

Preoperative blood neutrophil count predicts survival in hepatocellular carcinoma patients with living donor liver transplantation

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Background: The Milan criteria (MC) used to select patients for liver transplantation among patients with hepatocellular carcinoma (HCC) do not include tumor biology. Furthermore, systemic inflammatory markers have been identified to predict tumor biology. The present study investigated prognostic value of systemic inflammatory markers, including neutrophil count, in predicting the prognosis of patients with HCC undergoing living donor liver transplantation (LDLT).

Methods: We retrospectively analyzed data regarding peripheral blood inflammatory markers, as well as patient and tumor characteristics of patients with HCC who underwent LDLT. Univariate and multivariate analyses were performed to analyze variables associated with survival.

Results: A total of 103 patients with HCC who underwent LDLT were included. The 3- and 5-year recurrence-free survival (RFS) in patients with a high neutrophil count ($>2,640/\mu\text{L}$) were significantly lower than those in patients with a low neutrophil count ($\leq 2,640/\mu\text{L}$; 70.0% and 64.7% vs. 88.3% and 84.6%, respectively; $P=0.02$). Patients with a high neutrophil count also had lower 5-year overall survival (OS; 63.9% vs. 79.3%, $P=0.03$). In multivariate analysis, radiologic MC (hazard ratio [HR], 5.04; $P=0.02$) and neutrophil count (HR, 4.47; $P=0.04$) were independent factors predicting RFS. Among patients exceeding the MC, those with a high neutrophil count had significantly lower 5-year RFS than those with low neutrophil count (10% vs. 83%; $P<0.01$).

Conclusions: We demonstrated that high preoperative neutrophil count is associated with poor RFS and OS in patients with HCC undergoing LDLT.

Keywords: Milan criteria; Prognosis; Recurrence-free survival; Neutrophil; Protein induced by vitamin K absence-II

HIGHLIGHTS

- All patients with hepatocellular carcinoma (HCC) who underwent living donor liver transplantation (LDLT) were examined to investigate prognostic value of systemic inflammatory markers, including neutrophil count, in predicting the prognosis.
- Patients with a high neutrophil count ($>2,640/\mu\text{L}$) had lower recurrence-free survival (RFS) and overall survival (OS).
- Among patients exceeding the Milan criteria, those with a high neutrophil count had significantly lower 5-year RFS than those with low neutrophil count.
- High preoperative neutrophil count is associated with poor RFS and OS in patients with HCC undergoing LDLT.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the fourth most common cause of cancer-related death. More than 80% of patients with HCC have cirrhosis, and as such, the ideal treatment method for early unresectable HCC in such patients is liver transplantation (LT), which provides a cure for the underlying liver cirrhosis as well as complete tumor clearance.

Although results of LT for HCC in the early era of LT were unsatisfactory due to unsuccessful patient selection [1,2], introduction of the Milan criteria (MC; single tumor ≤ 5 cm or ≤ 3 tumors, each ≤ 3 cm, without vascular invasion) by Mazzaferro et al. [3] in 1996 allowed for improved results. The expected 5-year survival rates after LT for HCC meeting the MC are 65% to 80%, while the overall recurrence rate has been reported to be 8% after 4 years of follow-up. Currently, expanded criteria for patients with HCC have been proposed due to donor liver shortage and the reported nonsignificant differences compared with the MC in terms of post-LT survival, which have been externally validated [4-6].

The MC remain the most frequently adopted criteria for patient selection worldwide and have shown excellent results; however, the risk of post-LT HCC recurrence persists, with recurrence rates ranging from 6% to 17% [7]. The MC rely solely on preoperative radiologic characteristics, and there is a discrepancy in both tumor size and number when compared with histology [8-10]. In addition,

most importantly, because imaging provides little insight into tumor biology, the MC do not identify patients at a high risk of recurrence due to aggressive tumor, such as microvascular invasion and poorly differentiated tumor [9]. Although tumor biopsy is helpful to identify high-risk group of recurrence, biopsy for grading of HCC poses concerns over tumor seeding and risk of bleeding, especially in patients with underlying liver cirrhosis. Therefore, identifying noninvasive surrogate markers of HCC aggressiveness is essential to predict tumor biology.

There is growing evidence suggesting that inflammatory markers (resulting from a systemic inflammatory response) can be used to predict outcomes in various types of tumors, including HCC. For example, elevations in circulating neutrophils, neutrophil-to-lymphocyte ratio (NLR), and tumor-infiltrating neutrophils have been associated with poor outcomes in various cancers, including HCC [11-13]. Neutrophils make up the majority of peripheral leukocytes and are the first responders to sites of inflammation and disease progression. Previous studies reported that a high neutrophil count is associated with angiogenesis and immunosuppression, which facilitate tumor progression [14,15].

The aim of our study was to evaluate the predictive value of NLR and neutrophil count, in patients with HCC undergoing living donor liver transplantation (LDLT) and to determine the utility of these markers as a selection criteria for patients who would most benefit from LDLT.

METHODS

We retrospectively analyzed patients with HCC who underwent LDLT between 2008 and 2016. Data on patients' demographic and clinical characteristics, laboratory results, imaging findings, and explant pathology were collected retrospectively. We also collected data on the types of pretransplant therapies performed. Peripheral blood cell counts were determined on the day before or the day of transplant. Due to a shortage of donations, most patients underwent deceased donor liver transplantation (DDLT) when their condition deteriorated. We excluded DDLT recipients whose neutrophil count fluctuated more than that in LDLT recipients due to infection, bleeding, and/or medications. The NLR was defined as the absolute neutrophil count divided by the absolute lymphocyte count. There was no demonstrable infection in any of the

patients included in the study. Recurrence-free survival (RFS) was calculated from the date of LT to the date of confirmed radiologic recurrence or death. Overall survival (OS) was calculated from the date of LT to the date of death or last follow-up. This study was reviewed and approved by the ethical committee of our center.

Statistical Analysis

Continuous variables were expressed as median and range. Differences between categorical variables were calculated using the chi-square test. Univariate and multivariable analyses of between survivals and the study variables was performed using Cox proportional hazard models. All variables showing statistical significance in univariate analysis were included in the multivariate model. Survival differences were calculated using the Kaplan-Meier method with the log-rank test. Statistical analysis was performed using IBM SPSS ver. 21.0 (IBM Corp., Armonk, NY, USA). A P-value <0.05 was considered statistically significant.

RESULTS

Patient Characteristics

A total of 103 patients with HCC underwent LDLT during the study period. Among the 103 patients, 78.6% were

male and the median age was 53 years (range, 24–74 years). The majority of patients had an associated viral infection: 78.6% of patients had hepatitis B and 9.7% had hepatitis C virus infection. Preoperatively, 72.8% of patients fulfilled the MC. The majority of patients had Child-Pugh score A (71.8%) or B (21.3%) and the median model for end-stage liver disease score was 8 (range, 3–49). Pretransplant treatment was performed in 50 patients (48.5%). Transarterial chemoembolization was performed in 45 of patients (43.7%), radiofrequency ablation in nine patients (8.7%) and hepatic resection in six (5.8%).

Tumor Characteristics

Among the 103 patients included in the study, 70 (68.0%) fulfilled the MC based on final pathology of the explanted liver. Thus, there was a 4.8% discordance between radiologic and histologic evaluations. Seventeen patients (16.5%) were diagnosed as having poorly differentiated HCC, and microvascular invasion was found in 19.4%.

Survival Analysis and Predictors of RFS and OS

The median follow-up time was 50 months. At the end of the follow-up period, 22 patients (21.4%) died and 20 (19.4%) were confirmed to have tumor recurrence. The 3- and 5-year RFS rates were 81.4% and 79.5%, respectively, while the 3- and 5-year OS rates were 80.7% and 77.7%, respectively. Table 1 summarizes the results of factors associated with RFS. As shown, α -fetoprotein (AFP) >100 ng/

Table 1. The predictive factors that influence the recurrence-free survival

Variable	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Sex (male:female)	1.66 (0.49–5.66)	0.42		
Age (>60 yr)	0.74 (0.10–5.52)	0.77		
Hepatitis B virus (yes)	0.60 (0.18–2.04)	0.41		
AFP (>100 ng/mL) ^{a)}	4.44 (1.48–13.28)	0.008	3.28 (0.93–12.95)	0.09
PIVKA-II (>100 mAU/mL) ^{a)}	5.87 (1.72–20.05)	0.005	1.42 (0.35–5.85)	0.63
Radiologic Milan criteria (beyond)	4.83 (1.97–11.86)	0.001	5.04 (1.32–19.29)	0.02
Neutrophil (>2,640/ μ L) ^{b)}	2.74 (1.12–6.72)	0.03	4.47 (1.09–18.33)	0.04
NLR >2.4 ^{b,c)}	4.05 (1.47–11.17)	0.01		
Maximal tumor size (>2.8 cm) ^{b)}	3.11 (1.13–8.56)	0.03	0.88 (0.27–2.87)	0.88
ES grade III/IV	4.83 (2.01–11.69)	0.001	1.23 (0.38–3.96)	0.73
Microvascular invasion (yes)	8.52 (3.44–21.07)	<0.001	2.44 (0.46–12.93)	0.30
Macrovacular invasion (yes)	10.49 (4.2–26.27)	<0.001	5.60 (0.83–37.53)	0.08

HR, hazard ratio; CI, confidence interval; AFP, α -fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II; NLR, neutrophil-to-lymphocyte ratio; ES, Edmondson-Steiner.

^{a)}Value from previous studies; ^{b)}Median value was used to stratify the groups; ^{c)}NLR was excluded in multivariate model.

mL, protein induced by vitamin K absence or antagonist-II (PIVKA-II) >100 mAU/mL, beyond the MC, neutrophil count >2,640/ μ L, NLR >2.4, maximum tumor size >2.8 cm, poorly differentiated HCC, and presence of vascular invasion were significant preoperatively available predictors of poor RFS in univariate analysis. Only factors beyond the MC and neutrophil count >2,640/ μ L were found to be independent predictors of poor RFS in multivariate analysis. The association with recurrence for previous treatment history was also analyzed, but it was not significant (data not shown).

Univariate and multivariate analyses of factors affecting OS are shown in Table 2. AFP >100 ng/mL, PIVKA-II >100 mAU/mL, neutrophil count >2,640/ μ L, NLR >2.4, and presence of vascular invasion were significant preoperatively

available predictors of poor OS in univariate analysis. Only microvascular invasion and neutrophil count >2,640/ μ L were found to be an independent predictor of poor OS in multivariate analysis.

Prediction of HCC Recurrence and Survival Using Neutrophil Count

We analyzed HCC recurrence and patient survival using the preoperative factors found to be significant in multivariate analysis, namely radiologic MC and neutrophil count. Forty-two patients (40.8%) were found to have high neutrophil count (>2,640/ μ L). Median value of neutrophil count at the time of recurrence was 2,670/ μ L (range, 1,270–6,690/ μ L).

Table 2. The predictive factors that influence the overall survival

Variable	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Sex (male:female)	6.24 (0.84–46.46)	0.74		
Age (>60 yr)	1.63 (0.38–7.01)	0.51		
Hepatitis B virus (yes)	0.63 (1.87–2.14)	0.46		
AFP (>100 ng/mL) ^{a)}	3.05 (1.30–7.14)	0.01	3.21 (1.02–10.14)	0.05
PIVKA-II (>100 mAU/mL) ^{a)}	6.02 (2.52–14.35)	<0.001	2.48 (0.87–7.05)	0.07
Neutrophil (>2,640/ μ L) ^{b)}	2.54 (1.08–5.95)	0.03	2.80 (1.06–7.33)	0.04
NLR >2.4 ^{b)}	2.76 (1.12–6.78)	0.03		
ES grade III/IV	2.91 (1.22–6.95)	0.02	2.79 (1.06–7.33)	0.40
Microvascular invasion (yes)	6.21 (2.66–14.42)	<0.001	5.69 (1.77–18.26)	0.003
Macrovascular invasion (yes)	5.45 (2.22–13.42)	<0.001	0.80 (0.22–2.90)	0.73

HR, hazard ratio; CI, confidence interval; AFP, α -fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II; NLR, neutrophil-to-lymphocyte ratio; ES, Edmonson-Steiner.

^{a)}Value from previous studies; ^{b)}NLR was excluded in multivariate model.

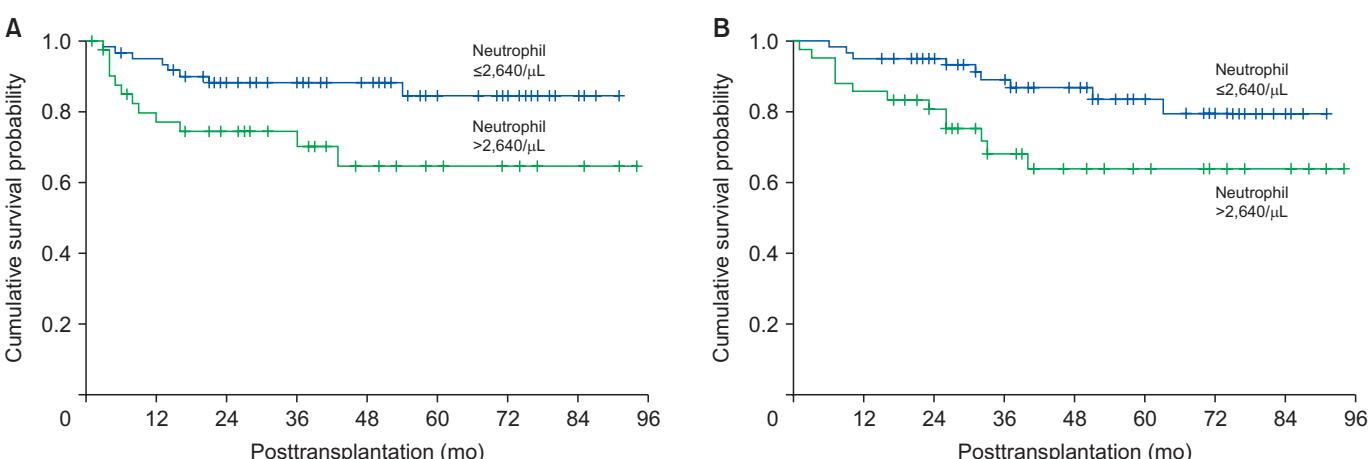


Fig. 1. Kaplan-Meier curve comparing recurrence-free survival (A) and overall survival (B) for patients classified according to the neutrophil counts.

The 3- and 5-year RFS in patients with high neutrophil count ($>2,640/\mu\text{L}$) were significantly lower than those in patients with low neutrophil count (70.0% and 64.7% vs. 88.3% and 84.6%, respectively; $P=0.02$) (Fig. 1A). Patients with high neutrophil count also had lower 5-year OS (63.9% vs 79.3%, $P=0.03$) (Fig. 1B). When categorizing patients according to the MC, patients with high neutrophil count with factors beyond the MC had significantly lower RFS than those with low neutrophil count (5-year survival, 10% vs. 83%; $P<0.01$) (Fig. 2A). There was no difference between patients with low and high neutrophil counts who fit the MC (5-year survival, 86% vs. 89%; $P=0.97$) (Fig. 2B). Neutrophil count was not associated with survival among within MC subgroup (data not shown).

When comparing the clinicopathologic characteristics of patients with high and low neutrophil counts, the incidence of high PIVKA-II, increased tumor size, presence of vascular invasion, and poor tumor differentiation, which represent tumor aggressiveness, tended to be more frequent in those with high neutrophil count, although not to a statistically significant degree (Table 3).

DISCUSSION

As the discrepancy between the number of LT candidates and that of potential donor livers is increasing, it is essential to allocate organs in a way that provides the best patient survival. With increasing experience around the world, it has been recognized that while results of LT in

patients meeting the MC are excellent, there are many patients with HCC beyond these criteria who also show favorable outcomes enough to have a chance of being cured with LT [4-6]. LDLT has emerged as the predominant form of LT in most of Asia due to poor organ donation rates. Therefore, the criteria for LT for HCC remain controversial, especially in the case of LDLT.

Considering the risks of LDLT, including donor safety, detecting the risk of HCC recurrence after LDLT seems

Table 3. Comparison of clinicopathological characteristics of patients in the low neutrophil and high neutrophil groups

Variable	Low neutrophil (n=61)	High neutrophil (n=42)	P-value
Age (yr)	53.0±7.3	53.7±6.5	0.58
Male sex	43 (70.5)	38 (90.5)	0.03
Radiologic Milan criteria (beyond)	18 (29.5)	10 (23.8)	0.65
AFP (>100 ng/mL)	15 (24.6)	5 (11.9)	0.09
PIVKA-II (>100 mAU/mL)	12 (19.7)	13 (30.9)	0.14
Maximum tumor size (>2.8 cm)	29 (47.5)	23 (54.8)	0.55
Tumor number (multiple)	27 (44.3)	18 (42.9)	1.00
Macrovascular invasion	4 (6.6)	5 (11.9)	0.48
Microvascular invasion	10 (16.4)	10 (23.8)	0.45
ES-grade (III, IV)	7 (11.5)	10 (23.8)	0.11
Underlying liver cirrhosis	58 (95.1)	34 (81.0)	0.05

Values are presented as mean±standard deviation or number (%).

AFP, α -fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II; ES, Edmondson-Steiner.

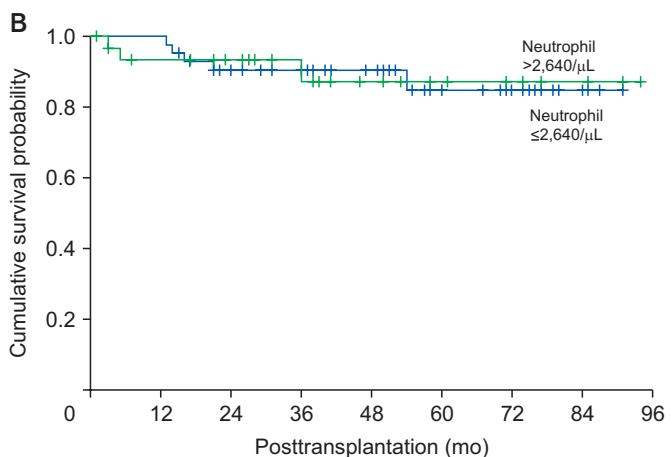
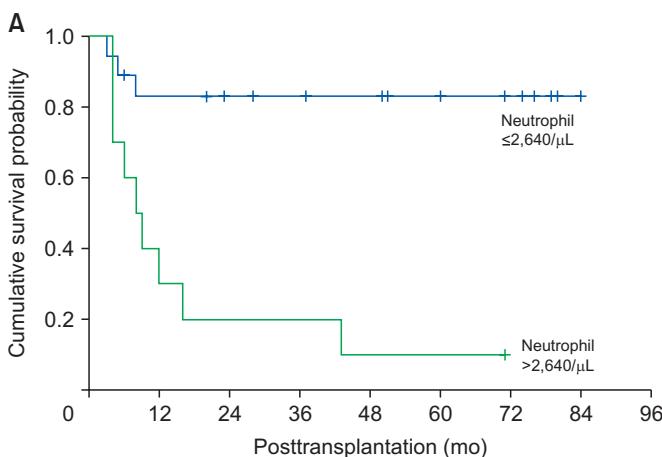


Fig. 2. Kaplan-Meier curve comparing recurrence-free survival for patients classified according to the neutrophil counts in within (A) and beyond (B) Milan criteria groups.

to be of paramount importance because it can provide a chance of cure with LT or, on the contrary, avoid the meaningless donation of a liver. Although expansion of the MC may be considered, a more complete understanding of tumor biology is required to exclude patients with aggressive tumor behavior. This study primarily investigated two factors associated with HCC recurrence and patient survival after LDLT. Well-known variables in previous studies, such as AFP, meeting the MC at LT, and tumor histologic characteristics of the explanted liver, were also analyzed [7,8,16-19]. In addition to these factors, inflammatory markers, which have recently been gaining interest, were investigated. Our study confirmed that there is still a relevant discrepancy between morphologic data provided by imaging studies and those of final histology, leading to understanding of a substantial proportion of patients.

Recent studies have demonstrated that systemic inflammation correlates with tumor progression [20,21]. Given that inflammatory cells induce changes in the tumor microenvironment that favor tumor progression by facilitating genomic instability and promoting angiogenesis, inflammatory cells are powerful tumor promoters in carcinogenesis. There is increasing evidence that inflammatory markers, particularly inflammation-based scores, which represent the systemic inflammatory state, can be used to predict cancer-specific prognosis and survival in various cancers.

Neutrophils traditionally are viewed as the first line of defense against pathogens, have a short lifespan, and are the most abundant subpopulation of peripheral leukocytes. Despite being a major component of the immune system, their contribution has often been overshadowed by that of other immune components, such as lymphocytes and macrophages. However, increasing evidence suggests that neutrophils may also play an important role in many aspects of tumor biology, ranging from tumor initiation to metastasis, including the ability to promote or prevent disease progression. Neutrophils can kill tumor cells via direct secretion of cytotoxic molecules or indirect modulation of T lymphocytes [22]. On the contrary, neutrophils also have a pro-tumor effect by their involvement in multiple steps in carcinogenesis.

Neutrophil infiltration has been found to be associated with the promotion of inflammation, contributing to tumor progression [23]. The presence of intratumoral neutrophils has been reported to be a poor prognostic factor after resection of HCC [24]. In addition to tumor-infiltrating neutrophils, circulating neutrophils alone have been asso-

ciated with outcomes [25]. However, the underlying mechanism between neutrophils and the tumor microenvironment is not well identified. Several studies have provided compelling evidence to understand this mechanism and have suggested that neutrophil count is associated with the release of chemokines and interleukins, including vascular endothelial growth factor and matrix metalloproteinase-9, which promote tumor invasion and metastasis [26]. In addition, a previous study showed that myeloid-derived suppressor cell levels, which play a critical role in the immune tumor microenvironment, are also positively related to neutrophil count.

The NLR can be measured as the ratio of peripheral blood neutrophils to lymphocytes and can be a valuable predictive marker in various solid tumors. The NLR also applies to HCC and has been shown to be an effective predictor of outcomes for various treatments. In similar studies, researchers have investigated use of the NLR as a prognostic factor in patients with HCC undergoing LT [12,27-29]. Motomura et al. [12] studied 158 patients with HCC who underwent LDLT and reported that an elevated NLR was related to significantly poor RFS. In addition, outcomes were poor in patients with a high NLR who exceeded the MC, and a high NLR was related to elevated peritumoral interleukin-17, which has been related to poor OS and high risk of recurrence, as it has been reported to promote tumor angiogenesis [30].

In our data analysis, neutrophil count was found to have a significant association with outcomes, whereas lymphocyte count did not. In addition, the NLR was associated with survival, but was not an independent predictive factor (data not shown). Therefore, in the present study, absolute neutrophil count was used as a predictor. Compared with patients who fulfilled the MC, those who exceeded the MC had more advanced tumors. Aggressive tumor behavior can be reflected in high neutrophil count. Thus, in the present study, elevated neutrophils was associated with worse outcome, especially poor RFS, in patients exceeding the MC, independent of traditional negative prognostic factors.

There are several limitations in this study. First, this was a single center, retrospective analysis, and the sample size was relatively small. Second, we did not evaluate postoperative change in neutrophil count because it is difficult to exclude other causes representing neutrophilia, including infection or steroid use. Third, validation of the cutoff value for neutrophil count was not performed. Fourth, we did not conduct molecular experiments to

identify the underlying mechanism of the role that neutrophils play in patients with HCC undergoing LDLT. Despite these limitations, to our knowledge, this is the first study to suggest that individual immune cells alone can predict the prognosis of patients with HCC undergoing LDLT.

Although our results should be interpreted with caution because survival analyses of small populations are less reliable, our data demonstrated that elevated preoperative neutrophil count is associated with poor RFS and OS in patients with HCC undergoing LDLT, as well as the possibility of using expanded MC combined with neutrophil count. If validated in a larger prospective study, preoperative neutrophil count may provide a simple method for identifying patients with poor prognosis.

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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