

IVIM-DWI Noninvasively Assesses Tumor-Infiltrating Lymphocytes and Correlates with Immunotherapy Prognosis in Previously Treated Recurrent or Metastatic Nasopharyngeal Carcinoma

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Title page

Title: IVIM-DWI Noninvasively Assesses Tumor-Infiltrating Lymphocytes and Correlates with Immunotherapy Prognosis in Previously Treated Recurrent or Metastatic Nasopharyngeal Carcinoma

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Abstract

Background: Immunotherapy is ineffective in recurrent or metastatic nasopharyngeal carcinoma (RM-NPC) refractory to standard chemotherapy. Therefore, new biomarkers must be considered for identifying patients with RM-NPC who may benefit from immunotherapy. To investigate the role of intravoxel incoherent motion diffusion-weighted MR imaging (IVIM-DWI) for noninvasively assessing tumor-infiltrating lymphocytes (TILs) and immunotherapy prognosis in RM-NPC refractory to standard chemotherapy.

Methods: 67 patients with RM-NPC refractory to standard chemotherapy were retrospectively identified. All patients underwent nasopharyngeal IVIM-DWI scans prior to immunotherapy. IVIM parameters, including the apparent diffusion coefficient (ADC), pure diffusion coefficient (D), pseudo-diffusion coefficient (D*), and perfusion fraction (*f*), were measured. Four TIL biomarkers (CD4, CD8, FOXP3, and CD20) were detected, and the histochemistry score (H-score) was calculated. A TIL-based immunoscore signature was also developed.

Results: The median follow-up period was 17 months. Among the 67 patients, 36 (53.73%) had died. The immunoscore was positively correlated with the overall survival (OS) ($P < 0.001$). Kaplan–Meier analysis indicated that the high-immunoscore group had significantly longer OS than that of the low-immunoscore group ($P < 0.001$). The ADC ($P = 0.016$) and D ($P = 0.003$) values were negatively correlated with the immunoscore, while the *f* value ($P = 0.011$) was positively correlated with it. Conversely, higher ADC ($\geq 0.74 \times 10^{-3} \text{ mm}^2/\text{s}$) and higher D ($\geq 0.50 \times 10^{-3} \text{ mm}^2/\text{s}$) were negatively associated with OS, while higher *f* (≥ 0.33) was positively associated with it (all $P < 0.05$).

Conclusion: IVIM parameters could noninvasively evaluate TIL infiltration and predict immunotherapy prognosis in RM-NPC.

Keywords: Nasopharyngeal carcinoma, Immunotherapy, Tumor-infiltrating lymphocytes, Prognosis, Intravoxel incoherent motion, Diffusion-weighted MR imaging

Background

Recurrence and metastasis are the chief reasons for treatment failure in nasopharyngeal carcinoma (NPC). Although standard systemic chemotherapy is available for NPC, the prognosis of recurrent or metastatic NPC (RM-NPC) is very poor [1]. Epstein–Barr virus infection is instrumental in NPC development, which often induces overexpression of programmed death ligand-1 (PD-L1), indicating the potential application of PD-1 inhibitor immunotherapy [2]. However, the immunotherapy exhibits unsatisfactory therapeutic effects for RM-NPC refractory to standard chemotherapy (objective response rates [ORRs] = 20.5–34%) [3-6].

Currently, only a few prognostic biomarkers, including PD-L1 expression, tumor mutation burden (TMB), and microsatellite instability, have been verified in randomized settings and applied in clinical practice [7]. Additionally, the predictive value of PD-L1 expression for immunotherapy response in NPC is not yet unambiguously established [3, 6]. In addition, TMB is not a helpful biomarker for immunotherapy because of a few genetic mutations [6]. Some recent studies have shown that the subtypes and densities of tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment (TME) are closely associated with the immunotherapy response [8]. In particular, the TIL-specific markers (e.g., CD4, CD8, and Forkhead Box Protein 3 [FOXP3]) play a critical role in the immune response [9]. Tumors with abundant CD8⁺ and CD4⁺ T cell infiltration are more likely to benefit from immunotherapy [7, 10]. However, the presence of FOXP3⁺ Treg is frequently correlated with a poor prognosis [11, 12]. Additionally, the abundant CD20⁺ B cell infiltration may promote an immune response and prolong survival [13-15]. In fact, immunohistochemical (IHC) detection of TIL

biomarkers to develop an immunoscore signature is one of the primary approaches for predicting immunotherapy outcomes [16-18].

Detection of TILs is generally an invasive and expensive procedure. Functional MRI could quantitatively evaluate molecular and cellular features within the TME [19]. In contrast to traditional DWI, multi-*b*-value diffusion weighted imaging (DWI) with intravoxel incoherent motion (IVIM) based on a biexponential model can differentiate true molecular diffusion from microcapillary perfusion. Quantitative functional parameters measured at IVIM-DWI exhibit broad prospects for the differential diagnosis of NPC [20, 21], evaluation of the therapeutic effects of chemoradiotherapy [22, 23], and prognosis prediction [24, 25]. In immunotherapy, IVIM-DWI could be used to quantitatively assess the PD-L1 expression status in lung and cervical cancers [26, 27]. Moreover, IVIM-DWI exhibits potential applicability in the assessment of response to immunotherapy in hepatoma carcinoma and colorectal cancer [28, 29]. However, the role of IVIM-DWI in the evaluation of TIL infiltration and immunotherapy response in NPC is still unclear.

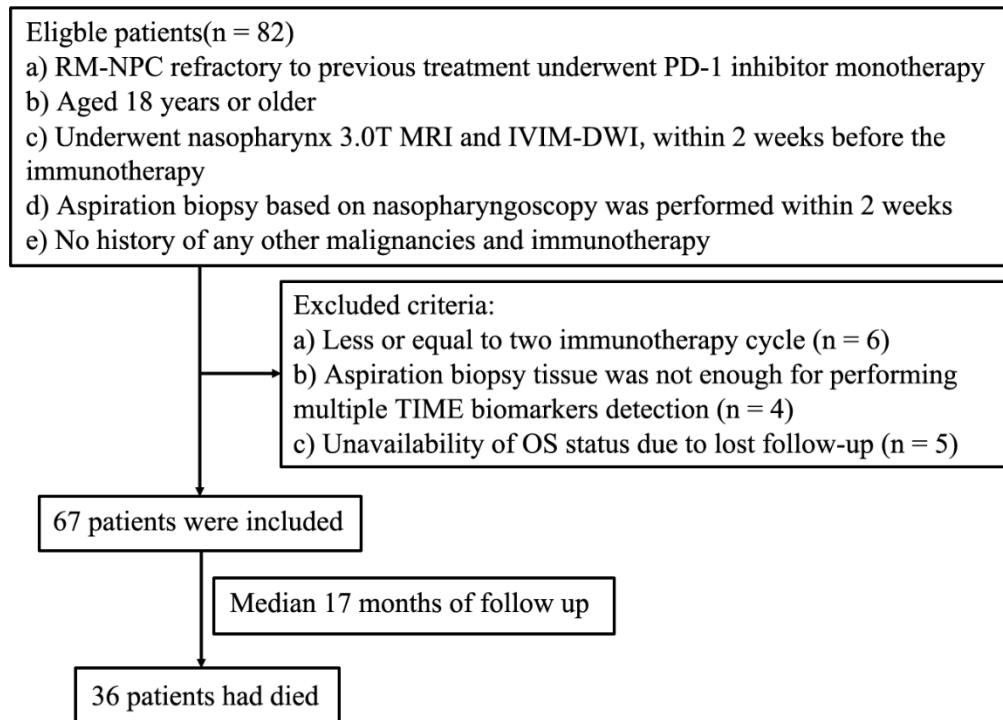
This study aims to develop a TIL-based immunoscore signature for predicting immunotherapy prognosis in previously treated RM-NPC, evaluate the association of IVIM-DWI parameters with the immunoscore and prognosis, and finally construct a merged model by integrating IVIM-DWI parameters with the immunoscore to predict immunotherapy prognosis.

Materials and methods

Patients

This retrospective study was approved by the Institutional Review Board of the Affiliated Cancer Hospital of Guangzhou Medical University, and the requirement for informed consent was waived. Between January 2021 and June 2023, 82 patients with RM-NPC accepted PD-1 blockade monotherapy. The patients were identified based on the following inclusion criteria: a) patients who resisted prior standard chemotherapy or patients in whom disease progression occurred within 6 months of completing adjuvant chemotherapy or chemoradiotherapy; b) aged 18 years or older; c) underwent nasopharyngeal and neck MRI, including IVIM-DWI scan, within 2 weeks before starting the immunotherapy; d) aspiration biopsy based on nasopharyngoscopy was performed within 2 weeks for histopathological examination prior to immunotherapy; and e) no history of any other malignancies and immunotherapy. The exclusion criteria were as follows: a) patients who underwent ≤ 2 immunotherapy cycles ($n = 6$); b) aspiration biopsy tissue was not sufficient to perform multiple TIL biomarker detections ($n = 4$); and c) unavailability of the OS status due to lost follow-up ($n = 5$). Based on these exclusion criteria, 15 patients were excluded from the study. Ultimately, 67 patients (men: 48; women: 19; median age: 50 years [range: 25–72 years]) were included, with a median follow-up of 17 months (range: 2–36 months). Notably, 36 of these 67 patients died (53.73%). The patient inclusion flowchart is displayed in **Fig. 1**.

Fig. 1. Flow chart of patient selection.



TIL biomarker detection and evaluation

Four TIL biomarkers (CD4, CD8, FOXP3, and CD20) and PD-L1 were detected in the biopsy samples taken from patients with primary NPC before they were started on immunotherapy using standard immunohistochemical (IHC) staining. The details of IHC staining methods are given in the Supplementary Materials.

The IHC staining images were jointly evaluated by two pathologists (X.H.X and Y.L.C., with 8 and 5 years of experience in tumor pathology, respectively). They were blinded to clinical information, including treatments and outcomes. Based on a previous study [30], the PD-L1 expression was quantized by calculating the combined positive scores (CPS):

$$\text{CPS} = ([\text{PD-L1 membrane staining positive tumor cells} + \text{PD-L1 membrane staining positive tumor-associated immune cells}] / \text{Total number of tumor cells}) \times 100.$$

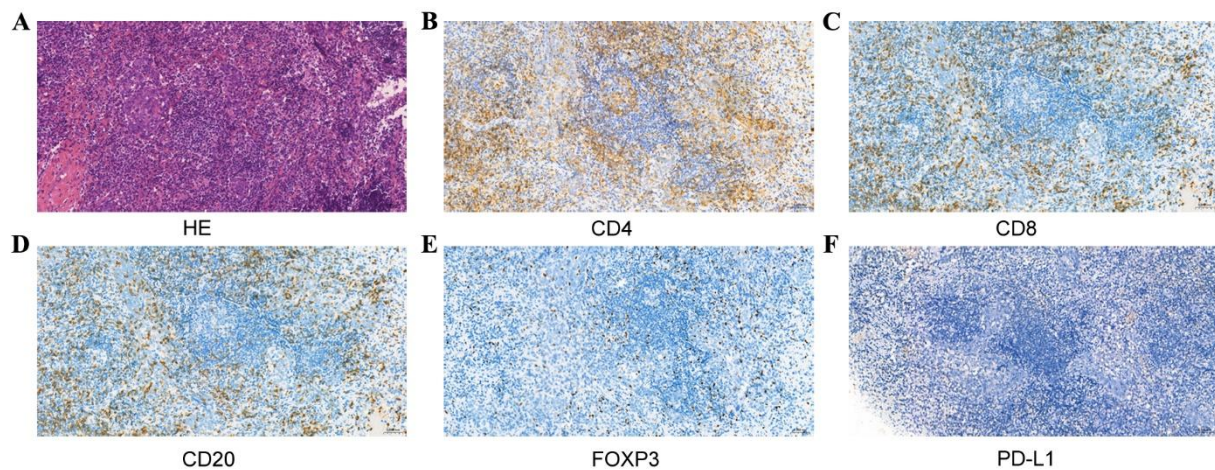
TIL infiltration in the stroma surrounding the stained cancer cells was quantitatively measured

and viewed at 200× magnification. As described in previous studies [18, 31, 32], TIL infiltration was quantized using the histochemistry score (H-score), which is the product of SI (staining intensity) and PP (percentage of positive cells). The SI was scored as 0, 1, 2, and 3 for negative, weak, moderate, and strong staining, respectively, while the PP was scored as 0 (<1%), 1 (1–10%), 2 (11–50%), 3 (51–80%), and 4 (>80%). The H-score for each TIL biomarker was calculated as follows:

$$\text{H-score} = \text{SI score} \times \text{PP score}.$$

TIL biomarkers and the PD-L1 expression score are shown in Supplementary **Fig. S1**.

Supplementary Fig. S1. Evaluation of TIL biomarkers and PD-L1 expression score



Acquisition of MR images

All patients underwent nasopharyngeal and neck MRI examinations prior to immunotherapy with 3.0T MRI (Discovery MR 750 w 3.0 T System, GE Healthcare Milwaukee, WI, USA) using an 8-channel head-and-neck combined coil (GE Healthcare). The MRI scans included the following sequences: 1) axial T1-weighted imaging (time of repetition [TR]/time of echo [TE] = 575/20 ms, slice thickness [ST] = 5 mm, gap = 5 mm, field of view [FOV] = 26 × 25 cm, matrix = 256 × 192, number of excitations (NEX) = 2); 2) axial T2-weighted imaging (TR/TE = 4000/90 ms, ST = 5 mm, gap = 5 mm, FOV = 26 × 25 cm, matrix = 256 × 192, NEX = 2); 3) coronal T2-weighted with fat suppression

(TR/TE = 2470/68 ms, ST = 5 mm, gap = 5 mm, FOV = 26 × 25 cm, matrix = 256 × 192, NEX = 1); and 4) axial and sagittal contrast-enhanced T1-weighted imaging (TR/TE = 506/14 ms, ST = 5 mm, gap = 5 mm, FOV = 26 × 25 cm, matrix = 256 × 192, NEX = 1). The contrast-enhancing agent was gadolinium–diethylenetriaminepentaacetic acid ([Gd-DTPA], Schering, Berlin, Germany), with a bolus dose of 0.1 mmol/kg. The IVIM-DWI protocol was as follows: multiple B-DWI (MB-DWI) with 13 b -values (0, 10, 20, 40, 60, 100, 120, 160, 200, 400, 600, 800, and 1,000 s/mm²) were applied using the following single-shot spin echo echoplanar imaging sequence: TR/TE = 4000/64.5 ms, ST = 5 mm, gap = 2 mm, FOV = 25 × 25 cm, matrix = 128 × 128, NEX = 2.0.

IVIM-DWI post-processing and functional parameter measurements

The multiple b -value DWI raw data were transferred to the GE-AW 4.6 workstation software (GE Healthcare) for post-processing. Based on the IVIM theory, the biexponential model can be expressed as follows [33] :

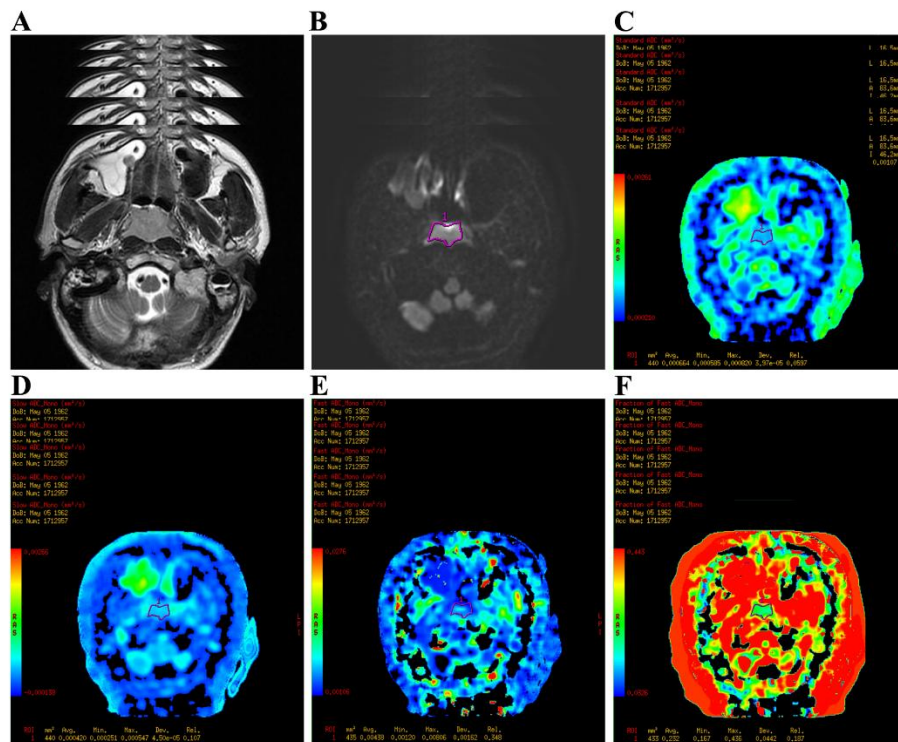
$$S_b / S_0 = (1 - f) \cdot \exp(-b \cdot D) + f \cdot \exp[-b \cdot (D + D^*)],$$

where S_0 is the signal intensity in the pixel without a diffusion gradient, and S_b is the signal intensity in the pixel with diffusion gradient b . Three IVIM functional maps (D , D^* , and f) can be reconstructed by fitting the MR signal acquired at 13 b -values to a bi-exponential model, where D represents a true diffusion coefficient related to pure molecular diffusion, D^* is the pseudo-diffusion coefficient (for microcirculation perfusion), and f refers to the perfusion fraction as reflected by the fraction of diffusion linked to microcirculation. In addition, as described in previous studies [30], b -values (0 and 1000 s/mm²) were used to generate the apparent diffusion coefficient (ADC) map.

The IVIM-DWI functional parameter measurements were independently performed on the GE-AW 4.6 workstation (GE Healthcare) by two radiologists (observer 1 (Y.F.T.) and observer 2 (J.X.Y.)),

with 5 and 10 years of experience in MRI diagnosis, respectively). Both radiologists were blinded to the clinical, pathological, and prognostic results of patients. They segmented the tumor whole-volume regions of interest (ROIs) by manually delineating the boundaries of the nasopharyngeal lesion slice by slice on DW images ($b = 800 \text{ s/mm}^2$). The regions of obvious necrosis, hemorrhage, air, and blood vessels were carefully avoided to contrast with T2-w images and enhanced T1-w images. These ROIs were automatically fitted to the corresponding slices on IVIM functional maps (ADC, D , D^* , and f) and were then manually adjusted in case of any mismatching. The mean ADC, D , D^* , and f values for each slice ROI were measured. In addition, the whole-volume ADC, D , D^* , and f values were calculated by averaging the measured values derived from all sections. The measured functional parameters from the two observers were averaged for the subsequent data analysis. The diagram for IVIM-DWI functional parameter measurements is displayed in **Fig. 2**.

Fig. 2 Measurements of IVIM-DWI functional parameters.



Immunotherapy and follow-up

In total, 67 RM-NPC patients underwent at least three cycles of PD-1 blockade monotherapy until they faced disease progression (Response Evaluation Criteria In Solid Tumors [RECIST] v1.1 criterion) [34] or intolerable toxicity or voluntarily discontinued the treatment. The immunotherapy was performed by injecting 3 mg/kg of PD-1 blockade once every 2 or 3 weeks. The patients were followed up once every 4–6 weeks based on the type of drugs being used and their adherence to drug regimens. The endpoint of this study was overall survival (OS), defined as the time from the initiation of immunotherapy to the last follow up or death from any cause, whichever is earlier. The OS status was determined from outpatient visits or via telephonic calls for those who could not go for regular hospital checkups.

Statistical analyses

All data processing and statistical analyses were performed using R software (version 4.0.2; <https://www.r-project.org>). Statistical significance was represented by $P < 0.05$. Continuous quantitative data were presented as the mean $\pm$ standard deviation (SD) or median (range). An independent *t*-test and a Mann–Whitney U-test were employed to analyze differences when the data obeyed the normal distribution and when they did not, respectively. Categorical variables were expressed as numbers (%) and Pearson's χ^2 test was executed to compare the differences. Univariate Cox regression was performed to analyze the relationships of TIL biomarkers with OS, while multivariate Cox analysis was executed to develop an immunoscore signature.

The interobserver reproducibility of IVIM parameter measurements between the two observers was assessed by Bland–Altman analysis and intraclass correlation coefficients (ICC). Spearman correlation analysis was applied to evaluate the relationships between IVIM parameters and the

immunoscore. The efficacy of IVIM parameters in classifying the immunoscore status was determined using ROC analysis, and the optimum cutoff values were determined by maximizing the Youden index. Next, the area under the ROC curve (AUC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated, and the AUC values were compared by performing the DeLong test. Survival curves of the immunoscore and IVIM parameters were plotted using the Kaplan–Meier method, and the differences were compared by the log-rank test.

Results

Patient characteristics

Among the 67 patients with RM-NPC, 23 (34.33%) received prior systemic chemotherapy, 5 (7.46%) received systemic chemotherapy combined with adjuvant therapy, 32 (47.76%) received concurrent chemoradiotherapy, and 7 (10.45%) received chemoradiotherapy combined with adjuvant therapy. However, they all became refractory to these previous lines of treatment. The patients underwent PD-1 blockade monotherapy with a median treatment cycle of six (range: 3–12 times). The median follow-up period was 17 months (range: 2–36 months). The detailed characteristics of the patients are summarized in **Table 1**.

Table 1 Patients' Characteristics

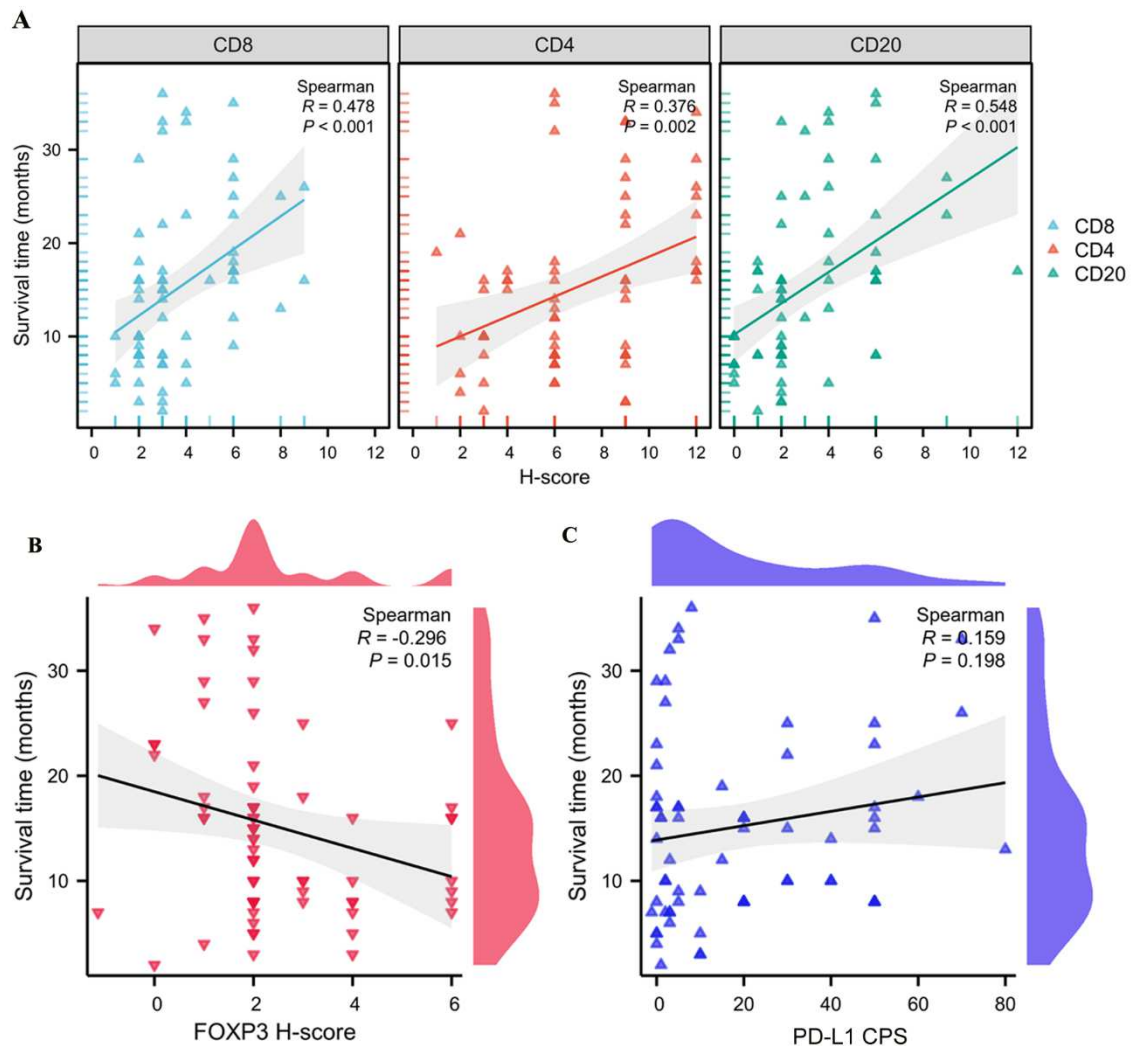
Characteristics	Value	No. (%)
No. of patients		67
Age (years)	Median	50
	Range	25–72
Gender	Male	48 (71.64)
	Female	19 (28.36)
ECOG	0	38 (56.72)
	1	29 (43.28)
Histology	Keratinizing	3 (4.48)
	Non-keratinizing	64 (95.52)
Disease status	Recurrent	11 (16.42)

Prior lines of treatment	Primary metastatic	56 (83.58)
	Systemic chemotherapy	23 (34.33)
	Systemic chemotherapy combined adjuvant therapy	5 (7.46)
	Concurrent radiochemotherapy	32 (47.76)
	Concurrent radiochemotherapy combined adjuvant therapy	7 (10.45)
Immunotherapy cycles	Median	6
	Range	3–12
PD-1 inhibitor	Triplimab	35 (52.24)
	Carelizumab	17 (25.37)
	Tislelizumab	11 (16.42)
	Sintilimab	4 (5.97)
Baseline serum EBV (IU/mL)	< 2000	25 (37.31)
	≥2000	38 (56.72)
	Unknown	4 (5.97)
Follow-up period	Median (months)	15
	Range (months)	2–36
OS statue at the final follow-up	Alive	31 (46.27)
	Dead	36 (53.73)

Abbreviations: ECOG: Eastern Cooperative Oncology; PD-1: Programmed death-1; EBV: Epstein–Barr virus; OS: Overall survival.

Prognostic value of TIL biomarkers and TIL-based immunoscore signature

As shown in **Fig. 3A-B**, the CD8 H-score ($R = 0.48$, $P < 0.001$), CD4 H-score ($R = 0.40$, $P < 0.01$), and CD20 H-score ($R = 0.54$, $P < 0.001$) were positively correlated with the OS. In contrast, the FOXP3 H-score was negatively correlated with the OS ($R = -0.35$, $P < 0.01$). However, no correlation was observed between PD-L1 expression and OS ($R = 0.13$, $P = 0.32$). The Spearman correlation curves for each TIL biomarker are shown in Supplementary **Fig. S2**.

Supplementary Fig. S2. Correlations between TIL H-score and OS.

As shown in **Table 2**, univariate Cox regression analysis indicated that the CD8 H-score (HR = 1.72 [95% CI: 1.30, 2.27], $P < 0.001$), CD4 H-score (HR = 1.22 [95% CI: 1.10, 1.37], $P < 0.001$), and CD20 H-score (HR = 1.47 [95% CI: 1.20, 1.79], $P < 0.001$) were positively associated with OS, while the FOXP3 H-score was negatively associated with it (HR = 0.79 [95% CI: 0.66, 0.93], $P = 0.006$). In contrast, OS was not significantly associated with PD-L1 expression (HR = 1.03 [95% CI: 0.89, 1.08], $P = 0.079$). Based on multivariate Cox analysis, the CD8 H-score (HR = 1.47 [95% CI: 1.10, 1.92], $P = 0.009$) and CD20 H-score (HR = 1.28 [95% CI: 1.03, 1.59], $P = 0.027$) were identified as independent prognostic factors.

Table 2 Univariate and multivariate Cox analyses of TIL biomarkers and PD-L1 for assessing relative risk for OS

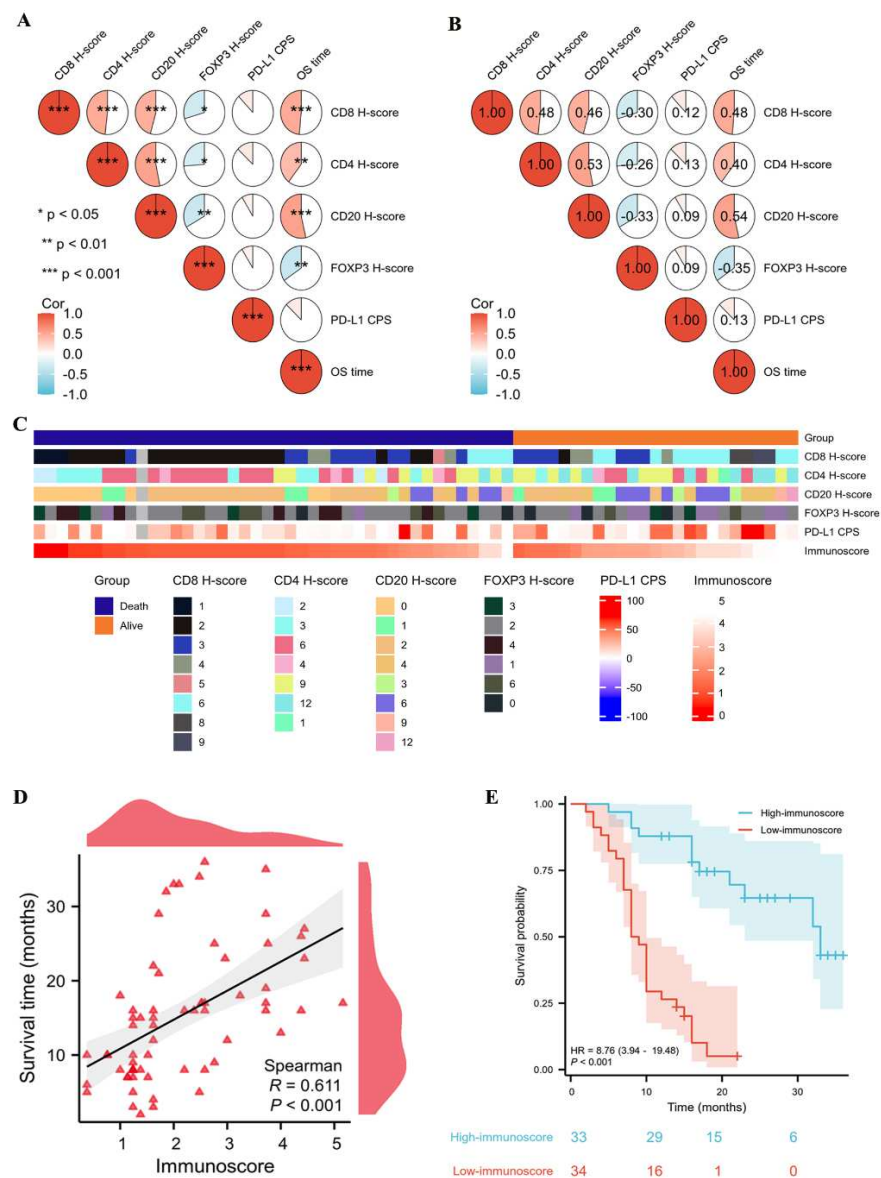
Variables	Univariate Cox analysis			Multivariate Cox analysis		
	β	HR (95%CI)	P-value	β	HR (95% CI)	P-value
CD8 H-score	0.54	1.72 (1.30–2.27)	<0.001	0.38	1.47 (1.10–1.92)	0.009
CD4 H-score	0.20	1.22 (1.10–1.37)	<0.001	0.04	1.04 (0.90–1.20)	0.595
CD20 H-score	0.38	1.47 (1.20–1.79)	<0.001	0.24	1.28 (1.03–1.59)	0.027
FOXP3 H-score	-0.24	0.79 (0.66–0.93)	0.006	-0.19	0.83 (0.68–1.01)	0.067
PD-L1 (CPS)	0.02	1.03 (0.89–1.08)	0.079	0.02	1.02 (1.00–1.04)	0.123

Abbreviations: HR: hazard ratio; CI: confidence interval; OS: overall survival; FOXP3: Forkhead Box Protein 3; TIL: tumor-infiltrating lymphocytes; PD-L1: programmed death ligand-1; CPS: combined positive score.

A TIL-based immunoscore signature was constructed based on the CD8 H-score and CD20 H-score, along with their weight coefficient (β):

$$\text{Immunoscore} = 0.38 \times \text{CD8 H-score} + 0.24 \times \text{CD20 H-score}.$$

The heat map presented the correlations of the TIL biomarkers and immunoscore with the OS status (**Fig. 3C**). The immunoscore was positively correlated with the OS ($R = 0.61$, $P < 0.001$) (**Fig. 3D**). The median immunoscore was used as a cutoff value for classifying patients into the following two groups: high-immunoscore ($n = 33$) and low-immunoscore ($n = 34$). Kaplan–Meier analysis (**Fig. 3E**) indicated that patients in the high-immunoscore group had significantly longer OS than those in the low-immunoscore group (HR = 8.76, [95% CI: 3.94, 19.48], $P < 0.001$).

Fig.3 Prognostic value of TILs and immunoscore in the prediction of immunotherapy OS.

Measurement repeatability of IVIM parameters and its association with immunoscore

Both observers reported excellent reproducibility of IVIM parameter measurements. The ICC of the ADC, D , D^* , and f values were 0.824 (95% CI: 0.729, 0.888), 0.885 (95% CI: 0.819, 0.928), 0.828 (95% CI: 0.735, 0.891), and 0.867 (95% CI: 0.792, 0.916), respectively. Bland–Altman analysis (Supplementary Fig. S3) revealed the absence of any measurement differences between the values reported by Observer 1 and Observer 2. The values of IVIM parameters measured by the two observers

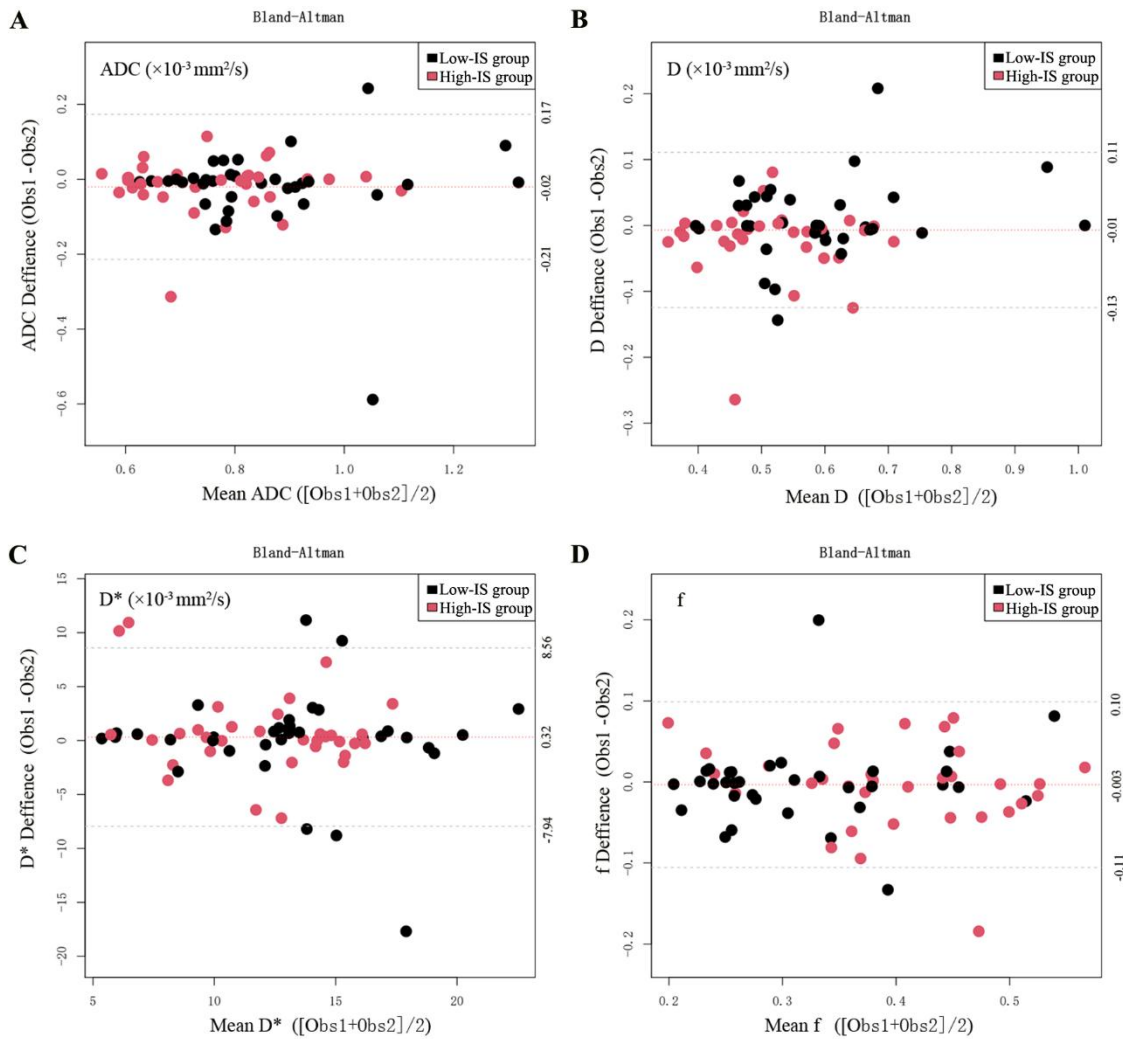
and a comparison of their differences are presented in Supplementary Table S1.

Supplementary Table S1. Difference analyses between the two observers for IVIM-DWI parameters measurements

Variables	Observer 1	Observer 2	Averaged	2.5% Difference	Mean Difference	97.5% Difference	P
ADC ($\times 10^{-3}$ mm ² /s)	0.79 $\pm$ 0.17	0.82 $\pm$ 0.17	0.81 $\pm$ 0.16	-0.21	-0.02	0.17	0.099
D ($\times 10^{-3}$ mm ² /s)	0.55 $\pm$ 0.13	0.56 $\pm$ 0.12	0.55 $\pm$ 0.122	-0.13	-0.01	0.11	0.358
D* ($\times 10^{-3}$ mm ² /s)	12.78 $\pm$ 6.48	12.45 $\pm$ 6.58	12.63 $\pm$ 3.82	-7.94	0.32	8.56	0.536
<i>f</i>	0.35 $\pm$ 0.10	0.36 $\pm$ 0.10	0.36 $\pm$ 0.10	-0.11	-0.003	0.1	0.602

ADC = apparent diffusion coefficients, D = true diffusion coefficient, D* = pseudo-diffusion coefficient, *f* = perfusion fraction, IVIM-DWI = intravoxel incoherent motion diffusion-weighted MR imaging.

Supplementary Fig. S3. Difference analyses of IVIM-DWI parameters between the two observers using Bland–Altman curve



The ADC ($R = -0.295$, $P = 0.016$) (Fig. 4A) and *D* ($R = -0.352$, $P = 0.003$) (Fig. 4B) values were

negatively correlated with the immunoscore. In addition, no significant correlation was observed for the D^* value ($R = -0.076$, $P = 0.543$) (**Fig. 4C**), while a positive correlation was observed for the f value ($R = 0.310$, $P = 0.011$) (**Fig. 4D**). As shown in **Fig. 4** and Supplementary **Table S2**, the ADC (0.76 ± 0.14 vs. $[0.86 \pm 0.17] \times 10^{-3} \text{ mm}^2/\text{s}$, $P = 0.01$) (**Fig. 4E**) and D (0.51 ± 0.10 vs. $[0.59 \pm 0.13] \times 10^{-3} \text{ mm}^2/\text{s}$, $P = 0.008$) (**Fig. 4F**) values of the high-immunoscore group were significantly lower than those of the low-immunoscore group. In contrast, no difference was observed in the D^* values of the two groups (12.05 ± 3.28 vs. $[13.19 \pm 4.25] \times 10^{-3} \text{ mm}^2/\text{s}$, $P = 0.226$) (**Fig. 4G**). However, the f value (0.40 ± 0.09 vs. $[0.32 \pm 0.09]$, $P < 0.001$) of the high-immunoscore group was significantly higher than that of the low-immunoscore group (**Fig. 4H**). As displayed in **Table 3** and **Fig. 4I**, the ADC, D , and f values yielded the AUC values of 0.669 (95% CI: 0.538, 0.800), 0.686 (95% CI: 0.559, 0.814), and 0.735 (95% CI: 0.611, 0.859) with the optimum cutoff values of $0.74 \times 10^{-3} \text{ mm}^2/\text{s}$, $0.50 \times 10^{-3} \text{ mm}^2/\text{s}$, and 0.33, respectively, for classifying patients into high- and low-immunoscore statuses. No statistically significant difference was noted among the AUC values of these IVIM parameters (all $P > 0.05$).

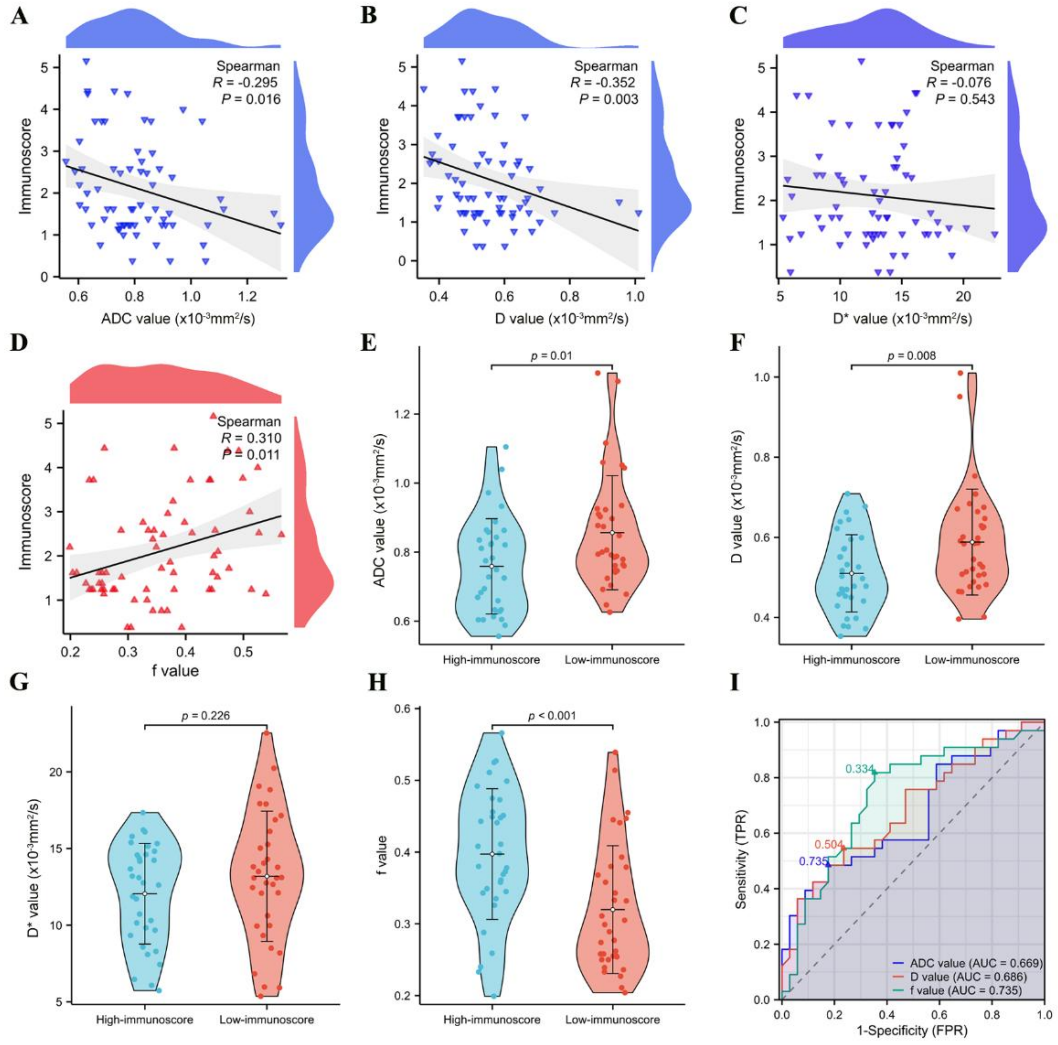
Fig 4. Associations of IVIM-DWI parameters with immunoscore.

Table S2. Differences Comparison of IVIM-DWI parameters between high and low-immunoscore groups

Variables	Total	High-immunoscore group	Low-immunoscore group	P
	(n = 67)	(n = 34)	(n = 33)	
ADC ($\times 10^{-3} \text{mm}^2/\text{s}$)	0.81 ± 0.16	0.86 ± 0.17	0.76 ± 0.14	0.011
D ($\times 10^{-3} \text{mm}^2/\text{s}$)	0.55 ± 0.12	0.59 ± 0.13	0.51 ± 0.10	0.008
D* ($\times 10^{-3} \text{mm}^2/\text{s}$)	12.63 ± 3.82	13.19 ± 4.25	12.05 ± 3.28	0.226
<i>f</i>	0.36 ± 0.10	0.32 ± 0.09	0.40 ± 0.09	<0.001

ADC = apparent diffusion coefficients, D = true diffusion coefficient, D* = pseudo-diffusion coefficient, *f* = perfusion fraction, IVIM-DWI = intravoxel incoherent motion diffusion-weighted MR imaging.

Table 3. Performance evaluation and AUC comparison of IVIM-DWI parameters for differentiating high-immunoscore and low-immunoscore statuses

Parameters	AUC (95% CI)	Cut off	P-value in compari son to ADC	P- value in compa rison to <i>D</i>	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV% (95%CI)	NPV% (95%CI)
ADC ($\times 10^{-3} \text{mm}^2/\text{s}$)	0.669 (0.538–0.800)	0.74	NA	0.671	48.48 (31.43–66.54)	82.35 (69.54–95.17)	72.73 (54.12–91.34)	62.22 (48.06–76.39)
D ($\times 10^{-3} \text{mm}^2/\text{s}$)	0.686 (0.559–0.814)	0.50	0.671	NA	54.55 (37.56–71.53)	76.47 (62.21–90.73)	69.23 (51.49–86.97)	63.41 (48.67–78.16)
<i>f</i>	0.735 (0.611–0.859)	0.33	0.451	0.591	81.82 (68.66–94.98)	64.71 (48.64–80.77)	69.23 (54.75–83.72)	78.57 (63.37–93.77)

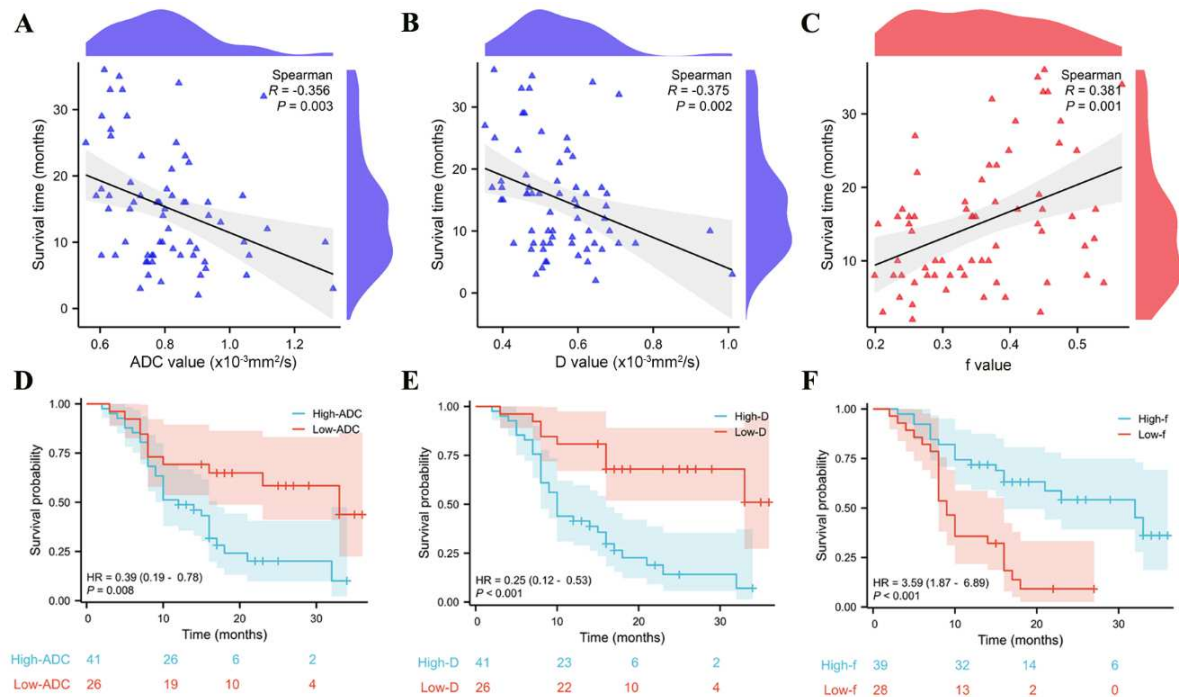
Abbreviations: AUC: area under the receiver operating characteristic curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; ADC: apparent diffusion coefficients; *D*: true diffusion coefficient; *f*: perfusion fraction; IVIM-DWI: intravoxel incoherent motion diffusion-weighted MR imaging.

Association of IVIM parameters with immunotherapy OS

The ADC ($R = -0.356$, $P = 0.003$) (**Fig. 5A**) and *D* ($R = -0.375$, $P = 0.002$) (**Fig. 5B**) values were negatively correlated with the OS, while the *f* value ($R = 0.381$, $P = 0.001$) was positively correlated (**Fig. 5C**). The status of IVIM parameters was classified as high and low based on the optimum cutoff values presented in **Table 3**. The Kaplan–Meier analysis indicated that patients in high-ADC (HR =

0.39 [95% CI: 0.19, 0.78], $P = 0.008$ (**Fig. 5D**) and high- D ($HR = 0.25$ [95% CI: 0.12, 0.53], $P < 0.001$) (**Fig. 5E**) groups showed significantly shorter OS than those in the low-ADC and low- D groups, while patients in the high- f group had longer OS than those in the low- f group ($HR = 3.59$ [95% CI: 1.87, 6.89], $P < 0.001$) (**Fig. 5F**).

Fig 5. Association of IVIM-DWI parameters with immunotherapy OS.



Discussion

This study developed a TIL-based immunoscore signature that could be used to categorize patients with RM-NPC refractory to chemotherapy into high-risk and low-risk groups with significantly different OS ($HR = 8.76$, [95% CI: 3.94, 19.48], $P < 0.001$). Several IVIM parameters (ADC, D , and f values) were found to be significantly correlated with immunoscore and demonstrated good performance for classifying patients into high- and low-immunoscore statuses. Additionally, patients with a higher f value (≥ 0.33) are more likely to benefit from immunotherapy and have a longer OS. In contrast, patients with higher ADC ($\geq 0.74 \times 10^{-3} \text{mm}^2/\text{s}$) and higher D values ($\geq 0.50 \times 10^{-3} \text{mm}^2/\text{s}$)

were found to be correlated with a poorer OS. These findings suggest that IVIM parameters could be employed to quantitatively evaluate TIL infiltration and noninvasively predict immunotherapy prognosis in RM-NPC.

The importance of TIL infiltration as a prognostic marker for immunotherapy is attracting increasing interest. Intratumoral CD4⁺ T, CD8⁺ T, and CD20⁺ B cell infiltration was positively correlated with OS, whereas FOXP3⁺ Treg infiltration was negatively correlated with it. In addition, an immunoscore signature was developed by incorporating independent predictive markers (CD8 and CD20). Patients with a high immunoscore had markedly longer OS than those with low immunoscores, suggesting that tumors with high TIL infiltration were more likely to benefit from the immunotherapy. These results support those reported by previous studies on NPC [32, 35], according to which immune cell activation and strong ligand–receptor activation are required for a favorable immunotherapy response.

Noninvasive methods to assess TIL infiltration are attracting increasing research interest. In contrast to conventional DWI, the bi-exponential model of IVIM demonstrated the heterogeneity of water diffusion and can differentiate between the contribution of perfusion and that of diffusion [36]. Previous studies have reported on the correlation of various IVIM parameters with the tumor immune status, such as that of the Ki-67 proliferation index in rhabdomyosarcoma [37], VEGF expression in hepatocellular carcinoma [38], and PD-L1 expression in lung and cervical cancers [26, 27]. In line with these findings, the present study also showed that the ADC and D values were markedly negatively correlated with the immunoscore in RM-NPC, while the f value was significantly positively correlated with it. In particular, the ADC, D , and f values afforded comparable performances for classifying patients into high- and low-immunoscore groups, with AUC values of 0.669, 0.686, and

0.735, respectively. Thus, it can be suggested that IVIM parameters are correlated with TIL infiltration in RM-NPC.

Recently, several studies have reported on the potential performance of IVIM-DWI for prognosis prediction in locally advanced NPC. Qamar et al. reported that a low pretreatment D value is correlated with prolonged disease-free survival (DFS) [39]. However, Qin et al. demonstrated that higher ADC and D values are markedly associated with higher OS [25]. The present preliminary study on immunotherapy in RM-NPC showed that patients with higher ADC and D values have significantly lower OS than those with lower ADC and D values. These findings seemed to be in contrast to those of Qin et al. for chemoradiotherapy in NPC [25]. Indeed, NPC with low ADC and D values generally represents aggravated diffusion restriction and high invasiveness [20, 21], which may increase the risks of recurrence and distant metastasis post-chemoradiotherapy and affect the prognosis. However, the response to immunotherapy may mainly rely on the TME status. The ADC and D values have been frequently reported to be negatively correlated with PD-L1 expression and TIL infiltration [26-28, 30]. Thus, tumors with low ADC and D values are more likely to benefit from immunotherapy and exhibit prolonged survival.

This study has several limitations. First, the study sample was relatively small because only patients with RM-NPC refractory to standard chemotherapy were included. Multicenter studies with large samples are needed to validate these study results. Second, the study has a selection bias that could not be eliminated because of its retrospective nature. PD-1 inhibitors and treatment cycles used for immunotherapy had discrepancies. In addition, the patient follow-up interval could not be standardized. Third, because of the limited availability of puncture tissue of NPC, only a few typical TIL-specific markers most likely to correlate with immunotherapy prognosis could be detected. More

markers need to be identified for comprehensively characterizing the complex heterogeneity of TILs. Finally, because no standardized protocol has yet been developed for choosing b -values in IVIM-DWI, this study was performed based on the recommendations of previous studies and used at least more than 10 b -values, at least four of which were $<200 \text{ s/mm}^2$ [22, 23, 39].

Conclusions

A TIL-based immunoscore was significantly associated with immunotherapy prognosis in RM-NPC refractory to previous chemotherapy sessions. Quantitative IVIM parameters were markedly correlated with the immunoscore and OS. The IVIM-DWI could be employed as a noninvasive imaging technique to quantitatively evaluate TIL infiltration and predict immunotherapy prognosis to aid clinical treatment decisions.

Abbreviations

ADC: Apparent Diffusion Coefficient

D: Pure Diffusion Coefficient

D*: Pseudodiffusion Coefficient

EBV: Epstein - Barr Virus

f : Perfusion Fraction

FOXP3: Forkhead Box Protein 3

Gd-DTPA: Gadolinium - Diethylenetriaminepentaacetic Acid

H: score Histochemistry Score

ICC: Intraclass Correlation Coefficient

IHC: Immunohistochemical

IVIM: Intravoxel Incoherent Motion

IVIM-DWI: Intravoxel Incoherent Motion Diffusion - Weighted MR Imaging

NEX: Number of Excitations

NPV: Negative Predictive Value

ORRs: Objective Response Rates

OS: Overall Survival

PD - L1: Programmed Death Ligand - 1

PPV: Positive Predictive Value

RECIST: Response Evaluation Criteria In Solid Tumors

RM - NPC: Recurrent or Metastatic Nasopharyngeal Carcinoma

ROI: Region of Interest

ST: Slice Thickness

TILs: Tumor Infiltrating Lymphocytes

TMB: Tumor Mutation Burden

TME: Tumor Microenvironment

TE: Time of Echo

TR: Time of Repeation

HR: Hazard Ratio

ORRs: Objective Response Rates

DWI: Diffusion Weighted Imaging

Declarations

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments. The study protocol was reviewed and approved by the Institutional Review Committee of the Affiliated Cancer Hospital & Institute of Guangzhou Medical University (GYZL-2024-KY44). As this was a retrospective study, the Ethics Committee approved a waiver of informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Xi Zhong conceptualized the study. Man Lu , Ziqing Sun and Xin Xin collected the CT and clinical data. Xi Zhong, Yuanlin Chen, Man Lu and Ziqing Sun wrote the manuscript. Yuanlin Chen and Zixuan Wang were responsible for the acquisition and processing of IHC data. Jinxue Yin and Yongfang Tang manually segmented the regions of interest. Jian Shang and Lidan Yang performed the statistical analyses. Xi Zhong, Donghui Zhang, and Jiangyu Zhang provided critical feedback and discussions. Xi Zhong edited the manuscript. Xi Zhong, Donghui Zhang, and Jiangyu Zhang supervised this study. All authors have read and approved the final manuscript.

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Figure Legends

Fig. 1. Flow chart of patient selection. Abbreviations: RM-NPC: recurrent or metastatic nasopharyngeal carcinoma; PD-1: programmed cell death protein-1; IVIM-DWI: diffusion weighted imaging with intravoxel incoherent motion; OS: overall survival.

Fig. 2. Measurements of IVIM-DWI functional parameters. (A) Slightly high-signal mass can be seen in T2W images. (B) Tumor whole-volume ROIs were placed slice by slice on DW images. (C-F) These ROIs were automatically fitted to the corresponding slices on ADC maps (C), D maps (D), D^* maps (E), and f maps (F). Abbreviations: ROI: region of interests; ADC: apparent diffusion coefficient; D : true diffusion coefficient; D^* : pseudo-diffusion coefficient; f : perfusion fraction; IVIM-DWI: intravoxel incoherent motion diffusion-weighted MR imaging.

Fig. 3. Prognostic value of TILs and immunoscore in the prediction of immunotherapy OS. (A) Spearman correlation heat map (P values) between the H-score of TIL biomarkers and OS. (B) Correlation coefficient (R) heat map between the H-score of TIL biomarkers and OS. (C) Heat map for presenting the correlations of TIL biomarkers and immunoscore with the OS status. (D) The immunoscore was significantly positively correlated with the OS. (E) Kaplan–Meier analysis indicated that patients in the high-immunoscore group have a significantly longer OS than that of the patients in the low-immunoscore group (HR = 8.76). Abbreviations: TIL: tumor infiltrating lymphocyte; OS: overall survival; HR: hazard ratio; H-score: histochemistry score; FOXP3: Forkhead Box Protein 3; PD-L1: programmed death ligand-1; CPS: combined positive score.

Fig. 4. Associations of IVIM-DWI parameters with immunoscore. (A, B) ADC and D values were significantly negatively correlated with the immunoscore. (C) D^* value showed no significant correlation with the immunoscore. (D) The f value was significantly positively associated with the

immunoscore. (E, F) The ADC and D values of the high-immunoscore group were significantly lower than those of the low-immunoscore group. (G) No difference was observed between the high-immunoscore and low-immunoscore groups in the D^* value. (H) The f value in the high-immunoscore group was significantly higher than that of the low-immunoscore group. (I) Performance evaluation of IVIM-DWI parameters for classifying the high-immunoscore status from the low-immunoscore status using ROC analysis. Abbreviations: IS: immune score; ADC: apparent diffusion coefficient; D : true diffusion coefficient; D^* : pseudo-diffusion coefficient; f : perfusion fraction; AUC: area under the ROC curve; TPR: true positive rate; FPR: false positive rate; IVIM-DWI: intravoxel incoherent motion diffusion-weighted MR imaging.

Fig. 5. Association of IVIM-DWI parameters with immunotherapy OS. ADC value (A) and D value (B) were negatively correlated with OS. (C) The f value was positively correlated with OS. Patients with a high ADC value (D) and a high D value (E) had shorter OS than those with low ADC and D values. (F) Patients with a high f value had longer OS than those with low f values. Abbreviations: OS: overall survival; HR: hazard ratio; ADC: apparent diffusion coefficient; D : true diffusion coefficient; D^* : pseudo-diffusion coefficient; f : perfusion fraction; IVIM-DWI: intravoxel incoherent motion diffusion-weighted MR imaging.

Supplementary Fig. S1. Evaluation of TIL biomarkers and PD-L1 expression score (200× magnification). (A) Hematoxylin–Eosin (H&E) staining: non-keratinizing undifferentiated carcinoma of the nasopharynx, tumor cell nests, and surrounding stroma infiltrated by immune cells, (B) IHC staining of the CD4 antibody: the infiltration abundance of CD4⁺ T cells was approximately 85% (score 4), and the staining intensity was strongly positive (score 3), the H-score of CD4⁺ T infiltration was 12, (C) IHC staining of CD8 antibody: Infiltrating abundance of CD8⁺ T cells was approximately 55%

(score 3), and staining intensity was strongly positive (score 3), the H-score of CD4⁺T infiltration was 9. (D) IHC staining of CD20 antibody: Infiltrating abundance of CD20⁺ B cells was approximately 60% (score 3), and the staining intensity was strongly positive (score 3), the H-score of CD4⁺ T infiltration was 9. (E) IHC staining of FOXP3 antibody: Infiltrating abundance of FOXP3⁺ Treg was approximately 15% (score 1), and the staining intensity was strongly positive (score 3), and the H-score of FOXP3⁺ Treg infiltration was 3. (F) IHC staining of PD-L1 antibody: Only a few tumor cells and infiltrating immune cells (such as lymphocytes and macrophages) membrane staining expression (CPS $\approx$ 1). Abbreviations: TIL: tumor infiltrating lymphocyte; PD-L1 : programmed death ligand-1; FOXP3: Forkhead Box Protein 3; IHC: immunohistochemical; H-score : histochemistry score; CPS: combined positive score.

Supplementary Fig. S2. Correlations between TIL H-score and OS. (A) H-score of CD8 ($R = 0.478$, $P < 0.001$), CD4 ($R = 0.376$, $P = 0.002$), and CD20 ($R = 0.548$, $P < 0.001$) were significantly positively associated with OS. (B) The H-score of FOXP3 was significantly negatively associated with OS ($R = -0.296$, $P = 0.015$). (C) PD-L1 CPS showed no significant correlation with OS ($R = 0.159$, $P = 0.198$). Abbreviations: TIL: tumor infiltrating lymphocyte; H-score: histochemistry score; OS: overall survival; FOXP3: Forkhead Box Protein 3; PD-L1: programmed death ligand-1.

Supplementary Fig. S3. Difference analyses of IVIM-DWI parameters between the two observers using Bland–Altman curve for the ADC value (A), D value (B), D^* value, and (C) and f value (D). The central red line represents the mean difference, the top ashy line shows the upper 95% limit of difference, and the bottom ashy line shows the lower 95% limit of difference. Abbreviations: Obs: observer; ADC: apparent diffusion coefficient; D : true diffusion coefficient; D^* : pseudo-diffusion coefficient; f : perfusion fraction; IS: immunoscore.

Supplementary Files

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