肝細胞癌におけるmonocarboxylate transpoter 4 (MCT4) の発現は、肝癌患者の予後推定因子である。

Aberrant expression of monocarbohydrate transporter 4 (MCT4) in tumor cells predicts an unfavorable outcome in patients with hepatocellular carcinoma

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Running head: MCT4 in HCC predicts unfavorable outcome

ABSTRACT

Background: The tumor cell microenvironment, which includes local oxygen saturation, pericellular pH and stromal cells, can modulate tumor progression.

Aims: This study determined the prognostic impact of infiltrating tumor-associated macrophages and the expression of monocarbohydrate transporter 4 (MCT4) and glypican 3 (GPC3) in hepatocellular carcinoma (HCC) clinical specimens.

Methods: A total of 225 cases of resected HCC were subjected to immunohistochemical analyses of CD68, CD204, MCT4 and GPC3. Immunoreactivities and other common clinicopathological parameters were subjected to univariate prognostic analyses for overall survival (OS, n = 225) and disease-free survival (DFS, n = 222). All variables with prognostic impact were further analyzed in multivariate analysis.

Results: Increased intratumoral infiltration of CD204-positive or MCT4-positive macrophages suggested shorter OS ($p = 0.015$ or $p = 0.001$, respectively), but DFS was not altered. The GPC3 score (with an emphasis on circumferential immunoreactivity) was correlated with shorter OS and DFS. Aberrant expression of MCT4 in HCC cells was observed in a subset of HCC cases (21%, 47/225). In those cases, significantly poorer OS ($p < 0.0001$) and DFS ($p = 0.0003$) were observed, and there was a positive correlation to the intratumoral infiltration of CD204- or MCT4-positive macrophages and the GPC3 score. Multivariate analysis showed that aberrant MCT4 expression in HCC cells was an independent prognostic factor for shorter OS ($p = 0.018$) and DFS ($p = 0.006$) after resection of HCC.

Conclusions: Aberrant expression of MCT4 in carcinoma cells serves as a novel, independent prognostic factor for HCC, indicating a poorer patient outcome.

Key words: MCT4, tumor-associated macrophages, hepatocellular carcinoma, prognosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is a prevalent cancer worldwide and the 5-year survival rate after surgery remains low with a high recurrence rate (1). Its incidence is increasing, mainly due to the increasing prevalence of advanced hepatitis C virus (HCV) infection (2). The tumor microenvironment is a critical factor that determines the biology of cancer cells. The microenvironment is a complex mixture of tumor cells, stromal cells, proteins expressed on and around the cells, extracellular matrix, pericellular oxygen tension and pH. Like many other solid cancers, the role of the tumor cell microenvironment is thought to be critical in progression of HCC (2). Macrophages (mφ) constitute a major component of the cellular infiltrate in tumor tissue, and tumor-associated mφ (TAMs) are known to be related to tumor progression and outcome (3, 4). Generally, TAMs show the M2 polarized phenotype and express CD204 and/or CD163 (5, 6). Like other solid cancers, the prognostic significance of intratumoral infiltration of mφ has been studied in HCC; however, conflicting results have been reported (7, 8, 9). Monocarboxylate transporters (MCTs) are proteins that facilitate the transmembrane transport of short-chain fatty acids, such as pyruvate and lactate, coupled with a proton, and they play a critical role in preventing intracellular acidosis associated with increased glycolysis (10). Among the MCTs, MCT4 is strongly expressed by monocytes and mφ (11) and might constitute a marker of enhanced glycolysis in mφ. Moreover, as MCT4 expression is regulated by HIF-1 signaling (12),

its enhanced expression in tumor cells might represent a hypoxic microenvironment in cancer tissue.

Previously, we described circumferential expression of cell surface glypican 3 (GPC3), an oncofetal GPI-anchored glycoprotein highly expressed in HCC. We concluded that it might indicate poor patient outcome (13), but the mechanism by which the circumferential GPC3 expression influenced disease progression has remained undefined. Based on a small number of HCC (30 cases), we suggested that there was a positive correlation between circumferential GPC3 expression and infiltration of TAMs (14). The data indicated a possible role of the tumor microenvironment in the GPC3 expression pattern or vice versa in HCC. The present retrospective study was initially aimed at assessing the prognostic significance of intratumoral infiltration of mφ, using CD68 (a pan-marker for mφ/monocyte), CD204, and MCT4 as markers, in surgically resected HCC samples and their relationships to the circumferential GPC3 expression score. Rather unexpectedly, we found that aberrant MCT4 expression in HCC cells, which was observed in a subset of HCC cases, predicted significantly worse prognosis of the patients.

MATERIALS AND METHODS

Study cohort

This study protocol was approved by the Institutional Review Board of the Faculty of Medicine, University of Miyazaki. A total of 225 Japanese patients (168 males and 57 females) were included in this study. All were diagnosed with HCC and had received partial hepatectomy at the University of Miyazaki Hospital from February 1999 to

March 2013. Patients' ages ranged from 18 to 86 years-old, with mean and median ages of 65.5 and 68, respectively. Clinicopathological data (summarized in Table 1) included tumor size, tumor multiplicity, recurrence, infection by hepatitis B virus (HBV) and HCV, serum α-fetoprotein (AFP) level, serum protein induced by vitamin K absence or antagonist II (PIVKA-II) level, Child-Pugh score, TNM stage, cancer of the liver Italian program (CLIP) score, Japan integrated staging (JIS) score, tumor morphology, vascular invasion, capsular invasion, and cirrhosis. Tumor grading and histological types were assessed according to World Health Organization classification.

The postoperative mean follow-up period was 3.6 years, with 13.8 years as the longest period, while the mean disease-free interval was 2.4 years. During the follow-up period, 75 patients (33%) died of HCC, and 20 patients (9%) died of unrelated (19 cases) or unknown (1 case) causes. Primary study endpoints were postoperative overall survival (OS) and postoperative disease-free survival (DFS). OS (n = 225) and DFS (n = 223) were defined as the time from the date of surgery to the date of death from HCC and to the date of initial detection of local recurrence or distant metastasis, respectively. The postoperative follow-up included abdominal ultrasonography or computed tomography study every 3 months and laboratory testing of AFP and/or PIVKA-II level at 1 to 3 month intervals. Patients underwent hepatic angiography, bone scintigraphy or chest computed tomography when clinically indicated. Fifty-five cases received neoadjuvant therapy within three months of surgery. Postoperative chemotherapy was performed for 41 patients, when portal vein invasion or metastasis was detected by pathologic studies. If cancer recurrence was confirmed, various treatments, including repeat hepatectomy, transcatheter arterial embolization, percutaneous ablation and radiation therapy were applied as deemed necessary.

Preparation of tissue samples and immunohistochemistry

Paraffin-embedded tissue sections with a thickness of 5 μm were fixed in 10% formalin for hematoxylin and eosin (HE) and immunohistochemical staining. Most of these sections included surrounding non-neoplastic liver tissue. They were immunostained for expression of CD68, CD204, MCT4 and GPC3. The primary antibodies used were as follows: rabbit polyclonal anti-MCT4 antibody (clone H-90, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-CD68 antibody (clone PG-M1, 1:100, Dako, North America, Inc.), mouse monoclonal anti-CD204 antibody (clone SRA-E5, 1:100, Trans Genic, Kobe, Japan), and mouse monoclonal anti-GPC3 antibody (GC33, 1 μg/mL) (13, 15). The staining was carried out on the Leica Bond-Max III automated immunostainer according to the manufacturer's instructions. Heat treatment for antigen retrieval was 10 min for CD68, 20 min for CD204, or 30 min for MCT4 and GPC3. Negative controls consisted of sections with omission of the primary antibody.

Intratumoral mφ counting and scoring system for GPC3 and MCT immunoreactivity in HCC cells

Infiltrating mφ with expression of CD68, CD204 or MCT4 were counted with a 40 × objective lens (high power field: HPF) at three representative areas randomly selected from the tumor portion, and the average number/HPF was calculated. To count mφ, immune-positive cells that were obviously larger than 10 μm in width were selected and counted. The GPC3 scoring system with an emphasis on circumferential immunoreactivity of HCC cells (A-Cm score) was described previously (13). For

MCT4, both HCC with focal aggregation(s) of MCT4-positive HCC cells (more than 20 cells) and with rather diffuse immunoreactivity were judged as positive, as long as readily visible membranous immunoreactivity was identified with a 4 × objective lens. In all immunohistochemical analysis, the evaluation was performed by two or three independent researchers (A.O., K.Y. and/or H. K.).

Statistical analysis

Fisher's exact test, $\chi 2$ test and Spearman's rank correlation test were used for assessment of the relationship between variables. OS and DFS were estimated using the Kaplan-Meyer method and groups were compared using the log-rank test. Cox proportional hazards regression models were used to calculate the hazard ratios (HRs) and 95% confidence of intervals (CIs). Patients were censored on the date of last contact or dying of causes other than HCC. The multivariable Cox proportional hazards regression analysis model was used to detect independent prognostic factors. Statistical significance was assumed if $p < 0.05$. Data were analyzed by STAT view 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Intratumoral infiltration of CD204- or MCT4-positive mφ in HCC tissues and patient prognosis

We conducted univariate analyses of the clinicopathological factors in HCC patients' OS (n = 225) and DFS (n = 222) in this study cohort. Most conventional prognostic factors such as recurrence, tumor size, multiplicity, serum AFP and

PIVKA-II levels, presence of liver cirrhosis, vascular invasion, tumor grade, and staging (TNM, CLIP and JIS) showed significant prognostic impact on both OS and DFS (Table 2). To analyze the number of TAMs with an M2 polarized phenotype in human HCC, an immunohistochemical study was undertaken using 225 cases of surgically resected HCC samples and antibody against CD204. The mean number ± standard deviation (SD) of CD204-positive TAMs (hereafter indicated as M2-mφ) per HPF was 20.0 ± 13.1. A representative example is shown in Figure 1A. Among 225 cases, 93 showed M2-mφ above average numbers and were designated as high M2-mφ cases.

Univariate analysis for OS and DFS revealed that high M2-mφ cases showed shorter OS ($p = 0.015$, Cox proportional hazards regression analysis) (Table 3). However, no relationship to DFS was observed. On the other hand, the number of intratumoral CD68-positive mφ did not show a prognostic impact in this study. Mφ and monocytes that were clearly immunoreactive with anti-MCT4 were also seen in HCC tissues, particularly around necrosis (Figure 1B). In addition, activated mφ forming epithelioid granulomas also showed strong MCT4 immunoreactivity (data not shown). MCT4-positive mφ were comparatively fewer in number between tumor cells compared to M2-mφ, showing 1.4 ± 2.8/HPF (Figure 1C). Notably, cases that showed intratumoral MCT4-positive mφ above average (62 cases) had shorter OS ($p = 0.001$), similar to the case for M2-mφ (Table 3). No relationship to DFS was observed.

Aberrant expression of MCT4 by HCC cells

During the course of the immunohistochemical analysis for intratumoral infiltration of MCT4-positive mφ, we found that a subset of HCC cases showed MCT4 immunoreactivity at the HCC cell surface. The immunopositive-HCC cells were found

either focally as groups of readily visible positive cells (Figure 2A) or nodularly in a substantial area (Figure 2B). Non-neoplastic hepatocytes were negative. The existence of groups of HCC cells with readily recognizable membranous MCT4 expression was judged as MCT4-positive in HCC (MCT4+ HCC). The MCT4+ HCC cases accounted for about 21% (47 cases) of the total analyzed cases, with diffusely positive immunoreactivity (>50% of HCC cells) in 8 cases. In focally positive cases, the MCT4-positive cancer cells often emerged near necrotic portions, frequently with M2-mφ and occasionally in nests isolated in fibrosing stroma (Figure 2C-E). In the remaining 178 cases, MCT4-immunoreactivity was hardly observed in HCC cells. MCT4+ HCC cases showed statistically significant correlations to higher intratumoral M2-mφ ($p = 0.0004$) and higher intratumoral MCT4-positive mφ ($p < 0.0001$).

Relationship of aberrant MCT4 expression to clinicopathological parameters and patient prognosis

The putative association between MCT4 expression in HCC cells and various clinicopathological factors was evaluated statistically (Supplemental Table 1). MCT4+ HCC correlated with higher AFP levels ($p = 0.001$, $\chi 2$ test), presence of vascular invasion ($p = 0.016$), and a less differentiated histology ($p < 0.0001$). MCT4+ HCC was also associated with advanced clinical stages. Furthermore, Kaplan-Meyer survival analysis and the log-rank test revealed that MCT4+ HCC was associated with significantly worse prognoses in both OS ($p < 0.0001$) and DFS ($p < 0.0001$) after resection of HCC (Figure 3). Interestingly, there was no statistically significant difference between HCT4+ HCC with a smaller positive area (< 20%; n = 31) and a larger positive area (≥ 20%; n = 16) in regard to the prognosis (Supplemental Figure 1).

Relationship of TAMs and MCT4 expression to circumferential GPC3 immunoreactivity

GPC3 is a well-known marker of HCC cells and in this study cohort, GPC3 immunoreactivity was observed in 78% (176/225) of HCC. Previously, we reported that there were several immunoreactivity patterns of GPC3 in HCC (membranous, canalicular, luminal, and intracytoplasmic) (13). We hypothesized that a GPC3 immunoreactivity scoring system with an emphasis on circumferential membranous pattern (A-Cm score) might be useful for predicting patient prognosis, as higher scores correlated to a poorer prognosis in 185 cases that overlapped with this study cohort (13). With an additional 40 cases (total 225 cases), we confirmed the above trend in this study (Supplemental Figure 2). Then, we analyzed the correlation between the GPC3 A-Cm score and intratumoral mφ number or MCT4+ HCC. The GPC3 score showed positive correlations to the number of M2-mφ ($p = 0.0003$, Spearman's rank correlation test) and MCT4+ HCC ($p < 0.0001$), but not to intratumoral MCT4-positive mφ ($p = 0.118$) (Supplemental Figure 3).

Aberrant MCT4 expression in HCC cells as a novel, independent prognostic factor for HCC

Finally, to compare all prognostic factors directly in terms of their impacts on patient OS or DFS, we carried out a multivariate analysis of eligible patients of all HCC cases (Table 4). Parameters that showed a statistically significant impact in the univariate analyses were incorporated into this analysis. Notably, MCT4+ HCC was an independent prognostic factor for both OS ($p = 0.018$) and DFS ($p = 0.006$) after

resection of HCC (Table 4). Other independent prognostic factors in this study cohort included tumor recurrence ($p = 0.006$), larger tumor size ($p = 0.048$), high AFP level ($p = 0.045$) for OS. With regard to DFS, prognostic factors included Child-Pugh score ($p = 0.002$), liver cirrhosis ($p = 0.038$) and TNM stage ($p = 0.030$).

DISCUSSION

In this immunohistochemical study of HCC (225 cases), tumors with increased intratumoral CD204-positive mφ (M2-mφ), MCT4-positive mφ and expression of MCT4 in HCC cells (MCT4+ HCC) were associated with an unfavorable patient outcome. To some extent, this work also suggested that M2-mφ number and MCT4+ HCC were correlated with each other and might also be correlated with a circumferential GPC3 expression pattern. In the multivariate analysis that included all statistically significant univariate prognostic parameters in this study cohort, we observed that MCT4+ HCC was an independent prognostic factor predicting decreased OS and DFS after resection of HCC. To the best of our knowledge, this study is the first to show the prognostic significance of MCT4 expression in HCC.

MCTs belong to the *SLC16* gene family that is composed of 14 members (10). Among MCTs, MCT1 and MCT4 are important proton symporters that regulate intracellular pH. They are believed to play a critical role in the maintenance of glycolytic metabolism through the proton-linked transmembrane transport of lactate. Upregulation of MCT1 and MCT4 has been reported in several solid tumors, such as gastrointestinal, gynecological, breast, prostatic, lung, head and neck carcinomas, melanoma, and central nervous system tumors (10, 16 - 21) and the prognostic

significance of MCT4 has been shown (10, 17, 19 - 21). In this study, we observed aberrant MCT4 expression in 21% of the HCC cases analyzed. The immunoreactivity pattern was largely focal, showing scattered groups of positive cells, and cases with rather diffuse immunoreactivity (positive in more than 50% of the cells) represented only 17% of MCT4+ HCC cases. However, regardless of the area of positivity, MCT4+ HCC cases showed significant prognostic impact. We also analyzed the expression of MCT1 in HCC cells (data not shown). However, there was no clear relationship between MCT1 expression and patient prognosis.

The question, then, is how MCT4+ HCC is associated with poor patient outcome. MCT4 expression is regulated by HIF-1 signaling (12). Therefore, it is reasonable to postulate that the existence of MCT4-expressing HCC cells represents enhanced activation of HIF-1 signaling that is critically involved in angiogenesis and chemoresistance of HCC cells (22, 23). Moreover, the expression of MCT4 might result in an acidic pericellular milieu by exporting intracellular lactate. Acidification might in turn modulate the tumor cell microenvironment in favor of malignant progression. Indeed, recent evidence suggests that acidity in the tumor microenvironment drives local invasion (24). As there was a positive correlation between MCT4+ HCC and M2-mφ infiltration in tumor tissue, MCT4-mediated alteration of the pericellular microenvironment might recruit TAMs, which might also be involved in an unfavorable outcome for patients. Alternatively, MCT4 might contribute to malignant progression of HCC via other functions than its capacity to work as a proton symporter. For example, MCT4 is reported to be colocalized with CD147; the latter induces expression of matrix metalloproteases (MMP) as indicated by its synonym, EMMPRIN (extracellular MMP inducer) (25 - 27). Therefore, expression of CD147 by MCT4+ HCC cells might

produce higher activities of pericellular MMP. In fact, expression of CD147 in HCC has been reported (28). The expression of CD147 in HCC cells might also induce the epithelial to mesenchymal transition, resulting in a more invasive phenotype (29). Finally, a recent study suggests that CD147 is associated not only with MCT4, but also with assembly of CD44, epidermal growth factor receptor, and drug transporters in the plasma membrane, having a role in the properties characteristic of cancer stem-like cells (30). Therefore, the MCT4-positive area in HCC might be an area enriched with cancer stem-like cells.

This study also suggests a possible role of CD204-positive M2-mφ in decreased OS after resection of HCC, which is in accordance with a presumed supporting role of TAMs in tumor progression (3, 4, 9). In accordance with a previous report (14), we observed a positive correlation between M2-mφ and circumferential membranous GPC3 immunostaining score (A-Cm score). Of note, this GPC3 immunostain score might also be higher in MCT4+ HCC. Currently, it is not known whether these correlations are simply epiphenomena in dedifferentiated tumors or represent a functional interrelationship between the parameters. We also analyzed the prognostic impact of MCT4-positive mφ in this study. While MCT4 is reported to be expressed abundantly by mφ (11), the number of intratumoral mφ clearly positive for MCT4 was much less than CD204-positive or CD68-positive mφ and most MCT4-positive mφ showed plump phagocytizing morphology and were enriched in/around necrotic areas. Although the precise nature of MCT4-positive mφ remains undefined, higher intratumoral MCT4-positive mφ showed a positive correlation to the existence of MCT4-positive HCC cells and also to decreased OS after HCC resection.

In summary, our study revealed that, in a subset of HCC cases, focal or diffuse

expression of MCT4 occurs in HCC cells. The aberrant MCT4 expression in HCC predicts decreased OS and DFS after resection of HCC and serves as a novel independent prognostic marker for HCC. For these MCT4-positive HCC cases, inhibition of MCT4 function could be a new and advantageous therapeutic approach.

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REFERENCES

1. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-17.
2. Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology* 2013; **144**: 512-27.
3. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A. Macrophage polarization in tumour progression. *Semin Cancer Biol* 2008; **18**: 349-55.
4. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* 2013; **23**: 277-86.
5. Komohara Y, Hasita H, Ohnishi K et al. Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma. *Cancer Sci* 2011; **102**: 1424-31.
6. Shigeoka M, Urakawa N, Nakamura T, et al. Tumor associated macrophage expressing CD204 is associated with tumor aggressiveness of esophageal squamous cell carcinoma. *Cancer Sci* 2013 May 4. doi: 10.1111/cas.12188.
7. Ding T, Xu J, Wang F, Shi M, Zhang Y, Li SP et al. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. *Hum Pathol* 2009; **40**: 381-9.
8. Li YW, Qiu SJ, Fan J et al. Tumor-infiltrating macrophages can predict favorable prognosis in hepatocellular carcinoma after resection. *J Cancer Res Clin Oncol* 2009; **135**: 439-49.
9. Shirabe K, Mano Y, Muto J, Matono R, Motomura T, Toshima T, Takeishi K, Uchiyama H, Yoshizumi T, Taketomi A, Morita M, Tsujitani S, Sakaguchi Y,

Maehara Y. Role of tumor-associated macrophages in the progression of hepatocellular carcinoma. Surg Today 2012;42:1-7.

10. Pinheiro C, Longatto-Filho A, Azevedo-Silva J, Casal M, Schmitt FC, Baltazar F. Role of monocarboxylate transporters in human cancers: state of the art. *J Bioenerg Biomembr* 2012; **44**: 127-39.
11. Moreau A, Le Vee M, Jouan E, Parmentier Y, Fardel O. Drug transporter expression in human macrophages. *Fundam Clin Pharmacol* 2011; **25**: 743-52.
12. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem 2006;281:9030-7.
13. Yorita K, Takahashi N, Takai H et al. Prognostic significance of circumferential cell surface immunoreactivity of glypican-3 in hepatocellular carcinoma. *Liver Int* 2011; **31**: 120-31.
14. Takai H, Kato A, Kato C et al. The expression profile of glypican-3 and its relation to macrophage population in human hepatocellular carcinoma. *Liver Int* 2009; **29**: 1056-64.
15. Ishiguro T, Sugimoto M, Kinoshita Y et al. Anti-glypican 3 antibody as a potential antitumor agent for human liver cancer. *Cancer Res* 2008; **68**: 9832-8.
16. Ho J, de Moura MB, Lin Y et al. Importance of glycolysis and oxidative phosphorylation in advanced melanoma. *Mol Cancer* 2012; **11**: 76.
17. Curry JM, Tuluc M, Whitaker-Menezes D et al. Cancer metabolism, stemness and tumor recurrence: MCT1 and MCT4 are functional biomarkers of metabolic symbiosis in head and neck cancer. *Cell Cycle* 2013; **12**: 1371-84.
18. Miranda-Goncalves V, Honavar M, Pinheiro C et al. Monocarboxylate transporters

(MCTs) in gliomas: expression and exploitation as therapeutic targets. *Neuro Oncol* 2013; **15**: 172-88.

19. Meijer TW, Schuurbiers OC, Kaanders JH et al. Differences in metabolism between adeno- and squamous cell non-small cell lung carcinomas: spatial distribution and prognostic value of GLUT1 and MCT4. *Lung Cancer* 2012; **76**: 316-23.
20. Gerlinger M, Santos CR, Spencer-Dene B et al. Genome-wide RNA interference analysis of renal carcinoma survival regulators identifies MCT4 as a Warburg effect metabolic target. *J Pathol* 2012; **227**: 146-56.
21. Nakayama Y, Torigoe T, Inoue Y et al. Prognostic significance of monocarboxylate transporter 4 expression in patients with colorectal cancer. *Exp Ther Med* 2012; **3**: 25-30.
22. Liao D, Johnson RS. Hypoxia: a key regulator of angiogenesis in cancer. *Cancer Metastasis Rev* 2007; **26**: 281–90.
23. Tong Y, Li QG, Xing TY, Zhang M, Zhang JJ, Xia Q. HIF1 regulates WSB-1 expression to promote hypoxia-induced chemoresistance in hepatocellular carcinoma cells. *FEBS Lett* 2013 Jun 19. doi:pii: S0014-5793(13)00464-X. 10.1016/j.febslet.2013.06.017.
24. Estrella V, Chen T, Lloyd M, et al. Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res* 2013; **73**: 1524-35.
25. Kataoka H, DeCastro R, Zucker S, Biswas C. Tumor cell-derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin, and 72-kDa gelatinase. *Cancer Res* 1993; **53**: 3154-8.
26. Biswas C, Zhang Y, DeCastro R et al. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin

superfamily. *Cancer Res* 1995; **55**: 434-9.

27. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *EMBO J* 2000; **19**: 3896-904.
28. Tang X, Guo N, Xu L, Gou X, Mi M. CD147/EMMPRIN: an effective therapeutic target for hepatocellular carcinoma. *J Drug Target* 2012 Aug 29. [Epub ahead of print]
29. Wu J, Ru NY, Zhang Y et al. HAb18G/CD147 promotes epithelial-mesenchymal transition through TGF-β signaling and is transcriptionally regulated by Slug. *Oncogene* 2011; **30**: 4410-27.
30. Dai L, Guinea MC, Slomiany MG, Bratoeva M, Grass GD, Tolliver LB, Maria BL, Toole BP. CD147-dependent heterogeneity in malignant and chemoresistant properties of cancer cells. *Am J Pathol* 2013; **182**: 577-85.

FIGURE LEGENDS

Figure 1. Representative immunohistochemistry of infiltrating mφ. **A.** Intratumoral infiltration of CD204-positive mφ. Bar, 100 μm. **B.** MCT4-positive mφ in tumor tissue (left panel) and perinecrotic area (right panel). Bar, 100 μm. **C.** Comparative immunohistochemistry of CD68-, CD204- and MCT4-positive mφ in serial sections of HCC tissue. Bar, 200 μm.

Figure 2. Expression of MCT4 in HCC cells. **A.** Focal immunoreactivity in small groups of HCC cells. Bar, 200 μm. T, tumor portion. N, non-tumor portion. Inset, higher manigification photo of NCT-positive HCC cells. **B.** Diffuse immunoreactivity pattern in HCC cells with average (left) or strong (right) intensity. T, tumor portion. N, non-tumor portion. Bars, 200 (left) and 100 μm (right). **C-E.** Representative examples of focal MCT4 immunoreactivity in HCC cells, showing perinecrotic pattern (C), isolated nest pattern (D) and association with CD204-positive mφ (E). Note that MCT4-positive HCC cells are rimmed by CD204-positive mφ (E). *, necrotic portion. Bars, 100 μm or 50 um (high magnification photo in D).

Figure 3. Kaplan-Meyer survival curves of MCT4+ HCC for OS (A) and DFS (B) after resection of HCC. *P* value was calculated by log-rank test.

Table 1. Demographic and baseline characteristics of all HCC patients

Variables		Number	Mean ± SD / median / range
Age (years)		225	65.5 ± 11.0 / 68.0 / 18 - 86
Gender: Male / Female		168 / 57	
HBV / HCV / both / none		68 / 90 / 4 / 71	
Newly onset / Recurrence		183 / 42	
Tumor diameter (cm)		225	4.8 ± 3.4 / 4.0 / 0.8 - 19
Tumor number, single / multiple		175 / 50	
Serum AFP (ng/ml)		225	12614 ± 89409 / 28 / 1 - 1121170
Serum PIVKA-II (mAU/ml)		223	7173 ± 34215 / 124 / 7 - 443000
Child-Pugh score, A / B		196 / 29	
Adjuvant therapy		41	
Tumor differentiation:			
well / moderate / poor		82 / 123 / 20	
Vascular invasion	+ / -	118 / 107	
Capsular invasion	+ / -	140 / 85	
Cirrhosis	+ / -	109 / 116	
Staging			
TNM	I / II / III / IV	23/80/81/41	
CLIP	0 / 1 / 2 / 3 / 4 / 5	75/96/29/17/8/0	
JIS	0 / 1 / 2 / 3 / 4 / 5	22/72/82/46/3/0	

AU, Anson unit;

Table 2. Univariate analysis of demographic and basaline characteristics for OS and DFS

Parameters	OS (n = 225)		DFS (n = 222)	
	HR (95% CI)	*p* value [c]	HR (95% CI)	*p* value [c]
Age (< 60 vs ≥ 60)	1.4 (0.8-2.2)	0.197	1.2 (0.9-1.9)	0.250
Gender (male vs female)	0.9 (0.6-1.6)	0.956	0.9 (0.6-1.3)	0.621
Recurrent (recurrent vs new)	2.6 (1.6-4.2)	0.0002	1.8 (1.2-2.8)	0.005
Tumor size (≥ 5cm vs < 5)	2.4 (1.5-3.8)	0.0002	1.7 (1.2-2.4)	0.003
Multiplicity (multiple vs single)	1.8 (1.1-3.1)	0.025	1.7 (1.2-2.6)	0.006
AFP [a] (≥ 14 vs < 14)	4.3 (2.3-8.1)	< 0.0001	2.2 (1.5-3.2)	< 0.0001
PIVKA-II [b] (≥ 40 vs < 40)	2.5 (1.4-4.4)	0.001	1.8 (1.2-2.7)	0.003
Child-Pugh score (B vs A)	1.7 (0.9-3.1)	0.082	2.2 (1.4-3.5)	0.0008
Adjuvant therapy (+ vs –)	1.8 (1.0-3.2)	0.033	1.8 (1.1-2.7)	0.010
Cirrhosis (+ vs –)	1.7 (1.0-2.6)	0.033	1.7 (1.2-2.4)	0.005
Capsular invasion (+ vs –)	1.6 (0.9-2.6)	0.086	1.1 (0.7-1.6)	0.693
Vascular invasion (+ vs –)	4.0 (2.3-6.9)	< 0.0001	2.4 (1.7-3.4)	< 0.0001
Tumor grade (mod. + poor vs well)	3.1 (1.7-5.6)	0.0001	1.9 (1.3-2.8)	0.001
TNM stage (III-IV vs I-II)	3.9 (2.2-6.8)	< 0.0001	2.9 (2.0-4.3)	< 0.0001
CLIP (≥ 2 vs ≤ 1)	4.1 (2.6-6.5)	< 0.0001	3.2 (2.2-4.7)	< 0.0001
JIS (≥ 2 vs ≤ 1)	3.6 (2.0-6.3)	< 0.0001	2.8 (1.9-4.2)	< 0.0001

a, ng/ml; b, mAU/ml; c, Cox proportional hazards regression analysis

Table 3. Univariate analysis of intratumoral mφ number for OS and DFS after resection of HCC

Parameters	OS (n = 225)			DFS (n = 222)		
	n	HR (95% CI)	*p* value [b]	n	HR (95% CI)	*p* value [b]
CD68-positive mφ						
≥ 14/HPF [a]	91	1.5 (0.9-2.4)	0.064	89	1.2 (0.8-1.7)	0.330
< 14/HPF	134			133		
CD204-positive mφ						
≥ 20/HPF [a]	93	1.8 (1.1-2.8)	0.015	91	1.2 (0.9-1.8)	0.271
< 20/HPF	132			131		
MCT4-positive mφ						
≥ 1.4/HPF [a]	62	2.2 (1.4-3.5)	0.001	60	1.4 (0.9-2.1)	0.068
< 1.4/HPF	163			162		

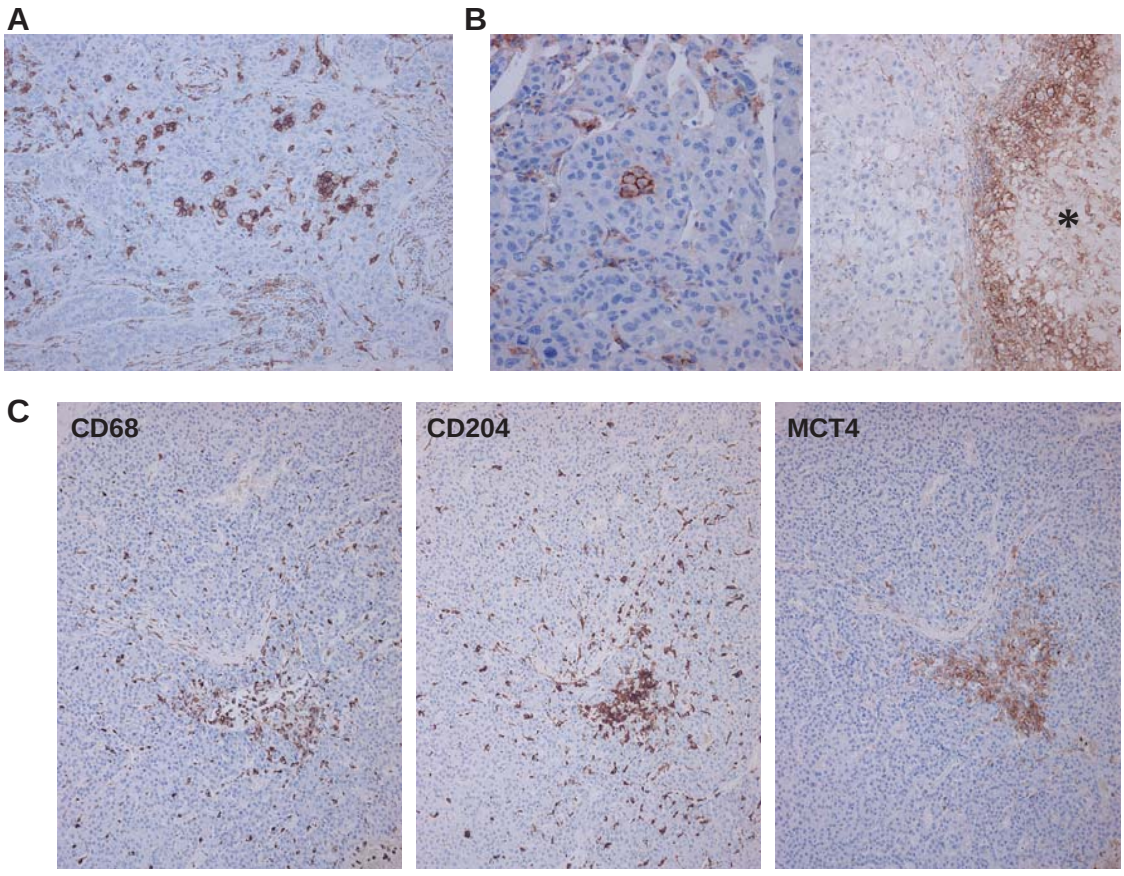
a, each numer represents mean value/HPF of all cases analyzed. b, Cox proportional hazards regression analysis

Table 4. Multivariate analysis of factors associated with OS and DFS in all eligible cases

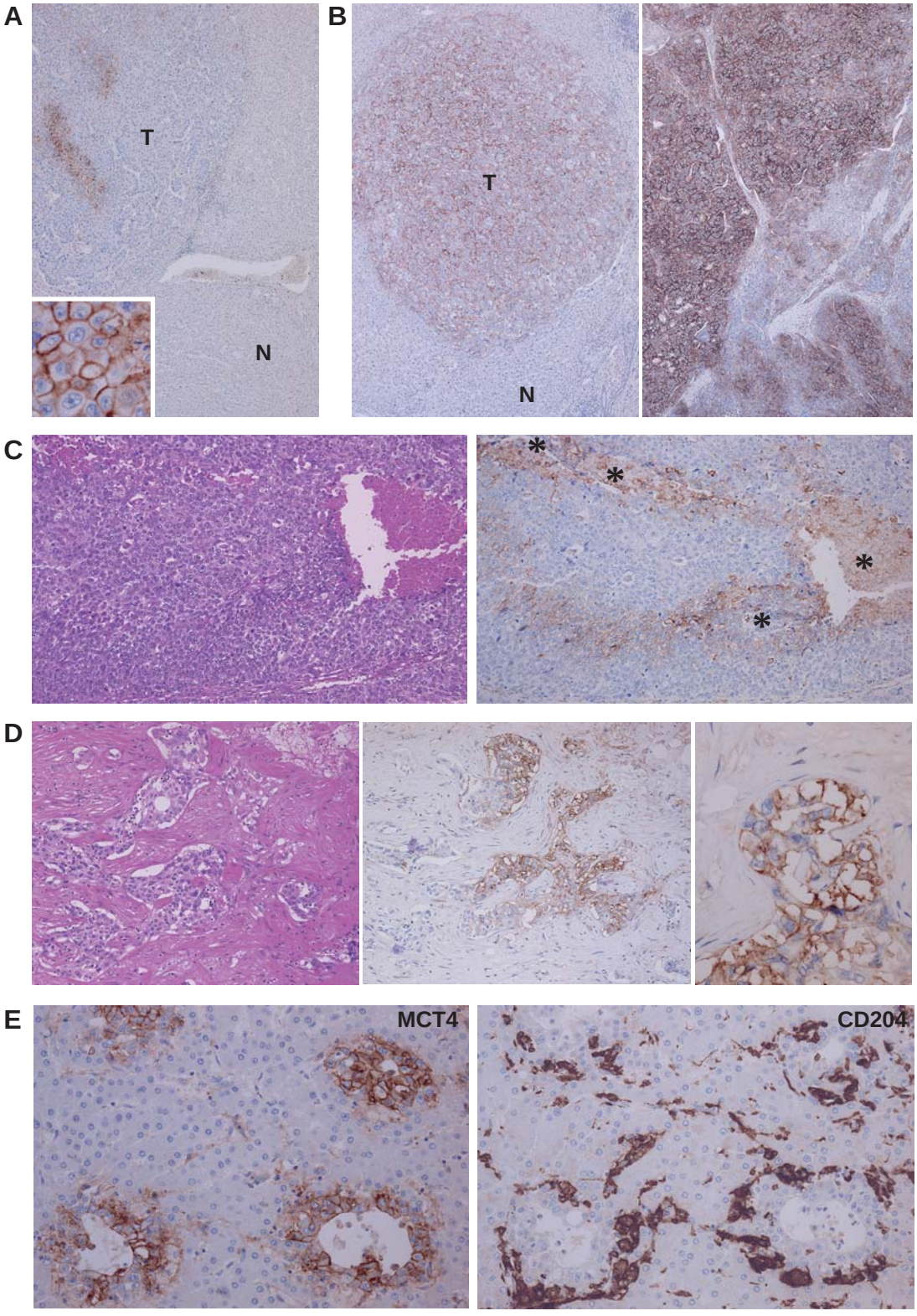
Parameters	OS (n = 225)		DFS (n = 222)	
	HR (95% CI)	*p* value [c]	HR (95% CI)	*p* value [c]
Recurrent (**recurrent** vs new)	2.2 (1.3-3.8)	**0.006**	1.4 (0.9-2.3)	0.109
Tumor size (≥ **5** vs < 5)	1.7 (1.0-3.0)	**0.048**	1.3 (0.9-2.0)	0.217
Multiplicity (**multiple** vs single)	1.3 (0.7-2.3)	0.438	1.3 (0.8-2.1)	0.240
AFP (≥ **14** vs < 14)	2.1 (1.0-4.3)	**0.045**	1.3 (0.8-2.0)	0.263
PIVKA-II (≥ **40** vs < 40)	1.8 (0.9-3.3)	0.059	1.5 (0.9-2.4)	0.066
Child-Pugh score (**B** vs A)	-	-	2.3 (1.3-3.8)	**0.002**
Adjuvant therapy (+ vs –)	1.2 (0.6-2.1)	0.626	1.3 (0.8-2.1)	0.332
Cirrhosis (+ vs –)	1.5 (0.9-2.5)	0.132	1.5 (1.0-2.3)	**0.038**
Vascular invasion (+ vs –)	1.9 (0.9-4.2)	0.127	1.1 (0.6-1.8)	0.779
Tumor grade (**mod.** + **poor** vs well)	1.2 (0.6-2.5)	0.521	0.9 (0.6-1.6)	0.925
TNM stage (**III-IV** vs I-II)	3.2 (0.4-22.7)	0.250	3.1 (1.1-8.6)	**0.030**
CLIP (≥ **2** vs ≤ 1)	1.5 (0.8-2.6)	0.184	1.5 (0.9-2.4)	0.074
JIS (≥ **2** vs ≤ 1)	0.3 (0.05-2.5)	0.296	0.5 (0.2-1.5)	0.223
CD204-positive mφ (**high** vs low) [a]	0.7 (0.4-1.3)	0.259	-	-
MCT4-positive mφ (**high** vs low) [b]	1.2 (0.7-2.1)	0.598	-	-
MCT4+ HCC (**positive** vs negative)	2.0 (1.1-3.5)	**0.018**	1.9 (1.2-2.9)	**0.006**
GPC3 score (A-Cm score ≥ **2** vs ≤ 1)	1.6 (0.9-2.9)	0.092	1.2 (0.8-1.9)	0.332

a, high: > 20/HPF; b, hign: 1.4/HPF; c, Cox proportional hazards regression analysis

Ohno et al, Figure 1



Ohno et al, Figure 2



Ohno et al, Figure 3