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High Expression of Macrophage Colony-Stimulating Factor-1 Receptor in Peritumoral Liver Tissue Is Associated with Poor Outcome in Hepatocellular Carcinoma After Curative Resection

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Key Words. Macrophage colony-stimulating factor-1 receptor • Hepatocellular carcinoma • Microenvironment • Peritumoral liver tissue • Prognosis

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ABSTRACT

Background. **Macrophage colony-stimulating factor 1 receptor (CSF-1R) expression in hepatocellular carcinoma (HCC) and its prognostic values are unclear. This study evaluated the prognostic values of the intratumoral and peritumoral expression of CSF-1R in HCC patients after curative resection.**

Methods. **Tissue microarrays containing material from cohort 1 (105 patients) and cohort 2 (32 patients) were constructed. Immunohistochemistry was performed and prognostic values of these and other clinicopathological data were evaluated. The CSF-1R mRNA level was assessed by quantitative real-time polymerase chain reaction in cohort 3 (52 patients).**

Results. **Both the CSF-1R density and its mRNA level were significantly higher in peritumoral liver tissue than in the corresponding tumor tissue. CSF-1R was distributed in a gradient in the long-distance peritumoral tissue microarray, with its density decreasing as the distance from the tumor margin increased. High peritumoral CSF-1R was significantly associated with more intrahepatic metastases and poorer survival. Peritumoral CSF-1R was an independent prognostic factor for both overall survival and time to recurrence and affected the incidence of early recurrence. However, intratumoral CSF-1R did not correlate with any clinicopathological feature. Peritumoral CSF-1R was also associated with both overall survival and time to recurrence in a subgroup with small HCCs (≤5 cm).**

Conclusions. **Peritumoral CSF-1R is associated with intrahepatic metastasis, tumor recurrence, and patient survival after hepatectomy, highlighting the critical role of the peritumoral liver milieu in HCC progression. CSF-1R may become a potential therapeutic target for postoperative adjuvant treatment.** ***The Oncologist*** 2010; 15:732–743

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of death from cancer worldwide [1, 2]. Although hepatectomy or radiofrequency ablation are the best methods for prolonging patient survival [2], a high postoperative recurrence rate is a major problem [3]. Conventional tumor–node–metastasis (TNM) classification is usually insufficient to predict HCC recurrence. Although biomarkers from HCC tumors have been extensively studied [4–7], the results have not been satisfying until recently.

Our recent studies found that a high expression level of peritumoral colony-stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor, and/or macrophage infiltration was associated with poor survival after hepatectomy [8, 9]. Together with other reports [10, 11], these findings imply that the peritumoral liver tissue might be a favorable substrate for intrahepatic micrometastasis and HCC progression. However, whether or not the receptor for CSF-1 (CSF-1R) expression is also enriched in the peritumoral liver tissue as well as the clinical significance of CSF-1R remain unclear.

CSF-1R, which is encoded by *c-fms* proto-oncogene, is a transmembrane receptor tyrosine kinase (RTK) [12]. Its ligand, CSF-1, is a cytokine mainly produced by tumor cells [13]. Expression of CSF-1R is found in primitive multipotent hematopoietic cells [14], mononuclear phagocytic lineage cells [15–17], the testis, uterus, ovary, placenta, and mammary glands, and the early phase of the developing prostate [18, 19]. CSF-1 binding to CSF-1R results in dimerization, autophosphorylation, and activation of signal transduction, and it causes monocytes and macrophages to differentiate, trophoblastic implantation, and mammary gland development [18, 20–22]. In pathological conditions, macrophages can be recruited by CSF-1. When appropriately activated through the CSF-1 and CSF-1R combination, macrophages secrete growth factors that are essential for the growth of a premetastatic niche and helpful for tumor growth or metastasis, resulting in a higher recurrence rate [23–25]. Inhibition of CSF-1R activity could therefore reduce macrophage infiltration and cause a delay in tumor progression, whereas macrophage recruitment is enhanced by overexpression of CSF-1 in the tumor, which correlates with accelerated tumor malignant transformation and angiogenesis [26–31]. High CSF-1R expression correlates with aggressive behavior in a variety of epithelial malignancies, including breast cancer, progressing prostate cancer, and ovarian cancer [18, 19, 31–35]. But the distribution of CSF-1R in HCC and its prognostic value are still unclear.

In this study, we investigated the distribution of CSF-1R in specimens obtained from HCC patients. The relationships among CSF-1R, macrophages, immunopositive-staining cells, and other clinicopathological variables and outcome in patients were studied as well.

Materials and Methods

Patients, Specimens, Follow-up, and Postoperative Treatment

In total, 105 patients (cohort 1) who underwent curative liver resection for pathology-proven HCC were examined in the present and previous studies [8, 36]. None of these patients received any preoperative anticancer treatment. Entire tumors with corresponding peritumoral liver tissues were collected. Tumor stage was determined according to the 2002 International Union Against Cancer TNM Classification system (Sixth Edition). The study was approved by the Zhongshan Hospital Research Ethics Committee. Informed consent was obtained from each patient according to the committee's regulations.

All patients were monitored until March 2008, with a median follow-up time of 31.7 months. The surgical techniques for liver resection were described previously [36–38]. Treatment modalities after relapse were administered according to a uniform guideline [37, 39, 40]. The overall survival (OS) time and time to recurrence (TTR) were defined as the interval between the dates of surgery and death or recurrence, respectively. If recurrence was suspected, computerized tomography scanning or magnetic resonance imaging was performed immediately; if recurrence was not diagnosed, patients were observed until death or the last follow-up. The detailed clinicopathological features of cohort 1 are listed in Table 1. At the time of the last follow-up, 49 patients had tumor recurrence and 38 patients had died, including 10 patients who died from liver failure without a record of tumor recurrence. The 1-, 3-, and 5-year OS rates were 83%, 59%, and 45%, respectively, and the 1-, 3-, and 5-year TTR rates were 73%, 55%, and 43%, respectively.

Tissue Microarray Construction, Immunohistochemistry, and Evaluation

Tissue microarray (TMA) construction was described in our previous reports [8, 36, 38]. Briefly, to construct TMA slides, two cores were taken from each representative formalin-fixed, paraffin-embedded tumor tissue and from tissue adjacent to the tumor within a distance of 10 mm. Cohort 1 TMA slides with 105 pairs of tumorous and matched peritumoral samples were constructed. Another 32 specimens including tumor and long-distance peritumoral liver tissue (at distances of 10 mm, 20 mm, and 30 mm from the tumor margin) were collected and constructed into a long-distance peritumoral TMA chip (cohort 2).

Table 1. The clinicopathological features of 105 patients from cohort 1

Features	Values/counts ($n = 105$)
Age (yrs), median (range)	51 (18–75)
Gender (male/female)	96/9
Preoperative ALT (U/L), median (range)	34.5 (2–187)
α-fetoprotein (ng/ml), median (range)	245 (0–60,500)
Liver cirrhosis (yes/no)	82/23
Hepatitis B history (yes/no)	90/15
Hepatitis B e antigen (positive/negative)	27/78
Tumor size (cm, mean ± SD)	6.5 ± 4.6
Encapsulation (complete/none)	45/60
Tumor differentiation (high/low)	94/11
Intrahepatic metastasis (yes/no)	23/82
Microvascular invasion (yes/no)	43/62
UICC TNM stage (I/II/IIIA)	68/18/19

Abbreviations: ALT, alanine aminotransferase; SD, standard deviation; TNM, tumor–node–metastasis; UICC, International Union Against Cancer Classification.
Based on Zhu XD, Zhang JB, Zhuang PY et al. High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma. J Clin Oncol 2008;26:2707–2716.

Immunohistochemistry was performed by a two-step method, as described in our previous reports [8, 36, 38]. The primary antibody (1:200 polyclonal rabbit anti-human CSF-1R; Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the slides were incubated overnight at 4°C in a wet chamber. The components of the EnVision+ detection system were applied with an anti-rabbit polymer (EnVision+/HRP/Mo; Dako, Glostrup, Denmark). Reaction products were visualized by incubation with 3,3′-diaminobenzidine. Negative controls were treated identically but with the primary antibody omitted.

The intensity of positive CSF-1R staining in the whole view was measured using a computerized image system composed of a Leica charged-coupled device camera, DFC500, connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions Ltd., Cambridge, U.K.). Under 200× magnification, images of four representative fields were captured using Leica QWin Plus Version 3 software. An identical setting was used for all images. The density of immunostaining was measured using Image-Pro Plus Version 6.2 software (Media Cybernetics Inc., Bethesda, MD), as described previously [8]. The integrated optical density (IOD) in each image was measured, and CSF-1R density was calculated as IOD/total area of each image. The TMAs were assessed independently by two observers (W.Q. Wang and X.D. Zhu), with a high correlation between them ($p < .001$).

Because it was impossible to separate the expression of CSF-1R on hepatocytes and macrophages by the computerized imaging system, the regions of most intense staining were scored by eye. The intensity of staining in the cytoplasm of hepatocytes was graded as 0, 1, 2, and 3 and then classified into low expression (grades 0 and 1) and high expression (grades 2 and 3). The number of infiltrated macrophages with positive CSF-1R staining in peritumoral liver tissue was also counted by eye. The TMAs were scored or counted independently by two observers, as described earlier.

Immunofluorescence Double-Staining of CSF-1R and CD68

Eight HCC samples were collected and frozen sections were made. Sections were rinsed three times in phosphate-buffered saline (PBS). Goat serum (10%) in PBS was used to block nonspecific protein binding. Sections were then incubated overnight at 4°C with CSF-1R antibody (1:50 polyclonal rabbit anti-human CSF-1R) and CD68 antibody (1:50 monoclonal mouse anti-human CD68; Abcam, Cambridge, MA). After rinsing to remove excess antibody, sections were incubated in both Cy3-conjugated affinipure goat anti-mouse IgG antibody (1:100 dilution) and Cy5-conjugated affinipure goat anti-rabbit IgG antibody (1:100 dilution) (Amersham Biosciences, Ltd., Little Chalfont, U.K.) and counterstained with 10 μg/ml 4′,6-diamidine-2′-phenylindole dihydrochloride to stain nuclei. Representative fields were acquired with a computerized imaging system as described earlier. Negative controls were treated identically but with the primary antibody omitted.

Quantitative Real-Time Polymerase Chain Reaction

CSF-1R and hypoxia-inducing factor (HIF)-1α mRNA levels were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) in frozen paired specimens obtained from cohort 3, which contained 52 HCC patients who underwent curative resection in 2008–2009. Total RNA was retracted by Trizol according to the protocol recommended by the manufacturer. Briefly, specimens were obtained during surgery as blocks of 1 cm^3, snap frozen in liquid nitrogen, and stored at −80°C. Then, CSF-1R mRNA was reverse transcribed and amplified using a SYBR PrimeScript RT-PCR kit (Invitrogen Life Technologies, Gaithersburg, MD). The primer sequences were as follows: CSF-1R forward, 5′-ACACTAAGCTCGCAATCCC-

3′; CSF-1R reverse, 5′-GTATCGAAGGGTGAGCTCAAA-3′; HIF-1α forward, 5′-GCTGACCCTGCACTCAAT-3′; HIF-1α reverse, 5′-GATCGAAGGAAGGTAACTGG-3′; β-actin forward, 5′-CATCTCTTGCTCGAAGTCCA-3′; β-actin reverse, 5′-ATCATGTTTGAGACCTTCAACA-3′. Gene expression was quantified by RT-PCR and relative gene expression values were calculated by the $\Delta\Delta$Ct method [41] using Sequence Detection System 2.1 software (Applied Biosystems, Foster City, CA). The result of CSF-1R or HIF-1α mRNA was normalized to the corresponding β-actin signal (ΔCt). Measurements were performed in triplicate.

Statistical Analysis

All statistical analyses were performed with SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL). Pearson's χ^2 and Fisher's exact tests were applied to compare qualitative variables, and quantitative variables were analyzed by Student's *t*-test or Spearman's ρ rank correlation coefficient determination. Clinicopathological features were compared between the two risk groups using the Mann-Whitney test. One-way analysis of variance (ANOVA) was applied to determine the distributional characteristics of the biomarker in peritumoral liver tissue. Kaplan–Meier analysis was applied to determine survival. The log-rank test was applied to compare patient survival between subgroups, and the Cox proportional hazard regression model was applied to perform the multivariate analysis. Receiver operating characteristic (ROC) curve analysis was applied to determine predictive value among parameters. $p < .05$ was considered statistically significant. X-tile Plots Version 3.6.1 (Yale University, New Haven, CT) [42] was used to find an optimal cutoff point for CSF-1R expression.

Results

Patterns of CSF-1R Expression and Distribution

As shown in Figure 1, CSF-1R staining was mainly in the cytoplasm and cytomembrane of tumor cells or hepatocytes. Sporadic stroma cells were also observed with positive staining. Some cells with darker staining were observed in between tumor cells or hepatocytes. Most of these cells also stained positive for CD68 (Fig. 2). The CSF-1R densities of intratumoral and peritumoral tissues were 0.011 $\pm$ 0.008 (range, 0.000–0.042) and 0.091 $\pm$ 0.019 (range, 0.007–0.688), respectively. CSF-1R expression was significantly higher in peritumoral liver tissue than in tumor tissue ($p < .001$).

As shown in Figure 1J, both the CSF-1R and HIF-1α mRNA levels were significantly higher in peritumoral liver tissue than in the corresponding intratumoral tissue ($p = .005$ and $p = .013$, respectively). The average power ($2^{-\Delta Ct}$) was 0.014 $\pm$ 0.003 and 0.007 $\pm$ 0.002 (mean $\pm$ standard deviation) for peritumoral and intratumoral CSF-1R, respectively, and 0.040 $\pm$ 0.007 and 0.024 $\pm$ 0.006 for peritumoral and intratumoral HIF-1α, respectively. CSF-1R expression correlated with HIF-1α expression in both intratumoral and peritumoral tissues ($r = 0.478$, $p = .001$ and $r = 0.397$, $p = .008$, respectively).

A gradient distribution of CSF-1R expression in peritumoral liver tissue was observed in the long-distance peritumoral TMA, with the expression of CSF-1R decreasing as the distance from the tumor margin increased (Fig. 1F–1I). The CSF-1R densities of the proximal, median, and distant peritumoral liver tissues and the matched intratumoral tissue were 0.012 $\pm$ 0.008 (range, 0.000–0.029), 0.008 $\pm$ 0.006 (range, 0.000–0.026), 0.006 $\pm$ 0.005 (range, 0.000–0.028), and 0.004 $\pm$ 0.004 (range, 0.000–0.025), respectively. CSF-1R expression differed significantly among the proximal, median, and distant distances of peritumoral liver tissue (one-way ANOVA, compared with proximal peritumoral CSF-1R expression; $p < .001$, $p = .01$, $p < .001$, respectively). The CSF-1R expression level at 10 mm correlated positively with those at 20 mm and 30 mm ($r = 0.825$, $p < .001$ and $r = 0.689$, $p < .001$, respectively).

Correlations with Clinicopathological Factors

The 85% value as the cutoff point for peritumoral CSF-1R density was obtained with an optimal *p* value, using the OS time as the marker, and it was located at the position of 0.08 (supplemental online Fig. S1A). The range of low CSF-1R density was 0.0065–0.076 and the range of high CSF-1R density was 0.13–0.69. There was not an optimal cutoff point for intratumoral CSF-1R expression (online supplemental Fig. S1B); therefore, the cutoff point was set at the median value. The distribution of peritumoral hepatocyte scoring is shown in supplemental online Figure S1C. The 66% value as the cutoff point for peritumoral macrophage counting was obtained with an optimal *p*-value, using the OS time as the endpoint (supplemental online Fig. S1D).

As shown in Table 2 (and supplemental online Tables S1 and S2), patients with a high peritumoral CSF-1R expression level were prone to have a large tumor size, high serum α-fetoprotein concentration, high TNM stage, and the presence of intrahepatic metastasis, whereas intratumoral CSF-1R expression did not correlate with any clinicopathological feature. Patients with cirrhosis (stage 4, $n = 38$) had a significantly lower peritumoral CSF-1R density than patients without cirrhosis (stages 1–3, $n = 62$; 0.044 versus 0.074, respectively; $p = .009$).

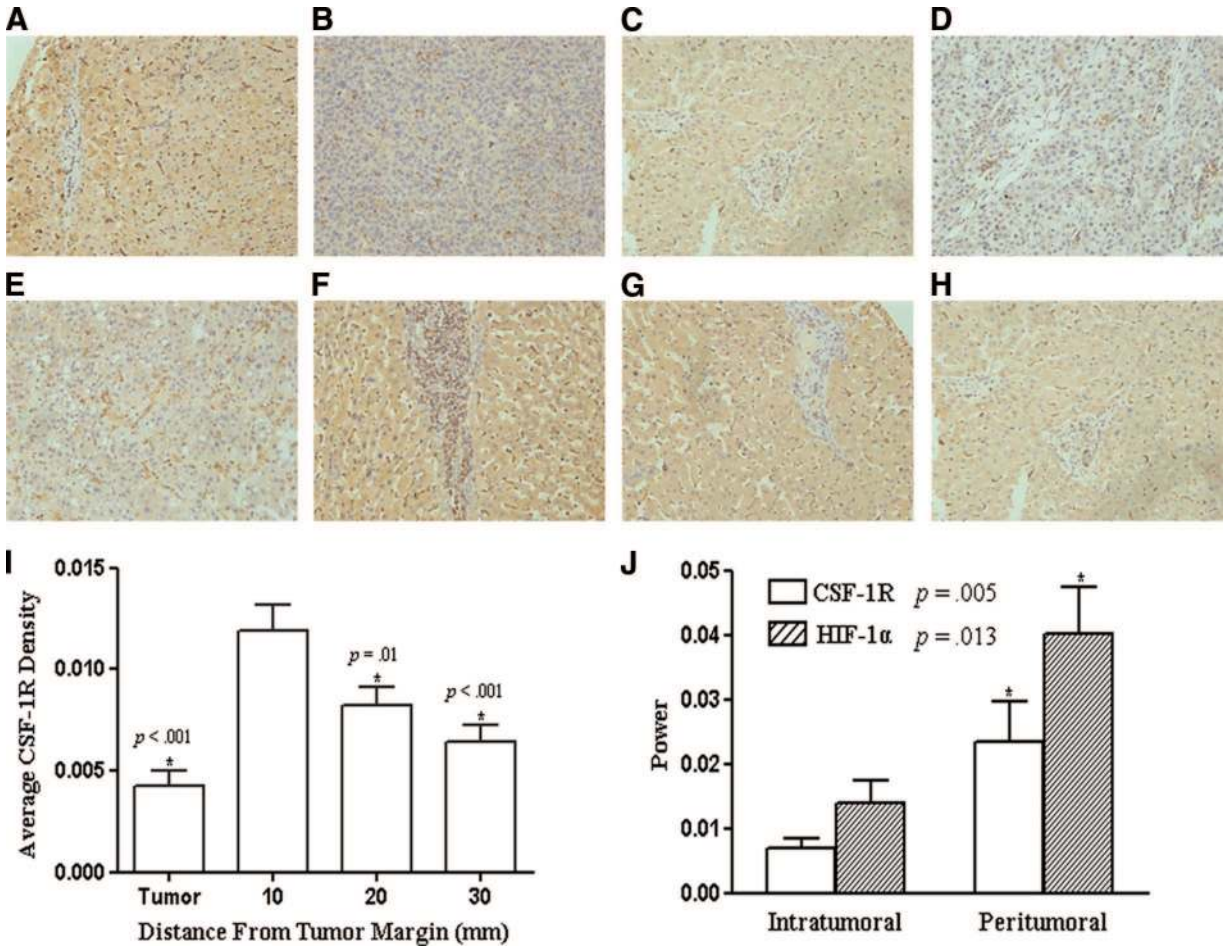

Figure 1. Patterns of CSF-1R expression and distribution. Representative strong (case 20, **(A)**, **(B)**) and mild (case 60, **(C)**, **(D)**) immunostaining of colony-stimulating factor 1 receptor (CSF-1R) in peritumoral **(A, C)** and intratumoral **(B, D)** tissue microarray (TMA) chips (200×). **(E–H):** Immunostaining of intratumoral **(E)** and peritumoral **(F–H)** CSF-1R in a long-distance peritumoral TMA chip. **(I):** CSF-1R expression differed in tumor and peritumoral liver tissue and presented a gradient distribution pattern in the latter. *$p < .05$, one-way analysis of variance, compared with proximal peritumoral CSF-1R density. **(J):** Quantitative real-time polymerase chain reaction analyses of CSF-1R and hypoxia-inducing factor 1α gene expression in intratumoral and peritumoral specimens. *$p < .05$, paired-samples *t*-test between the two intratumoral or peritumoral subgroups.

Correlation with Patient Outcome

On univariate analysis, tumor size, the presence of intrahepatic metastasis, and TNM stage were associated with the OS time and TTR. The presence of microvascular invasion, liver cirrhosis, and tumor differentiation was also associated with OS (Table 3). The median OS time and TTR for patients with a high peritumoral CSF-1R expression level were 8.4 months and 10.3 months, respectively, and these periods were significantly shorter than those for patients with a low CSF-1R expression level (>72 months and 75.3 months, respectively; $p < .001$ for both) (Fig. 3A, 3C). However, intratumoral CSF-1R expression was not associated with the OS time or TTR ($p = .621$ and $p = .882$, respectively) (Fig. 3B, 3D). Low peritumoral hepatocyte expression and macrophage count was also associated with a longer OS time ($p = .001$ and $p < .001$, respectively) (supplemental online Fig. S2A, S2B, S2E) and TTR ($p = .011$ and $p < .001$, respectively) (Fig. S2C, S2D, S2F).

The risk factors identified by univariate analysis were adopted in a multivariate Cox proportional hazard analysis. High peritumoral CSF-1R expression was an independent risk factor for OS (hazard ratio [HR], 12.861; $p < .001$) and TTR (HR, 3.879; $p = .002$) (Table 3). A high peritumoral hepatocyte expression level was an independent risk factor for OS (HR, 3.470; $p = .008$), and a high peritumoral macrophage count was an independent risk factor for TTR (HR, 2.483; $p = .005$) (Table 3).

We then used 12 months as the cutoff value to categorize tumor recurrences into early recurrence and late recurrence, as previously reported [43]. More patients with a high peritumoral CSF-1R density (than patients with a low peritumoral CSF-1R density) had an early recurrence (13 of 15 versus 23 of

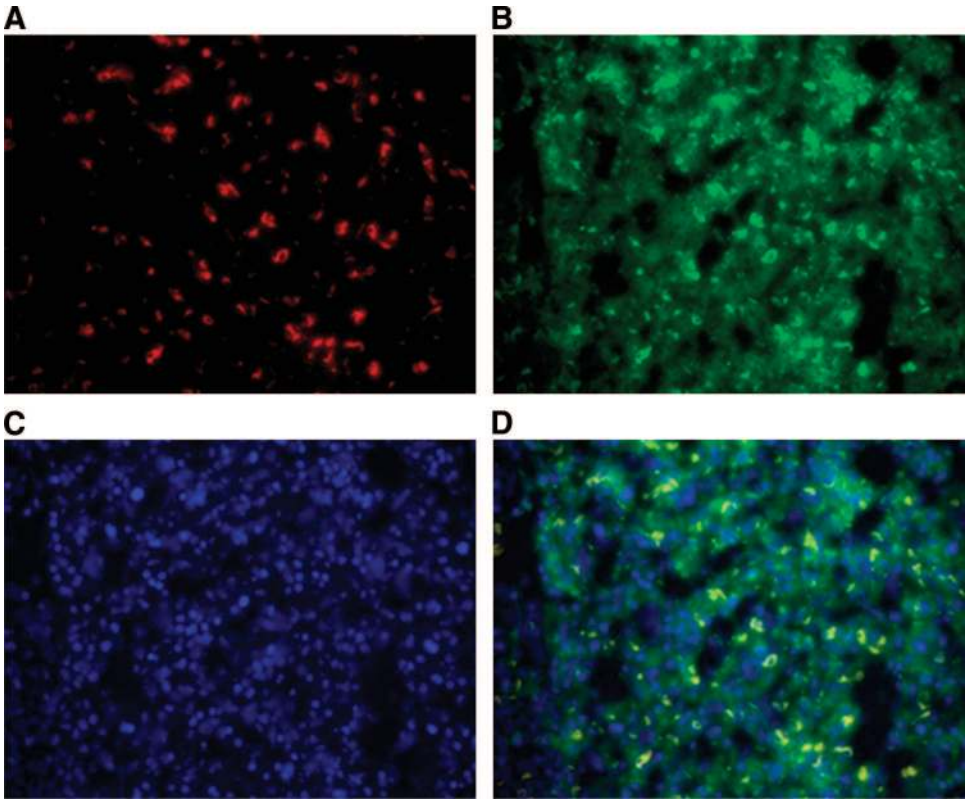

Figure 2. Immunofluorescence double staining of CD68 and colony-stimulating factor 1 receptor (CSF-1R) in eight hepatocellular carcinoma samples. CD68 staining used Cy3 ((**A**), red), CSF-1R staining used Cy5 ((**B**), green), and nucleus staining used 4′,6-diamidine-2′-phenylindole dihydrochloride ((**C**), blue) as substrates. **(D)** was merged from **(A)**, **(B)**, and **(C)**.

90 patients, respectively; $p < .001$) rather than a late recurrence (1 of 15 versus 6 of 90 patients, respectively; $p = .300$). To reduce the impact of tumor size on patient survival, we further investigated prognostic factors in the small HCC subgroup (maximum diameter ≤5 cm, $n = 57$). The peritumoral CSF-1R level also correlated with the OS time and TTR ($p = .027$ and $p = .007$, respectively) in this subgroup (supplemental online Fig. S3). Positive hepatitis B e antigen (HBeAg) correlated with TTR ($p = .013$), whereas it did not correlate with the OS time ($p = .130$).

ROC Analysis

Risk factors identified by multivariate analysis were adopted, and their predictive values were studied by ROC analysis (Table 4). Peritumoral CSF-1R, hepatocyte scoring, macrophage count, and intrahepatic metastasis precisely predicted death, and peritumoral CSF-1R, macrophage count, TNM stage, and tumor size precisely predicted early recurrence ($p < .05$ for all). However, microvascular invasion did not predict death ($p = .143$). The area under the curve of peritumoral CSF-1R was 0.645 (95% confidence interval [CI], 0.538–0.752; $p = .011$) for death and 0.666 (95% CI, 0.548–0.784; $p = .005$) for 1-year recurrence (Fig. 3E, 3F), and both the predictive values were >0.600.

Discussion

CSF-1R, as a member of the class III RTKs, is important in the malignant transformation of a tumor [44]. The extent of CSF-1R expression in some types of tumor is associated with high TNM grade and poor prognosis [18, 19]. However, the CSF-1R expression level in HCC is still unknown. Moreover, previous studies of CSF-1R mostly focused on tumor tissue, whereas its expression and significance in peritumoral tissue are not fully understood.

Our results revealed that expression of CSF-1R in peritumoral liver tissue was higher than that in the corresponding tumor tissue, and it was associated with poor outcome after resection of the primary tumor. We also found that CSF-1R was expressed not only in macrophages but also in peritumoral hepatocytes and HCC cells; the peritumoral hepatocyte expression of CSF-1R and macrophage infiltration were also associated with poor outcome for HCC patients. Overexpression of CSF-1R in the tumor is common in many types of cancer and correlates with a poor prognosis [18, 19, 31, 34, 35]. However, Kluger et al. [33] found that intratumoral CSF-1R expression was not an independent predictor of survival in breast cancer within a node-positive subset of patients. Our studies showed that there was significant correlation between peritumoral CSF-1R and a

Table 2. Relationship between intratumoral and peritumoral CSF-1R and clinicopathological features

Variables	Intratumoral CSF-1R density					Peritumoral CSF-1R density				
	High ($n = 53$)		Low ($n = 52$)			High ($n = 15$)		Low ($n = 90$)		
	n of patients	%	*n* of patients	%	*p*	*n* of patients	%	*n* of patients	%	*p*
Age, yrs[a]	50.6		52.2		.462	49.3		53.5		.058
Gender					.750[b]					.088[b]
Male	48	91	48	92		12	80	84	93	
Female	5	9	4	8		3	20	6	7	
Hepatitis B history					.381					.139[b]
Yes	47	89	43	83		11	73	79	88	
No	6	11	9	17		4	27	11	12	
HBeAg					.540					.466
Positive	15	28	12	23		5	33	22	24	
Negative	38	72	40	77		10	67	68	76	
ALT, U/L[a]	48.6		43.0		.431	44.0		47.7		.405
AFP, ng/dl[a]	2,818.11		4,995.59		.209	7,130.04		1,705.90		.031
Liver cirrhosis					.218					.012
Yes	44	83	38	73		8	53	74	82	
No	9	17	14	27		7	47	16	18	
Tumor size, cm[a]	6.42		6.52		.908	7.57		4.15		.020[c]
Intrahepatic metastasis					.774					.012
Yes	11	21	12	23		7	47	16	18	
No	42	79	40	77		8	53	74	82	
Tumor encapsulation					.063					.171[b]
Complete	18	34	27	52		4	27	41	46	
None	35	66	25	48		11	73	49	54	
Microvascular invasion					.607					.099
Yes	23	43	20	38		8	53	35	39	
No	30	57	32	62		7	47	55	61	
TNM stage					.324					.008[b]
I	35	66	33	63		6	40	62	69	
II	11	21	7	14		2	13	16	18	
IIIA	7	13	12	23		7	47	12	13	

$p < .05$ was considered statistically significant.
[a]Student's *t*-test.
[b]Twenty-five percent of all the cells have expected count <5; Fisher's exact test.
[c]Equal variances not assumed.
Abbreviations: AFP, α-fetoprotein; ALT, alanine aminotransferase; CSF-1R, colony-stimulating factor-1 receptor; HBeAg, hepatitis B e antigen; TNM, tumor–node–metastasis.

high incidence of intrahepatic metastasis; however, there was no correlation between intratumoral CSF-1R and any clinicopathological feature.

Recently, we reported that the expression levels of several angiogenesis factors and CSF-1 as well as macrophage infiltration are higher in peritumoral liver tissues [8, 9, 36]. Together with findings reported by other authors [10, 11], we propose that a unique condition in peritumoral liver tissue, which is associated with a specific type of HCC, provides a suitable substrate for the development and growth of intrahepatic metastasis or tumor recurrence.

The present study revealed that neither intratumoral nor peritumoral CSF-1R expression correlated with features related to hepatitis, including hepatitis B history, serum alanine aminotransferase, and the presence of HBeAg, except for cirrhosis scores. The possible reason may lie in the het-

Table 3. Univariate and multivariate analyses of factors associated with overall survival and time to recurrence

	OS				TTR			
		Multivariate				Multivariate		
Factors	Univariate *p*	Hazard ratio	95% CI	*p*	Univariate *p*	Hazard ratio	95% CI	*p*
Age: <51 yrs versus ≥51 yrs	.989			NA	.592			NA
Gender: female versus male	.087			NA	.075			NA
Hepatitis B history: no versus yes	.554			NA	.597			NA
HBeAg: negative versus positive	.424			NA	.337			NA
Liver cirrhosis: no versus yes	.036			NS	.053			NA
ALT: ≤75 U/L versus >75 U/L	.569			NA	.446			NA
AFP: ≤300 ng/dl versus >300 ng/dl	.035			NS	.119			NA
Tumor differentiation: low versus high	.028			NS	.298			NA
Tumor size: ≤5 cm versus > 5 cm	<.001			NS	<.001	2.218	1.153–4.268	.017
Tumor number: single versus multiple	.771			NA	.178			NA
Tumor encapsulation: none versus complete	.141			NA	.073			NA
Microvascular invasion: no versus yes	.019	2.461	1.224–4.947	.011	.266			NA
Intrahepatic metastasis: no versus yes	<.001	2.665	1.296–5.480	.008	.002			NS
TNM stage: I versus II versus IIIA	<.001			NS	<.001	2.319	1.124–4.783	.023
Peritumoral CSF-1R density: low versus high	<.001	12.861	5.556–29.768	<.001	<.001	3.879	1.652–9.109	.002
Intratumoral CSF-1R density: low versus high	.621			NA	.882			NA
Peritumoral hepatocytes scoring: low versus high	.001	3.470	1.393–8.645	.008	.011	1.975	.952–4.098	.067
Peritumoral macrophages counting: low versus high	<.001	1.824	.896–3.713	.097	<.001	2.483	1.325–4.653	.005

Abbreviations: AFP, α-fetoprotein; ALT, alanine aminotransferase; CI, confidence interval; CSF-1R, colony-stimulating factor-1 receptor; HBeAg, hepatitis B e antigen; NA, not adapted; NS, not significant; OS, overall survival; TTR, time to recurrence.

erogeneous baseline expression level of CSF-1R, which is associated with other genetic heterogeneity among individuals. However, the centripetal distribution of CSF-1R expression in peritumoral liver tissue implies that the tumor may be a stimulator.

This peritumoral condition may be caused by hypoxia in hepatocytes resulting from direct compression by the primary tumor. Another possibility is that free radicals created by tumor hypoxia penetrate into the peritumoral liver milieu, strongly influencing the HIF-1α activity of hepatocytes [45], and then promote CSF-1R expression. The qRT-PCR results for HIF-1α suggest that a hypoxic microenvironment might explain CSF-1R expression in both intratumoral and peritumoral tissues. However, the tumor itself can be regarded as a stimulator of chronic inflammation as a nonhealing wound that causes a reaction in peritumoral hepatocytes, which produce a number of cytokines and recruit macrophages to the tumor margin. Therefore, the higher expression level of CSF-1R in peritumoral tissue may represent more macrophage infiltration and a

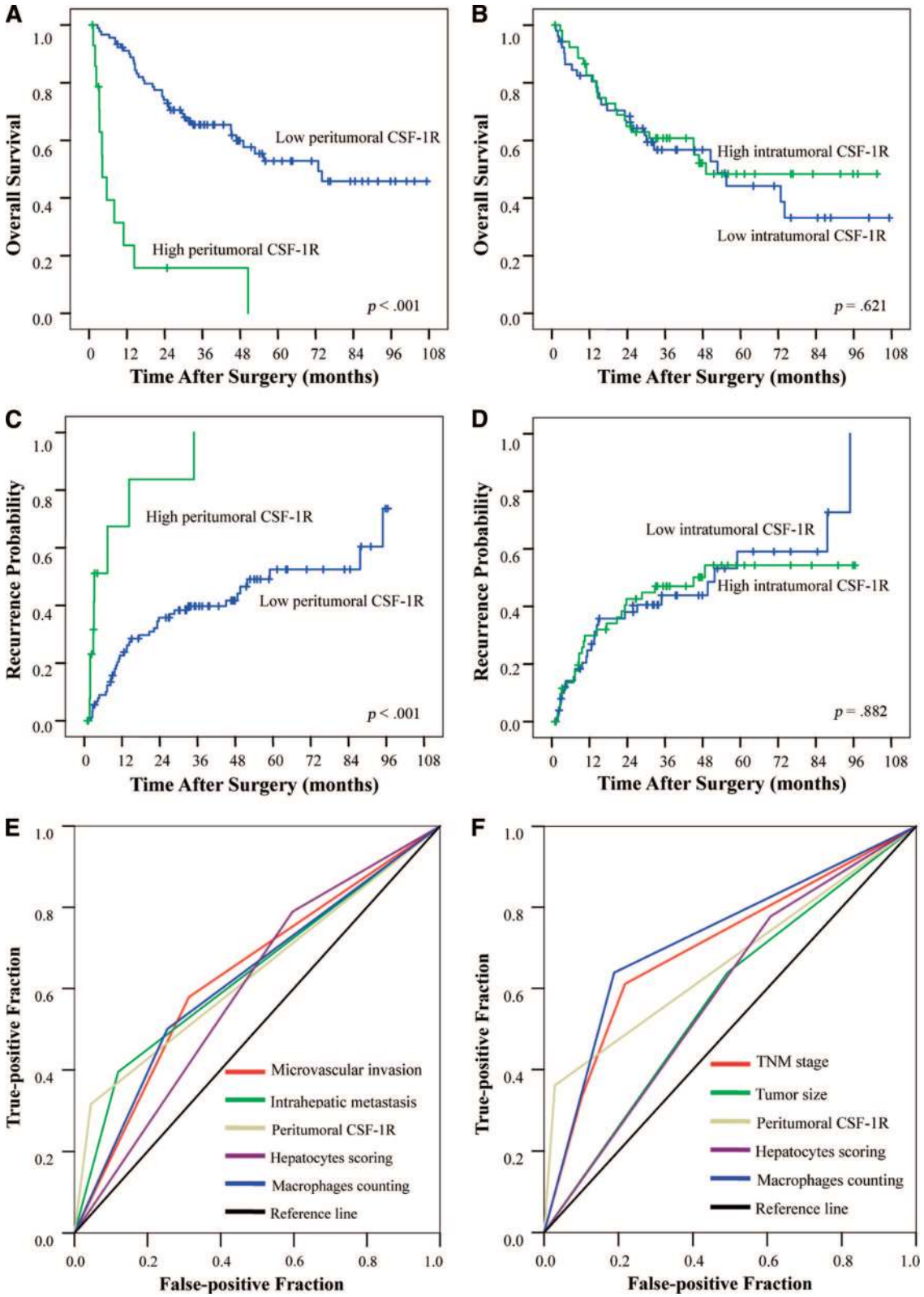

Figure 3. Cumulative overall survival (OS) and time to recurrence (TTR) curves of patients (cohort 1) with low or high macrophage colony-stimulating factor-1 receptor (CSF-1R) density. Low peritumoral CSF-1R density was associated with a longer OS time and TTR **(A, C)**, whereas intratumoral CSF-1R density was associated with neither the OS time nor the TTR **(B, D)**. **(E, F):** Receiver operating characteristic curve: All the other adopted factors predicted death and 1-year recurrence precisely ($p < .05$ for all), whereas microvascular invasion could not predict death ($p = .143$).

Table 4. Prognostic values of variables for death and 1-year disease recurrence by ROC analysis

Variable	Area under curve	95% CI	*p*-value
Death			
Peritumoral CSF-1R	0.645	0.538–0.752	.011
Peritumoral hepatocyte scoring	0.596	0.485–0.707	.021
Peritumoral macrophage counting	0.623	0.509–0.737	.037
Intrahepatic metastasis	0.672	0.567–0.778	.002
Microvascular invasion	0.583	0.473–0.693	.143
1-year recurrence			
Peritumoral CSF-1R	0.666	0.548–0.784	.005
Peritumoral hepatocyte scoring	0.585	0.472–0.697	.156
Peritumoral macrophage counting	0.725	0.618–0.833	.000
TNM stage	0.702	0.592–0.812	.001
Tumor size	0.617	0.503–0.731	.049

Abbreviations: CI, confidence interval; CSF-1R, colony-stimulating factor-1 receptor; ROC, receiver operating characteristic; TNM, tumor–node–metastasis.

more intense reaction of hepatocytes responding to hypoxia or "inflammation stimulation." This hypothesis is supported by a study that showed strong expression of CSF-1R by macrophages infiltrating near prostate cancers and that the most intense and uniform staining for lesions within the prostate occurred in areas of high-grade carcinoma [19], which is similar to our observation. Recently, it was reported that both the paracrine and autocrine loops involving CSF-1R contribute to a more aggressive phenotype in human breast and ovarian cancer [31, 46], which is consistent with our results.

Although conflicting results on the clinical significance of intratumoral CSF-1R have been reported, the present findings suggest that CSF-1/CSF-1R may be a good target to block to prevent intrahepatic metastasis of HCC, especially in the adjuvant setting, because peritumoral liver tissue remains and becomes a major site for postoperative recurrence.

In summary, our present study shows that a high expression level of CSF-1R in peritumoral liver tissue is a strong predictor of postoperative survival in patients with HCC. Thus, this marker may be clinically useful for evaluating prognosis. Moreover, the expression of CSF-1R in residual liver after hepatectomy may play a critical role in promoting recurrence, invasion, and metastasis. With the recent development of new inhibitors that target RTKs and monoclonal antibodies to these receptors, CSF-1R may become a new target of therapy in HCC.

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