



Regulatory T cell and activated natural killer cell infiltration in hepatocellular carcinoma: immune cell profiling using the CIBERSORT

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Background: Hepatocellular carcinoma (HCC) is understood to be an immunogenic tumor caused by chronic liver disease. Emerging research has indicated close interaction between various immune cells and tumor cells. Immunophenotyping, which has shown potential predictive value for the prognosis of various human malignancies, might allow responsive and non-responsive patients to be identified based on the extent and distribution of immune cell infiltration. Several novel immunotherapeutic approaches have been trialed and have shown promising efficacy. However, the efficacy of immunotherapies in HCC is limited by several factors. This study aimed to investigate tumor-infiltrating immune cells in HCC.

Methods: Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) allows immune cell profiling analysis by deconvolution of gene expression microarray data. In this study, we analyzed the proportions of immune cells in 14 paired samples of HCC tissues obtained from GSE84402 in Gene Expression Omnibus (GEO) database.

Results: In the 14 paired samples, HCC tissues showed significant infiltration by regulatory T cells (Tregs), activated natural killer (NK) cells, and M0 macrophages ($P < 0.001$, $P = 0.007$ and $P = 0.001$, respectively), which were validated in CIBERSORT with the P value set at ≤ 0.05 . In four paired samples identified from those selected by CIBERSORT, HCC tissues were found to have significant Treg and activated NK cell infiltration compared to non-tumor tissues ($P = 0.007$ and $P = 0.015$, respectively). Additionally, Pearson correlation analysis revealed Tregs to be positively correlated with activated NK cells (Correlation coefficient = 0.41).

Conclusions: HCC tumor tissues were markedly infiltrated by Tregs and activated NK cells, which should be considered as candidate therapeutic targets in HCC multidisciplinary treatments.

Keywords: Hepatocellular carcinoma (HCC); regulatory T cells (Treg); natural killer cells (NK cells); tumor-infiltrating immune cells

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Introduction

Hepatocellular carcinoma (HCC) is understood to be an immunogenic tumor caused by chronic liver disease. HCC develops when the liver becomes chronically inflamed and the intra-hepatic immunosuppressive microenvironment occurs (1-3). Emerging research has indicated close interaction between various immune cells and tumor cells. Many molecular mechanisms involved in the biological properties of tumor cells during hepatocarcinogenesis also have considerable implications on the immune system (4).

Although a variety of tumor-associated antigens are expressed in HCC, no immune therapies have proved beneficial in the treatment of HCC (5). Several novel immunotherapeutic approaches have been trialed and have shown promising efficacy (6,7). However, the efficacy of immunotherapies in HCC is limited by several factors. More focused studies into how tumor response can be accurately assessed and predicted, as well as how the immunosuppressive effects of the tumor microenvironment can be overcome, are urgently needed (8,9). Immunophenotyping, which has shown potential predictive value for the prognosis of various human malignancies (10,11), might allow responsive and non-responsive patients to be identified based on the extent and distribution of immune cell infiltration (10,12,13). Bioinformatic analysis of tumor-infiltrating immune cells in HCC tissues have been addressed in previous studies (14,15). In a study reported by Rohr-Udilova *et al.*, total B cells, memory B cells, T follicular helper cells and M1 macrophages were strongly infiltrated into HCC (14). Since the efficacy of immunotherapy for HCC is not satisfactory (16), the supplementary assessment of tumor-infiltrating immune cells should be addressed further.

Recent reports have demonstrated that deconvolution of gene expression data by Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) can provide crucial information regarding immune cell composition in HCC (14,17). In this analysis, we selected microarray dataset GSE84402 with 14 paired tumor and the corresponding non-cancerous tissues and aimed to investigate the relative proportions and distribution of tumor-infiltrating immune cells in HCC patients using CIBERSORT. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-5830>).

Methods

Data source

The GSE84402 series comprising 14 pairs of HCC tissues and the corresponding non-cancerous tissues was obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). Double-stranded complementary DNA (cDNA) was synthesized using 5 µg of total RNA from each sample, before amplification, labeling with biotin, and hybridization to GeneChip Human Genome U133 Plus 2.0 Array (18). As declared by Wang *et al.*, ethical approval was obtained from the Zhongshan Hospital Research Ethics Committee, and written informed consent was obtained from each patient (18).

The GSE84402 series matrix file and platform documents (GPL570) were downloaded. The Perl programming language was used to translate gene probe IDs to gene symbol ID. The limma package (19,20) in R program was used to normalized the gene expression levels of the tumor and non-tumor tissue samples from HCC patients. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

CIBERSORT analysis

Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT), created by Newman *et al.*, is an analytical tool that allows the abundance of member cell types in a mixed cell population to be estimated using gene expression data (17). CIBERSORT gene signature matrix, termed LM22, contains 547 genes and distinguishes 22 human hematopoietic cell phenotypes, including 7 T cell types, naïve and memory B cells, plasma cells, NK cells, and myeloid subsets. CIBERSORT was used to obtain the immune cell profile of each sample from GSE84402, with the number of permutations set at 100 (17). *Table 1* shows the complete samples from GSE84402. For each sample, 22 types of immune cell, along with CIBERSORT metrics including the Pearson correlation coefficient, CIBERSORT P value, and root mean squared error (RMSE), were quantified. The CIBERSORT P value represents the statistical significance of the deconvolution results across all cell subsets and can be used to filter out deconvolution with less significant fitting accuracy. From

Table 1 Comparison of CIBERSORT immune cell fractions between tumor and non-tumor tissues from HCC patients

| Immune cell type | CIBERSORT fraction in % of all infiltrating immune cells (mean ± SD) | | |
|------------------------------|--|-------------------|---------|
| | Tumors (n=14) | Non-tumors (n=14) | P value |
| T cells CD8 | 0.140±0.071 | 0.192±0.073 | 0.075 |
| T cells CD4 naive | 0.0±0.0 | 0.0±0.0 | 1.0 |
| T cells CD4 memory resting | 0.071±0.082 | 0.088±0.066 | 0.553 |
| T cells CD4 memory activated | 0.002±0.008 | 0.0±0.001 | 0.408 |
| T cells follicular helper | 0.074±0.030 | 0.066±0.030 | 0.545 |
| T cells regulatory (Tregs) | 0.066±0.040 | 0.011±0.017 | <0.001 |
| T cells gamma delta | 0.004±0.009 | 0.012±0.017 | 0.109 |
| B cells naive | 0.013±0.017 | 0.020±0.027 | 0.454 |
| B cells memory | 0.022±0.026 | 0.008±0.016 | 0.133 |
| Plasma cells | 0.065±0.025 | 0.061±0.022 | 0.658 |
| NK cells resting | 0.008±0.016 | 0.006±0.009 | 0.709 |
| NK cells activated | 0.040±0.021 | 0.017±0.019 | 0.007 |
| Macrophages M0 | 0.043±0.041 | 0.0±0.003 | 0.001 |
| Macrophages M1 | 0.090±0.030 | 0.090±0.033 | 0.965 |
| Macrophages M2 | 0.143±0.081 | 0.169±0.054 | 0.351 |
| Monocytes | 0.033±0.024 | 0.056±0.034 | 0.056 |
| Dendritic cells resting | 0.074±0.059 | 0.043±0.024 | 0.085 |
| Dendritic cells activated | 0.003±0.007 | 0.0±0.0 | 0.160 |
| Mast cells resting | 0.003±0.011 | 0.001±0.003 | 0.505 |
| Mast cells activated | 0.081±0.055 | 0.125±0.144 | 0.318 |
| Eosinophils | 0.0±0.0 | 0.0±0.0 | 1.0 |
| Neutrophils | 0.026±0.020 | 0.033±0.015 | 0.313 |

Italic P values indicate significance between individual groups. HCC, hepatocellular carcinoma.

the samples analyzed, 8 non-tumor samples and 7 tumor samples were identified when a CIBERSORT P value ≤ 0.05 was required. *Table 2* lists the samples selected. SPSS 23.0 software (SPSS Inc., Chicago, USA) was employed to compare CIBERSORT immune cell fractions between tumor and non-tumor tissues from HCC patients.

The pheatmap package in R program, (<https://cran.r-project.org/web/packages/pheatmap/>) was used to perform heatmap analysis of immune cells in tumor and non-tumor tissues. Correlation analysis of immune cells was conducted using the corrplot package (<https://cran.r-project.org/web/packages/corrplot/index.html>). Four paired samples from the samples selected by CIBERSORT

were used to calculate the difference between candidate infiltrating immune cells.

Results

Comparison of CIBERSORT immune cell fractions

The CIBERSORT immune cell fractions of the tumor and non-tumor tissues from HCC patients from GSE84402 were compared (*Table 1*). The tumor tissues showed significant infiltration by regulatory T cells (Tregs), activated natural killer (NK) cells, and M0 macrophages compared to non-tumor tissues ($P < 0.001$, $P = 0.007$, and $P = 0.001$, respectively, *Table 1* and *Figure 1A*).

Table 2 Comparison of CIBERSORT-selected immune cell fractions between tumor and non-tumor tissues from HCC patients

| Immune cell type | CIBERSORT fraction in % of all infiltrating immune cells (mean ± SD) | | |
|------------------------------|--|------------------|--------------|
| | Tumors (n=8) | Non-tumors (n=7) | P value |
| T cells CD8 | 0.164±0.066 | 0.198±0.080 | 0.422 |
| T cells CD4 naive | 0.0±0.0 | 0.0±0.0 | 1.0 |
| T cells CD4 memory resting | 0.043±0.065 | 0.058±0.047 | 0.64 |
| T cells CD4 memory activated | 0.005±0.011 | 0.001±0.002 | 0.372 |
| T cells follicular helper | 0.069±0.033 | 0.077±0.031 | 0.675 |
| T cells regulatory (Tregs) | 0.052±0.035 | 0.008±0.009 | <i>0.007</i> |
| T cells gamma delta | 0.007±0.011 | 0.020±0.019 | 0.152 |
| B cells naive | 0.018±0.018 | 0.023±0.030 | 0.479 |
| B cells memory | 0.017±0.028 | 0.0±0.001 | 0.132 |
| Plasma cells | 0.069±0.031 | 0.064±0.028 | 0.759 |
| NK cells resting | 0.005±0.012 | 0.002±0.004 | 0.578 |
| NK cells activated | 0.042±0.027 | 0.015±0.019 | <i>0.05</i> |
| Macrophages M0 | 0.047±0.049 | 0.0±0.0 | <i>0.025</i> |
| Macrophages M1 | 0.104±0.029 | 0.094±0.039 | 0.605 |
| Macrophages M2 | 0.126±0.053 | 0.155±0.050 | 0.326 |
| Monocytes | 0.031±0.028 | 0.055±0.033 | 0.184 |
| Dendritic cells resting | 0.082±0.065 | 0.035±0.019 | 0.09 |
| Dendritic cells activated | 0.003±0.006 | 0.0±0.001 | 0.31 |
| Mast cells resting | 0.0±0.0 | 0.002±0.004 | 0.369 |
| Mast cells activated | 0.093±0.059 | 0.154±0.183 | 0.444 |
| Eosinophils | 0.0±0.0 | 0.0±0.0 | 1.0 |
| Neutrophils | 0.021±0.010 | 0.032±0.012 | 0.097 |

Italic P values indicate significance between individual groups. HCC, hepatocellular carcinoma.

From the samples analyzed, 8 non-tumor and 7 tumor samples were selected when the CIBERSORT P value was set at ≤ 0.05 . *Table 2* compares the CIBERSORT immune cell fractions of the tumor and non-tumor tissues from HCC patients. Consistent with above, there was significant infiltration by regulatory T cells, activated NK cells, and M0 macrophages in HCC tumor tissues ($P=0.007$, $P=0.05$, and $P=0.025$, respectively; *Table 2* and *Figure 1B*). The landscape and heatmap of immune cells in the tumor and non-tumor tissue samples are presented in *Figure 2*.

Comparison of CIBERSORT immune cell fractions in paired samples

Four paired tumor and non-tumor samples from HCC patients (GSM2233092/GSM2233093, GSM2233098/GSM2233099, GSM2233110/GSM2233111, and GSM2233112/GSM2233113) were identified from the samples selected by CIBERSORT. As shown in *Figure 3*, infiltration by regulatory T cells and activated NK cells was more significant in tumor tissues than in non-tumor tissues ($P<0.01$ and $P<0.05$, respectively; *Figure 3A*). Moreover,

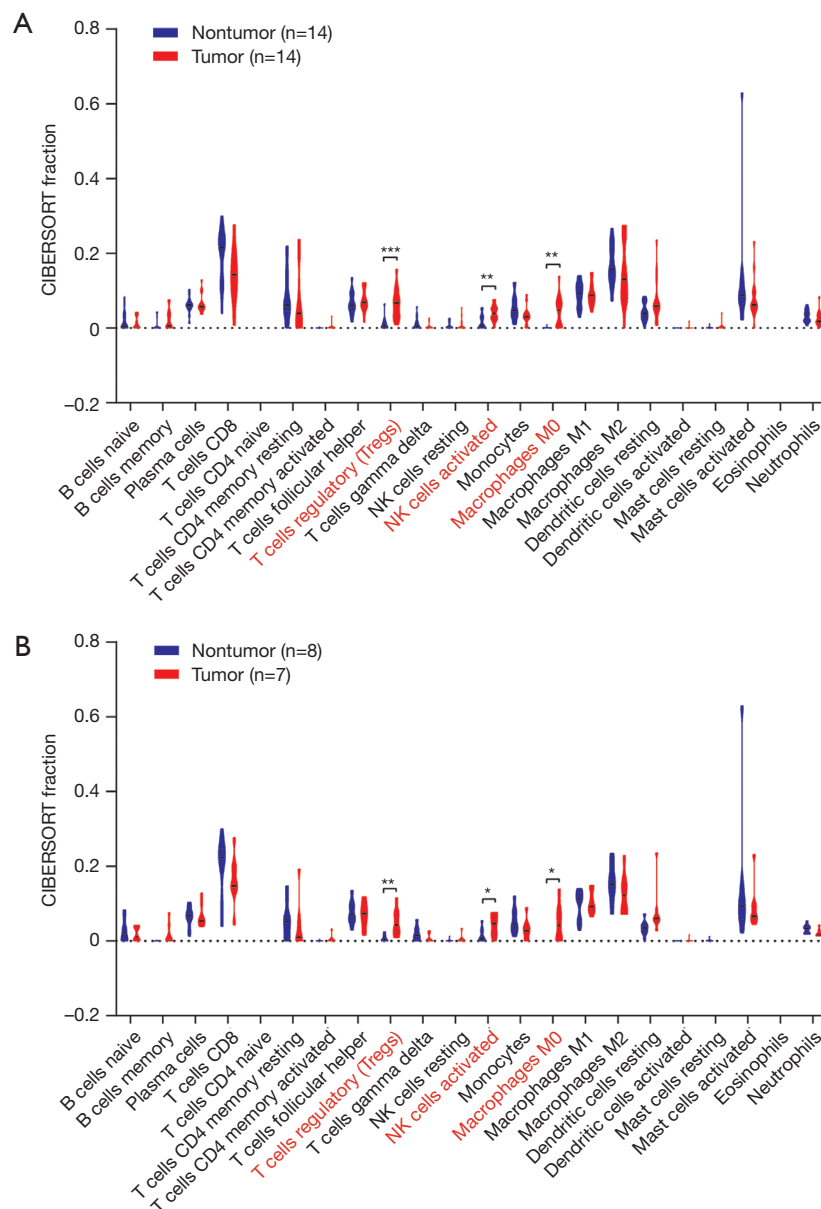


Figure 1 CIBERSORT immune cell fractions of tumor and non-tumor tissues from all samples (A) and selected samples (B) in hepatocellular carcinoma patients in the GSE84402 series. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the levels of regulatory T cells and activated NK cells were significantly increased in each tumor tissue sample compared with those in the corresponding non-tumor tissue sample ($P = 0.007$ and $P = 0.015$, respectively; *Figure 3B,C*).

Correlation of immune cells

Figure 4 shows a correlation map displaying the Pearson

correlation values for each comparison between the immune cells in the CIBERSORT-selected samples. As shown in *Figure 4*, regulatory T cells had a positive Pearson correlation value of 0.77 with resting dendritic cells and 0.46 with macrophages M0 cells. Furthermore, activated NK cells had positive Pearson correlation values of 0.67 and 0.41 with memory B cells and regulatory T cells, respectively.

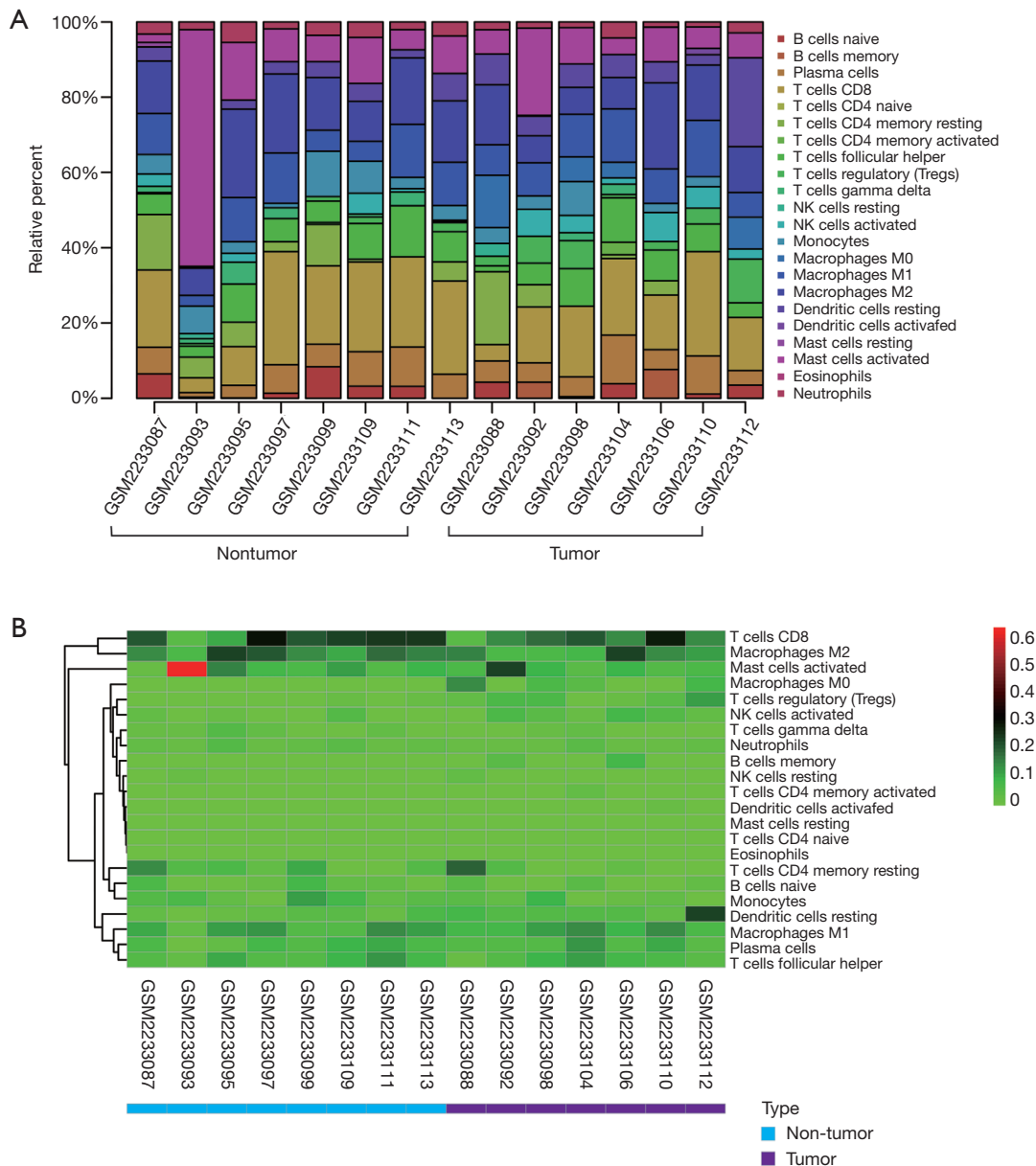


Figure 2 Landscape (A) and heatmap (B) of immune cells between tumor and non-tumor samples in samples selected by CIBERSORT.

Discussion

Several computational tools, including microarray microdissection with analysis of differences (MMAD) (1,21), linear least-square regression (LLSR) (22) and digital sorting algorithm (DSA) (23), have been used to deconvolute complex gene expression profiles mixtures to infer cellular composition. However, their sensitivities to experimental noise and cell types, and high unknown mixture content

limited the utility for tumor infiltrating immune cells assessment. CIBERSORT is a widely useful approach for high throughput characterization of tumor infiltrating immune cells assessment from complex tissues (24).

Tumor-infiltrating immune cells have been associated with the aggressiveness of many cancers (25,26), and their prognostic correlation with HCC has been extensively investigated (14,27,28). In HCC patients, abundant T lymphocytes (29,30), B cells (31), NK cells (32), and

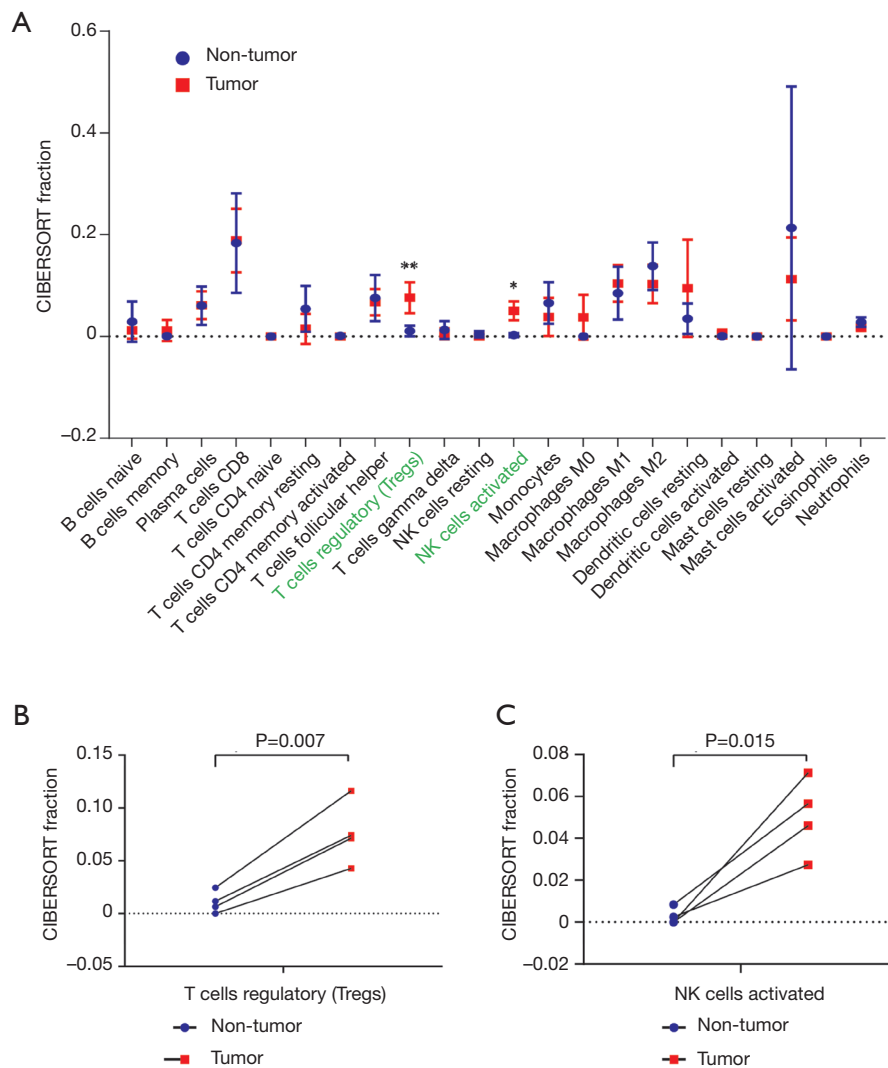


Figure 3 Validation for CIBERSORT immune cell fractions in four pairs of tumor and non-tumor tissues. *, $P < 0.05$; **, $P < 0.01$.

dendritic cells (33,34) have been found to contribute to favorable prognosis. In contrast, high levels of regulatory T cells (35), neutrophils (36,37), monocytes (38), and CXCR3+ subtype B cells (39) have been associated with poor outcomes in HCC patients. Moreover, a link has been reported between the proportions of different types of macrophages and the prognosis of HCC (40,41). Therefore, uncovering the diverse composition of tumor-infiltrating immune cells in tumor immunology might be helpful in overcoming the limitations and barriers faced by immunotherapies in HCC (42).

In this analysis, we found significant infiltration by Tregs and activated NK cells in HCC tumor tissues. In the

GSE84402 series, 13 out of 14 cases were HBsAg positive. Consistent with our findings, a previous study revealed that Tregs in tumor tissues displayed higher frequencies and more suppressive phenotypic functions than those in peritumoral and peripheral tissues (43). The higher Treg levels in patients with chronic hepatitis B (CHB) exerted a suppressive effect on the specific immune responses induced by HBV antigens, as well as by HCC tumor antigens. Furthermore, Tregs could inhibit tumor immunosurveillance against HCC, which potentially plays a role in the immunopathogenesis from CHB to HCC (44). Tregs have crucial involvement in forming and maintaining the hepatic inhibitory microenvironment, as well as in the

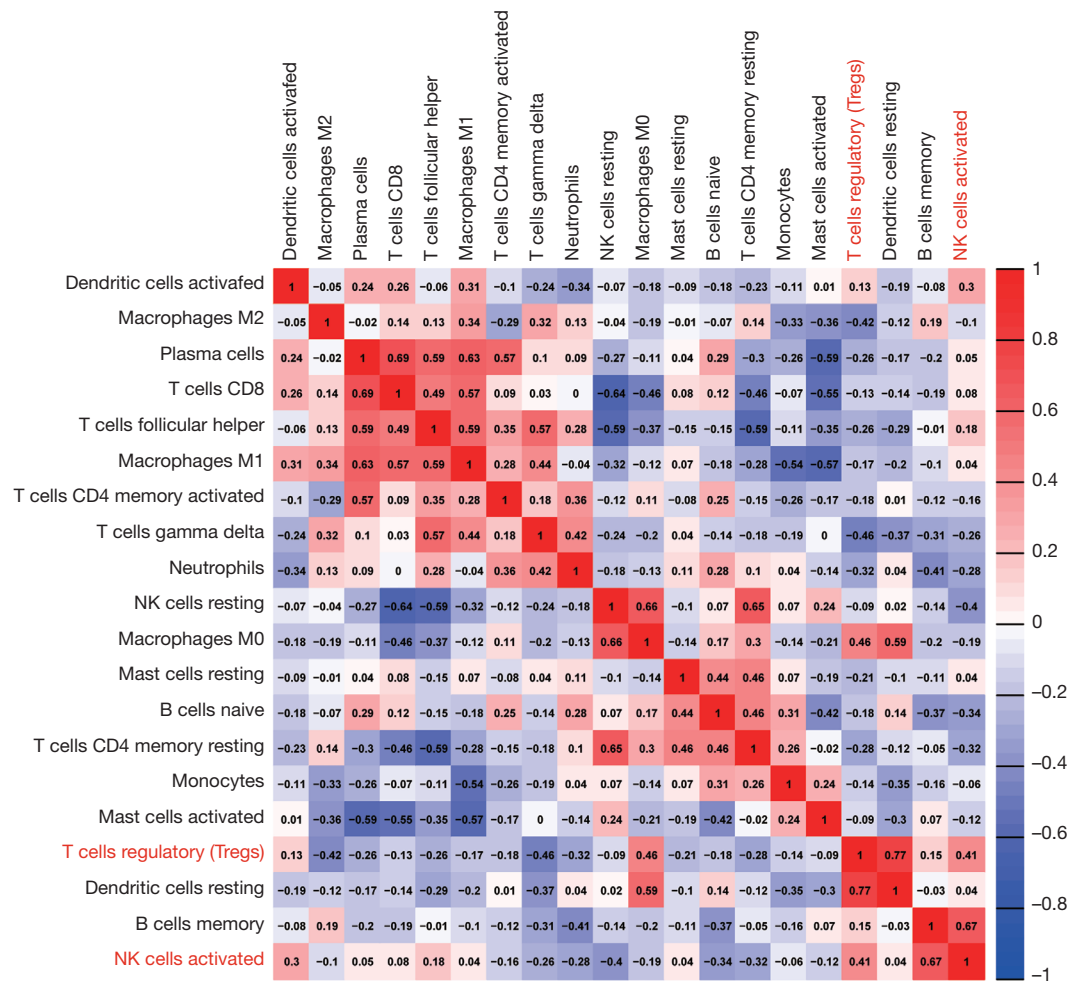


Figure 4 Correlation of immune cells in the samples selected by CIBERSORT.

development of cirrhosis, the transformation of cirrhosis to HCC, and HCC progression and metastasis (45). Moreover, various studies have demonstrated that high quantities of tumor-infiltrating Tregs are considered to be an unfavorable prognostic indicator for advanced tumor biological behaviors and poor survival in HCC patients (46-49).

NK cell regulation of antigen-specific T lymphocytes occurs during viral infection. When NK cells become depleted, the number of antigen-specific T lymphocytes increases; therefore, these NK cells may reduce T cell levels, leading to a weaker antitumor immune response (50). NK cell activator has been shown to induce liver inflammation by significantly increasing the levels of infiltrating lymphocytes, along with concurrently increasing apoptosis and proliferation of hepatocytes; consequently, epithelial-to-

mesenchymal transition of hepatocytes is accelerated (51). Decreased NK cell counts have also been reported in HCC patients (52). NK cell deficiency is believed to be a key mechanism underlying tumor cell evasion of the host's immune system. In HCC, NK cells appear to have reduced function. A growing bank of evidence suggests that impaired NK cell function leads to the body's failure to eliminate tumor cells, which indicates that tumor cells could be killed more effectively through enhancing the activity of dysfunctional NK cells (32,53). Our results also showed that Tregs were positively correlated with activated NK cells. Current evidence showed that Tregs are closely linked to the homeostasis of NK cells and attenuate the sensitivity of target cells through minimizing interleukin (IL)-2 to NK cells (50). In contrast, in chronic myeloid leukemia,

the degree of NK cell differentiation was closely and inversely correlated with the proportion of Treg cells (54). Considered these conflicting results regarding the correlation of tumor-infiltrating NK cells with Tregs, more mechanism investigations on tumor-infiltrating NK cells in HCC development should be carried out in future.

Our study has some limitations. The primary is that this study is based on relatively small samples, the external validation of our results should be considered. Secondly, the associations between Tregs and NK cells and prognosis in HCC patients were not addressed in this study. Thirdly, this study was a preliminary bioinformatic analysis, no experimental data was available. Even though, the CIBERSORT approach to analyzing HCC samples from the GSE84402 series revealed that tumor tissues were markedly infiltrated by Tregs and activated NK cells. Therefore, these cells should be considered as candidate therapeutic targets in multidisciplinary treatment for HCC.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <http://dx.doi.org/10.21037/atm-20-5830>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-20-5830>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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