Cross-Match as an Immuno-Oncological Risk Factor for Hepatocellular Carcinoma Recurrence and Inferior Survival After Living Donor Liver Transplantation: A Call for Further Investigation

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ABSTRACT

BACKGROUND: The success of immunotherapy for patients with hepatocellular carcinoma (HCC) suggests that immune dysregulation occurs in HCC patients. This warrants an immuno-oncological risk assessment in the platform of liver transplantation.

METHODS: This retrospective single-center study analyzed risk factors for—particularly cross-matching performed through conventional complement-dependent cytotoxicity cross-match tests—and the outcomes of HCC recurrence following living donor liver transplant.

RESULTS: A total of 71 patients were included. The median follow-up period was 29.1 months; 17 (23.9%) patients had posttransplant HCC recurrence, and their 1-, 3-, and 5-year-survival rates were 70.6%, 25.7%, and 17.1%, respectively, which were inferior to those of patients without HCC recurrence (87.0%, 80.7%, and 77.2%, respectively; $P < .001$). In addition to microvascular invasion, positive cross-match results for B cells at 37°C (B- 37°C) or T cells at 4°C (T- 4°C) were associated with inferior overall survival in multivariable analysis after adjustment for tumor status beyond Milan criteria and elevated alpha-fetoprotein levels. Rejection alone cannot be the mechanism underlying the effects of positive cross-match results on patient outcomes. Adjusted survival curves suggested that positive cross-match B- 37°C or T- 4°C was associated with inferior recurrence-free and patient survival, but the robustness of the finding was limited by insufficient power.

CONCLUSIONS: Additional large-scale studies are required to validate positive cross-match as an immuno-oncological factor associated with HCC recurrence and inferior patient survival.

KEYWORDS: Hepatocellular carcinoma, liver transplantation, recurrence, risk factor, immuno-oncology, cross-match

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Introduction

Hepatocellular carcinoma (HCC) is the fourth commonest cause of cancer-related deaths worldwide.[1] Liver transplantation is its most definitive treatment option, as it removes the tumor and cirrhotic tissue field prone to future carcinogenesis.[1,2] The Milan criteria were first established to define tumor burden restrictions for liver transplantations to achieve excellent long-term outcomes.[3] The risk of HCC recurrence, however, affects 15% to 20% of related patients.[4] These patients still have an unfavorable prognosis,[5] even in the era of immunotherapy.[6-9] Therefore, HCC recurrence continues to be an issue following transplantation.[10]

Risk factors for HCC recurrence after liver transplantation were thoroughly reviewed and summarized by Filgueira.[4] Most factors related to tumors and patients are shared between curative resection and liver transplantation. Potential risk factors that are unique to transplantation include waiting time, donor age, ischemia time, piggyback technique, and immunosuppression.[4] The general belief that immunosuppression being a risk factor[11] and the success of immunotherapy[6,7] in HCC treatment suggests immune dysregulation occurs in patients with HCC. Specifically, this can occur due to an immunosuppressive tumor microenvironment or to chronic liver disease, resulting in a change to the net hypofunctionality of immune cells.[8] Cell quantity changes, such as the neutrophil-lymphocyte ratio, a marker of systemic inflammatory response, partly indicate the flavor,[12] although acellular serum may also contribute to the overall dysregulation process.

Therefore, we assume that the existence of serological biomarkers reflects immunological risk factors for patients with HCC. Whether this serological dysregulation interacts with healthy donor lymphocytes and further defines the risk of posttransplant recurrence is unknown.

In this study, we hypothesized that immune dysregulation, particularly positive cross-match, may have an effect on HCC recurrence and patient survival after transplantation. By using the platform of living donor liver transplantation, a common practice in Asia used to solve the problem of scarcity of deceased

donors, we analyzed the risk factors of being predisposed to HCC recurrence following liver transplantation.

Methods

Subjects

Cases of patients who received living donor liver transplantation for HCC between March 2001 and May 2019 at National Taiwan University Hospital were collected. Patients who derived the grafts from deceased donors, did not receive a transplant for HCC, or did not receive cross-match tests were excluded. We reviewed medical charts to retrospectively collect patients' demographic data. The diagnosis of HCC prior to liver transplant was confirmed through the execution of histopathological tissue examination or observation of a typical contrast-enhanced image pattern.[13] The University of California San Francisco (UCSF) criteria constituted the basis for HCC selection for this transplant series; these criteria are outlined as follows: a single tumor nodule with a diameter of up to 6.5 cm or 3 or fewer tumors—with the largest tumor having a diameter of ⩽4.5 cm and the sum of the tumor diameters being ⩽8 cm—without extrahepatic metastasis.[14,15] The policy for patient follow-up for, detection of, and management of HCC recurrence was as described previously.[13] The Institutional Review Board of National Taiwan University Hospital, Taipei, approved the study (NTUH REC: 201410006RINA). Because this was a retrospective study using chart review, the mentioned board waived the need for informed consent.

Cross-matching

Cross-matching was performed through conventional complement-dependent cytotoxicity tests at the hospital laboratory. The technique involved isolating donor B and T lymphocytes, which were then separately tested against recipient serum. Complement was added to the mixed recipient serum and donor lymphocytes, and lymphocyte lysis was observed at 4°C and 37°C, respectively.[16] Before July 2013, cross-match tests were performed mostly in living donor but not in deceased donor liver transplant. Subsequently, antibody-mediated rejection became an emerging issue, and cross-match tests came to be routinely performed. Cross-match test results did not influence the decision for liver transplant or the immunosuppression regimen.

Immunosuppression

The immunosuppression regimen included tacrolimus as the backbone of immunosuppressive drug therapy, steroids, and routine basiliximab induction. The target trough level of tacrolimus was maintained at 4 to 5 ng/mL, and the corresponding dosing was titrated monthly or bimonthly. Low-dose steroid therapy was also used in the maintenance phase. The maintenance level of immunosuppressants was not different between patients with or without a prior history of HCC. High-dose corticosteroid therapy (pulse therapy) was used to treat acute T-cell-mediated rejection when diagnosed using liver biopsy. Additional immunosuppressants—mycophenolate mofetil or everolimus—were added after rejection episodes.

Demographic parameters

Demographic information was collected, including sex, age, hepatitis B virus (HBV) or hepatitis C virus (HCV) status, history of alcohol use, liver cirrhosis, prior hepatectomy, tumor status at transplant (beyond Milan criteria and microvascular invasion), alpha-fetoprotein (AFP) level at transplant, waiting period for transplant, donor and recipient relationship, human leukocyte antigen (HLA) mismatches, occurrence and date of first biopsy-proven rejection, and B- and T-cell cross-match test results. A single-antigen assay for the detection of donor-specific antibodies (DSAs) was not performed before liver transplant. All living donors were relatives and were further classified as offspring (children) or nonoffspring. The maintenance level of tacrolimus (FK) was the trough level measured at the last visit.

Outcome measurement

The event date was the recurrence of HCC. The patients were followed up until death or December 31, 2019.

Statistical analysis

The Student t test, the χ^2 test, Fisher's exact test, or the Mann-Whitney U test was used, where appropriate, for the comparison of variables. Data were expressed as mean ± standard deviation, median (interquartile range [IQR]), or number (percentage) when appropriate. Cumulative survival rates were estimated using the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazard model was used for univariable and multivariable analyses, the latter of which was employed to adjust for potential confounding factors, to identify prognostic factors, and to depict adjusted survival curves. Statistical significance was considered when the 2-sided P value was $< .05$. Analyses were performed using SPSS version 21.0 (IBM Corporation, Armonk, NY, USA).

Results

Demographic characteristics

A total of 71 living-related liver recipients with a pretransplant diagnosis of HCC and cross-match data were identified among the 668 patients who received liver transplantation before the end of the follow-up in December 2019 (Figure 1). The median follow-up time was 29.1 months, and 17 (23.9%) patients had posttransplant HCC recurrence during the follow-up period. Table 1 shows the demographic characteristics of the patients. Compared with the group without HCC recurrence, the HCC recurrence group had a shorter median follow-up time, a higher

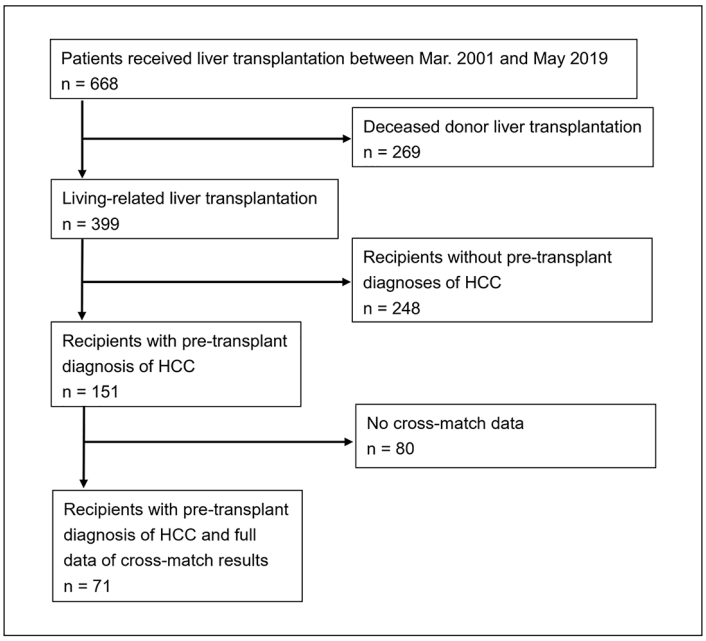

Figure 1. Schematic representation of the patient selection process. HCC indicates hepatocellular carcinoma.

incidence of prior hepatectomy, a worse HCC status beyond Milan criteria at transplant, more microvascular invasion of HCC at explant, higher serum AFP levels (and higher frequency of elevation >400 ng/mL) at transplant, and a higher maintenance FK level. The 2 groups did not differ in terms of the following variables: sex, age, HBV status, HCV status, history of alcohol use, presence of liver cirrhosis, waiting period for transplant, offspring donor grafts, high degrees (>3) of HLA mismatches in grafts, or occurrence rate of biopsy-proven rejection. Compared with 54 patients without recurrence, these 17 patients had a higher frequency of positive cross-match B- 4°C, B- 37°C, and T- 4°C, and lower T- 37°C, but the differences were without significance (Table 1).

Recurrence-free and overall survival

The 1-, 3-, and 5-year recurrence-free survival rates in the 71 living-related liver transplant recipients were 82.8%, 70.0%, and 61.3%, respectively. The 1-, 3-, and 5-year survival rates in the with- and without-HCC recurrence groups were 70.6%, 25.7%, and 17.1%, and 87.0%, 80.7%, and 77.2%, respectively (Figure S1). Crude patient survival in the without recurrence group was higher than that in the recurrence group ($P < .001$).

Significance of positive cross-match for HCC recurrence and patient survival

The Kaplan-Meier curves for recurrence-free and overall survival in patients with and without positive cross-match are shown in Figure 2. Crude recurrence-free survival in the positive group was lower than that in the negative group for B- 37°C ($P = .046$) and T- 4°C ($P = .026$). Crude patient survival in the positive group was lower than that in the negative group for B- 37°C ($P = .031$) and T- 4°C ($P = .065$). Table S1 presents the characteristics of variables selected on the basis of positive cross-match results for B cells at 37°C or T cells at 4°C. The positive group exhibited a higher incidence of prior hepatectomy and a poorer tumor status (beyond Milan, microvascular invasion, and AFP > 400 ng/mL) than did the negative group, although the differences did not reach statistical significance. The average FK level was maintained at 5.6 ng/mL in both groups. Biopsy-proven rejection occurred in 13 (18.5%) patients, all of whom were diagnosed as having acute T-cell-mediated rejection. Figure S2 illustrates Kaplan-Meier curves for the effects of positive cross-match results on rejection-free survival, indicating no significant difference between the groups.

Univariable risk factor analysis of recurrence-free and overall survival

Hepatocellular carcinoma status beyond the Milan criteria at transplant, microvascular invasion, elevated AFP levels, and positive cross-match results for B cells at 37°C or T cells at 4°C were factors associated with both recurrence-free and overall survival. Table 2 provides details about the corresponding hazard ratios (HRs) and 95% confidence intervals (CIs). For recurrence-free survival, the HR for positive cross-match results for B cells at 37°C was 2.58 (95% CI: 0.98-6.78; borderline significance) and that for positive cross-match results for T cells at 4°C was 3.35 (95% CI: 1.08-10.40). For overall survival, the HR for positive cross-match B- 37°C was 2.38

Table 1. Demographics of HCC patients receiving living donor liver transplantation.

	ALL N=71	NO RECURRENCE N=54	RECURRENCE N=17	*P* VALUE
Median follow-up time, months (IQR)	29.1 (14.6-58.0)	34.9 (17.0-62.9)	20.4 (9.8-29.0)	.031[a]
Sex (male, %)	51 (71.8)	38 (70.4)	13 (76.5)	.762
Age (mean, SD, years)	58.0 (6.6)	57.2 (6.7)	58.9 (6.1)	.528
HBV (n, %)	32 (45.1)	21 (38.9)	11 (64.7)	.093
HCV (n, %)	33 (46.5)	28 (51.9)	5 (29.4)	.163
Alcohol (n, %)	4 (5.6)	3 (5.6)	1 (5.9)	1.000
Cirrhosis (n, %)	65 (91.5)	51 (94.4)	14 (82.4)	.144
Prior hepatectomy (n, %)	16 (22.5)	8 (14.8)	8 (47.1)	.016
Tumor status at transplant				
Beyond Milan (n, %)	22 (31.0)	10 (18.5)	12 (70.6)	<.001
Microvascular invasion (n, %)	12 (16.9)	3 (5.6)	9 (52.9)	<.001
Alpha-fetoprotein at transplant				
Mean (SD) ng/mL	454.7 (2508.6)	89.3 (284.7)	1615.1 (5038.1)	<.001
>400 ng/mL (n, %)	7 (9.9)	1 (1.9)	6 (11.1)	.001
Waiting time period to transplant (median months, IQR)	4.4 (3.0-8.8)	4.3 (3.0-8.4)	4.4 (2.9-15.2)	.947[a]
Grafts from offspring donors (n, %)	54 (76.1)	43 (79.6)	11 (64.7)	.327
HLA mismatch >3 (n, %)	9 (12.7)	6 (11.1)	3 (17.6)	.439
Occurrence of biopsy-proven rejection (%)	13 (18.3)	9 (16.7)	4 (23.5)	.496
Cross-match				
+B- 4°C (n, %)	29 (40.8)	20 (37.0)	9 (52.9)	.270
+B- 37°C (n, %)	18 (25.4)	11 (20.3)	7 (41.2)	.112
+T- 4°C (n, %)	10 (14.1)	6 (11.1)	4 (23.5)	.237
+T- 37°C (n, %)	5 (7.0)	4 (7.4)	1 (5.9)	1.000
Last FK level (ng/mL) (mean, SD)	5.6 (2.9)	5.1 (2.4)	7.1 (3.7)	.012
Everolimus use (n, %)	9 (12.7)	6 (11.1)	3 (17.6)	.439

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; IQR, interquartile range; SD, standard deviation.
[a]Mann-Whitney U test.

(95% CI: 1.06-5.37) and that for T- 4°C was 2.54 (95% CI: 0.91-6.78; borderline significance).

Multivariable risk factor analysis of recurrence-free and patient survival

Table 3 presents results obtained from a multivariable analysis adjusted for tumor factors, cross-match positivity, and last FK level. Microvascular invasion was a strong covariate in both recurrence-free and patient survival. Elevated AFP levels played a significant role in recurrence-free survival but not in patient survival. Accordingly, HCC status beyond the Milan criteria at transplant became nonsignificant in both survival analyses. The adjusted HR for positive cross-match B- 37°C or T- 4°C was 1.91 (95% CI: 0.67-5.46) for recurrence-free survival and 2.16 (95% CI: 0.92-5.05) for patient survival. Figure 3 displays the adjusted survival curves for positive cross-match B- 37°C or T- 4°C, indicating inferior recurrence-free and patient survival; however, robustness of the analysis was limited by insufficient power.

Discussion

From a statistical perspective, traditional risk factors such as tumor status beyond Milan criteria, microvascular invasion, or elevated AFP carry a robust and large effect size in terms of

recurrence-free and overall survival. These might potentially obscure other "minor" factors with a smaller effect size (eg, "immunological" factors in this study), making them difficult to express as robust. Stratification is one solution, but in this sense, numerous events are required to demonstrate significance in the absence of those strong, traditional risk factors. This may result in a dilemma. In our study results, although the "immunological" factor of cross-match was not as robust as the traditional ones, positive cross-match B- 37°C or T- 4°C does imply insidious biological mechanisms of HCC recurrence that are unique to liver recipients and warrant further investigation.

From the perspective of immuno-oncology, immunological biomarkers are there to be discovered. For example, the high expression of CIITA (HLA II gene transactivator)-related major histocompatibility complex (MHC) II molecules in HCC tissue is linked to longer recurrence-free survival after curative resection.[17] Whereas normal hepatocytes do not express HLA class I and II, HCC cells strongly upregulate HLA class I while remaining negative for HLA class II.[18] The absence of HLA class II expression in HCC cell lines is correlated with a lack of CIITA expression.[18] This supports the hypothesis that MHC-II positive tumor cells could be recognized by the host immune system and help establish a protective immune response.[19]

Briefly, a cross-match involves placing recipient serum (potentially containing donor-specific anti-HLA antibodies) onto donor lymphocytes (containing HLAs).[20] A cytotoxic reaction (deemed "positive") suggests the presence of preformed DSAs.[17] T cells do not constitutively express HLA class II, so the result of a T-cell cross-match generally reflects antibodies to HLA class I only.[20] B cells, by contrast, express both HLA class I and II, so a positive B-cell cross-match may be due to antibodies directed against HLA class I, II, or both.[20] However, assays are not specific and are frequently confounded by cross-reactive epitope groups (CREGs).[21] Cross-reactive epitope groups are shared HLA epitopes through which a single antibody can react to several HLA molecules.[21] We could

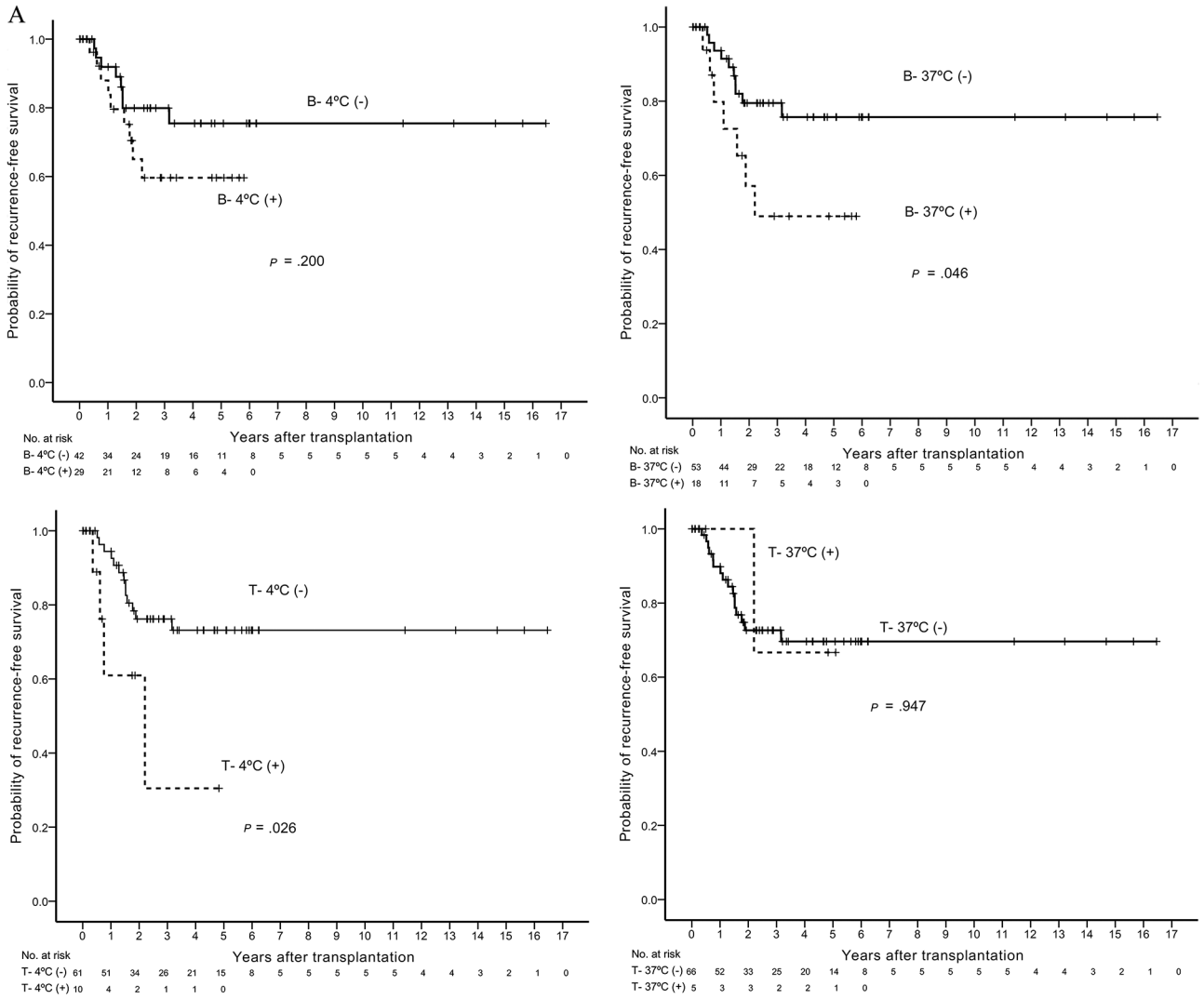

Figure 2. *(Continued)*

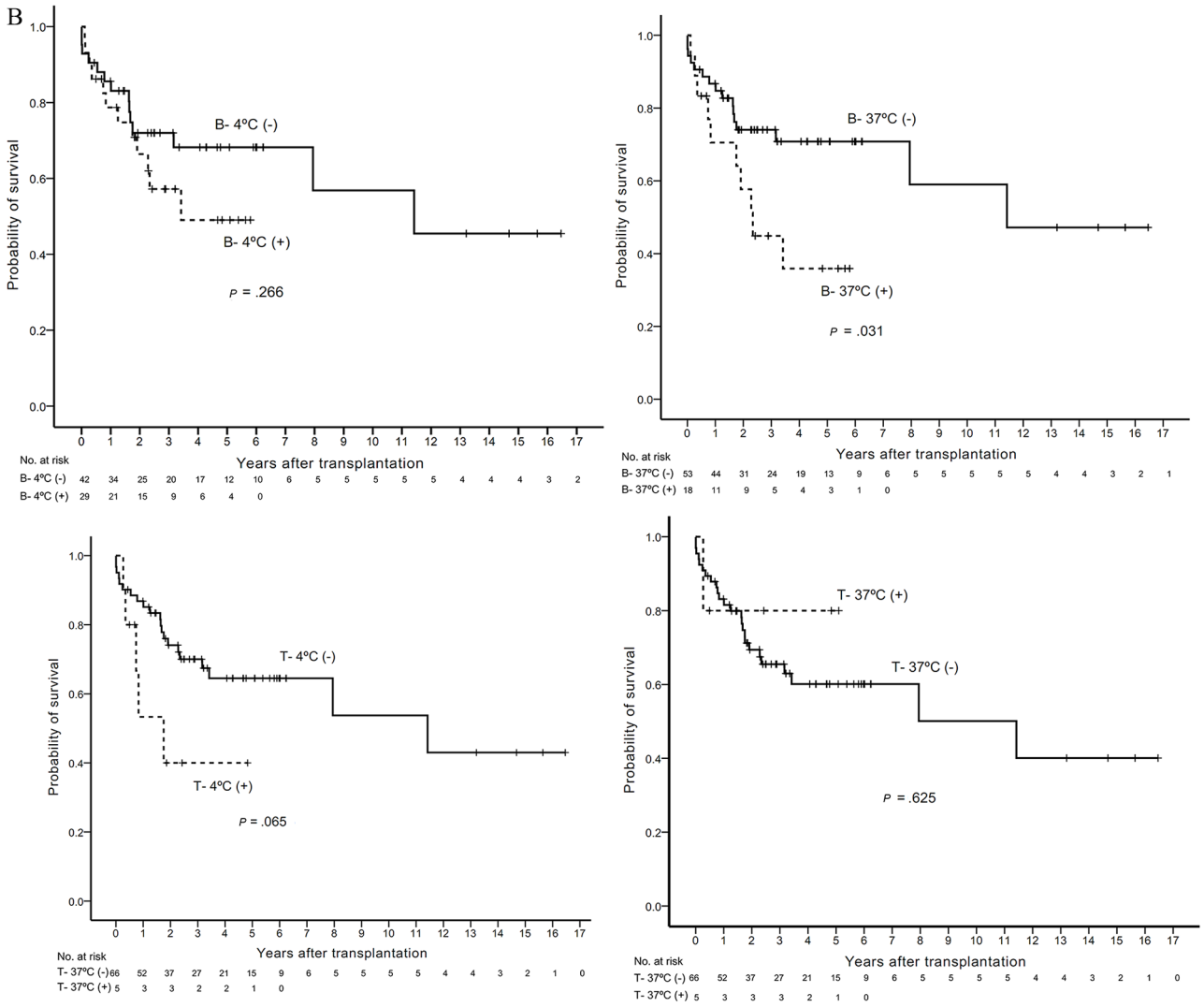

Figure 2. Significance of positive cross-match in recurrence-free survival and overall survival in patients with HCC. (A) Kaplan-Meier curves for recurrence-free survival and (B) Kaplan-Meier curves for overall survival. HCC indicates hepatocellular carcinoma.

not determine whether CREGs had confounding effects in our study, although their influence is possible. The role of these nonspecific interactions as an immuno-oncological risk factor may have vanished in the single-antigen assay conducted for the detection of specific DSAs. Because our study revealed that positive cross-match T- 37°C played no role in recurrence-free or overall survival, we can assume that HLA-I is not involved in the underlying biological mechanism of posttransplant HCC recurrence, if it even exists. Although no validated immunological biomarkers for HCC are currently in clinical application, transplant oncology should be a good platform to start on.[9]

Tumor-derived exosomes can interact with immune effector cells in the tumor microenvironment and in the circulation, delivering negative signals to these cells and interfering with their antitumor functions.[22] Hepatocellular carcinoma–derived exosomes have been reported to attenuate the cytotoxicity of T cells and natural killer cells as well as promote immunosuppressive M2 macrophages and regulatory B cells.[23] Exosomes could regulate complement activity and contribute to the pro- and anti-inflammatory immune balance.[24] Exosomes derived from pancreatic ductal adenocarcinoma can display a large repertoire of tumor antigens that induce autoantibodies and exert a decoy function against complement-mediated cytotoxicity.[25] The interaction of tumor-reactive immune cells, which are probably extinct in tumor-bearing patients but otherwise circulate in healthy live liver donors, with HCC-derived exosomes during cross-matching might be a plausible biological mechanism explaining our observation. In addition to traditional risk factors (viz, tumor grade and microvascular invasion), cross-match reactivity may serve as a reference for immunosuppressive regimen adjustment and aggressive tumor surveillance after liver transplant.

The limitations of this study are as follows. The single-center setting results are based primarily on living donor liver transplantation and might not be exploitable to deceased liver transplantation. Furthermore, this small cohort could reduce the power of the results and model robustness. Further large-scale studies are required to validate our findings.

Table 2. Univariable risk factor analysis of recurrence-free and patient survival after living donor liver transplantation.

VARIABLE	RECURRENCE-FREE SURVIVAL		OVERALL SURVIVAL	
	HR, 95% CI	*P* VALUE	HR, 95% CI	*P* VALUE
Male sex	1.44 (0.47-4.4)	.522	2.31 (0.79-6.72)	.124
Age	1.04 (0.96-1.13)	.286	1.05 (0.98-1.13)	.143
HBV	2.29 (0.85-6.20)	.103	1.22 (0.55-2.70)	.622
HCV	0.43 (0.15-1.23)	.115	0.90 (0.41-2.01)	.809
Alcohol	1.06 (0.14-8.03)	.954	0.78 (0.11-5.79)	.808
Cirrhosis	0.38 (0.11-1.32)	.128	0.67 (0.20-2.24)	.511
Prior hepatectomy	2.86 (1.10-7.43)	.031	1.54 (0.68-3.51)	.305
Beyond Milan	5.87 (2.05-16.84)	.001	2.20 (1.00-4.86)	.052
Microvascular invasion	8.99 (3.40-23.80)	<.001	3.33 (1.40-7.92)	.007
Alpha-fetoprotein >400 ng/mL	11.82 (4.17-33.48)	<.001	3.64 (1.44-9.20)	.006
Graft from offspring donors	0.53 (0.20-1.44)	.214	0.78 (0.34-1.80)	.555
HLA mismatch >3	2.33 (0.67-8.11)	.185	1.98 (0.67-5.81)	.216
Occurrence of biopsy-proven rejection	1.25 (0.41-3.84)	.697	1.37 (0.57-3.28)	.482
Cross-match				
+B- 4°C	1.85 (0.71-4.80)	.207	1.57 (0.70-3.50)	.271
+B- 37°C	2.58 (0.98-6.78)	.055	2.38 (1.06-5.37)	.037
+T- 4°C	3.35 (1.08-10.40)	.036	2.49 (0.91-6.78)	.074
+T- 37°C	0.93 (0.12-7.06)	.947	0.61 (0.08-4.52)	.629
Last FK level	1.28 (1.11-1.48)	.001	1.15 (1.03-1.29)	.016
Everolimus use	1.17 (0.34-4.09)	.802	0.61 (0.18-2.05)	.422

Abbreviations: CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; HR, hazard ratio.

Table 3. Multivariable risk factor analysis of recurrence-free and overall survival after living donor liver transplantation.

VARIABLE	RECURRENCE-FREE SURVIVAL		OVERALL SURVIVAL	
	HR, 95% CI	*P* VALUE	HR, 95% CI	*P* VALUE
Beyond Milan	2.12 (0.62-7.31)	.233	1.17 (0.48-2.85)	.727
Microvascular invasion	7.86 (2.71-22.84)	<.001	2.86 (1.11-7.39)	.030
Alpha-fetoprotein >400 ng/mL	5.50 (1.35-22.36)	.017	2.23 (0.69-7.15)	.178
+B- 37°C or T- 4°C	1.91 (0.67-5.46)	.223	2.16 (0.92-5.05)	.076
Last FK level	1.16 (0.96-1.40)	.130	1.08 (0.95-1.24)	.247

Abbreviations: CI, confidence interval; HR, hazard ratio.

Conclusions

In addition to traditional risk factors (tumor status beyond Milan criteria at transplant, microvascular invasion, and elevated AFP), positive cross-match B- 37°C or T- 4°C might suggest inferior recurrence-free and patient survival, but the robustness of this result was limited by insufficient power. Additional large-scale studies are required to validate this as an immuno-oncological factor associated with HCC recurrence and inferior patient survival.

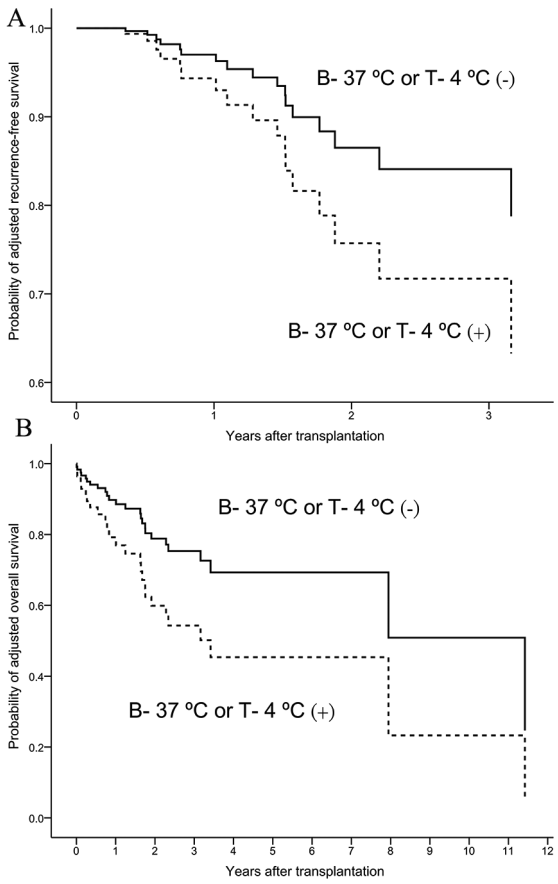

Figure 3. Adjusted survival curves for positive cross-match in (A) recurrence-free and (B) overall survival.

Author's Note

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Author Contributions

C.-M.H. drafted the manuscript and with R.-H.H., P.-H.L., and M.-C.H. designed the study. C.-M.H., R.-H.H., and Y.-M.W. were involved in data processing. C.-M.H. and R.-H.H. performed statistical analysis. C.-M.H. was the director responsible for general organization and instruction. All authors read and approved the final version of the manuscript.

Availability of Data and Material

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

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Supplemental Material

Supplemental material for this article is available online.

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