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Research Article

The Prognostic Value of Stromal Tumor-Infiltrating Lymphocytes in Intrahepatic Cholangiocarcinoma

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ABSTRACT

Objective: We conducted this study to assess the predictive value of tumor-infiltrating lymphocytes (TILs) for progression-free survival (PFS) and overall survival (OS) in intrahepatic cholangiocarcinoma (ICC) patients after complete resection.

Methods: Sixty-eight patients with resectable ICC were included in this study. We studied stromal TIL density and scored it by staining sections from surgically resected ICC patients with hematoxylin and eosin (HE). The clinical data and prognosis of ICC patients were obtained by searching clinical and follow-up records.

Results: A stromal TIL negative status was an independent predictor of poor OS (HR=2.84, 95% CI 1.48-5.46, P<0.01) and poor PFS (HR=2.88, 95% CI 1.44-5.76, P<0.01). Low stromal TIL density was associated with high CA125 (P=0.03) and CA19-9 (P<0.01) levels. Patients with a stromal TIL negative status tended to develop tumors with a high level of CA19-9 (P=0.05), high differentiation (P=0.02), a large diameter (P=0.05), a positive bile duct/vascular cancer embolus (P=0.03) and positive satellite nodules (P=0.02).

Conclusion: Our data suggest that stromal TILs play an important role in predicting the PFS and OS of ICC patients after complete resection.

Introduction

Intrahepatic cholangiocarcinoma (ICC) refers to an adenocarcinoma that originates in the secondary bile duct and its branch epithelium. ICC is reported to account for 10-15% of primary liver malignancies and is the second most common hepatic malignancy following hepatocellular carcinoma [1, 2]. The incidence and mortality rate of ICC have continued to increase globally in recent years [1-3]. Surgical resection is the most effective way to treat early-stage ICC [4]. Nevertheless, due to the high recurrence rate, the prognosis of ICC after curative resection is still very poor [5]. Therefore, other prognostic factors must be identified to predict progression-free survival (PFS) and overall survival (OS) in patients with ICC after complete resection, which may be valuable in developing treatment strategies and selecting appropriate treatment options for individual patients.

In recent years, it has been shown that tumor-infiltrating immune cells have significant predictive value for many solid tumors [6-10]. A study evaluating tumor-infiltrating lymphocytes (TILs) in pancreatic ductal adenocarcinoma (PDAC) showed that the presence of CD4 and CD8 TILs served as a good index of patient prognosis after surgical resection [11, 12]. Another study found that stromal TILs could be a useful index to predict liver metastasis and the OS of patients with PDAC after complete resection [13]. However, the study of stromal TILs in ICC has not yet been reported.

Recently, the International TILs Working Group performed a standardized hematoxylin and eosin (HE) staining section analysis of stromal TILs in breast carcinoma as the primary target parameter [10]. This conclusion was also confirmed in a study of PDAC [13]. However, the prognosis of ICC patients with complete resection generally tends to

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be much worse. It has been found that the tumor microenvironment has an inherent immunosuppressive effect and many mechanisms to evade immune surveillance [14-16]. The density of TILs in breast cancer tissues may differ significantly from that in ICC tissues. Tougher criteria may be more appropriate for ICC. Therefore, we analysed the predictive value of stromal TILs and clinical parameters for PFS and OS after complete resection in ICC patients. We conducted this study to assess the predictive value of TILs and clinical parameters for PFS and OS in ICC patients after complete resection.

Methods

I Ethics Statement

The current study was authorized by the Clinical Ethics Committee of Henan Provincial People's Hospital. We did not obtain written consent from the participants because this study was a retrospective study. We performed all methods in accordance with relevant guidelines or regulations. Furthermore, we analysed all participants' data anonymously.

II Study Population and Design

All patients in this study were registered at Henan Provincial People's Hospital (Zhengzhou, China) between January 2010 and December 2016. This study enrolled patients with ICC who underwent complete resection and were confirmed by pathology. Participants whose preoperative examination revealed that the tumor invaded surrounding organs or blood vessels, distant metastases or local lymph node metastasis were excluded. In addition, the cut edge of ICC patients must have been confirmed to be negative by a postoperative pathological examination. However, three patients who were found to have peritoneal metastasis during surgery that was not detected in the preoperative examination were also enrolled. Five ICC patients were excluded from the study because they underwent transcatheter arterial chemoembolization or ultrasound-guided radiofrequency ablation. We followed up the PFS and OS of 106 ICC patients after complete resection. Complete follow-up data were obtained from only 83 ICC patients. TIL assessment was performed on tumor specimens from 83 ICC patients. Fifteen ICC patients' permanent HE-stained sections were not available, so their TILs could not be assessed. All 68 ICC patient specimens were pathologically evaluated to determine tumor differentiation, lymph node metastasis and surgical margins. We determined the pathological staging of ICC according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging system [17].

III Determination of Postoperative Recurrence or Metastasis

During the postoperative follow-up, regular laboratory tests, including tumor markers, computed tomography (CT) scans, magnetic resonance examinations, ultrasonography, and positron emission tomography (PET)-CT, were performed. If new lesions were found in the liver or other organs by the CT scan, magnetic resonance examination, ultrasonography or PET-CT and there was no clear evidence of other cancer metastasis or recurrence elsewhere, we considered these patients to be relapsed or metastatic.

IV Stromal TIL Assessment

TILs were evaluated in each patient by collecting HE-stained sections from surgically resected specimens from the pathology department. The area assessed for stromal TILs was within the boundaries of the invasive tumor. Immune infiltration adjacent to normal tissue or intraductal growth types was not included. TILs in the tumor areas with squeezing artifacts, necrosis or regressive transparency were also excluded. Stromal TILs were considered all stromal monocytes that did not come into direct contact with cancer cells. We report different levels of stromal TILs as none, low, medium or high (scores 0, 1, 2 and 3, respectively).

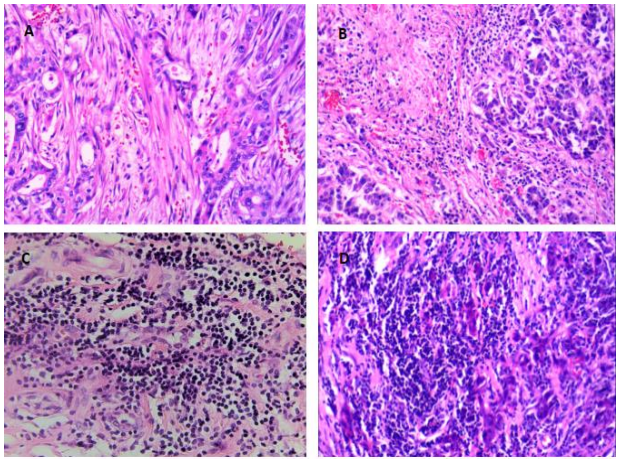

Figure 1: A) Stromal TIL-negative group (score 0): almost no lymphocytes have infiltrated the stroma of the tumor tissue; **B)** Stromal TIL-negative group (score 1): a small amount of lymphocytes have infiltrated the stroma of the tumor tissue; **C)** Stromal TIL-positive group (score 2): more lymphocytes have infiltrated the stroma of the tumor tissue; **D)** Stromal TIL-positive group (score 3): infiltration of a large number of lymphocytes between the mesenchymal and tumor cells [200× magnification, H & E-stained sections. (A-D)].
TIL: Tumor-Infiltrating Lymphocytes.

Figure 1 (A-D) shows representative images of different levels of stromal TILs. Lymphocytes are mostly small lymphocytes, 5-8 mm in diameter, with round nuclei, shallow concaves on one side, dense chromatin, and a small cytoplasm. Lymphocytes are very different from dendritic cells (DCs) (10-20mm) and other monocytes (14-20mm) and are easily distinguishable, especially for experienced pathologists. ICC patients were classified as TIL negative (none to low) and TIL positive (medium to high) according to the pathological score. We evaluated the different regions and reported the average of the highest prefrontal levels in patients with heterogeneous stromal cell density in a single tumor section. Two experienced pathologists were unaware of the clinical results and independently assessed the stromal TILs. The final score was obtained after the two pathologists had consistently assessed the score. If the percentage of stromal TILs varied, we selected the higher grade to represent the final grade.

V Statistical Analysis

We analysed the corresponding variables by the Mann-Whitney U-test, the X^2 test, Kaplan-Meier curve analysis, the log-rank test, multivariate Cox regression and other methods. Based on the univariate analysis,

variables with P<0.05 were included in the multivariate logistic regression analysis to determine the independent variables. All analyses were performed using SPSS 22.0 statistical software (SPSS, IL, USA). P<0.05 was considered statistically significant.

Results

I Patient Characteristics

A total of 68 patients who had primary ICC and underwent complete resection were enrolled, including 38 males and 30 females, aged 35-80 years, with an average age of 60.28 years. All participants' diagnoses were confirmed clinically and pathologically. Our follow-up data showed that 45 patients died at the end of follow-up. There were 38 patients with clear causes of death, including 24 relapses or metastases. According to the scoring method shown in (Figure 1), we calculated the information of all patients. The quantitative clinical characteristics are shown in (Table 1), and the clinical factors of the classification are shown in (Table 2). Overall, 32 patients were positive for TILs, and the remaining 36 patients were negative for TILs.

Table 1: Comparison of quantitative factors between TIL-negative group and TIL-positive group.

Factors	TIL-Negative		TIL- Positive		P value
	Mean	Standard deviation	Mean	Standard deviation	
Age (years)	61.56	6.42	58.25	10.91	0.14
AFP (U/mL)	69.32	331.92	37.73	69.51	0.59
CA125 (U/mL)	221.18	214.00	72.37	100.91	0.03
CA19-9 (U/mL)	3097.67	9942.69	184.79	252.32	0.00
Platelets (×10^3/mL)	231.14	67.70	193.19	117.90	0.39
Lymphocytes (×10^3/mL)	1.53	0.54	1.55	0.60	0.89
Neutrophils (×10^3/mL)	4.87	2.12	6.33	3.71	0.32
Monocytes (×10^3/mL)	0.41	0.23	0.39	0.18	0.58
Diameter (cm)	6.22	2.76	5.70	3.51	0.49

TIL: Tumor-Infiltrating Lymphocyte.

Table 2: Univariate analysis of clinical characteristics based on stromal TILs.

Characteristics		TILs-Negative		TILs- Positive		P value
		N=36	%	N=32	%	
Sex	Male	21	58	17	53	0.66
	Female	15	42	15	47	
Age (years)	<65	23	64	22	69	0.67
	≥65	13	36	10	31	
CA125 (U/mL)	<35	14	39	12	38	0.91
	≥35	22	61	20	62	
CA19-9 (U/mL)	<39	16	19	7	47	0.05
	≥39	20	81	25	53	
Location	Left lobe	12	33	7	22	0.29
	Right lobe	24	67	25	78	
Differentiation	Poor	16	44	6	19	0.02
	Well to moderate	20	56	26	81	
Diameter (cm)	<5.5	14	31	20	56	0.05
	≥5.5	22	69	12	44	
LN metastasis	Negative	28	78	28	87	0.29
	Positive	8	22	4	13	
AJCC stage	<IIb	22	61	21	66	0.70
	≥IIb	14	39	11	34	
Nerve invasion	Negative	31	86	25	78	0.39
	Positive	5	14	7	22	
Bile duct/vascular	Negative	18	64	24	41	0.03
cancer embolus	Positive	18	36	8	59	
Satellite nodules	Negative	21	58	27	84	0.02
	Positive	15	42	5	16	

TILs: Tumor-Infiltrating Lymphocytes; LN: Lymph Node; AJCC: American Joint Committee on Cancer.

II Comparison of OS-Related Clinical Variables after Hepatectomy

In the univariate analysis, the CA125 level, the CA19-9 level, and stromal TILs were all significant prognostic factors for OS (all p<0.05, Table 3). In the multivariate analysis, high levels of CA125 (HR=0.47, 95% CI 0.24-0.91, p=0.02), high levels of CA19-9 (HR=0.44, 95% CI 0.21-0.94, p=0.03) and negative TILs (HR=2.84, 95% CI 1.48-5.46, p<0.01) were important independent predictors of poor OS (Table 3). TIL density was also associated with OS (Figure 2).

Table 3: Univariate and multivariate analyses of clinical data in relation to PFS and OS after complete resection.

Parameter		N	Progression-free survival			Overall survival		
			Univariate analysis P value	Multivariate analysis HR(95% CI)	P value	Univariate analysis P value	Multivariate analysis HR(95% CI)	P value
Sex	Male	38	0.74			0.51		
	Female	30						
Age (years)	<65	45	0.45			0.63		
	≥65	23						
CA125 (U/mL)	<35	26	0.516			0.04	1	0.02
	≥35	42					0.47(0.24-0.91)	
CA19-9 (U/mL)	<39	23	0.02	1	0.11	<0.01	1	0.03
	≥39	45		0.54(0.26-1.14)			0.44(021-0.94)	
Differentiation	Poor	22	0.72			0.78		
	Well to moderate	46						
Diameter (cm)	<5.5	34	0.51			0.86		
	≥5.5	34						
LN metastasis	Negative	56	0.67			0.12		
	Positive	12						
AJCC stage	<IIb	43	0.89			0.27		
	≥IIb	25						
Nerve invasion	Negative	56	0.69			0.59		
	Positive	12						
Bile duct/vascular cancer embolus	Negative	42	0.90			0.29		
	Positive	26						
Satellite nodules	Negative	48	0.63			0.45		
	Positive	20						
Stromal TILs	Negative	36	<0.01	1	<0.01	<0.01	1	<0.01
	Positive	32		2.88(1.44-5.76)			2.84(1.48-5.46)	

TILs: Tumor-Infiltrating Lymphocytes; LN: Lymph Node; HR: Hazard Ratio; CI: Confidence Interval.

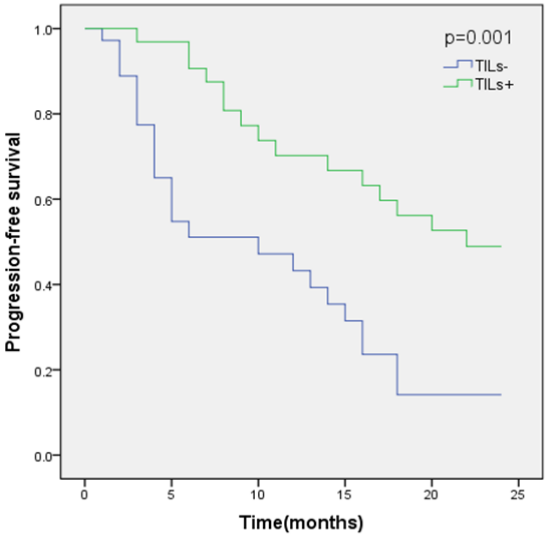

Figure 2: Graph of progression-free survival based on TIL groups.
TIL: Tumor-Infiltrating Lymphocyte.

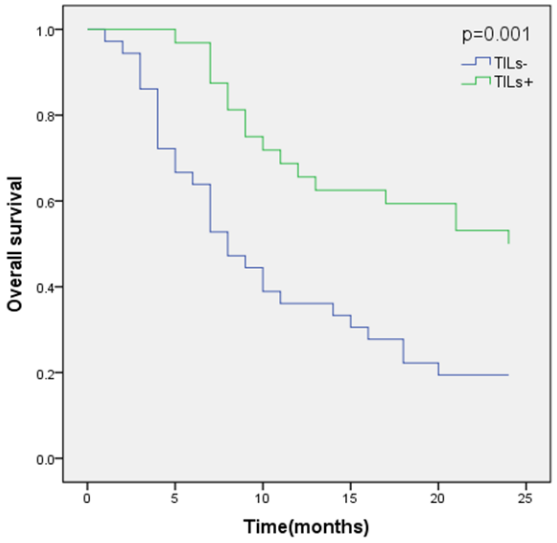

Figure 3: Graph of overall survival based on TIL groups.
TIL: Tumor-Infiltrating Lymphocyte.

III Comparison of PFS-Related Clinical Variables after Hepatectomy

In the univariate analysis, the CA19-9 level and stromal TILs (both $p<0.05$) were important prognostic factors for PFS after hepatectomy (Table 3). We selected the above variables as independent risk factors in the multivariate analysis, which showed that a stromal TIL negative status (HR=2.88, 95% CI 1.44-5.76, $p<0.01$) was an important independent predictor for PFS (Table 3). The PFS curve also demonstrated the predictive value of stromal TILs (Figure 3).

IV Clinical Parameters for a TIL Negative and TIL Positive Status in ICC Patients

As shown in (Table 1), the difference in the tumor markers CA125 and CA19-9 between the two groups was statistically significant ($P=0.03$, $P<0.01$). There were no significant differences between the two groups in terms of clinical quantitative parameters such as age, AFP, platelets, lymphocytes, neutrophils, monocytes and diameter.

Comparisons of the categorical variables in the TIL-negative and -positive groups indicated that the CA19-9 level ($P=0.05$), differentiation ($P=0.02$), tumor diameter ($P=0.05$), bile duct/vascular cancer embolus ($P=0.03$) and satellite nodules ($P=0.02$) showed significant differences according to stromal TIL density (Table 2). There were no significant differences in sex, age, the CA125 level, tumor location, lymph node (LN) metastasis, AJCC stage or nerve invasion between the TIL-negative group and the TIL-positive group (Table 2).

Discussion

We conducted the current study by evaluating pathological sections to confirm the prognostic value of stromal TILs in ICC patients whose primary lesions were completely resected. The results showed that negative stromal TILs were a poor prognostic index for the OS of ICC patients ($P<0.01$), which was consistent with the results from other tumors, such as breast cancer, lung cancer and colorectal cancer [6-10]. Such results show that lymphocytes may play an important role in restricting the development of tumors. Our data also showed that 52.94% (36/68) of ICC patients had a stromal TIL negative status, which suggests that many ICC patients may have a poor prognosis after complete resection. These results may lead to a poor prognosis in patients with ICC. As the tumor tissue of ICC is poorly vascularized, lymphocyte infiltration is limited, which may lead to a poor prognosis in ICC patients.

Moreover, recurrence or metastasis was the main cause (66.17%) of death in ICC patients whose primary lesions were completely resected. Therefore, it is of great importance for us to identify an effective predictor of PFS after complete resection. Our data showed that low levels of stromal TILs were also associated with PFS ($P<0.001$). The stromal TIL density reflects the environmental state of the tumor's immunogenicity. A lack of an effective immune environment will facilitate recurrence or metastasis. Studies have shown that cancer cells can form an immunosuppressive environment conducive to immune escape through a series of mechanisms that inhibit TIL infiltration (e.g., inhibiting IFN-I signaling) [18]. Ogiya's study demonstrated that immune escape plays a role in tumor progression by comparing the stromal TILs of patients with primary and metastatic breast cancer [19]. The above analysis showed that the stromal TILs were closely related to metastasis, OS and recurrence after complete resection. Therefore, we further compared TIL-negative and TIL-positive patients. Our data indicated that a low level of stromal TILs is related to a high level of CA19-9, a large tumor diameter, high differentiation, a positive bile duct/vascular cancer embolus and positive satellite nodules. This phenomenon can be explained by the lack of an effective immune environment. Unconstrained tumor progression often promotes high levels of serum tumor markers, increases tumor burden and tumor differentiation, and promotes bile duct/vascular cancer embolus and satellite nodules.

Studies have shown that the 1, 3 and 5-year survival rates of patients are related to tumor stage, LN metastasis, vascular invasion and tumor number at the time of surgery [20, 21]. Vascular invasion and lymph node metastasis indicate a higher possibility of postoperative recurrence, while a tumor size larger than 5cm, tumor number, vascular invasion and peripheral nerve invasion suggest early recurrence [20-22]. Our study did not reach the above conclusions; such disagreement may be because of individual differences, the cutoff value or the grouped criterion for analysis. Although our data are not completely consistent with the above reports, the overall trend of relevant data is consistent. Moreover, our data also indicate that other clinical parameters, such as CA19-9 ($P=0.03$) and CA125 ($P=0.02$) levels, which are clinically widely used serum markers as prognostic indicators, are also independent predictors of poor OS in ICC patients.

Conclusion

Our study demonstrated that the stromal TIL status was an important indicator of OS and PFS in ICC patients. Lymphocytes may be a potential treatment for ICC. However, the present study did not examine the inhibitory effect of lymphocytes on tumor tissues at the cellular and molecular levels or evaluate the role of certain lymphocytes in ICC. Further studies are needed to study the inhibitory effect of lymphocytes on tumor tissues at the cellular and molecular levels and to elucidate the specific mechanism by which certain lymphocytes affect ICC cells. Overall, as a simple, inexpensive, convenient and effective prognostic indicator, stromal TILs play a favorable role in predicting PFS and OS in patients with ICC after complete resection.

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Availability of Data and Materials

Please contact the corresponding author with requests for data and materials.

Author Contributions

This study was designed by Haibo Yu and Yuting He. Yuting He, Kunfu Dai and Xiaopei Hao conducted the HE specimen collection and data acquisition; Li Zhen and Xu Mei conducted the evaluation of tumor-infiltrating lymphocytes; Yuting He, Kungfu Dai, Zhen Li, Mei Xu, Haibo Yu conducted the statistical analysis and interpreted the data. Haibo Yu and Yuting He wrote the manuscript. All authors reviewed and accepted the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of Henan Provincial People's Hospital, (2015) Ethics Review No. (27). The research was conducted in accordance with the principles of the 1964 Declaration of Helsinki and its later amendments.

Consent for Publication

Not Applicable.

Competing Interests

None.

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