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Evolutionary Distance Predicts Recurrence After Liver Transplantation in Multifocal Hepatocellular Carcinoma

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Background. Liver transplantation (LTx) is a potentially curative treatment option for hepatocellular carcinoma (HCC) in cirrhosis. However, patients, where HCC is already a systemic disease, LTx may be individually harmful and has a negative impact on donor organ usage. Thus, there is a need for improved selection criteria beyond nodule morphology to select patients with a favorable outcome for LTx in multifocal HCC. Evolutionary distance measured from genome-wide single-nucleotide polymorphism data between tumor nodules and the cirrhotic liver may be a prognostic marker of survival after LTx for multifocal HCC. **Methods.** In a retrospective multicenter study, clinical data and formalin-fixed paraffin-embedded specimens of the liver and 2 tumor nodules were obtained from explants of 30 patients in the discovery and 180 patients in the replication cohort. DNA was extracted from formalin-fixed paraffin-embedded specimens followed by genome wide single-nucleotide polymorphism genotyping. **Results.** Genotype quality criteria allowed for analysis of 8 patients in the discovery and 17 patients in the replication set. DNA concentrations of a total of 25 patients fulfilled the quality criteria and were included in the analysis. Both, in the discovery ($P = 0.04$) and in the replication data sets ($P = 0.01$), evolutionary distance was associated with the risk of recurrence of HCC after transplantation (combined $P = 0.0002$). In a univariate analysis, evolutionary distance ($P = 7.4 \times 10^{-6}$) and microvascular invasion ($P = 1.31 \times 10^{-5}$) were significantly associated with survival in a Cox regression analysis. **Conclusions.** Evolutionary distance allows for the determination of a high-risk group of recurrence if preoperative liver biopsy is considered.

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Hepatocellular carcinoma (HCC) is the sixth most common cancer and third leading cause of death with a rising incidence in western countries.[1-3] For patients with advanced cirrhosis, liver transplantation (LTx) is an attractive and oncological potentially curative treatment option, because it removes the tumor, preneoplastic lesions and

treats the underlying cirrhosis.[4,5] The oncological success of LTx for HCC critically depends on the biology of the underlying tumor disease. Liver transplantation is curative if the disease is indeed localized to the liver. However, if the HCC is a systemic disease at the point of transplantation, this approach is no longer curative, and patients may in fact be harmed by the procedure.

As of now, the best and clinically accepted selection criteria for LTx in the setting of HCC are morphological criteria, that is, the size and number of nodules in the liver, macrovascular invasion and of evidence of extrahepatic metastases. These criteria have been incorporated into the Milan criteria, for which an incidence of tumor recurrence of approximately 10% and 5-year survival rates of over 75% have been reported.[6-8] Several subsequent studies have reported good outcomes for patients that were transplanted outside these criteria, leading to the expansion of transplantation guidelines to the so-called University of California San Francisco (UCSF) criteria[9-12] and Bologna criteria.[13]

The limitations of purely morphological criteria have been recognized,[14] because (i) morphology may not adequately capture the tumor biology and growth dynamics, and (ii) the morphological assessment itself has limitations as small tumor nodules may be overlooked and posttransplantation staging may not be congruent to preoperative imaging studies. Indeed, incorrect staging is a well-known problem and one of the major challenges in today's LTx.[5,12,15,16] Thus, there is an ongoing search for an improvement of selection criteria. An attractive option is an assessment of tumor biology through response to neoadjuvant or bridging therapy. Transarterial chemoembolization (TACE) combined with or without other locoregional therapies has been reported as a tool to stratify patients with more favorable tumor biology. Several studies showed significantly better survival rates of patients with tumor response after TACE before LTx.[17,18] This method is, however, not universally accepted, as TACE itself has a distinct risk profile and selection criteria for patients for bridging therapy have not been prospectively validated.

As an alternative to imaging or neoadjuvant treatment response, noninvasive or invasive biomarkers could provide a supplementary approach to judge tumor biology.[19-22] Across several cancer types, it was shown that tumors with higher levels of genetic diversity are correlated with a poorer clinical prognosis.[23-25] Furthermore, comparisons between metastatic and primary tumors have so far revealed high genetic

divergence.[26-29] Understanding tumor genomics and biology has allowed critical advancements in patient stratification in other malignancies that have yet not been translated to HCC. We chose the setting of multifocal HCC, because the sampling of more than 1 tumor nodule allows a more refined assessment of the dynamics of tumor mutagenesis, and this setting also harbors the greatest clinical need for better patient selection. Thus, to contribute toward such an improved selection system, we present a retrospective analysis of evolutionary distance between tumor nodules and the cirrhotic liver with clinical and histopathological data in LTx for multifocal HCC.

MATERIALS AND METHODS

Patients and Phenotypes

Histopathological specimens were obtained from a total of 210 patients with multifocal HCC transplanted between 2005 and 2014 at transplant centers University Hospital Schleswig-Holstein Campus Kiel, Charité Campus Virchow Klinikum Berlin, University Hospital Regensburg, University Hospital Muenster, and Hannover Medical School. Informed consent was obtained from all patients, and the study was approved by the local ethics committee of the University Hospital Schleswig-Holstein Campus Kiel. For each patient, a formalin fixed paraffin embedded (FFPE) specimen from 2 different tumor locations and from the explanted cirrhotic liver was retrieved from the pathology archives. Clinical data was obtained from the respective routine clinical documentation. Median patient follow up was 1634 [1091-2137] days after transplantation. Assessed parameters were sex, age at time of LTx, type of concurrent liver disease, hepatitis and type of hepatitis, type of bridging therapy, response to bridging therapy, lab-MELD at time of LTx, retransplantation, length of follow-up, type of immunosuppression, Tumor Nodes Metastasis (TNM) status according to the 7th Edition, tumor grading, number and cumulative size of tumor lesions (≥8 cm), tumor stage related to up to 7, Milan and UCSF criteria, AFP-level before LTx (≥20, ≥200, ≥400 ng/mL), tumor recurrence and localization of the recurrent disease, survival and tumor-free survival. After LTx, tumor staging of the liver explant was carried out using a histopathological examination. Tumor recurrence was diagnosed by positive histology, elevated AFP >400 ng/ml in combination with hyper vascularized lesions detected by MRI or CT or hypervascularized lesions

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accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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detected by 2 different methods, including MRI or CT according to the guidelines of the federal German Medical Association. Patients and tumor characteristics are shown in Table 1. Patients with a survival below 60 days after transplantation were excluded from the analysis to avoid confusion of perioperative mortality with long-term survival determined by tumor biology, which was the focus of this analysis.

DNA Extraction and Genotyping

Genomic DNA was extracted from 3 to 4 (5-µm-thick) sequential sections for each of the FFPE samples after deparaffinization.[30] Wax was removed from the specimens by adding 500-µL heptane to a 2-mL microcentrifuge tube containing 3 to 4 sections of paraffin-embedded tissue and incubation at room temperature for 10 minutes to dissolve the wax. Then 25 µl of Methanol (100%) were added, and the tube was vortexed for 10 seconds. The supernatant was removed after centrifugation at 12 000-16 000 rpm in a microcentrifuge for 2 minutes. One milliliter of 96% ethanol was added, and the tube was vortexed for 10 seconds. After proteinase K treatment, DNA was extracted from the resulting pellet with the commercially available AllPrep DNA/RNA FFPE kit (Qiagen, Hilden, Germany), using the manufacturer's protocols. The quality of extracted DNA was examined by agarose gel electrophoresis and ethidium bromide staining. Extracted DNA was quantified using the PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA) and normalized to a concentration of 50 ng/µL. Genotyping on Illumina Chip HumanOmniExpress-24 v1.0 arrays (Illumina Inc., San Diego, CA) was performed according to the manufacturer's instructions and as reported previously.[31]

Bioinformatic and Statistical Analysis

The raw data were processed with Illumina GenomeStudio V2011.1 (Genotyping Module v1.9.4). We used the reference panel with 96 normal samples in the discovery panel and with 816 normal samples in the replication panel to normalize raw data and generate log ratios. All genotyped samples were retained for this step. Single-nucleotide polymorphism (SNP), Log R Ratios (LRR) and B allele frequencies were calculated from the GenomeStudio software (Illumina Inc., San Diego, CA) with default settings for each panel separately. For subsequent analyses, only individuals were retained, where all 3 processed samples fulfilled the quality criteria of a standard deviation of the LRR less than 0.3 and standard deviation of B allele frequency less than 0.05. This lead to the analysis of 8 samples in the discovery step and 17 samples in the replication step.

Genomic aberrations in tumor samples were detected using OncoSNP v2.1.[32] We analyzed each sample separately for autosomal chromosomes and with a maximum of 12 different tumor states. Phylogenies of the 2 HCC nodules and nontumorous control liver tissue for each patient were calculated using Medicc.[33] Only copy number regions supported by at least 40 SNPs were used for calculating the minimal event distances. From each phylogenetic tree, the distance from the control tissue to the branch point (X) and the length of the branches connecting the 2 tumors was extracted (Y=Y1 + Y2, Figure 1).

Joint analysis of clinical variables and measures of phylogenetic distance was performed using R (www.r-project.org).[34] Comparisons of distances between groups were performed using the Wilcoxon test and the Student t test. Predictors of survival after transplantation were performed using Cox regression.

RESULTS

Evolutionary Distance and Tumor Recurrence

For the discovery panel, paraffin embedded samples from explants of 30 patients who were transplanted for multifocal HCC between 2010 and 2014 were obtained at Kiel University Hospital. After DNA extraction from 3 samples from each patient (tumor 1 [TU1], tumor 2 [TU2] and nontumoral

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TABLE 1.

Clinical characteristics of the study population

Category	Parameter	Discovery	Replication	Total
Demographics	Age (years)	55.2 [43-61]	58 [53-62]	56.7 [52-62]
	% male	62.5	88.2	80
Transplantation data	MELD score	11.5 [8-14]	11 [10-16]	11 [9-16]
	% HCV	12.5	35.3	28
	% HBV	12.5	41.2	32
Tumor characteristics	T-stage	2 [2-3]	2 [2-3]	2 [2-3]
	% within MILAN	0	11.8	8
	% within UCSF	12.5	41.2	32
	Tumor grading	2 [1.75-2]	2 [2-3]	2 [2-2]
	% MVI	37.5	29.4	32
	AFP levels	216 [6.6-1141]	64 [15-107]	64 [7-451]
	Number of nodules	4 [3-5]	6 [2-6]	4 [3-6]
	% with nodule Ø >5 cm	37.5	52.9	48
	% with cumulative Ø >8 cm	75	64.7	68
Outcome	Number of patients	8	17	25
	Number of patients with tumor recurrence	5	6	11
	Time to recurrence (days)	344 [94-395]	356 [233-380]	355 [187-391]
	Follow-up time (days)	699 [590-1239]	1734 [1254-2179]	1634 [1091-2137]

For numerical variables, the median and the interquartile range is provided. USCF and Milan criteria are based on pathological explant analysis.
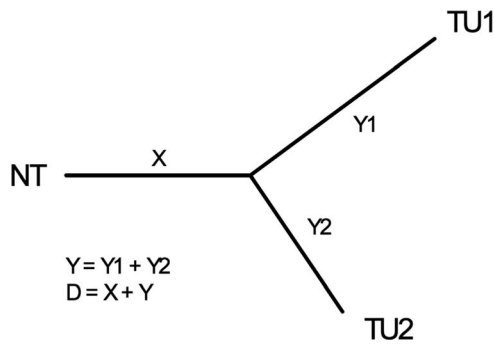
FIGURE 1. Illustration of the evolutionary distance measures. NT denotes the cirrhotic liver without tumor, that is, the "nontumoral tissue" of the patient. The 2 samples HCC nodules per patient are denoted as TU1 and TU2. Distances are calculated as Y, denoting the total distance between the 2 HCC nodules, and D, denoting the total evolutionary distance spread in the patient. The calculated phylogenetic tree defined the distance from NT to the branch point (X) and the distances of TU1 and TU2 to the branch point.

[NT] cirrhotic liver tissue), a total of 16 patients fulfilled the quality criterion of 1 to 30 µg of total DNA and a A260/280 ratio of 1.5:2. After genotyping of the corresponding 48 DNA samples (16 * 3) on Illumina OnmiExpress chips (Illumina Inc. San Diego, Ca., USA), the quality control threshold of a standard deviation of the LRR < 0.3 after processing using OncoSNP v2.1[32] was applied to each sample. By employing the quality criterion to all 3 samples per patient, a total of 8 patients were usable for the analysis of evolutionary distance. For the replication panel, 180 patients from centers at university hospitals Kiel, Berlin, Regensburg, Muenster and Hannover from an extended time interval (between 2005 and 2014) were extracted. Using the same quality criteria for extracted DNA, 336 samples corresponding to 112 patients were hybridized on Illumina OmniExpress chips. Subsequent filtering of genotype quality using a standard deviation of the LRR less than 0.3 yielded a total of 17 patients with 3 samples matching the genotype quality requirement. The characteristics of the patients passing these criteria are provided in Table 1.

Two measures of evolutionary distance between the 3 samples per patient were analyzed as illustrated in Figure 1. In the reconstructed tree, X corresponds to the distance of the normal tissue to the branching point to the tumor nodules. The

parameter Y was calculated as Y1 + Y2 and corresponds to the path distance between the 2 HCC nodules. These 2 parameters of evolutionary distance were utilized to classify patients. As shown in Figure 2, panels A and B, recurrent tumors are predominantly located in the right upper quadrant if X and Y are plotted in a patient-based analysis, that is, tumors with a more rapid evolution both as measured as distance from nontumorous liver and between nodules were more prone to recurrence after LTx. For formal testing, the total evolution distance D was compared between patients with recurrent and nonrecurring HCC using a single-sided Wilcoxon test. Both in the discovery ($P = 0.0357$) and in the replication data sets ($P = 0.0101$), a significantly lower evolutionary distance was noted. In a post hoc analysis of the total cohort (Figure 2, right panel), P value of 0.000199 was obtained.

Predictors of Recurrence-free Survival

The overall 3-year survival in the study cohort was 58% (**Figure S1, SDC,** http://links.lww.com/TP/B598). The qualitative analysis of HCC recurrence was followed by an analysis of survival depending on clinical, histopathological criteria and evolutionary distance (D). In a univariate analysis, evolutionary distance (likelihood ratio test, $P = 7.4 \times 10^{-6}$), microvascular invasion (MVI) ($P = 1.31 \times 10^{-5}$), the number of nodules with a diameter > 5 cm ($P = 0.00643$) and match of the UCSF criteria ($P = 0.000509$) were significantly associated with survival in a Cox regression analysis (Table 2). The impact of MVI and evolutionary distance on recurrence-free survival is depicted in the respective Kaplan-Meier analyses using MVI (Figure 3, panel A), evolutionary distance (panel B) and both parameters (panel C).

DISCUSSION

In this report, we demonstrate that both, evolutionary distance (D) and MVI, are strong predictors of survival after LTx for multifocal HCC.

The transition from preneoplastic lesions to cancer is characterized by a sequence of genomic events that differ not only between tumor entities but also within the same type of tumor, as recently also shown in the The Cancer Genome Atlas analysis of liver cancer.[35] Although ultimately, an individualized understanding of the functional impact of the genomic alterations in each cancer will hold the key to individualized
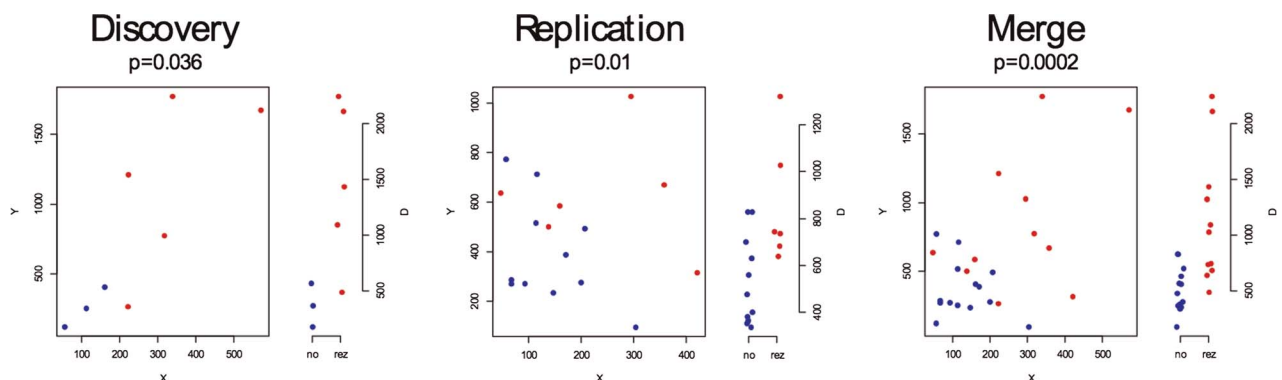
FIGURE 2. Association of measures of evolutionary distance with tumor recurrence after transplantation for the discovery, replication and total data sets. In each category, the left panel shows the distance between nodules (Y) and the distance from the nontumorous liver (X). Patients without recurrence are denoted in blue, patients with tumor recurrence in red. The association of total evolutionary spread and recurrence is provided in the right panels for each category. The significance level as provided by the Wilcoxon test is also noted for each category.

TABLE 2.

Univariate test of predictors of recurrence in a Cox regression analysis

Category	Parameter	Nominal P	HR	SE	95% CI
Demographics	Age	0.12	0.9999	7.519×10^{-5}	0.9997-1
	Sex	0.26	0.4398	0.6824	0.1155-1.675
Transplantation	MELD score	0.50	0.953	0.07429	0.8239-1.102
	Presence of HCV infection	1	0.9966	0.67802	0.2639-3.764
	Presence of HBV infection	1	1	0.62798	0.2922-3.425
Tumor characteristics	T-stage	0.093	1.949	0.3877	0.9114-4.167
	With MILAN	0.12	1.269×10^{-8}	7.829×10^{3}	0-Inf
	Within UCSF	0.00051	7.8×10^{-10}	1.246×10^{4}	0-Inf
	Tumor grading	0.64	0.7744	0.5456	0.2658-2.256
	Presence of MVI	1.3×10^{-5}	22.34	0.8234	4.449-112.2
	AFP level	0.054	1	6.588×10^{-6}	1-1
	No. nodules	0.0064	1.624	0.1893	1.12-2.353
	Presence of nodule Ø >5 cm	0.024	4.203	0.6837	1.101-16.05
	Presence cumulative Ø >8 cm	0.00069	1.196×10^{9}	1.250×10^{4}	0-Inf
Evolutionary distance	D	7.4×10^{-6}	1.003	0.0008	1.002-1.005

For each variable, the uncorrected nominal P value is provided.

therapy, global measures of genomic alterations such as evolutionary distance may be attractive tools in a clinical context. As follows, measurement of evolutionary distance could add to other recently proposed diagnostic tools for a better tumor assessment of HCC patients on the waiting list. For example, several studies were able to show, that pretransplant ^{18}F-FDG-PET provides very useful information on biological tumor viability and posttransplant outcome.[36-40] Furthermore, significantly better survival rates of patients with tumor response after TACE before LTx were shown by some authors.[17,18,41] In this study, evolutionary distance was used to measure a genomic alteration in human HCC between the preneoplastic cirrhotic liver and the tumor nodules. Evolutionary distance has recently been employed in a clonal analysis of liver cancer tracking the genealogy of clones within liver cancer nodules.[23] Here, we use evolutionary distance[32,33] of copy number variation in HCC as measured by SNP arrays for the first time as a prognostic marker in cancer. This measure of genomic alterations does not carry a direct functional interpretation, but its global nature may be a suitable integrator of a multitude of mutational pathways as shown by the strong correlation with survival in this study. Therefore, evolutionary distance combined with tumor stratifying tools like ^{18}F-FDG-PET and TACE-response might help to stratify patients on the waiting list before LTx.

FFPE samples have been used in the past years for increasingly complex genomic analyses.[30,42,43] The use of these samples has also enabled this study, as patients with LTx for multifocal HCC could be recruited. However, our report also shows the limitations of FFPE tissue if used across institutions and with samples dating back as far as 2005. A second reason for the limited use of the samples could be found in a tumor-necrosis after an earlier bridging therapy, like TACE or percutaneous ethanol injection, before transplantation. After all stages of quality control, only 25 of 210 patients could be used for the final analysis. These quality constraints have certainly limited the statistical power of our report, but likely do not introduce a systematic biological bias in our analysis. Nevertheless, in the future, tumor biopsies of fresh nonnecrotic tissue in advance of bridging therapies could result in a higher DNA quality to perform the genomic analysis and measure the patient's specific evolutionary distance
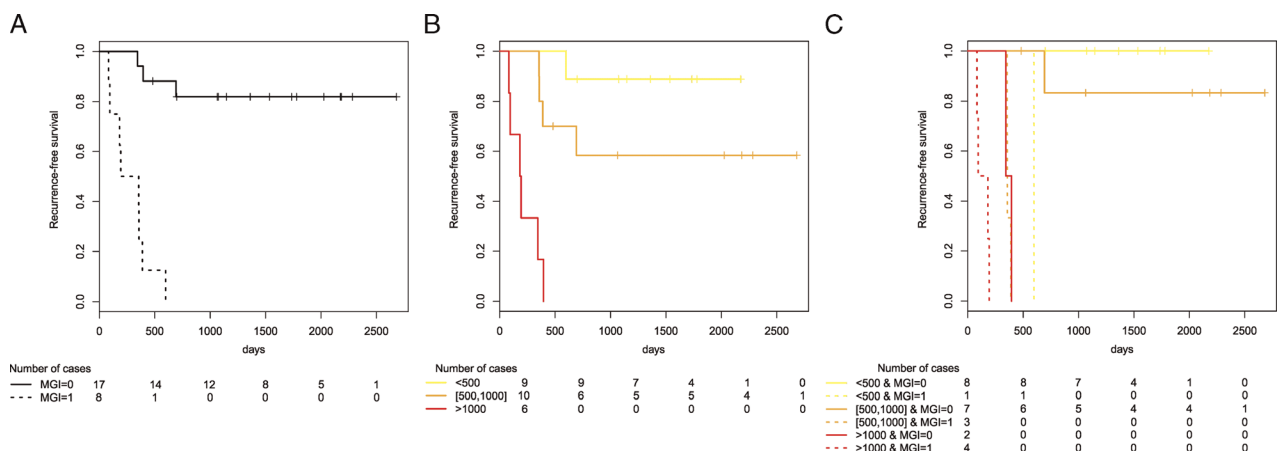

FIGURE 3. Kaplan-Meier plot of predictors of survival after LTx for multifocal HCC. Panel A depicts the survival in patients with tumors without (solid line) and with MVI (dotted line). Panel B shows 3 categories of total evolutionary distance (D) as noted in the figure legend. Panel C combines the 2 predictors in 6 categories.

between the tumor lesions and nontumorous tissue. Here it is possible to extend the approach of this study using 2 tumor nodules from 1 patient to more than 2 tumor nodules, if a patient has an intrahepatic tumor spread of more than 2 tumor nodules, because the predictor could be defined to be the length of all branches of the evolutionary tree. In this study, we only analyzed 3 samples (1 nontumorous tissue and 2 tumor tissues), as the acquisition and isolation of the samples was associated with higher financial costs. A measurement of evolutionary distance after an isolation of tumor DNA of more than 2 nodules could be analyzed in future studies. An analysis to investigate in how far a higher evolutionary distance correlates with a higher tumor growth activity was not possible in this study due to a retrospective study design. This point should be analyzed in a prospective multicenter-study in the future. A standardized imaging and could be enhanced by a 18F-FDG-PET.

Our study confirms the need for better selection of patients for LTx for multifocal HCC. In fact, the survival in the high-risk groups as defined by genomics or MVI might have a systemic tumor disease as survival is worse than under ablation therapy (Figure 3). Thus, some of these patients may be harmed by LTx despite the improved liver function as the ensuing immunosuppression lowers the immunological barrier for tumor spread. This situation is compounded by organ shortage and the high cost of LTx. Our results regarding MVI are confirmatory in nature: MVI was reported as a predictor for survival after recurrence after HCC resection.[22,44,45] Microvascular invasion, however, cannot be reliably assessed in biopsies and therefore genomic measures with good prognostic spread, such as evolutionary distance may be considered.

Our data add to the ongoing discussion about the ethical and practical problems of pretransplantation liver biopsy in HCC, which would be needed for assessment of evolutionary distance or other molecular markers. In this context, the risk of tumor cells seeding along the needle track and the potential for complications are relevant problems. Recently, Fuks et al[46] showed that a biopsy of HCC tumors before LT by CT- and ultrasound-guided fine needle aspiration neither negatively influenced the short-term nor the long-term outcomes of patients qualifying for LT. In their series of 75 biopsied patients, failure of the biopsy occurred in only 5% and complications occurred only in 2.5% and never discarded patients from LT.

In summary, we show that both MVI and evolutionary distance are strong predictors of survival after LTx for multifocal HCC. Microvascular invasion and evolutionary distance, each and in combination, differentiate groups wide drastically in different survival after the transplantation. Our data add to the ongoing discussion about liver biopsy before LTx and may contribute to a reevaluation of this intervention and a potentially better selection of organs and patients for LT.

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