ORIGINAL ARTICLE

Role of circulating microRNAs to predict hepatocellular carcinoma recurrence in patients treated with radiofrequency ablation or surgery

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Abstract

Background: Loco-regional treatments have improved the survival of patients with early hepatocellular carcinoma (HCC), but tumor relapse is a frequent event and survival rates remain low. Moreover, conflicting evidences address early HCC patients to surgery or radiofrequency ablation (RFA), with the clinical need to find predictive non-invasive biomarkers able to guide treatment choice and define patients survival.

Methods: Two independent case series of treatment-naïve HCC patients treated with local RFA, and a cohort of 30 HCC patients treated with liver surgery were enrolled.

On the basis of literature evidence, we customized a panel of 21 miRNAs correlated with relapse and prognosis after local curative treatment of HCC.

Results: Expression levels of let-7c predict tumor relapse after RFA; we also investigated the same panel in a small cohort of HCC patients undergoing surgery, finding no statistically significance in predicting tumor relapse or survival. Moreover, interaction test indicated that let-7c expression levels are predictive for identifying a subset of patients that should be addressed to surgery.

Conclusion: Results from this study could predict prognosis of early HCC patients, helping to address early HCC patients to surgery or RFA treatment.

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignancy of the liver, accounting for >80% of liver cancers.[1,2] It is ranked as the sixth most common cancer, the first leading cause of cancer-related death in many parts of the world, and it has been estimated as the fourth leading cause of cancer-related death worldwide.[3,4] HCC often arises in patients with underlying liver disease, through a complex multistep process involving inflammatory damage, fibrotic and cirrhotic liver, and it is associated with risk factors such as liver cirrhosis, metabolic liver disease, alcohol consumption and exposure to dietary toxins, hepatitis B (HBV) and C (HCV) infections.[3,5] Staging of the disease takes into account several parameters, *i.e.* the number and extent of the tumor nodules, the grade of liver dysfunction and performance status of the patient, with the need of a multidisciplinary approach involving experts in hepatology, hepatobiliary surgery, oncology and radiology; as a consequence, several staging systems have been constructed, of those the Barcelona Clinic

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LAY SUMMARY

Hepatocellular carcinoma (HCC) is one of the most common cancers, estimated as the fourth cause of cancer-related death worldwide. Although loco-regional treatments have improved the survival of patients with early disease, tumor relapse is a frequent event and survival rates remain low. Moreover, conflicting evidences address patients affected by early HCC to surgery or radiofrequency ablation (RFA), with the clinical need to find predictive biomarkers able to guide treatment choice and define patients survival. Micro-RNAs (miRNAs) are short non-coding nucleotides able to regulate gene expression at a post-transcriptional level. Deregulation in miRNAs expression has been established as a key regulator in HCC onset, progression and relapse, and have been widely investigated as HCC biomarkers. In this study, we evaluated the clinical value of a panel of circulating miRNAs in patients with early HCC undergoing RFA. By a training cohort and a validation cohort, we established that expression level of let-7c is able to predict tumor relapse after ablation therapy; we also investigated the same panel in a small cohort of HCC patients undergoing surgery, finding no statistically significance in predicting tumor relapse or survival. Moreover, interaction test indicated that let-7c expression levels are predictive for response to therapy, identifying a subset of patients that should be addressed to surgery. Once validated in larger prospective cohorts, the results from this study could predict prognosis of early HCC patients, helping to address early HCC patients to surgery or RFA treatment.

Liver Cancer (BCLC) is the most widely used.[3,6–8] Moreover, liver function is often evaluated through the Child-Pugh or the Model of End-stage Liver Disease (MELD) scores, while a scoring by Eastern Cooperative Oncology Group (ECOG) considers the overall well-being of the patients through a 5-points scale.[5]

Radiofrequency ablation is an effective and safe therapeutic option for early stage, unresectable and non-transplantable patients with <3 cm diameter nodules.[9–11] On the other hand, tumor relapse in treated patients are at high rates, and the results of several studies and meta-analysis comparing different approaches for these subset of patients have highlighted the great importance of patient stratification to select which is the best treatment choice.[12–15]

Even though a plethora of biomarkers able to predict tumor relapse have been investigated, to date no one has been validated to be useful in clinical practice.[16]

Micro-RNAs (miRNAs) are highly conserved small endogenous non-coding RNAs of approximately 19–25 nucleotides, which main function is to regulate gene expression at a post-transcriptional level through binding to the 3' untranslated region UTR of target mRNA, leading to translation blockade or mRNA degradation.[17]

miRNAs are recognized as deregulated biomarkers in several diseases such as viral infections, nervous system disorders, cardiovascular diseases, diabetes and cancer.[18] Several evidences have highlighted miRNAs as possible biomarkers for cancer diagnosis, prognosis, disease monitoring and therapeutic targeting,[18–20] and their stability in biological fluids enhance their potential as reliable non-invasive biomarkers.[21] More than other malignancies, HCC revealed distinct aberrant miRNAs profiles, and a number of studies have highlighted their role in disease onset and progression, such as HCC diagnosis, prognosis and even immune-microenvironment remodeling and drug resistance.[22–26]

In this context, circulating miRNAs have been investigating also as predictor of relapse-free survival (RFS) and overall survival (OS) of HCC patients treated with RFA, highlighting a possible role for these biomarkers also in these subset of patients.[27,28]

In this study, the potential clinical value of a panel of circulating miRNAs in predicting relapse-free survival of HCC patients treated with potential curative treatment was evaluated.

Materials and methods

Patient cohorts

Two independent case series of HCC patients treated with local RFA as a first-line treatment were enrolled and retrospectively analyzed. The first, training cohort, was composed of 41 consecutive patients enrolled and treated between April 2015 and January 2018 at the Internal Medicine Department of Degli Infermi Hospital, Faenza, Italy. The second, validation cohort, was composed of 69 consecutive patients enrolled and treated between June 2018 and March 2019 at Liver Unit, Sant'Orsola University Hospital of Bologna, Italy, and at the Internal Medicine Department, Degli Infermi Hospital, Faenza, Italy.

RFA was performed in patients under conscious sedation and local anesthesia. The RFA electrode using the Cool-Tip system was inserted into the tumor nodule under ultrasound percutaneous guidance.

During the ablation procedures, the creation of an echo–cloud complex on ultrasound image was carefully monitored to decide whether the index tumor was completely covered by the echo–cloud complex. When the coverage of index tumor by echo–cloud complex seemed to be incomplete, operators repositioned RFA electrodes after the initial session of ablation to achieve complete coverage of index tumor by the echo–cloud complex.

Moreover, contemporary to these cohorts, a cohort of 30 HCC patients that underwent liver surgery was enrolled at Liver Unit, Sant'Orsola University Hospital of Bologna, Italy, and Surgery Department of Morgagni-Pierantoni Hospital, Forlì, Italy.

Patients selection and procedures were performed by a multidisciplinary team according to the following inclusion

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criteria: Age ≥18 years; maximum diameter of tumor ≤3 cm; maximum number of tumor nodules ≤2; absence of extrahepatic metastasis; Child Pugh A-B7; ECOG 0–1. Patients were evaluated one month after therapeutic loco-regional treatment, every three months in the first year and every 6 months in for the following years by triphasic scanning technique.

The study was approved by the CEROM Ethical Committee (study code IRST-B088) and by the Ethical committee of University of Bologna (Study code 123/2017/O/Tess).

miRNA panel selection

On the basis of literature evidence, we customized a panel of 21 miRNAs correlated with relapse, relapse-free survival, and prognosis after local curative treatment of HCC, which demonstrated evaluability in human plasma or serum. In particular, the panel was composed of miR-34a-5p, miR-200c-3p, miR-26a-5p, miR-29a-3p, miR-122-5p, miR-155-5p, miR-221-3p, miR-148a-3p, miR-15a-5p, miR-15b-5p, miR-125b-5p, miR-30b-5p, miR-222-3p, miR-17-5p, let-7c, let-7d, let-7e, let-7f, let-7g, miR-140-3p, miR-21-5p.[27–38] For the selection of housekeeping (HSKs) genes, miR-484 and miR-106a-5p resulted as the most stable miRNAs by a GeNorm software (v 3.2) analysis in a fraction of the case series, and were included in the panel. Furtherly, the panel was implemented with cel-miR-39 as an extraction and miRNA amplification control. The final 24 miRNAs arrays were pre-spotted in 96-well custom qPCR plates, evaluated in duplicated and analysed through the ΔCt method. The expression levels of targets were then correlated with clinical outcome of patients.

Molecular analyses

For all patients, just before the tumor local ablation or surgery, a 3 mL EDTA-tube of peripheral blood was collected. Blood tubes were centrifuged at 2500 rpm for 15 min, and the obtained plasma were stored at −80 °C until use.

Circulating miRNAs expression: miRNAs extraction was performed starting from 400 μL of plasma through the miRVANA Paris kit (Thermo Fisher, Monza, Italy), with the spike-in cel-miR-39 at a final concentration of 0.1 mM mirNAs retrotranscription was executed through the TaqMan Advanced cDNA Synthesis kit (Thermo Fisher, Monza, Italy), and custom plates were run on a 7500 Real-Time PCR System (Thermo Fisher), with the following thermal cycling: a single step activation of 95 °C was followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 30 s (annealing/extension). Data was analysed with Thermo Fisher Cloud application software.

Gene expression analyses were performed using the ΔCt method, considering the fold change (Fc) of a single target normalized to the median value of the expression of its two housekeepings. Not all miRNAs were evaluable in every patient, and data were acquired in duplicated, considering for gene expression analyses only the miRNAs with both evaluable technical replicates with <0.5 cycle difference.

Statistical analyses

Association between categorical variables was assessed using the Fisher's exact test, when appropriate.

aRFS was defined as the time interval between the day of radiofrequency ablation and the day of any documented relapse (local or distant). Local relapse was defined as a tumor relapse in the same RFA-treated nodule, while distant relapse a new intra- or extra-hepatic nodule. All relapse-free survival (aRFS) were

Table 1 Clinico-pathological features of patients

	Training RFA cohort (n = 41)	Validation RFA cohort (n = 69)	Surgery cohort (n = 30)	P-value between RFA cohorts	p-value between training RFA and surgery cohort
	N (%)	N (%)	N (%)		
Gender					
Male	36 (87.8)	57 (82.6)	27 (90.0)		
Female	5 (12.2)	12 (17.4)	3 (10.0)	0.58	1.00
Etiology					
Hcv	19 (46.3)	38 (55.0)	17 (56.7)		
Hbv	7 (17.1)	6 (8.7)	4 (13.3)		
Other	15 (36.6)	25 (36.3)	9 (30.0)	0.38	0.68
Treated nodule (cm)					
<2	28 (68.3)	33 (47.8)	9 (30.0)		
>2	13 (31.7)	36 (52.2)	21 (70.0)	0.04	0.001
Child-Pugh					
A	40 (97.6)	54 (78.3)	30 (100.0)		
B	1 (2.4)	15 (21.7)	0 (0.0)	0.004	1.00
BCLC					
0	22 (53.6)	38 (55.0)	15 (50.0)		
A	19 (46.4)	31 (45.0)	15 (50.0)	0.88	0.81
Number of HCC nodules					
1	32 (78.0)	51 (73.9)	25 (83.3)		
2	9 (22.0)	18 (26.1)	5 (16.7)	0.65	0.76
AFP					
<20	35 (85.4)	55 (79.7)	21 (70.0)		
>20	4 (14.6)	14(20.3)	9 (30.0)	0.28	0.06
Platelets count (n x mL)					
<100	21 (51.2)	37 (53.6)	9 (30.0)		
>100	20 (48.8)	32 (46.4)	21 (70.0)	0.84	0.09
ALT					
<NV	13 (31.7)	42 (60.9)	12 (40.0)		
>NV	22 (68.3)	27 (39.1)	18 (60.0)	0.02	1.00
LESION RELAPSE					
YES	10 (24.4)	18 (26.1)	9 (30.0)		
NO	31 (75.6)	51 (73.9)	21 (70.0)		
EXTRALESION RELAPSE					
YES	19 (46.3)	21 (30.4)	17 (56.7)		
NO	22 (53.7)	48 (69.6)	13 (43.3)		

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estimated by the Kaplan–Meier method and curves were compared by the log-rank test. Unadjusted and adjusted hazard ratios (HRs) by baseline characteristics were calculated using the Cox proportional hazards model.

X-tile 3.6.1 software (Yale University, New Haven, CT) was used to determine the cutoff value for baseline levels of each miRNAs.

All p values were based on two-sided testing. MedCalc package (MedCalc® version 16.8.4, Bruges, Belgium) was used for the statistical analysis.

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Clinicopathological features of patients

The aim of this analysis was to examine the association between baseline expression levels of circulating miRNAs and (aRFS) in patients with early HCC treated with radiofrequency.

For each patient of the three cohorts, baseline serum levels of relevant clinical biomarkers (*i.e.* alpha-fetoprotein, albumin, alanine aminotransferase ALT and aspartate aminotransferase AST, neutrophils and lymphocytes with relative ratio NLR, creatinine) were evaluated.

A total of 140 consecutive patients affected by early HCC was analysed. RFA training and validation cohorts were composed of 41 and 69 patients, respectively; the surgery cohort was composed of 30 patients. Median age were 69 (range 27–88) and 66 (range 49–84) for the training and validation RFA cohorts, respectively, while it was 67 (range 40–82) for the surgery cohort. The patient characteristics for the three cohorts are shown in Table 1. The three cohorts had no overlapping patients.

RFA training cohort population had a median follow-up of 11.3 months (95%, 10.0–41.9) and at 12 months, 21 patients relapsed (51.2%). RFA validation and surgery cohorts had a median follow-up of 11.0 (95% 6.4–13.3) and 59.2 months (95% 21.0–59.2), respectively; at 12 months, 31 (44.9%) and 6 (20.0%) patients relapsed, respectively.

Circulating miRNA levels in relation to patient clinical outcome

After ROC curve for evaluating the best cut-off for every miRNAs (Supplementary table 1), the expression levels of the custom panel of circulating miRNAs were evaluated in relation to aRFS.

Training cohort

At univariate analyses, of the 21 analysed miRNA, we found that high levels of miR-17-5p (HR 4.02, 95% 1.1–14.5, p = 0.0332), miR-155-5p (HR 4.9, 95% 1.4–17.0, p = 0.0123), miR-34a-5p (HR 2.91, 95% 1.2–7.0, p = 0.0169), let-7c (HR 27.67, 95% 5.80–132.06, p < 0.0001), and let-7e (HR 3.15, 95% 1.2–8.1, p = 0.017) were significantly associated to aRFS (Fig. 1). No other clinical or circulating biomarkers were significantly related to HCC relapse (Table 2).

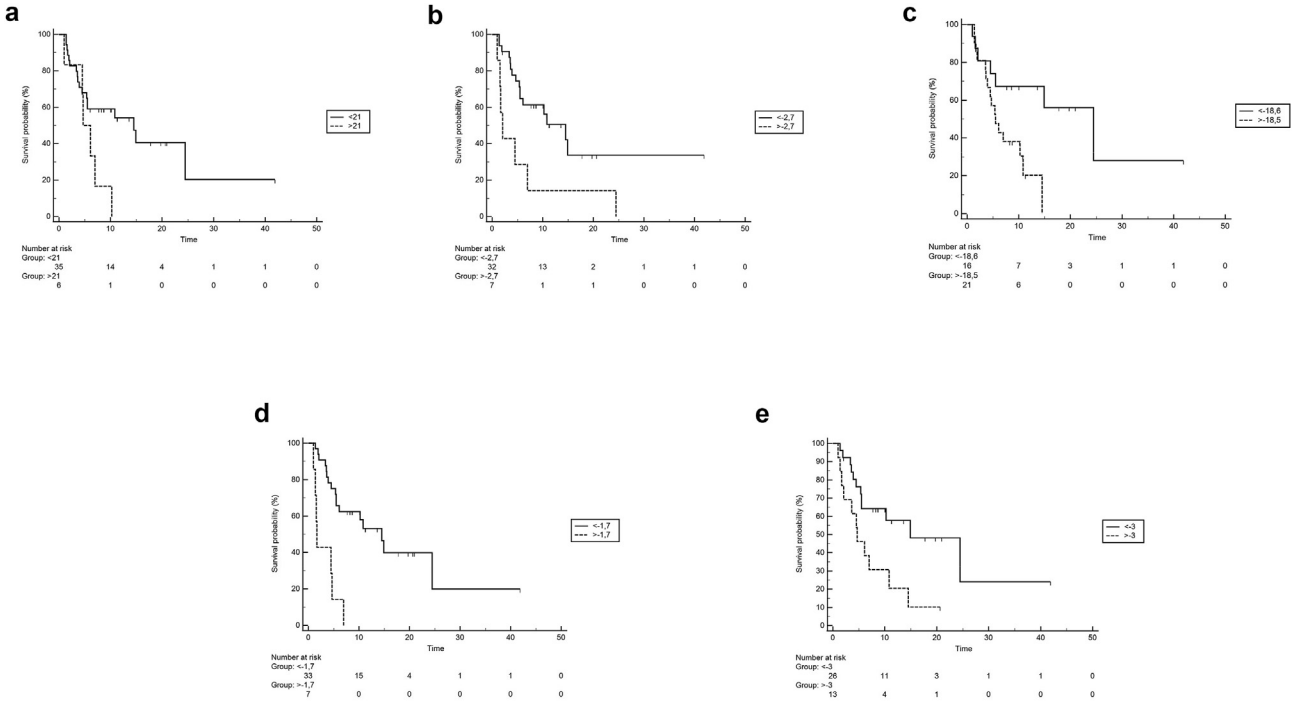

Figure 1 Relapse-free survival of patients, considering any relapse (aRFS), by miRNAs able to predict tumor relapse in the training cohort. (a) RFS for miR-17-5p; (b) RFS for mir-155-5p; (c) RFS for miR-34a-5p; (d) RFS for let-7c; (e) RFS for let-7e

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Table 2 Association of clinical parameters and circulating miRNAs with tumor relapse in the training cohort

	HR (95% CI)	p-value
Gender		
Male	1	
Female	2.76 (0.66–11.55)	0.1632
AFP		
<20	1	
>20	4.11 (0.86–19.54)	0.0752
Albumin		
<35	1	
>35	1.21 (0.29–5.01)	0.7895
Alanine-aminotransferase		
NV	1	
>NV	2.24 (0.90–5.61)	0.0831
BCLC		
0	1	
A	1.44 (0.64–3.23)	0.3750
Creatinine C.V.	3.50 (0.54–22.46)	0.1861
Size of largest nodule		
<2 cm	1	
>2 cm	1.47 (0.60–3.62)	0.3942
Aetiology		
HCV	1	
HBV	0.55 (0.19–1.59)	
Others	0.74 (0.30–1.86)	0.5392
Hemoglobin C.V.	1.01 (0.82–1.24)	0.9021
INR C.V.	0.89 (0.06–13.04)	0.9345
Lymphocytes C.V.	1.00 (0.99–1.00)	0.9262
MELD C.V.	0.98 (0.80–1.20)	0.9848
Neutrophils count	1.00 (0.99–1.00)	0.6844
NLR	1.07 (0.88–1.30)	0.4650
Number of nodule		
1	1	
2	2.43 (0.81–7.23)	0.1101
Platelets		
<100	1	
>100	0.66 (0.29–1.50)	0.3289
miR-34a-5p		
<-18.5	1	
>-18.5	2.92 (1.21–7.02)	**0.0169**
miR-200c-3p		
<-13	1	
>-13	2.00 (0.86–4.65)	0.1052
miR-26a-5p		
<7	1	
>7	0.55 (0.20–1.52)	0.2516
miR-29a-3p		
<1	1	
>	2.54 (0.85–7.62)	0.0959
miR-122-5p		
<1.6	1	
>1.6	1.61 (0.70–3.71)	0.2610
miR-155-5p		
<-2.7	1	
>-2.7	4.90 (1.41–17.03)	**0.0123**
miR-221-3p		
<50	1	
>50	3.31 (0.86–12.63)	0.0804
miR-15a-5p		
<81	1	
>81	1.36 (0.57–3.25)	0.4884
miR-15b-5p		
<3.5	1	
>3.5	0.38 (0.13–1.16)	0.0983
miR-125b-5p		
<-2.5	1	
>-2.5	0.79 (0.34–1.80)	0.5692
miR-30b-5p		
<3.5	1	
>3.5	0.72 (0.30–1.68)	0.4436
miR-222-3p		
<-1.7	1	
>-1.7	0.43 (0.17–1.10)	0.0811
miR-17-5p		
<21	1	
>21	4.02 (1.12–14.50)	**0.0332**
let-7c		
<-1.7	1	
>-1.7	27.67 (5.80–132.06)	**<0.0001**
let-7d		
<-4.8	1	
>-4.8	0.84 (0.36–1.98)	0.6924
let-7e		
<-3	1	
>-3	3.15 (1.23–8.10)	**0.0170**
let-7f		
<-1.8	1	
>-1.8	0.37 (0.13–1.05)	0.0633
let-7g		
<2.7	1	

(*continued on next column*)

(*continued*)

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Table 2 (*continued*)

	HR (95% CI)	p-value
>2.7	3.06 (0.90–10.40)	0.0737
miR-140-3p		
<-5.4	1	
>5.4	0.62 (0.21–1.77)	0.3687
miR-21-5p		
<11	1	
>11	0.67 (0.27–1.63)	0.3755

AFP: alpha-fetoprotein, NV=normal Value; BCLC: Barcelona Clinic Liver Cancer; C.V.:; MELD: Mayo End Stage Liver Disease.
The positive results are mentioned in bold.

After adjusting for clinical covariates (alpha fetoprotein, size of nodule, number of nodule and sex), let-7c > -1.7 and alphafetoprotein >20 ng/mL resulted as independent prognostic factors for poorer aRFS (HR = 17.80, 95% CI 2.70–117.18, p = 0.0027; HR = 11.56, 95% CI 2.09–63.98, p = 0.005, respectively).

ROC curve analysis showed an area under the curve (AUC) of 0.71 (95% CI 0.60–0.81; p = 0.0012) with a sensitivity of 36.8% and specificity of 100%; Positive Predictive Value of 100% and Negative Predictive Value of 53.8%.

All the miRNAs associated with relapse, except miR-34a-5p, resulted also significantly associated with distant relapse (Table 3 and Fig. 2). No miRNAs were significantly related to local relapse (Table 3).

Validation cohort

In the validation cohort, the 5 miRNAs that were significantly associated with aRFS in the training cohort were analyzed. At univariate analyses, miR-17-5p (HR 3.37, 95% 0.7–16.1, p = 0.007) and let-7c (HR 2.09, 95% 1,03–4,24, p = 0.02) confirmed to be significantly associated with aRFS, while let-7e and miR-34a-5p did not confirm their role in predicting aRFS (Fig. 3). miR-155-5p was not analyzed because there was only one patient with high expression levels. As for the training cohort, no other clinical markers were associated with aRFS. By the same multivariate analysis of training cohort we confirmed that high levels of let-7c remains the only biomarker able to predict aRFS of patients (HR 3.06, 95% 1.17–7.99, p = 0.0216).

Surgery cohort

In the cohort of patients undergoing surgery, we performed the same molecular analyses on circulating miRNAs designed for the other cohorts. In this patient cohort, we found no miRNAs either clinical or other circulating biomarkers significantly associated with aRFS (Tab 3). In particular, for let-7c the HR (Ref < -1.7) was 0.69 (95% IC 0.27–1.74; p value 0.403) (Fig. 4). After adjusting for clinical covariates (alpha fetoprotein, size of nodule, number of nodule and sex), let-7c > -1.7 remains no significant (HR = 0.81, 95% CI 0.22–2.98, p = 0.7496).

Table 3 Association between circulating miRNAs and site of tumor relapse in the training cohort

	Distant relapse		Local relapse	
	HR (95% IC)	P value	HR (95% IC)	P value
miR-17-5p				
<21	1		1	
>21	7.78 (1.85–32.6)	**0.0050**	3.25 (0.57–18.53)	0.1841
miR-155-5p				
<-2.7	1		1	
>-2.7	4.11 (1.12–15.00)	**0.0325**	1.48 (0.56–2.65)	0.781
miR-34a-5p				
<-18.6	1		1	
>-18.6	2.39 (0.91–6.27)	0.0749	1.16 (0.33–4.09)	0.8122
let-7c				
<-1.7	1		1	
>-1.7	9.33 (2.14–40.59)	**0.0029**	3.96 (0.65–20.09)	0.1351
let-7e				
<-3	1		1	
>-3	4.29 (1.50–12.28)	**0.0066**	1.70 (0.41–6.95)	0.4569

The positive results are mentioned in bold.

With the aim to assess the predictive role of let-7c for patients undergoing RFA, we performed an interaction test between data from the RFA and surgery cohorts. By this analysis, we confirmed the negative predictive role of let-7c for patients treated with RFA, who should be addressed to surgical treatment (p = 0.045). The forest plot (Fig. 5) highlighted that the choice of surgery conferred a better aRFS compared to RFA in patients with let-7c > -1.7 (HR 0.34; p = 0.0008).

Discussion

In this study, we evaluated a custom panel of circulating miRNAs in relation to aRFS of HCC patients treated with local RFA. Through a training and an independent validation cohort, we established the role of let-7c in predicting aRFS after local curative treatment. Furthermore, through the interaction test of the expression levels of let-7c of these cohorts with respect to a small case series of HCC patients undergoing surgical treatment, we established the predictive role of let-7c circulating levels for RFA treatment, identifying a subset of patients who should be addressed to surgical treatment.

miRNAs are a fraction of non-coding RNAs with short nucleotide sequence (18–22 nucleotides) that are involved in cancer onset, progression and patient prognosis, as well as

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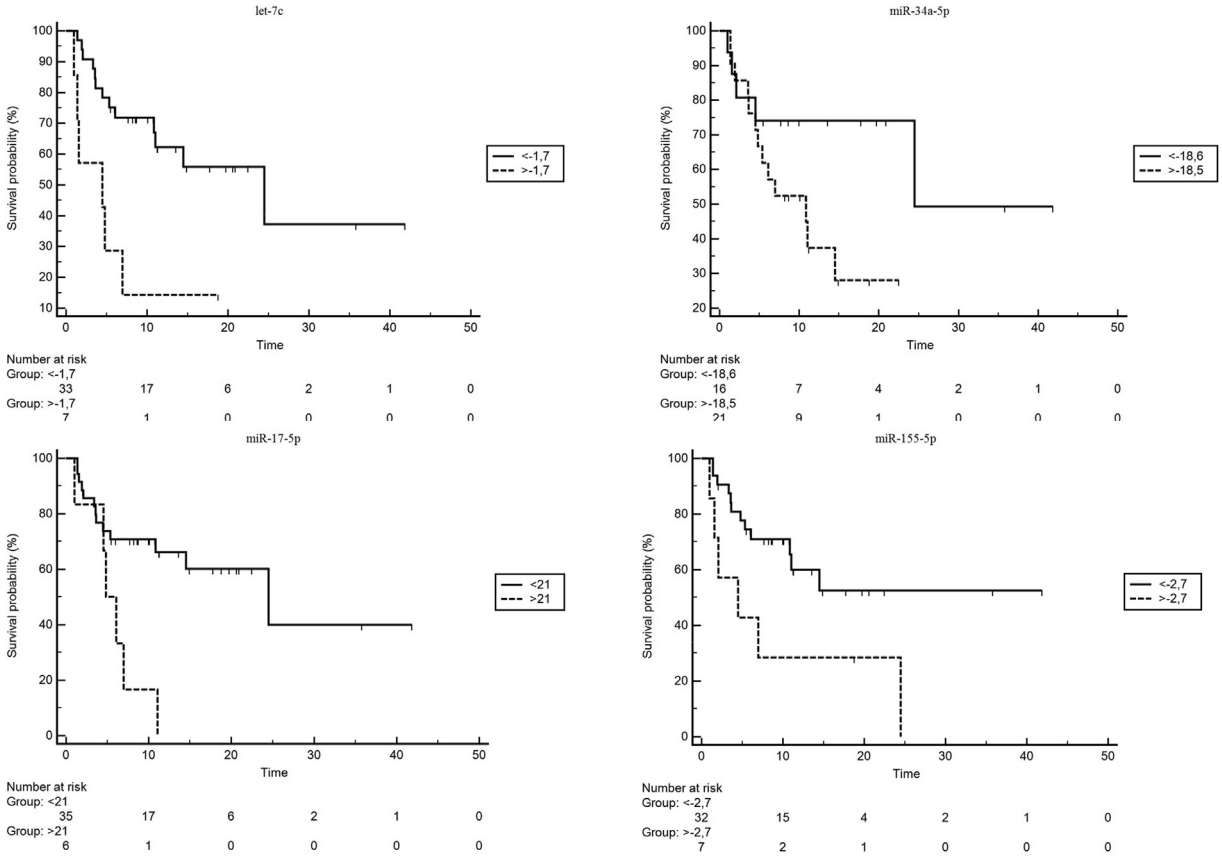

Figure 2 Relapse-free survival, only considering distant relapse, of patients in the training cohort by miRNAs expression

therapeutic targets for several malignancies, including HCC.[22,23,25] As detectable in serum/plasma of patients, miRNAs are recognized as important liquid biopsy biomarkers for diagnosis, prognosis prediction and disease monitoring for cancer patients.[21]

Local radiofrequency ablation is an effective treatment for patients with HCC nodules of <3 cm diameter, but tumor relapse in the treated nodule or with a formation of a new one is a frequent event in a considerable percentage of patients.

Moreover, several studies and meta-analyses compared the efficacy of RFA treatment and surgical treatment in terms of DFS and OS, with no clear differences established.[39–42] Thus, in these patients setting there is a need to find and validate clinical or biological markers able to serve as a clinical tool to address patient treatment.

let-7c is one of the 9 miRNAs of the miRNA let-7 family, a highly conserved miRNA family firstly discovered in *C.elegans* as a developmental gene[43,44]; miRNAs of let-7 family are generally recognized as anti-oncomiRs, targeting several pathways involved in carcinogenesis.[45] As in other malignancies, also in HCC let-7 family has been highlighted to act in a plethora of cellular mechanisms to prevent cancer progression and self-renewal, such as targeting of B-cell lymphoma 2 like protein 1 (Bcl-xL) and EMT process.[46,47] Moreover, circulating levels of mir-7a/7c/7d-5p were associated with disease progression in liver chronic hepatitis C patients,[48] while another study clustered this miRNA family to predict patient prognosis.[49]

A number of studies found low levels of let-7c in HCC compared to non-tumoral tissues,[50–52] and *in-vitro* studies highlighted that let-7c inhibits cellular proliferation and induces cellular apoptosis targeting CD25A, PI3K/Akt/FoxO and Wnt signalling.[47,53,54] On the other hand, even though let-7c is able to target the oncogenic transcription factor c-Myc, it has been showed that c-Myc is able to down-regulate the expression of let-7 family, highlighting how cellular miRNAs could have different functions in carcinogenesis.[50,55,56]

Consistent with these data, we found that most of the patients from the two independent cohorts had low levels of circulating let-7c, confirming that this miRNA is generally down-regulated in HCC patients, also in the subset of patients with early HCC. On the other hand, we found that high levels of let-7c are able to predict patient relapse after local curative treatment. Consistent with our data, a study by Shi et al. demonstrated that high tissue levels of let-7 family is able to predict prognosis of patients affected by early HCC; in particular, they found that let-7c overexpression was correlated with vein invasion and TNM

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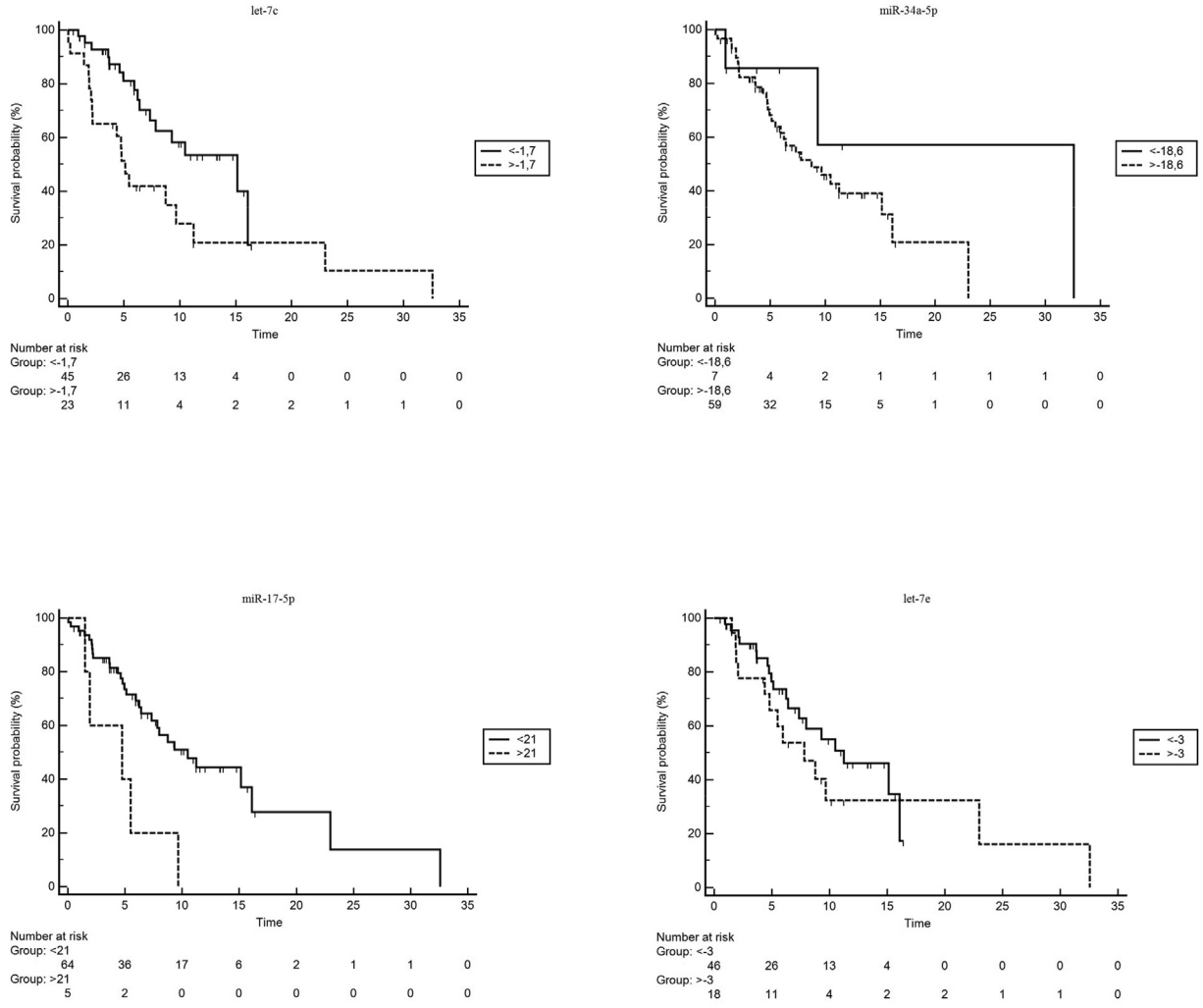

Figure 3 Relapse-free survival, considering any relapse, of patients in the validation cohort by miRNAs expression

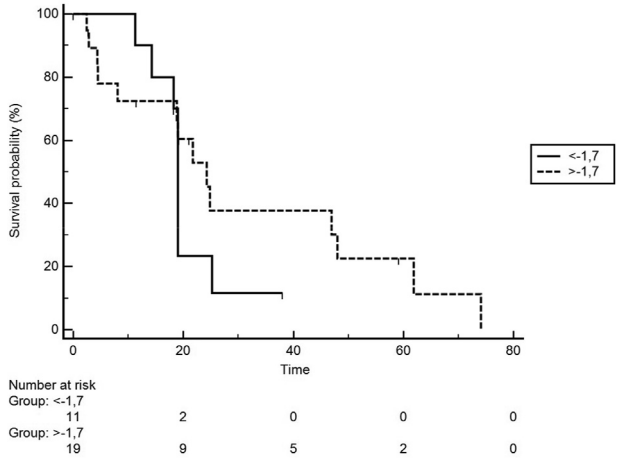

Figure 4 Relapse-free survival, considering any relapse, of patients in the surgery cohort by let7-c expression

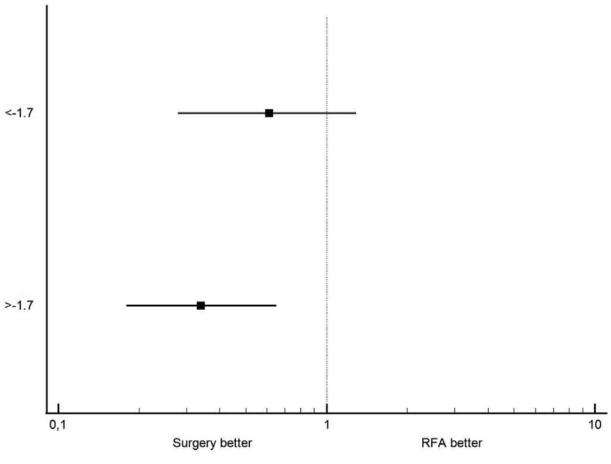

Figure 5 Forrest plot to predict the best treatment choice by let-7c expression

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stage, and with post-operative OS of patients.[57] This suggest that let-7c, even though recognized as an anti-oncomiR in HCC, could have a relevant role in the HCC cellular mechanisms, especially in the relapse after curative treatment of early stage patients.

A relevant aspect of the present study is that the miRNA panel was established based on literature evidences of HCC relapse after curative treatment, thus a specific validation was performed, without exploring large panels often correlated with many parameters.

Some limitations rely in this study, *e.g.* the difference in patient characteristics between RFA and surgery cohort; for this reason the prognostic and/or predictive role of miRNA should be validated in a randomized trial. Second, the relative short follow-up of the patients did not allow us to have mature data on overall survival.

Conclusion

To the best of our knowledge, this is the first study highlighting that the pre-treatment expression levels of a single biomarker predict HCC relapse after RFA loco-regional treatment, and could be able to establish a treatment choice for early HCC patients.

Novelty and impact statement

Tumor relapse after local radiofrequency ablation (RFA) is a frequent event in early hepatocellular carcinoma (HCC) patients. Through a liquid biopsy approach in a training and validation patients cohort, we establish that a single-biomarker expression is able to predict patients prognosis, and to address patients to RFA or surgery.

Authors' contributions

Conception and design: Andrea Casadei Gardini, Matteo Canale.

Acquisition of data (acquired and managed patients): All Authors.

Analysis and interpretation of data: Andrea Casadei Gardini, Matteo Canale.

Writing, review, and/or revision of the manuscript: Andrea Casadei Gardini, Matteo Canale.

All the authors have approved the manuscript.

Financial support

No support.

Conflicts of interest

A.C.G. reports receiving consulting fees from AstraZeneca, Bayer, Eisai, MSD, Ipsen, IQVIA, lectures fees from Eisai, Ipsen, Merck Serono, Roche.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.hpb.2021.06.421.

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