B cells in HCC tissue was obviously higher than that in the LC and normal

liver groups. At the same time, the antigenicity of tumour cells in the centre of the tumour tissue is relatively strong, so the number of B-cells in tumour surrounding tissues was obviously lower than that in tumour solid tissue.

The number of NK cells in all of the tissues was significantly lower than that of Mφ. The proportion of Mφ and NK cells was 3.6–9.8 : 1 but there is some relationship between them: homing, differentiation and activation of the precursor NK cells rely on the existence of normal KC in the liver sinusoids. Cytokines such as IL-1 secreted by KC can be taken as the lymphocyte chemotactic factor to concentrate hepatic NK cells in the tumour. The interaction of KC and liver sinusoids endotheliocyte and adherent molecules expressed on the surface of endotheliocyte strengthen the adhering function between the precursor NK cells and the endotheliocyte. TNF-α and IFN-β secreted by KC facilitate the differentiation and activation (18). At the same time, the activated NK cells can secrete a large number of cytokines to function as anti-tumour and to regulate various functions of Mφ (11) as had been demonstrated in the present study; there was a linear positive correlation between the number of Mφ and NK cells in HCC, which proved that there was a synergistic effect between Mφ and NK cells in preventing the growth or development of malignant tumour.

CD_{68} was expressed not only on residential Mφ such as KC, but also on migrating Mφ (19). Morphological observations will help to distinguish these two cell types. For example, migrating Mφ are usually oval and contain an abundance of cytoplasm while KC usually have spindle or stellate-shaped cytoplasm and partly adhere to the sinusoidal endothelial cells. But it was difficult to distinguish these two cell types accurately by light microscopy as they had the same roles in the host defense mechanism; so in this study CD_{68} positive Mφ included these two types of cells. The results showed that the number of Mφ in HCC was significantly lower than that in paraneoplastic, LC and normal liver tissues. The number of Mφ decreased successively with the decrease of the HCC differentiation. But in well-differentiated HCC, the shape and number was almost the same as non-cancerous tissues. The number of Mφ in paraneoplastic, LC and normal liver tissues also showed no statistical difference in the present study. There are some possible reasons for the results. Under normal conditions, migrating Mφ migrates from blood to the liver sinusoids and develop into KC. The blood space in well-differentiated HCC is similar to normal sinusoids of the liver lobule, so it could maintain a normal internal environment similar to that of normal sinusoids to support the KC. But with the decrease of the HCC differentiation, sinusoid structure was destroyed which affected the existence of KC (20). The mechanism responsible for the tumouricidal activities of Mφ is not completely known. Mφ may execute their anti-tumour effect by increasing the production of some cytotoxic molecules such as NO, TNF-α and IFN-γ which may inhibit the growth of tumour by damaging cellular DNA and inducing apoptosis.

The number of NK and GrB positive lymphocytes in the cases of stages I and II were significantly higher than those of stages III and IV ($p < 0.05$) in HCC. It meant that NK and GrB-expressing cells in HCC regressed with development of clinically advanced TNM stages ($p < 0.05$). NK cell and GrB-expressing lymphocytes can be regarded as an index of judging HCC patients' prognosis. Meanwhile, as the cells of HCC transform malignantly, GrB may have certain influence and function in the natural progress of the tumour. It has been inferred that during the clinical progress in HCC, some sensitive sub-clone may have been killed by tumour immunity while the remaining sub-clone may resist the cytotoxic attack by CTL and NK cells. It can even withstand the attack of the apoptotic signal and gain stronger survival ability to escape the immunity surveillance. This aids hepatocarcinogenesis and development of HCC. The mechanism is similar to immunologic escape through Fas/FasL and this may be another molecular mechanism for the tumour to escape the immunity surveillance.

The number of B cells in the cases of stage I were significantly higher than those of stage II ($p < 0.05$) in both HCC and paraneoplastic tissues ($p < 0.05$). It proved that with the gradual progress of the tumour, the status of the humoral immunity will become poorer. The tumour immunity of incipient HCC may rely mainly on humoral immunity early but on cellular immunity later. The number of T lymphocytes, NK cells and GrB-expressing cells in the cases with metastasis within 15 months in HCC tissues were significantly lower than in those without metastasis ($p < 0.01$). The cellular immune status in the group with metastasis was much poorer than in those without metastasis. T lymphocytes and NK cells have the function of killing tumour cells and can suppress the metastasis of the HCC cells. The decreasing of immunocytes might be a risk factor predicting tumour metastasis.

The number of Mφ in the cases with metastasis within 15 months of HCC was significantly lower than in those without metastasis ($p < 0.05$). Previous studies also found that Mφ might play an important role in controlling occurrence and progression of liver metastasis by causing tumour cell apoptosis (21–22).

In summary, as HCC progresses, T lymphocytes, NK cells, B lymphocytes and GrB-expressing lymphocytes in hepatic local tissue reduce gradually. It indicates that T lymphocytes, NK cells, B lymphocytes, Mφ and GrB-expressing cells might be important markers in estimating the special anti-tumour immune status and a useful factor in predicting the prognosis of HCC patients.

REFERENCES

1. O'Beirne JP, Harrison PM. The role of the immune system in the control of hepatocellular carcinoma. Eur J Gastroenterol Hepatol 2004; **16:** 1257–60.
2. Liu Y, Poon RT, Hughes J, Feng X, Yu WC, Fan ST. Chemokine receptors support infiltration of lymphocyte subpopulations in human hepatocellular carcinoma. Clin Immunol 2005; **114:** 174–82.

3. Zhang J, Dong B, Liang P, Yu X, Su L, Yu D et al. Significance of changes in local immunity in patients with hepatocellular carcinoma after percutaneous microwave coagulation therapy. Chinese Medical J 2002; **115:** 1367–71.

4. Keehn CA, Saeed S, Bickle K, Kholil FK, Morgan MB. Expression of insulin-like growth factor-I receptor in primary cutaneous carcinomas. J Cutan Pathol 2004; **31:** 368–72.

5. Bricard G, Bouzourene H, Martinet O, Rimoldi D, Halkic N, Gillet M et al. Naturally Acquired MAGE-A10- and SSX-2-Specific CD8+ T Cell Responses in Patients with Hepatocellular Carcinoma. J Immunol 2005; **174:** 1709–16.

6. Gong Q, Ou Q, Ye S, Lee WP, Cornelius J, Diehl L et al. Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. J Immunol 2005; **174:** 817–26.

7. Chen GG, Lau WY, Lai PB, Chun YS, Chak EC, Leung BC et al. Activation of Kupffer cells inhibits tumor growth in a murine model system. Int J Cancer 2002; **99:** 713–20.

8. Naito M, Hasegawa G, Ebe Y, Yamamoto T. Differentiation and function of Kupffer cells. Med Electron Microsc 2004; **37:** 16–28.

9. Claria J, Titos E. Kupffer cell. Gastroenterol Hepatol 2004; **27:** 264–73.

10. Luo D, Vermijlen D, Kuppen PJ, Wisse E. MHC class I expression protects rat colon carcinoma cells from hepatic natural killer cell-mediated apoptosis and cytolysis, by blocking the perforin/granzyme pathway. Comp Hepatol 2002; **1:** 2–9.

11. Luo DZ, Vermijlen D, Ahishali B, Triantis V, Plakoutsi G, Braet F, et al. On the cell biology of pit cells, the liver specific NK cells. World J Gastroenterol 2000; **6:** 1–11.

12. Lee JY, Chae DW, Kim SM, Nam ES, Jang MK, Lee JH et al. Expression of FasL and perforin/granzyme B mRNA in chronic hepatitis B virus infection. J Viral Hepat 2004; **11:** 130–5.

13. Vermijlen D, Luo D, Froelich CJ, Medema JP, Kummer JA, Willems E et al. Pit cells exclusively kill P815 tumor cells by the perforin/granzyme pathway. Comp Hepatol 2004; **14 (Suppl 1):** 58.

14. Wowk ME, Trapani JA. Cytotoxic activity of the lymphocyte toxin granzyme B. Microbes Infect 2004; **6:** 752–8.

15. Lees RK, Ferrero I, MacDonald HR. Tissue-specific segregation of TCRgamma delta+ NKT cells according to phenotype TCR repertoire and activation status: parallels with TCR alphabeta+NKT cells. Eur J Immunol 2001; **31:** 2901–9.

16. Garia-Barcina M, Bidaurrazaga I, Neaud V, Bioulac-Sage P, Balabaud C, Vidal-Vanaclocha F et al. Variations in the expression of cell-adhesion molecules on liver-associated lymphocytes and peripheral-blood lymphocytes in patients with and without liver metastasis. Int J Cancer 1995; **61:** 475–9.

17. Ioachim HL, Decuseara R, Giancotti F, Dorsett BH. FAS and FAS-L expression by tumor cells and lymphocytes in breast carcinomas and their lymph node metastases. Pathology Research and Practice 2005; **200:** 743–51.

18. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T et al. The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses. Immunol Rev 2000; **174:** 35–46.

19. Sharifi S, Hayek J, Khettry U, Nasser I. Immunocytochemical staining of Kupffer and endothelial cells in fine needle aspiration cytology of hepatocellular carcinoma. Acta Cytol 2000; **44:** 7–12.

20. Xidakis C, Kolios G, Valatas V, Notas G, Mouzas I, Kouroumalis E. Effect of octreotide on apoptosis-related proteins in rat Kupffer cells: a possible anti-tumour mechanism. Anticancer Res 2004; **24:** 833–41.

21. Miyagawa S, Miwa S, Soeda J, Kobayashi A, Kawasaki S. Morphometric analysis of liver macrophages in patients with colorectal liver metastasis. Clin Exp Metastasis 2002; **19:** 119–25.

22. Jewell AP. Is the liver an important site for the development of immune tolerance to tumours? Med Hypotheses 2005; **64:** 751–4.