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ORIGINAL ARTICLE

A possible role of microRNAs as predictive markers for the recurrence of hepatocellular carcinoma after liver transplantation

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Conflicts of interest
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Summary

With favourable 5-year survival rates up to 75%, liver transplantation (LT) is the treatment of choice for hepatocellular carcinoma (HCC). Nonetheless, tumour recurrence after LT remains a challenge. The aim of this retrospective study was to develop a predictive score for tumour recurrence after LT by combining clinical parameters with HCC biomarkers (microRNA). A microRNA (miRNA) microarray analysis was used to compare miRNA expression patterns in tissue samples of 40 patients with and without HCC recurrence after LT. In a screening cohort (n = 18), the miRNA analysis identified significant differences in the expression of 13 miRNAs in patients with tumour recurrence. Using the most significant miRNAs in this screening cohort, we could develop a predictive score, which combined the expression levels of miR-214, miR-3187 and the Milan criteria, and we could define low- and high-risk groups for tumour recurrence and death. The above score was evaluated in a second and independent cohort (n = 22). In contrast to the Milan criteria alone, this score was significantly associated with tumour recurrence. Our analysis indicated that the use of a specific miRNA expression pattern in combination with a limited tumour burden as defined by the Milan criteria may lead to a more accurate prediction of tumour recurrence.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death worldwide, with an increasing incidence in Western countries [1–3]. Eighty per cent of HCC incidence occurs in cirrhosis, which is mainly caused by chronic viral hepatitis and alcoholic liver disease [1]. However, in only 20–30% of all patients is curative therapy possible [1,4]. Liver transplantation (LT) is often the treatment of choice for HCC and is associated with high rates of long-term survival [5,6]. Despite the careful selection of patients with

HCC for LT using the Milan criteria (a single tumour ≤5 cm or up to three tumours, each ≤3 cm, with no macrovascular invasion) [5], tumour recurrence rates of up to 25% remain an unsolved problem [7].

However, the Milan criteria used for the allocation for LT are based primarily on radiologic findings and do not consider tumour biology [8]. Furthermore, in up to 25% of the patients, the size of the tumour, the number of tumours and the vascular invasion are underestimated in radiological imaging [9]. Therefore, the combination of clinical–radiological findings with biomarkers, such as microRNAs (miRNA), could be a valuable tool for selecting transplant candidates.

MicroRNAs are small, noncoding RNAs that are responsible for the regulation of targeted gene expression [10]. Recent studies have shown that the dysregulation of certain miRNAs plays a crucial role in tumourigenesis and cancer progression. In HCC, various studies have identified dysregulated miRNAs and their effects on prognosis, tumour progression and recurrence [11,12]. Knowledge regarding dysregulated miRNAs and their target genes in HCC such as β-catenin, fibroblast growth factor receptor-1 (FGF-1), matrix metalloproteinase (MMP) and mTOR [13–15] may lead to the development of novel therapeutic strategies [16].

Few studies exist regarding the identification of HCC recurrence-related miRNA expression after LT. A miRNA signature consisting of miR-19a, miR-886-5p, miR-126, miR-223, miR-24 and miR-247 was observed to be an independent predictor of recurrence-free survival after LT [17]. In another study, miR-155 was found to be upregulated in patients with HCC recurrence after LT compared with patients without HCC recurrence. Furthermore, miR-155 was found to correlate with the invasiveness of HCC cells [18]. Barry *et al.* [19] defined a biomarker consisting of 67 miRNAs that outperformed the Milan criteria for assessing the risk of tumour recurrence after LT.

However, there is still considerable heterogeneity in the published dysregulated miRNAs and miRNA signatures, which is most likely caused by multiple factors, including differences in underlying liver disease (viral, alcohol or mixed aetiologies) and different tumour stages [20].

The aim of the present study was to identify a recurrence-specific pattern of miRNAs and combine these miRNAs with clinical markers (e.g. Milan criteria and AFP) to develop a high predictive score for disease-free survival after LT for HCC.

Methods

Patients and samples

This was a retrospective analysis, which was approved by the local Ethics Committee of the University Hospital of Frankfurt, Germany (Institutional Review Board No. 342/13). A total of 92 patients undergoing LT for HCC in our surgical department between 2007 and 2012 were included. The data collected were demographic and clinicopathologic features, including age, gender, serum AFP levels before LT, highest AFP level during the waiting period (peak AFP), radiologic criteria, intra- and postoperative course, tumour recurrence rate, overall survival and disease-free survival data. The Milan criteria (a single tumour ≤5 cm or up to three nodules, each ≤3 cm and no macrovascular invasion) and UCSF criteria (University of California-San-Francisco criteria; a solitary tumour ≤6.5 cm or ≤3 nodules with the largest lesion ≤4.5 cm and a total diameter ≤8 cm) were determined by CT scan or MRI in initial imaging and by the histopathologic evaluation of explanted livers.

For the miRNA analysis (microarray analysis and PCR) of tissue samples (explanted livers), all patients ($n = 40$) fulfilled the following enrolment criteria: (i) HCC staged within the Milan or UCSF criteria ($n = 72$) in histopathologic examinations of the explanted livers, (ii) availability of survival data ($n = 91$), (iii) presence of viable formalin-fixed paraffin-embedded (FFPE) tumour material with a tumour necrosis rate <30% ($n = 47$) and (iv) sufficient quality of extracted RNA for further quantitative real-time reverse-transcription PCR (Q-RT-PCR, $n = 40$) and a minimum follow-up of 12 months ($n = 80$). An experienced pathologist (M.L.H.) confirmed the diagnosis of HCC. Grading and staging were assessed according to the current tumour–node–metastases (TNM) classification of malignant tumours. A pathologist reviewed all of the specimens microscopically. The tissue areas with a tumour cell content of more than 95% were macrodissected and used for further analyses.

Isolation of miRNA

Total RNA was extracted from tumour FFPE specimens using the RNeasy FFPE Kit (Qiagen, Hilden, Germany) following the manufacturers protocol. RNA quantification was performed using a Nanodrop 2000 spectrometer (Thermo Fisher Scientific, Wilmington, DE, USA) using 100–300 ng of total RNA for further analyses.

Microarray analysis

A global miRNA expression profiling analysis of 1105 mature miRNAs using GENECHIP® MIRNA ARRAY v2.0 (Affymetrix, Santa Clara, CA, USA) was performed following the manufacturer's protocol. The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO series accession

number GSE64989 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64989).

Real-time PCR miRNA expression analysis

TaqMan® MicroRNA Q-RT-PCR assays (Applied Biosystems, Darmstadt, Germany) were used to quantify miRNAs according to the manufacturer's protocol. Expression was analysed for five miRNAs (hsa-miR-140, hsa-miR-214, hsa-miR-455, hsa-miR-3188 and hsa-miR-3187) and one endogenous control (U6). Samples were analysed in triplicate, and ΔCT values were calculated using the endogenous control.

Bioinformatics and statistical analyses

The clinical and biochemical characteristics of the patients were expressed as the mean ± standard deviation or as the median and range as appropriate. Unless indicated otherwise, all tests were two-tailed, and P values <0.05 were considered significant.

The statistical analysis of the miRNA profiles was performed using the statistical computing environment R (R Foundation for Statistical Computing, Vienna, Austria). Additional software packages (GENEPLOTTER, GPLOTS) were obtained from the Bioconductor project. Replicate correlation was calculated using Pearson's correlation coefficient and depicted as a scatterplot. Unsupervised hierarchical clustering was performed for miRNAs with a standard deviation ≥1.0 across all samples using the Manhattan distance method and the average linkage method. For the supervised analysis, expression intensity and variance filtering were used to reduce the dimensions of the microarray data. The data were filtered by an intensity filter (gene intensity >100 in at least 25% of the samples, if the group size is equal) as well as a variance filter (an interquartile range of log2 intensities >0.5, if the group size is equal). After the filtering process, P-values were calculated with the two-sample t-test (variance = equal) to identify miRNAs expressed differentially between the two groups. For multiple testing problems, a false discovery rate (FDR) was used [21].

Pearson's correlation or the Spearman test was applied as appropriate for the calculation of the correlations between two variables. Fisher's exact test was used to analyse the differences in the categorical variables. For the independent variables, we used the Mann–Whitney U-test and the Kruskal–Wallis test. Associations of the Milan criteria and AFP levels with tumour recurrence-free survival were estimated by the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. For the Kaplan–Meier analysis, patients with different AFP levels were divided into three groups as follows: 1st AFP <20 ng/ml, 2nd AFP 20–400 ng/ml and 3rd AFP >400 ng/ml [22].

To investigate the risk factors for tumour recurrence, univariate and multivariate Cox regression analyses were used and expressed as a Concordance Index. Time-dependent receiver operating characteristic (survivalROC) curves and the area under the curve were used to determine feasibility at a time point of 5 years using the method of Heagerty *et al.* [23].

All data were analysed with SPSS, version 22 (IBM, Armonk, NY, USA) and R.

DIANA-MIRPATH v.3.0 (DIANA Tools, Athens, Greece) was used as a computational predictive model to calculate potentially targeted genes and pathways using the microT-CDS database (a P value threshold of 0.05, MicroT threshold of 0.8, and FDR correction and conservative stats were applied) [24]. The depicted pathways are derived from the KEGG database [25].

Results

Association of clinical parameters with disease-free survival after LT

Patient demographics are summarized in Table S1. During the study period, we performed 92 LTs in 22 women and 70 men with HCC as an underlying disease with a mean age of 57 years. Hepatitis C virus infection, which was noted in 42.4% of the patients, was the most common aetiology of liver cirrhosis. As a bridging therapy for tumour control during the waiting period before LT, 78 patients underwent transarterial chemoembolization (TACE). The minimum follow-up time was 24 months. Of the 92 patients included in this study, 22 had recurrent tumours with a median HCC recurrence-free survival of 10 months (3–55 months). The median survival with HCC recurrence in this subgroup was 25.5 months.

The prognostic values of different clinicopathological features were analysed with a univariate analysis. AFP level, tumour size, number of tumour nodules, microvascular invasion and patients outside of the Milan criteria were significantly associated with tumour recurrence (Table 1). The AFP level was seven times higher in the recurrent group (47.1 ng/ml) compared with patients without tumour recurrence (6.55 ng/ml). The majority of the patients had well-differentiated or moderately differentiated tumours, and only nine patients had poorly differentiated HCC. All nine patients developed tumour recurrence. Microvascular invasion was more frequently observed in patients with HCC recurrence ($P < 0.001$).

Other features, such as the neutrophil–lymphocyte ratio, were not associated with tumour recurrence.

Figure 1 shows the disease-free survival rates according to the Milan criteria and AFP levels. According to the

Table 1. Clinical and pathological parameters of patients with no hepatocellular carcinoma (HCC) recurrence versus HCC recurrence following liver transplantation.

	No HCC recurrence (n = 70)	HCC recurrence (n = 22)	P
Age years, mean (±SD)	56 (6.53)	59 (5.14)	0.147
Gender (female:male)	20:50	2:20	0.098
Serum AFP ng/ml, (range)	6.55 (1.1–3872)	47.1 (3.2–60 500)	<0.001
<20 ng/ml	53	6	<0.001
20–400 ng/ml	12	10	<0.001
>400 ng/ml	5	6	<0.001
Peak serum AFP, mean (range)	10.65 (2–15 509)	50.3 (3.2–60 500)	<0.001
Neutrophil–lymphocyte ratio, mean (range)	2.65 (0.3–27.5)	1.94 (0.79–8.55)	0.336
Waiting period in months, mean (±SD)	10.51 (8.33)	8 (7.51)	0.155
Number of patients with TACE (n)	60	18	0.408
TACE sessions per patient, mean (±SD)	5.29 ± 3.64	4.26 ± 2.88	
Pathology			
Tumour size, cm (range)	2.3 (0–8.3)	3.8 (0–24)	<0.001
>2 nodes	13	13	0.001
Microvascular invasion	1	7	<0.001
Grading G3	0	9	<0.001
Milan criteria fulfilled	53	5	<0.001
UCFS criteria fulfilled	59	13	<0.001

post-transplant pathology reports, 58 patients were within the Milan criteria, of whom five experienced HCC recurrence within the first 2 years after LT. As defined by their peak AFP levels during the waiting period, the patients were divided into three groups as follows: <20 ng/ml (n = 53), 20–400 ng/ml (n = 22) and >400 ng/ml (n = 11) (Table 2). The median HCC recurrence-free survival durations for these groups were 35, 30 and 8 months, respectively. The group of patients with AFP levels <20 ng/ml had significantly longer median HCC recurrence-free survival than the patients with AFP levels from 20 to 400 ng/ml or >400 ng/ml.

Before miRNA analysis, the patients chosen for miRNA analysis did not show any significant differences in age, gender, primary indication for LT, labMELD, tumour size and number of tumour nodules, and HCC recurrence-free survival (Table 2).

Global miRNA array analysis identified differential miRNA expression in patients with and without tumour recurrence after LT

Array analysis was performed using the Affymetrix GeneChip® miRNA microarray and FFPE tissue from 18 patients (screening cohort) who underwent LTX for HCC (recurrent group, n = 8; nonrecurrent group, n = 10). After global filtering for a standard deviation ≥1.0, a panel of 527 of 1105 miRNAs was used to perform an unsupervised hierarchical clustering analysis (UCA). UCA did not show clustering of the patients with HCC recurrence versus those without it (Fig. S1). However, the nonrecurrent group showed a high variance in miRNA expression patterns.

A supervised analysis comparing patients with and without tumour recurrence showed the upregulation of 84 miRNAs and downregulation of 130 miRNAs in the recurrence group. Candidate biomarker miRNAs were extracted using a P-value <0.10 as a cut-off, identifying five downregulated (miR-371, miR-939, miR3187, miR-3188 and miR-3197) and eight upregulated miRNAs (miR-let-7d, miR-let-7i, miR-140, miR-214, miR-455, miR-494, miR-1260 and miR-4284, see Table 3). Figure S2 shows a heat map of these miRNAs, which were differentially expressed with a cut-off P-value <0.10 between the recurrence and nonrecurrence groups.

In 34 of 74 patients within the Milan or UCSF criteria, we could not perform a miRNA analysis because of a tumour necrosis rate >30% (n = 17), extracted RNA of low quality (n = 6), or patient death within the first 12 months after LT, which prevented the assessment of tumour recurrence (n = 11).

Expression of miRNAs was associated with HCC recurrence after liver transplantation

For technical validation of the microarray data, we performed qRT-PCR for five miRNAs selected on the basis of P values (the lowest P values were selected). We included two downregulated miRNAs (miR-3187 and miR-3188) and three upregulated miRNAs (miR-140, miR-455 and miR-214) in a comparison between the recurrent and nonrecurrent groups (Fig. 2). Technical validation was

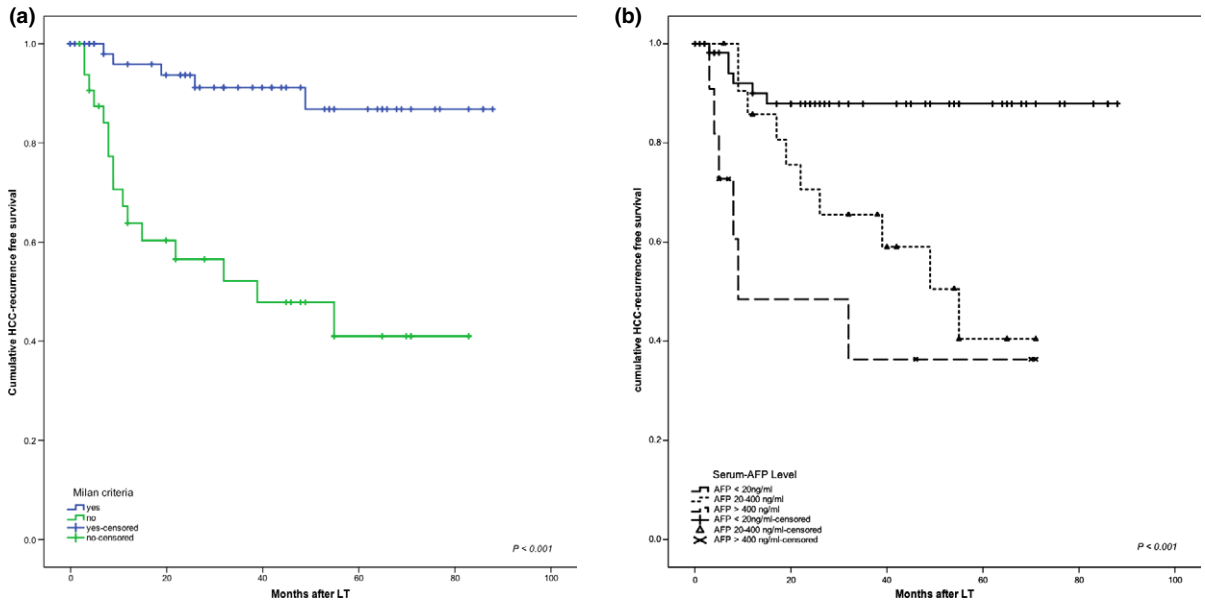

Figure 1 Association of the Milan criteria (a) and the serum AFP levels (b) with hepatocellular carcinoma recurrence-free survival following liver transplantation by Kaplan–Meier curves and the log-rank test.

performed in the same group of patients (n = 18, screening cohort) who were used for the prior array. Compared with the nonrecurrent group, the expression of miR-214 and miR-455 was significantly higher in the recurrent group, and the expression of miR-3187 was significantly lower (Fig. 2). There was no significant difference in the expression of miR-140. Unfortunately, miR-3188 could not be validated due to technical difficulties. The assay for this miRNA did not produce valid data.

miRNAs and Milan criteria as predictors of tumour recurrence after LT

Therefore, four miRNAs (miR-140, miR-214, miR-455 and miR-3187) differentially expressed in the microarray were analysed in the screening cohort for their predictive power. To evaluate the predictive potential of the selected miRNAs for tumour recurrence, we performed a univariate Cox regression analysis. We found that miR-214, miR-455 and miR-3187 correlated with the patient tumour recurrence and tumour recurrence-free survival (Table 4).

Although the patient numbers of the screening cohort were small, an explorative multivariate Cox proportional hazard regression was used to evaluate the significance of independent prognostic factors for patient tumour recurrence-free survival that were found to be significant in the univariate Cox regression analysis, including the serum AFP levels, the Milan criteria and the miRNAs. We found that two of the miRNAs (miR-214 and miR-3187) and the Milan criteria were significantly associated with tumour recurrence-free survival and may be used as independent prognostic factors (Table 5). Serum AFP levels and miR-455 were not identified as predictors in the multivariate Cox regression analysis in our screening cohort. For the next step, we developed a predictive score using the data (regression coefficient) from the multivariate Cox regression analysis as follows:

$$\begin{aligned}\text{Predictive Score} = {} & \\ & -2.033 \times [\text{miR-214}] + 4.217 \times [\text{miR-3187}] \\ & + 5.985 \times [\text{Milan in} = 1/\text{Milan out} = 2]\end{aligned}$$

As a cut-off, we used survivalROC methods at a follow-up time of 60 months. We defined the low- and high-risk groups for tumour recurrence and death with cut-off values $\geq$36 and <36, respectively (Fig. 3).

Validation of the findings in an independent cohort

To validate the findings from the screening cohort, a second and independent cohort (validation cohort) of 22 patients (recurrent group, n = 6; nonrecurrent group, n = 16) was analysed. High expression of miR-214 (P = 0.021) and low expression of miR-3187 (P = 0.006) were significantly associated with HCC recurrence after LT (Fig. 4a).

To evaluate the predictive potential of the selected miRNAs for tumour recurrence, we performed univariate tests (Mann–Whitney U-test) and a univariate Cox regression analysis of the patients of the validation cohort (n = 22).

Table 2. Clinical and pathological parameters of the cohort with microRNA (miRNA) and without miRNA analysis.

	miRNA analysis cohort (n = 40)	No-miRNA analysis cohort (n = 52)	P
Age years, mean (range)	56.13 (42–69)	57.13 (40.69)	0.425
Gender (female:male)	10:30	12:40	0.511
Serum AFP ng/ml, (range)	432.36 (1.1–6097.0)	2094 (1.6–60 500)	0.700
Bridging therapy			
TACE (n)	32	46	0.128
Others (n)	4	5	
No therapy (n)	2	3	
Pathology			
Tumour size, cm	2.55	2.35	0.105
>2 nodes	13	13	0.527
Microvascular invasion	4	4	0.647
Grading G3	5	4	0.601
Milan criteria fulfilled	34	24	0.572

Table 3. Differential microarray profiling in recurrent and nonrecurrent hepatocellular carcinoma after liver transplantation with P values <0.10.

microRNAs	FC	P	Alignments	Status
hsa-miR-140-3p_st	2.5	0.024	16:69966984–69967083 (+)	Up
hsa-miR-3187-3p_st	−4.6	0.044	19:813584–813653 (+)	Down
hsa-miR-455-3p_st	1.7	0.060	9:116971714–116971809 (+)	Up
hsa-miR-3188_st	−3.3	0.062	19:18392887–18392971 (+)	Down
hsa-miR-214_st	2.3	0.064	1:172107938–172108047 (−)	Up
hsa-miR-3197_st	−2.5	0.064	21:42539484–42539556 (+)	Down
hsa-miR-1260b_st	2.1	0.069	11:96074602–96074690 (+)	Up
hsa-miR-371b-5p_st	−3.5	0.075	19:54290931–54290996 (−)	Down
hsa-miR-4284_st	2.0	0.084	7:73125647–73125727 (+)	Up
hsa-miR-494_st	1.7	0.084	14:101495971–101496051 (+)	Up
hsa-miR-939_st	−2.6	0.089	8:145619364–145619445 (−)	Down
hsa-let-7d_st	3.1	0.094	4:11370451–11370545 (+)	Up
hsa-let-7i_st	1.5	0.099	12:62997466–62997549 (+)	Up

We found that miR-214 and miR-3187 were correlated with the patient tumour recurrence (Table 4). Furthermore, we evaluated the score combining miR-244 and miR-3187 with the Milan criteria, as previously described. In contrast to the Milan criteria alone, this score was significantly associated with recurrence-free survival in the Cox regression analysis (Table 5), which validated the predictive information of miR-244 and miR-3187. Using this score to define high- and low-risk groups, the Kaplan–Meier analysis also showed a significant difference between the groups in the validation cohort (P = 0.009, Fig. 4b). In the low-risk group within the Milan criteria, only two patients experienced tumour recurrence following LT. In the survivalROC analysis, our predictive score showed a higher sensitivity and specificity for tumour recurrence after LT, with an AUC of 0.885 (Fig. 4c) compared with the Milan criteria (AUC = 0.600) and AFP levels (AUC = 0.703).

Due to the lack of published mechanistic data on miR-3187, we used an *in silico* approach to identify the potential gene targets of this miRNA. A DIANA-MIRPATH v3.0 enrichment analysis of miRNA target genes predicted seven KEGG pathways to be potentially targeted by miR-3187. The highest number of target genes [9] was found within regions related to focal adhesion (P = 0.016) and the regulation of the cytoskeleton pathway (P = 0.016; Fig. S3).

Discussion

Hepatocellular carcinoma recurrence following LT is associated with poor long-term survival, and its prediction remains an unresolved issue [26,27]. Against the background of organ shortages, improvements in the prognostic tools for predicting outcomes after LT for HCC are necessary [7]. Our study indicated that a combination of tumour size and distribution (e.g. the Milan criteria) with a specific miRNA pattern may be a promising prognostic tool for the risk stratification of tumour recurrence following LT.

Initially, we identified differences in miRNA expression patterns between recurrent and nonrecurrent HCC using a microarray analysis in a screening cohort. After the identification of candidate biomarker miRNAs, four selected miRNAs (miR-140, miR-214, miR-455 and miR-3187) were technically validated in the screening group and in a second independent validation group.

After performing a Cox regression analysis in the screening cohort, we were able to develop a score that considers biological criteria (miRNA expression) in conjunction with the Milan criteria that significantly correlate with HCC recurrence-free survival. Although the sample size was small, we could confirm our predictive score in a second independent cohort. In this group, the predictive score was able to predict tumour recurrence more accurately than the Milan criteria alone or serum AFP levels. However, several limitations of our study must be mentioned. This study

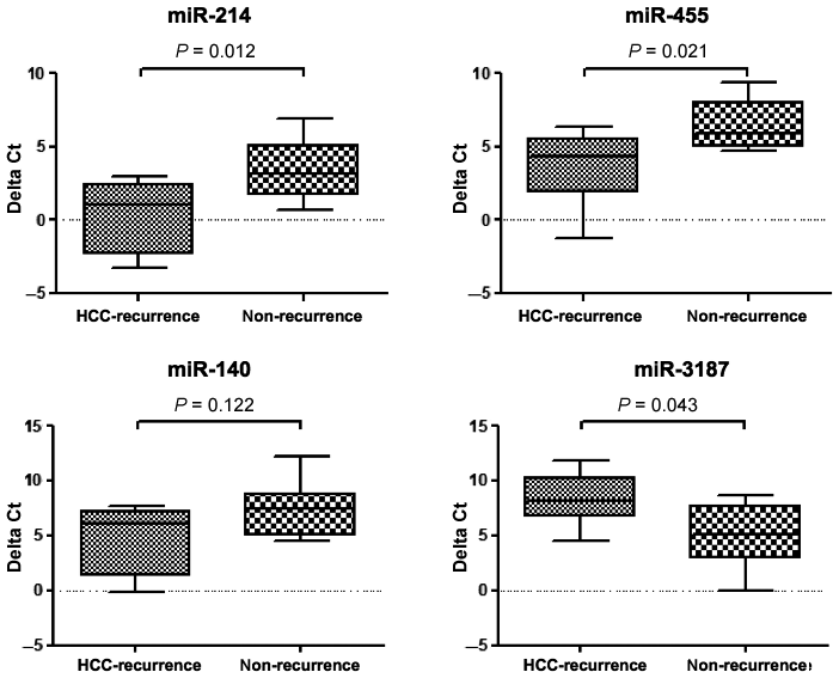

Figure 2 Technical validation of the microarray data for selected miRNAs. For technical validation, we performed qRT-PCR for selected miRNAs (miR-140, miR-214, miR-455 and miR-3187) and made comparisons between the recurrent and nonrecurrent groups of the screening cohort (screening group). Δ-CT values are in opposite direction to the regulation of the analysed miRNAs.

was performed in a retrospective, single-centre setting with a relatively small sample size. Furthermore, the miRNA expression patterns were obtained from the explanted livers of our LT recipients after hepatectomy. Therefore, the predictive value of our study is only valid for the analysis of the specimens collected at that time. Whether our predictive model may also have the same significance analysing HCC core-needle biopsies at the time of diagnosis is unclear because the miRNA expression patterns may change over time due to bridging therapies, such as transarterial chemoembolization [28,29]. Nevertheless, it has been shown that miRNA analysis is also feasible from HCC fine-needle biopsies, which can be performed before LT [30]. In our centre, 56.5% of the patients had a biopsy for HCC before LT. However, in 17.3% of the patients, the biopsy was negative for HCC (see Table S1). Although we were able to perform a miRNA analysis from the biopsy samples [30], the number of patients who had a biopsy-proven HCC before LT and fulfilled the enrolment criteria was too low in our study (55% in the screening cohort and 54% in the validation cohort).

Moreover, different studies have shown that cell-free and cellular miRNAs can be measured in the serum and that circulating miRNA levels are affected in HCC [20,31–34], which may lead to a highly relevant predictive signature at a very early time point in therapy. Circulating miRNAs in HCC may have a great potential to become diagnostic and prognostic tools for HCC in the future, but the research on circulation miRNAs is in its infancy [35].

In patients presenting with HCC in cirrhosis with preserved hepatic function, the available data are insufficient to answer the question of whether this group of patients benefits from LT or may be treated by liver resection with similar overall and disease-free survival [36]. Data from a large national cancer database analysing 3340 patients with clinical stages I and II HCC (American Joint Committee on Cancer (AJCC) show median survival periods for LT and liver resection of 127.9 and 56.7 months in stage I, respectively, whereas in stage II patients, the median survivals are 110.8 and 42.8 months, respectively ($P < 0.0001$) [37]. Because the existing criteria and scores merely define the limits of acceptable survival data by retrospective analyses of available radiological tumour distribution data [5,9,38], the tumour biology of the individual patient is insufficiently recognized. Actual data indicate that a subgroup of patients outside the Milan criteria and a well-defined downstaging algorithm may result in a low HCC recurrence rate and an excellent 5-year intent-to-treat survival of 56.1% vs. 63.3% ($P = 0.29$) compared with patients primarily presenting with a tumour distribution within the Milan criteria [39]. To our knowledge, the score presented in this manuscript was the first approach to combining

Table 4. Analysis of the four microRNAs in recurrent and nonrecurrent hepatocellular carcinoma with variance test analysis and their association with tumour recurrence-free survival in the univariate Cox regression analysis.

	Variance test (Mann–Whitney *U*-test)			Univariate Cox regression analysis	
	Nonrecurrent (mean, SD)	Recurrent (mean, SD)	*P*	HR [95% CI]	*P*
Screening cohort					
miR-140	4.779 (3.071)	7.351 (2.516)	0.122	0.822 [0.655–1.032]	0.091
miR-214	0.384 (2.413)	3.309 (1.986)	0.012	0.763 [0.592–0.982]	0.036
miR-455	3.560 (2.524)	6.421 (1.666)	0.021	0.699 [0.525–0.929]	0.013
miR-3187	8.200 (2.316)	4.957 (2.833)	0.043	1.769 [1.127–2.777]	0.013
Validation cohort					
miR-140	3.107 (3.221)	3.372 (3.999)	1.000	1.209 [0.890–1.642]	0.225
miR-214	2.230 (1.639)	5.075 (2.415)	0.021	0.734 [0.456–1.181]	0.203
miR-455	4.542 (2.280)	6.886 (3.173)	0.154	0.824 [0.544–1.249]	0.362
miR-3187	8.643 (3.128)	4.664 (2.189)	0.006	1.664 [0.977–2.834]	0.061

Table 5. Multivariate Cox regression analysis for tumour recurrence (backward stepwise regression).

	Coefficient	HR [95% CI]	*P*
Screening cohort			
miR-214	−2.033	0.131 [0.02–0.80]	0.027
miR-3187	4.217	67.8 [1.5–2980]	0.029
Milan criteria	5.958	387 [1.4–106 800]	0.038
Concordance index (SE)	0.981 (0.113)		
Validation cohort	0.118	1.125 [1.014–1.25]	0.027
Concordance Index (SE), Predictive Score	0.869 (0.159)		0.027
Concordance Index (SE), only Milan criteria	0.640 (0.095)		0.345

Other variables tested in the model: serum AFP, miR455.

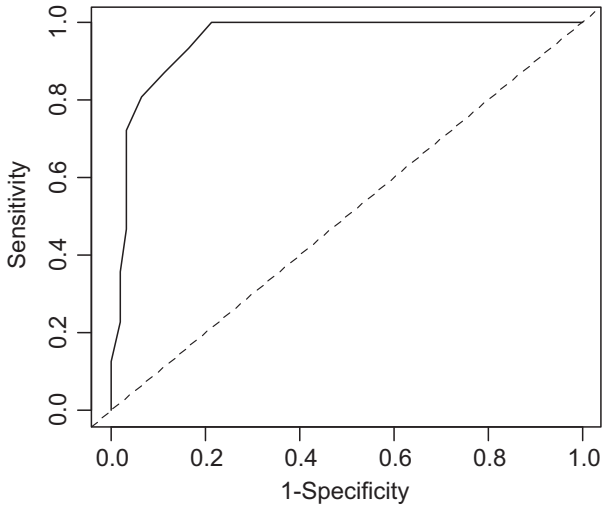

Figure 3 Time-dependent receiver operating characteristic curves (ROC) at a follow-up of 60 months suggesting a cut-off of 36 for the predictive score in the screening cohort. Based on that analysis, we defined low- and high-risk groups for tumour recurrence and death with cut-off values ≥36 and <36, respectively.

miRNA expression with the Milan criteria for the prediction of HCC recurrence following LT. This approach may close the gap between clinical tumour presentation and individual patient tumour biology as indicated by miRNA distribution patterns.

Two (miR-140 and miR-214) of the four validated miRNAs in our study were previously reported to be involved in HCC formation. MiR-140 could be identified as an HCC-related tumour suppressor miRNA by controlling nuclear factor kappa B (NF-κB) activity [40]. The downregulation of miR-140-5p was found to correlate well with multiple tumour nodules, tumour invasion and poor prognosis [41]. Because the clinical criteria of tumour characteristics such as the Milan, the UCSF and the expanded Asan criteria show a very heterogeneous picture of recurrence rates and long-term survival [42], these findings further highlight the role of biomarkers and their potential to unmask unfavourable tumour biology in individual patients. Dysregulation of miR-214 plays an important role in the field of tumour angiogenesis [43], and the downregulation of miR-214 in HCC tumour samples compared with benign adjacent liver tissue is associated with tumour progression and poor outcomes [14,15]. The ectopic expression of miR-214 in human HCC cell lines significantly inhibited cell proliferation, and in murine experiments, this ectopic expression suppressed tumour formation and tumour growth in nude mice. Hepatoma-derived growth factor could be identified as a target gene of miR-214. Furthermore, the ectopic expression of miR-214 or the antagonism of hepatoma-derived growth factor suppresses tumour angiogenesis, thereby suppressing tumour growth [14]. Another potential target gene of miR-214 is FGF-1, which is overexpressed in HCC [15,44]. Wang *et al.*

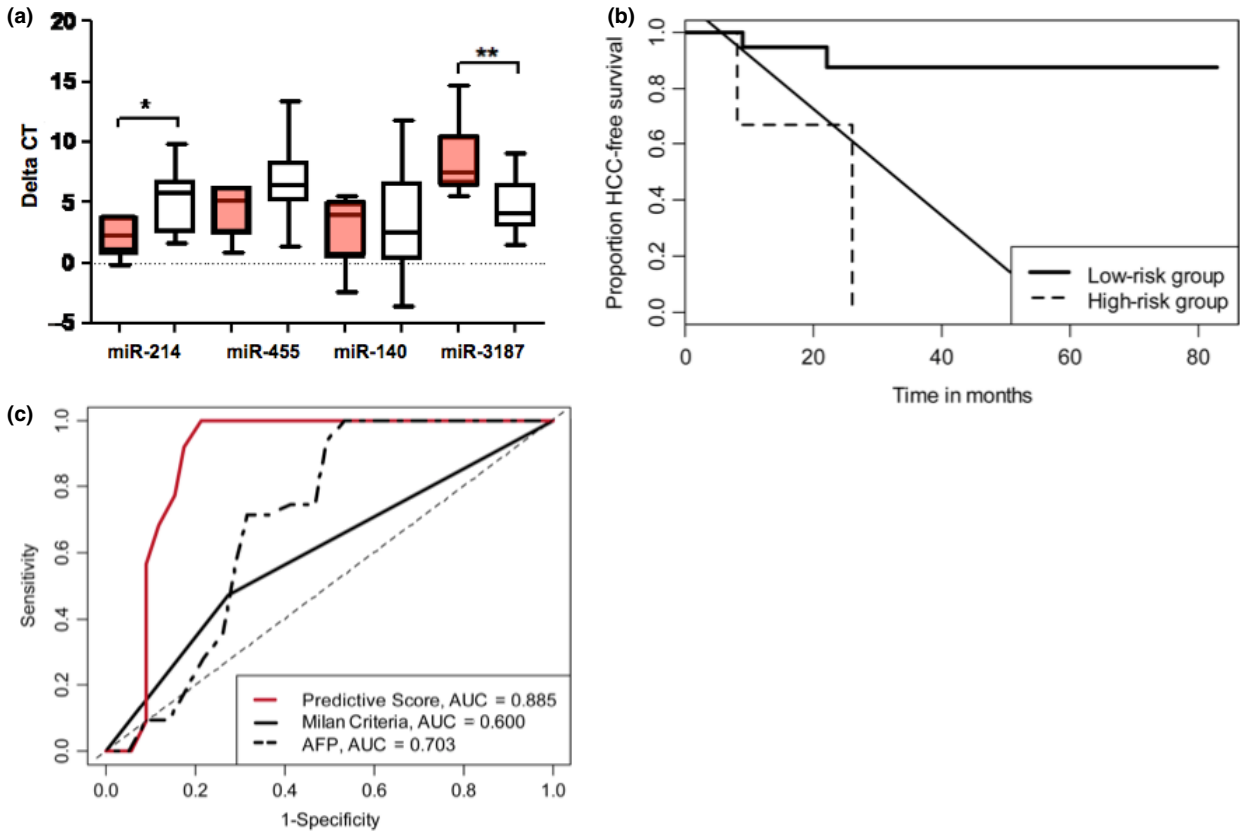

Figure 4 Validation of the miRNA expressions and the predictive score in an independent cohort of 22 patients. (a) QRT-PCR validation of different miRNA patterns between recurrent (red boxes) and nonrecurrent (white boxes) hepatocellular carcinoma (HCC) in an independent cohort. The expression of miR-214 and miR-3187 is associated with tumour recurrence after liver transplantation. ΔCT values are in opposite direction to the regulation of the analysed miRNAs. $*P < 0.05$; $**P < 0.01$ (b) Kaplan–Meier analysis shows HCC recurrence-free survival between low- and high-risk groups with a cut-off value of 36 for tumour recurrence in the validation cohort. (c) Our predictive score was associated with HCC recurrence-free survival in survivalROC analysis with a higher sensitivity and specificity compared with the Milan criteria and AFP levels (cut-off value 36).

[15] showed that the transfection of human HCC tumour cell lines with miR-214 resulted in the inhibition of cell invasion through the downregulation of FGF-1. Recently, it was shown that miR-214 is able to regulate the β-catenin pathway and hepatic cancer stem cells [45]. The identification of the target genes of miR-214 highlights the potential of miRNAs for anti-HCC therapy.

The role of miR-455 in HCC is unknown; however, in colorectal cancer cells, the overexpression of miR-455 inhibits proliferation and invasion by targeting RAF proto-oncogene serine/threonine protein kinase [46]. Different studies have shown that HCC development and progression are associated with the activation of the Raf1/MAP kinase pathway in humans [47,48].

Regarding miR-3187, there has been no report concerning its relationship to HCC or the molecular mechanism underlying the role of miR-3187 in tumour recurrence. However, the computational pathway analysis showed an enrichment of genes potentially influenced by miR-3187 within the region related to focal adhesion, as well as the regulation of the actin cytoskeleton. These cell functions have been associated with cancer development in general and were frequently found to be dysregulated in HCC previously [49–52]. However, the current pathway analyses should be treated with caution because they are based only on an *in silico* approach and are not yet supported by mRNA gene expression data.

Conclusion

In conclusion, our data support the hypothesis that the combination of miRNA expression levels and tumour distribution criteria (the Milan criteria) correlates with HCC recurrence in patients following LT. An upcoming study will validate our score in the HCC population in a second LT centre. Furthermore, the assays for detecting the identified miRNAs in blood samples should be evaluated and

established. In times of severe organ shortages in several countries, a new predictive score may be an effective tool for patient selection depending on low- or high-risk scores for HCC recurrence before LT.

Authorship

JL and JP-O: performed study, analysed data and wrote paper. CD: performed research/study. AAS and EH: analysed data. SZ: collected data, analysed data. MLH: contributed important reagents, analysed data. CM: designed study. M-WW: designed study. SZ: collected data. WOB: designed study. FU: designed study, analysed data and wrote paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Unsupervised hierarchical clustering of the miRNA expression patterns between the recurrence (R1-R8) and nonrecurrence groups (NR1-NR10). High expression levels are in red, low expression levels are in green.

Figure S2. Supervised hierarchical clustering of the miRNA expression patterns with a cut-off $P < 0.10$. MiRNA expression patterns are compared between the recurrence (R1-R8) and nonrecurrence groups (NR1-NR10). High expression levels are in red, low expression levels are in green.

Figure S3. The use of the *in silico* approach to identify potential gene targets of miR-3187. The DIANA-MIRPATH v3.0 enrichment analysis of miRNA target genes predicted seven KEGG pathways to be potentially targeted by miR-3187. The highest number of target genes was found within the regulation of cytoskeleton (a, $P = 0.016$) and the focal adhesion (b, $P = 0.016$) pathways.

Table S1. Clinicopathological features of the cohort.

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