

The clinical and prognostic significance of CD14⁺HLA-DR^{-/low} myeloid-derived suppressor cells in hepatocellular carcinoma patients receiving radiotherapy

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Abstract Myeloid-derived suppressor cells (MDSCs) are key player in mediating systemic immunosuppression, and their accumulation and expansion in the periphery and tumor have been iteratively observed in patients with various types of cancer. It has been reported that CD14⁺HLA-DR^{-/low} MDSCs are increased in hepatocellular carcinoma (HCC) patients; however, the clinical significance of MDSC alteration in HCC patients after treatment is poorly studied. In this study, we examined the frequency of MDSCs in 92 HCC patients, 14 chronic liver disease patients without HCC, and 22 healthy controls by flow cytometric analysis. The associations between the clinical features and the frequency of MDSCs were analyzed. In particular, we further examined the prognostic impact of MDSCs on the overall survival of HCC patients receiving radiation therapy. The frequency of MDSCs in HCC patients was significantly increased and correlated with tumor stage, size, burden, and Child-Pugh classification but not with biochemical parameters of liver function. In HCC patients who received radiation therapy, the frequency of MDSCs after treatment significantly decreased and was inversely correlated with overall survival time. In multivariate analysis, only post-treatment MDSC ratio and Child-Pugh classification were correlated with the prognosis of HCC patients. Patients with a high frequency of MDSCs after

radiotherapy should be closely followed, and the inhibition of MDSCs may improve the prognosis of patients.

Keywords Myeloid-derived suppressor cells · Hepatocellular carcinoma · Radiation therapy · Overall survival

Introduction

Liver cancer is one of the most common cancers and the third most frequent cause of cancer-related death worldwide [1]. Hepatocellular carcinoma (HCC) as a major type accounts for 70 to 85 % of primary liver cancers [2]. Current treatment options include surgical resection, chemotherapy, radiofrequency ablation (RFA), and transcatheter arterial chemoembolization (TACE) [3–5]. Recently, radiotherapy is growingly important in the management of HCC due to the innovation of three-dimensional conformal radiation therapy (3D-CRT) and intensity-modulated radiation therapy (IMRT) [6]. Despite these treatments, tumor recurrence rate of HCC patients is still high and the prognosis is dismal [7, 8]. Therefore, novel anti-tumor treatments for HCC are urgently needed. In this regard, immunotherapies have represented a great potential to overcome the aforementioned limitations and prolong the survival time of HCC patients [9, 10]; however, systemic immune suppression is often observed in patients with HCC and must be overcome before immunotherapies get success in clinic.

Recent research has been focused on myeloid-derived suppressor cells (MDSCs) that play a key role in mediating immune suppression in the host [11, 12]. MDSCs are immature myeloid cells with immunosuppressive activity and observed to accumulate in tumor-bearing mice and humans with different types of cancer, including HCC [13–15], where they potentially inhibit T cell effector function via multiple mechanisms

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and consequently promote tumor development [12, 16]. Importantly, intervening strategies targeting the various aspects of MDSCs, including their development, expansion/activation, function, and turnover, have demonstrated the anti-tumor effects in pre-clinical animal models [17], indicating its potentially therapeutic value in clinic. Regarding human MDSCs, they are commonly defined as suppressor cells with CD11b⁺CD33⁺HLA-DR^{-/low} phenotype and can be divided into granulocytic CD14⁻CD15⁺ and monocytic CD14⁺CD15⁻ subtypes [11, 16]. In previous studies, it has been documented that CD14⁺HLA-DR^{-/low} MDSCs are increased in HCC patients that can suppress the function of T cells through the induction of regulatory T cells (Tregs) [13] and concomitant ablation of these MDSCs and Tregs in peripheral blood mononuclear cells (PBMCs) of HCC patients significantly rescues the effector functions of CD4⁺ and CD8⁺ T cells in vitro [15]. Furthermore, MDSC ratio has been reported to be prognostic for HCC recurrence in patients receiving curative RFA therapy with MDSC frequency after treatment inversely correlating with recurrence-free survival [14]. Therefore, CD14⁺HLA-DR^{-/low} MDSCs are of clinical significance in HCC, and deeper elucidation of inhibitory mechanism of MDSCs and controlling their function is very important to develop more effective immunotherapy for HCC.

In this study, we investigated the clinical parameters associated with the frequency of MDSCs in patients with HCC and the prognostic role of MDSCs in advanced HCC patients receiving radiotherapy to validate the significance of targeting MDSCs.

Material and methods

Subjects and blood samples

Ninety-two histologically confirmed HCC patients without other systemic diseases and 14 hepatitis patients undergoing percutaneous liver biopsy to evaluate the disease severity recruited at Department of Oncology of Beijing Chao-Yang Hospital as well as 22 healthy blood donors were included in this study. All patients received complete staging according to the criteria of the seventh edition of the American Joint Committee on Cancer (AJCC). We obtained the whole blood from HCC patients prior to surgery, immunotherapy, radiotherapy, chemotherapy, or any other treatment. Samples were analyzed within 3 h of collection.

Of the 61 advanced HCC patients, 43 patients who received 3D-CRT or IMRT alone were followed up for at most 2 years. They received less than a total dose of 60 Gy by conventional fractionation of 2 Gy/fraction and five fractions a week in 4–6 weeks. Post-treatment blood samples were obtained in 3–4 weeks.

Antibodies and flow cytometry

For the surface marker, fresh heparinized peripheral blood was added to the following monoclonal antibodies (all from Beckman Coulter, Inc): PE-Cy7-conjugated CD14 (clone RMO52), fluorescein isothiocyanate (FITC)-conjugated HLA-DR (clone Immu357), and PE-Cy5-conjugated CD3 (clone UCHT1) with IgG2a-PE-Cy7, IgG2b-FITC, and IgG1-PE-Cy5 mouse isotype control used as controls. After incubation for 30 min at 4 °C in the dark, the cells were lysed with RBC Lysis/Fixation Solution (Beckman Coulter, Inc). Before intracellular staining, the cells were permeabilized with Cytofix/Cytoperm Kit (BD Biosciences) and stained with antibody Alexa Fluor 488-conjugated CD3 ξ (clone 6B10.2; sc-1239 AF488 from Santa Cruz) or isotype-matched control antibody (normal mouse IgG1 Alexa Fluor[®] 488; sc-3890 from Santa Cruz). Cells were analyzed using a FC500 MPL flow cytometer (Beckman Coulter) and FlowJo analysis software (Treestar).

MDSC isolation and proliferation

PBMCs were isolated from 20 ml of whole blood taken from three patients with stage IV HCC by Histopaque-1077 (Sigma) density gradient centrifugation and then costained with anti-HLA-DR/CD14/CD3 antibodies. CD14⁺HLA-DR^{-/low} cells and CD3⁺ T cells were purified by MoFloTM XDP cell sorting system (Beckman Coulter). The purity of all isolated cell populations was >95 % (data not shown). For functional analysis, purified T cells were labeled with 2 mM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen Molecular Probe) according to the manufacturer's instructions, and consequent CFSE-labeled T cells (50,000/well) were cocultured at various ratios with CD14⁺HLA-DR^{-/low} MDSCs in the absence or presence of CD3/CD28 beads (Miltenyi Biotec) in 96-well round-bottomed plates (Corning, Costar). After 72 h, proliferation was measured by CFSE dilution as described previously [18, 19].

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD) and analyzed using the Statistical Package for the Social Sciences version 18.0 (SPSS). Parametric (paired or unpaired *t* test and Fisher's exact test) or nonparametric (Mann–Whitney *U*) were used for univariate analysis of the two groups classified according to the frequency of MDSCs. The cumulative survival rates were calculated by the Kaplan–Meier method, and the differences in survival rates were analyzed by the log-rank test. The multivariate analysis of independent prognostic factors for survival was performed by the Cox proportional hazard model. Statistical significance was set at $p < 0.05$.

Results

Circulating CD14⁺HLA-DR^{-/low} cells are significantly increased in HCC patients correlating with disease stage

We used whole blood to analyze the percentage of CD14⁺HLA-DR^{-/low} MDSCs. As shown in Fig. 1a, the CD14⁺ monocytes were divided into CD14⁺HLA-DR⁺ cells and CD14⁺HLA-DR^{-/low} MDSCs based on HLA-DR expression. Consistent with previous study, the frequency of CD14⁺HLA-DR^{-/low} MDSCs was apparently higher in patients with HCC than healthy donors (mean 16.79 vs 9.33 %, $p=0.0002$; Fig. 1b). The absolute number of circulating CD14⁺HLA-DR^{-/low} MDSCs in peripheral blood was also significantly increased in these patients (mean 41.3 vs 10.2 cells/ μ l, $p<0.0001$; Fig. 1c). The frequency of MDSCs was correlated with the stage of HCC where patients with advanced disease had a significantly higher level of circulating MDSCs compared to that with early disease (18.91 vs

12.61 % for stages III and IV ($n=61$) and stages I and II ($n=31$), $p=0.0005$; Fig. 1d). The representative dotplots were shown in Fig. 1a. Intriguingly, no significant difference in MDSC frequency was seen between hepatitis patients without HCC and healthy controls, although the frequency of MDSCs in former was mildly higher.

To confirm the suppressive activities of CD14⁺HLA-DR^{-/low} MDSCs, we cocultured CD14⁺HLA-DR^{-/low} MDSCs sorted from PBMCs of HCC patients with autologous CD3⁺ T cells in the absence or presence of stimulation conferred by beads coated with anti-CD2/-CD3/-CD28 monoclonal antibodies. We observed that CD14⁺HLA-DR^{-/low} MDSCs suppressed proliferation of autologous CD3⁺ T cells in a dose-dependent manner (Fig. 2b). The representative histograms were shown in Fig. 2a. In addition, we found that the CD3 ξ expression of CD3⁺ T cells was decreased significantly in the peripheral blood of HCC patients ($n=20$) compared with healthy donors ($n=20$; Fig. 2c).

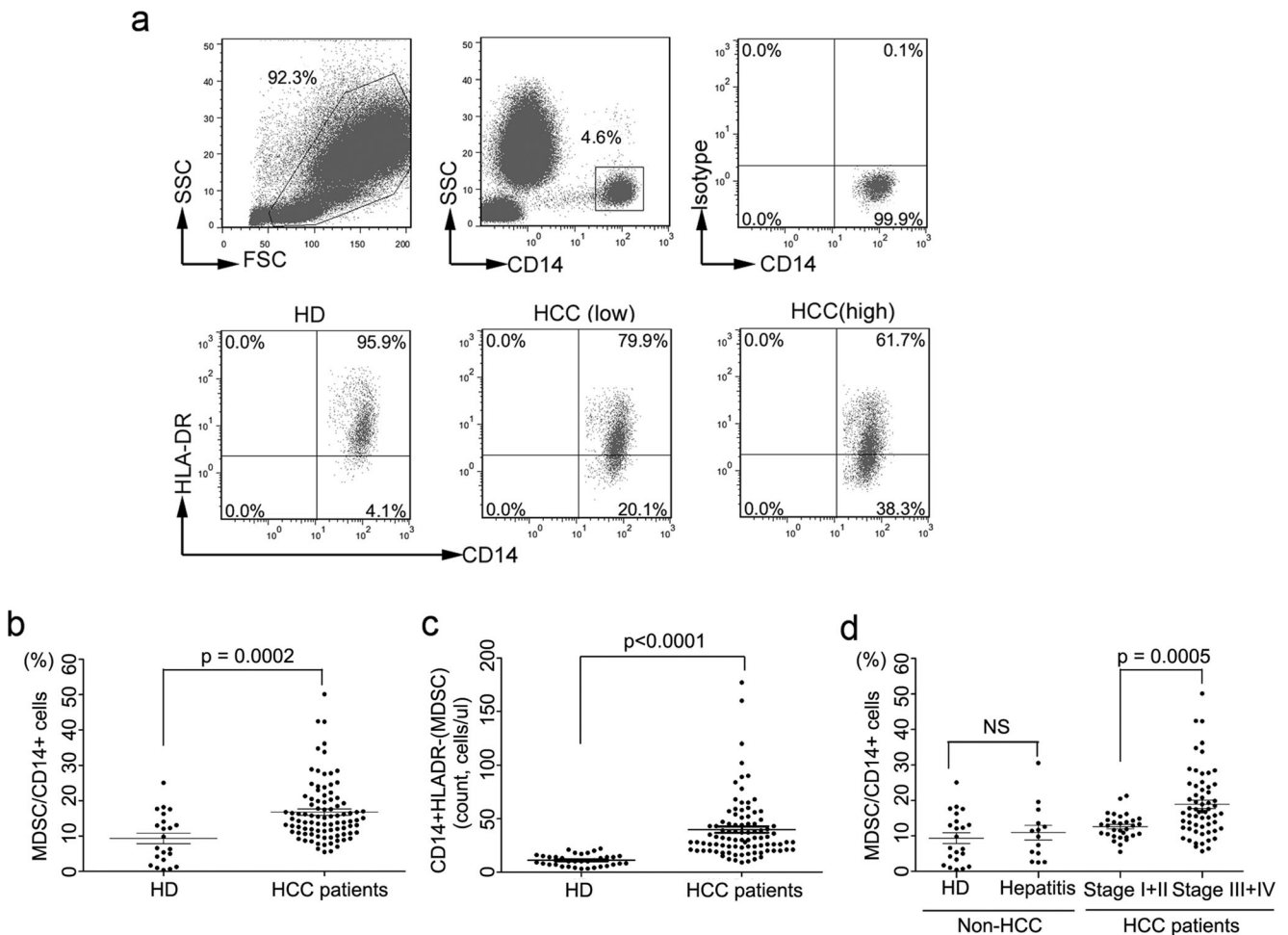


Fig. 1 Frequency and number of CD14⁺HLA-DR^{-/low} cells are significantly increased in the peripheral blood of HCC patients. **a** The representative dotplots showing CD14⁺HLA-DR^{-/low} MDSCs in the peripheral blood of healthy donors (HDs) and two HCC patients. **b** The frequency of

CD14⁺HLA-DR^{-/low} MDSCs in the peripheral blood of HD and HCC patients. **c** Absolute number of CD14⁺HLA-DR^{-/low} MDSCs in the peripheral blood of HD and HCC patients. **d** The frequency of CD14⁺HLA-DR^{-/low} MDSCs in HD, patients with hepatitis, and HCC patients of different stages

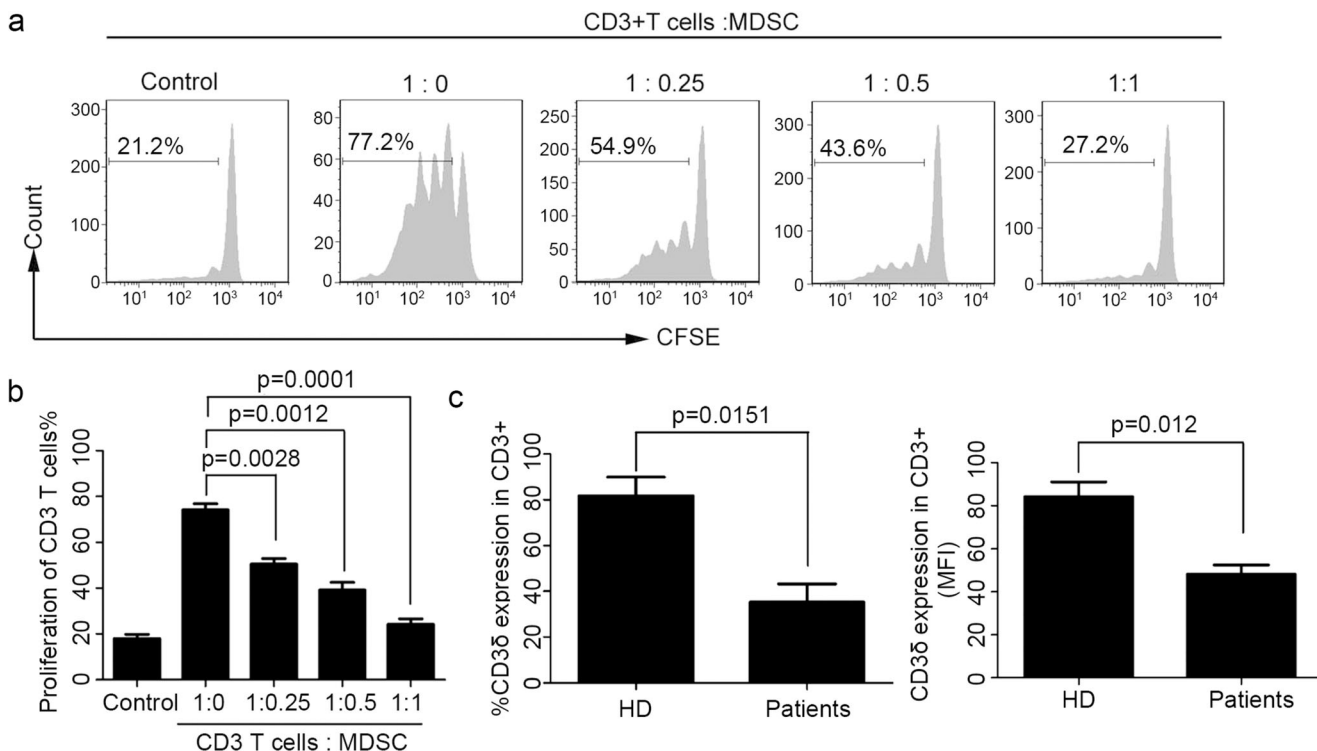


Fig. 2 Circulating CD14⁺HLA-DR^{-low} cells from HCC patients effectively suppress the proliferation of autologous CD3⁺ T cells. **a** Proliferation of CD3⁺ T cells was measured by dilution of CFSE staining intensity using flow cytometry. Representative histograms for each ratio of CD3⁺ T cell and MDSCs are shown. **b** The frequencies of

CFSE^{-low} cells are shown (*n* = 3; bars SD). **c** The percentage of CD3 ξ expression and the mean fluorescence intensity (MFI) of histograms of CD3 ξ expression on CD3⁺ T cells was decreased significantly in the peripheral blood of HCC patients (*n* = 5) compared with healthy donors (*n* = 5)

Characteristics of clinical parameters related to the MDSC frequency in HCC patients

The HCC patients were divided into two groups by the threshold of an MDSC frequency of 14.6 % that is the average + 2 SD of the MDSC frequency in non-HCC patients. Characteristics of clinical parameters of HCC patients are presented in Table 1, including age, gender, tumor-node-metastasis (TNM), Child-Pugh tumor size, alpha-fetoprotein (AFP), aspartate aminotransferase (AST), alanine transaminase (ALT), and tumor multiplicity. There was no significant difference between the two groups with regard to age, gender, AFP, AST, ALT, and GGT. Compared to that with lower MDSC frequency, patients with higher MDSC frequency had more advanced disease (TNM I/II vs II/IV, 24/22 vs 7/39, *p* = 0.0003), bigger tumor size (5.09 vs 7.39 cm, *p* < 0.0001), worse Child-Pugh classification (A/B/C 30/14/2 vs 15/24/7, *p* = 0.0055), and more tumor nodules (solitary/multiple, 25/21 vs 9/37, *p* < 0.0001).

Frequency of CD14⁺HLA-DR^{-low} cells is associated with overall survival in HCC patients

The frequency of MDSCs was examined before and after curative 3D-CRT or IMRT therapy in 43 followed-up

patients. The average frequency of CD14⁺HLA-DR^{-low} MDSCs before treatment (17.37 %) was reduced to

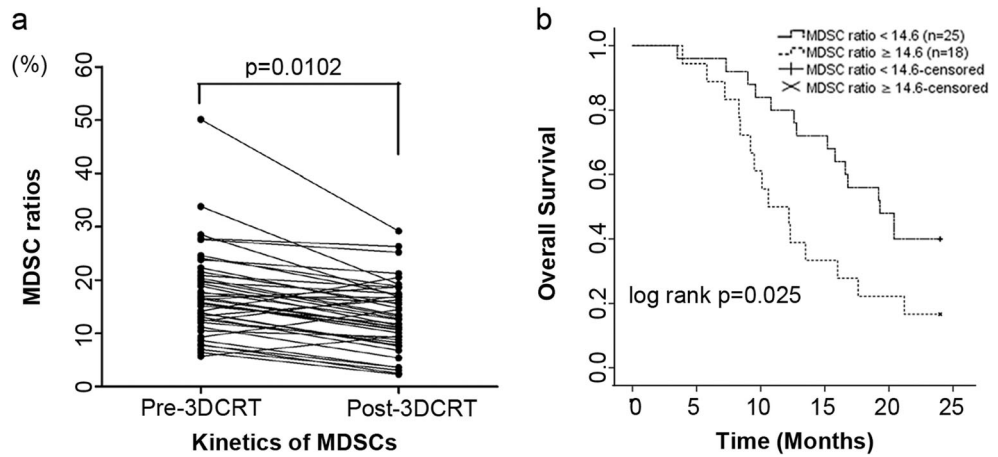
Table 1 Clinical parameters and MDSCs

Clinical parameters	MDSC frequency <14.6 % (<i>n</i> = 46)	MDSC frequency ≥14.6 % (<i>n</i> = 46)	<i>p</i> value
Age (year)	54.00 ± 10.12	54.00 ± 10.61	0.9489
Gender	34/12	38/8	0.449
TNM (I + II/III + IV)	24/22	7/39	0.0003
Child-Pugh (A/B/C)	30/14/2	15/24/7	0.0055
Tumor size (cm)	5.09	7.39	<0.0001
AFP (ng/ml)	539.34	1426.56	0.166
HBV/HCV	38/8	40/6	0.773
ALT (IU/l)	69.35	85.5	0.406
AST (IU/l)	68.6957	97.28	0.151
GGT (IU/l)	118.20	138.76	0.553
Tumor multiplicity (solitary/multiple)	25/21	9/37	0.001

Fisher’s exact test and Mann–Whitney *U* test were used for univariate analysis of two groups that were classified according to the frequency of MDSCs

AFP alpha-fetoprotein, HBV hepatitis B virus, HCV hepatitis C virus, ALT alanine aminotransferase, AST aspartate aminotransferase, GGT glutamyltranspetidase

Fig. 3 **a** In 43 advanced HCC patients who received curative radiotherapy (3D-CRT or IMRT), the frequency of MDSCs was significantly decreased after treatment ($p = 0.0102$). **b** Kaplan–Meier curve for overall survival after curative radiotherapy. The overall survival time of HCC patients with higher level of MDSCs was shortened



13.21 % after therapy ($p = 0.0102$; Fig. 3a). In addition, overall survival was significantly shortened in the group with high frequency of post-MDSC frequency ($p = 0.025$; Fig. 3b). We then analyzed the risk factors for prognosis in these patients (Table 2). In univariate analysis, Child-Pugh B, tumor size ≥ 6 cm, AFP ≥ 500 ng/ml, ALT ≥ 40 UI/l, AST ≥ 40 UI/l, pre-treatment MDSC ratio ≥ 14.6 %, and post-treatment MDSC ratio ≥ 14.6 % were significantly associated with worse prognosis in these HCC patients. In multivariable analysis, only post-treatment MDSC ratio ≥ 14.6 % (hazard ratio (HR) 2.838, 95 % confidence interval (CI) 1.379–5.837, $p = 0.005$) and Child-Pugh B (HR 3.290, 95 % CI 1.483–7.300, $p = 0.003$) were found to be risk factors.

Discussion

As an important immune regulator, MDSCs can block T cell function and facilitate tumor formation [20, 21]. Recent research into the onset, progression, metastasis, recurrence, and prognosis of HCC is especially interested in MDSCs due to its significant role in immune suppression and resistance to traditional therapies, as reported in several published studies [13–15, 22]. The involvement of MDSCs in progression of malignant disease is further supported by experiment observations that targeting MDSCs can indeed affect angiogenesis, an essential pre-requisite for tumor growth [23, 24]. These data suggest that CD14⁺HLA-DR^{-low} MDSCs may be of clinical significance for HCC.

Table 2 Cox multivariate analysis for overall survival in 3D-CRT-treated HCC patients

Variable	Univariate		Multivariate	
	<i>p</i> value	HR (95 % CI)	<i>p</i> value	HR (95 % CI)
Age (≥ 50 vs < 50)	0.691	0.861 (0.412–1.799)	0.892	
Gender (male vs female)	0.233	1.796 (0.687–4.696)	0.629	
TNM (stage IV vs stage III)	0.327	1.430 (0.700–2.921)	0.997	
Child-Pugh (B vs A)	0.009	2.853 (1.295–6.284)	0.003	3.290 (1.483–7.300)
Tumor size (≥ 6 vs < 6 cm)	0.046	2.659 (1.016–6.962)	0.281	
AFP (≥ 500 vs < 500 ng/ml)	0.026	2.424 (1.110–5.297)	0.345	
ALT (≥ 40 vs < 40 UI/l)	0.035	2.220 (1.058–4.660)	0.69	
AST (≥ 40 vs < 40 UI/l)	0.406	0.736 (0.357–1.518)	0.643	
Tumor multiplicity (Multiple vs solitary)	0.054	2.305 (0.987–5.382)	0.102	
Pre-MDSC frequency (≥ 14.6 vs < 14.6 %)	0.041	2.257 (1.035–4.924)	0.149	
Post-MDSC frequency (≥ 14.6 vs < 14.6 %)	0.015	2.414 (1.185–4.920)	0.005	2.838 (1.379–5.837)

HR hazard ratio, CI confidence interval, AFP alpha-fetoprotein, ALT alanine aminotransferase, AST aspartate aminotransferase

In the present study involving 92 HCC patients, 14 hepatitis patients, and 22 healthy controls, we investigated the impact of frequency of CD14⁺HLA-DR^{-/low} MDSCs on clinical parameters and radiotherapy effects. Consistent with previous studies [13–15], we found that HCC patients had a significantly higher level of CD14⁺HLA-DR^{-/low} MDSCs compared to the healthy controls. Such an increase in MDSC frequency, however, was not revealed in hepatitis patients. The findings have biological plausibility, since CD14⁺HLA-DR^{-/low} cells serve as immunity suppressors and higher level of CD14⁺HLA-DR^{-/low} MDSCs indicates the severer systemic immunosuppression and therefore increases the predisposition of carcinogenesis.

In addition to increased percentage and number of MDSCs in HCC patients, we observed that the MDSC frequency was correlated with the progression of HCC (tumor stage, size, and burden and Child-Pugh scores) but not biochemical parameters of liver function. The frequency of CD14⁺HLA-DR^{-/low} MDSCs has been also reported to be correlated with tumor progression in other cancers, such as melanoma, prostate cancer, and bladder cancer [25–27]. As MDSC frequency did not increase in patients with hepatitis, it is very likely that expansion of CD14⁺HLA-DR^{-/low} MDSCs was mostly derived from the tumor environment itself but not from inflammation or fibrosis of liver tissue around the tumor [14]. This speculation is supported by the finding that frequency of circulating CD14⁺HLA-DR^{-/low} MDSCs decreased in most patients with curative treatment in this study. We also found reduced CD3 ξ expression on CD3⁺ T cells in the peripheral blood of HCC patients, indicating that CD14⁺HLA-DR^{-/low} MDSCs may inhibit the activation of T cells by decreasing the expression of CD3 ξ signaling chain. MDSCs highly express arginase 1 that catabolizes L-arginine consequently reducing the raw materials for CD3 ξ synthesis, thus leading to blocked transmission of T cell antigen recognition signals and suppressed proliferation of T cells [28].

We also examined the change of MDSC frequency before and after curative therapy in 43 followed-up patients receiving radiotherapy (3D-CRT or IMRT) and found that frequency of CD14⁺HLA-DR^{-/low} MDSCs significantly decreased after treatment. Although radiotherapy has been widely used clinically to treat primary and metastatic malignancies in a large part of cancer patients, its effect remains to be elucidated on the tumor immune microenvironment. Regarding the impacts of radiotherapy on MDSCs, previous studies showed that radiotherapy could decrease the numbers of immunosuppressive MDSCs in cancer patients with primary or metastatic diseases and as an independent immune parameter decreased MDSCs after treatment predicted the good response in those patients [29, 30]. Consistent with these reports, we also observed that the frequency of CD14⁺HLA-DR^{-/low} MDSCs after radiotherapy was negatively correlated with the prognosis of HCC patients since patients with higher level of post-treatment

MDSCs had an evidently shortened overall survival. Thus, the findings support that the alterations in MDSC frequency can be viewed as a prognostic factors for cancer patients [14, 31].

Radiotherapy with accurate delivery of the irradiation dose has been widely applied in recent years; however, many patients suffer from local tumor recurrences following radiotherapy [32, 33]. Therefore, attempts to improve the efficacy of radiotherapy via combination treatment with other treatment regimens are urgently pursued. The findings that radiotherapy attenuated the accumulation of circulating MDSCs in HCC patients supports the exploration of combined treatment of radiotherapy and immunotherapy for advanced HCC since decreased immunosuppression by radiotherapy provides a permissive setting for an optimal effect of immunotherapy administered sequentially. This notion has been elegantly validated by recent studies demonstrating the impressively synergistic anti-tumor efficacy of radiotherapy and immune checkpoint inhibitors (anti-PD1, anti-CTLA4, and anti-PD-L1 antibodies) in pre-clinical animal models [34, 35]. In this aspect, patients with decreased MDSC after radiotherapy may be best suited for this combined treatment, which should be evaluated in future clinical trials.

In sum, we conclude that MDSCs levels may be an important predictor for radiotherapy effects of HCC and have direct association with tumor progression or resolution. MDSCs could be used a biomarker for prognosis of HCC, highlighting the clinical significance of MDSCs. Considering the sample inadequacy, the exact role of MDSCs in human HCC requires further larger studies to confirm.

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Compliance with ethical standards The study protocol was approved by the Ethics Committee of Beijing Chao-Yang Hospital. All participants provided written informed consent.

Conflicts of interest None

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