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# MiR-3201 as a Prognostic Blood Biomarker for Curative Treatments in Hepatocellular Carcinoma

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## Abstract

**Background:** Hepatic resection, radiofrequency ablation (RF), and liver transplantation (LT) represent the only available curative treatments for early stage hepatocellular carcinoma (HCC). Various studies showed that the 5-year overall survival (OS) rate reaches ~70% after resection and ~60% after RF. **Objective:** To improve the success rate of curative therapies and consequently the OS, an improvement in patients' selection and management should be pursued. In this regard, microRNAs (miRNAs) can be helpful prognostic biomarkers. **Materials and Methods:** In this retrospective study, a miRNA array profiling was performed on 34 HCC blood samples which is collected before therapy (T0), 1 month (T1), and 6 months (T2) after curative treatments (resection and RF) to identify noninvasive biomarker candidates for therapy response and OS. MiRNAs were validated in 80 blood HCC samples using quantitative real-time PCR (qRT-PCR). Patients were divided into complete responder (CR) and partial responder and progressive disease (PRPD). **Results:** Among the selected miRNAs, miR-3201 is significantly associated with treatment response in the validation phase, showing a 23% reduction ($P = .026$) in CR compared to PRPD. MiR-3201 was able to distinguish CR from PRPD (area under the curve [AUC] = 0.69, 71% sensitivity, 70% specificity, $P = .0036$). Furthermore, lower levels of miR-3201 were associated with longer OS (hazard ratio [HR] = 2.61, $P = .0006$). **Conclusions:** Blood miR-3201 could be used as a prognostic biomarker for curative therapy response and OS in HCC.

## Keywords

microRNA, blood, circulating miRNA, biomarker, hepatocellular carcinoma, liver cancer, HCC, resection, radiofrequency

## Abbreviations

AUC, area under the curve; BCLC, Barcelona Clinic Liver Cancer; CR, complete responder; DFS, disease-free survival; EASL, European Association for the Study of the Liver; FC, fold change; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; LT, liver transplantation; miRNAs, microRNAs; mRECIST, modified Response Evaluation Criteria In Solid Tumors; NAFLD, non-alcoholic fatty liver disease; OS, overall survival; PRPD, partial responder and progressive disease; REMARK, REporting recommendations for tumour MARKer prognostic studies; RF, radiofrequency ablation; RIN, RNA integrity number; ROC, receiver operating characteristic; RT-qPCR, quantitative real-time PCR

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## Introduction

Primary liver cancer is one of the most common tumors in the world, ranking second as the most frequent cause of cancer-related death, with an estimated global incidence rate per 100,000 person-years of 9.3 and a corresponding mortality rate of 8.5 in 2018.[1–3] Hepatocellular carcinoma (HCC) represents approximately 75% of the total cases of primary liver cancer, having an incidence strongly correlated to the male gender and advanced age.[4,5] Almost all cases of HCC are associated with a known etiology, most frequently chronic viral hepatitis (B and C), non-alcoholic fatty liver disease (NAFLD), genetic and hereditary disorders, environmental toxins such as aflatoxin exposure, metabolic diseases including diabetes mellitus and obesity, and dietary and lifestyle factors like alcohol consumption and smoking.[6,7] Despite the variety of etiologies, several factors promote the development of chronic damage during their long clinical course, often leading to liver cirrhosis, which represents one of the primary predisposing factors of HCC.[8,9] Indeed, one-third of cirrhotic patients develop HCC during their lifetime.[4]

In contrast to the reduction of death rates observed for many cancer types, HCC mortality continues to increase by ~2% to 3% per year due to the late diagnosis which hampers the efficacy of the currently available therapies.[10,11] Only 40% of patients with HCC (Barcelona Clinic Liver Cancer [BCLC] stages 0 and A) are eligible for liver transplant, surgical resection, or tumor ablation therapies, the potentially curative treatments.[12,13] Liver transplantation (LT) removes both the tumor and cirrhosis, with a 5-year overall survival (OS) rate of 70% and 6% to 18% of HCC recurrence.[14,15] Hepatic resection is a recommended option in patients with small lesions and preserved liver function, providing a 5-year OS rate of 60% to 70% and median disease-free survival (DFS) of 2 years with a low risk of early recurrence in 76.5% of patients.[16–19] Radiofrequency ablation (RF) is a minimally invasive therapy applicable to patients with small lesions. Around 60% of patients treated with RF can benefit from a 5-year survival, or even more patients (76%) if selected according to the BCLC guidelines.[20–22] The detection of lesions at early stages coupled with a personalized therapeutical protocol for each patient may represent a strategy to improve patient survival. The assessment of blood-based biomarkers at the time of diagnosis may help clinicians in a more precise patient stratification providing at the same time additional information for better clinical management.

In this context, circulating microRNAs (miRNAs) represent a valuable tool supporting conventional clinical practices.[23,24] They are small noncoding RNAs with a length of 19 to 22 nucleotides involved in the post-transcriptional gene silencing of their target genes.[25,26] Besides circulating miRNAs having a value of noninvasiveness, ease of measurement, and cost-effectiveness, they are stable and not degraded in blood, which makes them suitable as diagnostic, predictive, and prognostic biomarkers.[27,28] MiRNAs are generally studied in plasma and serum. However, blood can represent an interesting source since it contains additional noncoding RNAs derived from immune system cells.

In this study, we assessed the blood miRNA expression at the time of HCC diagnosis, at 1 month and 6 months after therapy to identify miRNAs associated with a complete response to curative treatments and OS, which can help clinicians in more accurate patient management.

## Materials and Methods

The reporting of this study conforms to REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines.[29]

### Study Design

The present retrospective study was organized into 2 phases.

*Discovery phase (phase 1)*: Thirty-four blood samples collected from 13 patients at the time of diagnosis (T0), 1 month (T1), and 6 months (T2) after curative therapy (resection and RF) were analyzed through microarray profiling. Six months after therapy, the patients were categorized, according to modified Response Evaluation Criteria In Solid Tumors (mRECIST) as complete responder (CR), partial responder (PR), and progressive disease (PD). Kruskal–Wallis test was used to determine gene expression differences in microarray analysis between CR patients *versus* PR + PD (PRPD) patients. In the discovery group, miRNAs associated with complete response to curative treatments were selected as blood biomarker candidates for subsequent validation.

*Validation phase (phase 2)*: MiRNA candidates selected in the discovery phase were tested by quantitative real-time PCR (RT-qPCR) in other 80 HCC blood samples from 39 patients treated with resection and RF at the 3 considered time points. MiRNA expression determined at T0, T1, and T2 was associated with the response to therapy and patient survival.

### Patients

Between 2012 and 2017, 52 consecutive patients referring to the Liver Center who were diagnosed with HCC according to the European Association for the Study of the Liver (EASL) criteria were enrolled for the study. Blood samples were collected at the time of HCC diagnosis (T0), 1 month (T1), and 6 months (T2) after curative treatments (hepatic resection [n = 18] and RF [n = 34]). Six months after therapy, 73% of patients had a complete response to treatment, while 27% did not respond. The end of the follow-up period was June 2021 (median follow-up: 48 months). The clinical and demographic features of the groups are shown in Table 1.

All the patients provided written informed consent for the blood samples and associated clinical data collection. Patient anonymity has been preserved. The investigation was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the regional ethical committee (Comitato Etico Regionale Unico FVG, No. 14/2012 ASUITS).

**Table 1.** Clinical Characteristics of the Enrolled Patients.

| | Discovery | | | Validation | | |
|---|---|---|---|---|---|---|
| | Tot (n = 13) | CR (n = 9) | PRPD (n = 4) | Tot (n = 39) | CR (n = 29) | PRPD (n = 10) |
| Age mean (95% CI) | 71.8 (66.2-75.1) | 73.6 (72.5-75.1) | 67.9 (55.8-80.0) | 70.4 (67.7-73.0) | 69.6 (66.3-72.9) | 72.8 (68.3-77.3) |
| Gender | | | | | | |
| Female | 2 | 2 | 0 | 7 | 5 | 2 |
| Male | 11 | 7 | 4 | 32 | 24 | 8 |
| Etiology | | | | | | |
| Alcohol or metabolic | 8 | 5 | 3 | 16 | 11 | 5 |
| Viral | 4 | 4 | 0 | 12 | 9 | 3 |
| Mixed | 1 | 0 | 1 | 11 | 9 | 2 |
| Scores | | | | | | |
| CTP A/B | 12/1 | 9/0 | 3/1 | 33/6 | 24/5 | 9/1 |
| BCLC 0/A | 3/10 | 2/7 | 1/3 | 6/33 | 4/25 | 2/8 |
| No. of lesions | | | | | | |
| Single <2cm | 2 | 2 | 0 | 4 | 4 | 0 |
| Single <5cm or 3 nodules ≤3cm | 9 | 6 | 3 | 27 | 20 | 7 |
| Large single or multi | 2 | 1 | 1 | 8 | 5 | 3 |
| AFP (ng/mL) | | | | | | |
| <20 | 10 | 7 | 3 | 25 | 19 | 6 |
| 20 to 400 | 3 | 2 | 1 | 6 | 3 | 3 |
| >400 | 0 | 0 | 0 | 2 | 2 | 0 |
| Treatment | | | | | | |
| Resection | 4 | 1 | 3 | 14 | 9 | 5 |
| RF | 9 | 8 | 1 | 25 | 20 | 5 |

Abbreviations: AFP: alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; CR: complete responder; CTP: Child–Turcotte-Pugh; PRPD: partial responder and progressive disease; RF: radiofrequency ablation.

### *Sample Collection*

Fasting whole blood samples were collected from each patient during clinical visits. Peripheral blood (3 mL per patient) was collected in Tempus™ Blood RNA Tubes containing 6 mL of Stabilizing Reagent (Thermo Fischer Scientific) and subsequently frozen at – 80 °C for long-term storage.

### *RNA Isolation and Quality Assessment*

Total RNA was isolated from Tempus™ Blood RNA Tubes using the MagMAX™ for Stabilized Blood tubes RNA Isolation Kit (Thermo Fischer Scientific) following the manufacturer protocol. The quality of the total RNA extracted was assessed with the Agilent RNA 6000 Nano Kit (Agilent Technologies) using the 2100 Bioanalyzer Instrument (Agilent Technologies). Samples with an RNA integrity number (RIN) less than 6 were discarded from the subsequent profiling experiments.

### *RNA Microarray Profiling and Analysis*

MiRNA profiles were analyzed through the Affymetrix GeneChip® microRNA 3.0 Array (Affymetrix®, Thermo Fischer Scientific) as previously described.[30]

### *qRT-PCR validation*

Fifty nanograms of total RNA were reverse-transcribed using the qScript microRNA cDNA Synthesis Kit (QuantaBio) according to manufacturer instructions. Samples were analyzed through qRT-PCR using the PerfeCTa SYBR® Green SuperMix (QuantaBio) in a CFX-96 thermal cycler (Bio-Rad Laboratories) according to manufacturer instructions. All reactions were run in duplicate in a 25μL reaction. MiRNA primers were purchased from Metabion International AG (Metabion) or Sigma-Aldrich (Merck KGaA). MiR-486 was used as an endogenous normalizer. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ formula.

### *Statistical analysis*

The Mann–Whitney U test was used to compare the differences between the 2 independent groups. For multiple comparisons, the Kruskal–Wallis test in a one-way ANOVA procedure was used. The receiver operating characteristic (ROC) curves were plotted to estimate the discriminatory potential of the miRNAs. Survival curves were plotted according to the Kaplan–Meier method. Analyses were performed using NCSS 11 Software (2016) (NCSS, LLC, ncss.com/software/ncss).

## Results

### MiRNA Screening in the Discovery Cohort

HCC blood samples collected from 13 patients at T0 (n = 13), T1 (n = 13), and T2 (n = 8) were analyzed through the Affymetrix GeneChip® miRNA 3.0 Array build on miRBase release 20. Nineteen miRNAs were differently expressed ($P < .05$) between CR and PRPD patients treated with curative therapies at all considered time points (Table 2 and Figure 1), with miR-370 and miR-3201 being the top-scoring miRNA in terms of both differential expression and $P$ value ($P < .001$). MiR-370 showed a higher expression in CR compared to PRPD (fold change (FC) = 6.47), while miR-3201 was higher in PRPD compared to CR (FC = 5.95) (Table 2).

**Table 2.** Differentially Expressed miRNAs Between CR and PRPD Patients of the Discovery Cohort at all Considered Time Points.

| ID | CR Avg. ($log_2$) | PRPD Avg. ($log_2$) | FC (linear) | $P$ value |
|---|---|---|---|---|
| hsa-miR-370 | 4.34 | 1.65 | 6.47 | .0003 |
| hsa-miR-769-3p | 4.72 | 2.54 | 4.52 | .0174 |
| hsa-miR-574-5p | 7.52 | 5.76 | 3.39 | .0441 |
| hsa-miR-134 | 3.41 | 1.87 | 2.89 | .0272 |
| hsa-miR-664a-5p | 6.42 | 4.93 | 2.81 | .0318 |
| hsa-miR-30c-1-3p | 4.05 | 2.62 | 2.7 | .0133 |
| hsa-miR-18a | 9.09 | 7.68 | 2.67 | .0175 |
| hsa-miR-1255b | 7.21 | 5.81 | 2.64 | .0121 |
| hsa-miR-15a | 10.98 | 9.58 | 2.64 | .0420 |
| hsa-miR-3128 | 2.64 | 1.26 | 2.61 | .0101 |
| hsa-miR-122 | 6.55 | 5.25 | 2.45 | .0438 |
| hsa-miR-2276 | 5.12 | 3.93 | 2.28 | .0258 |
| hsa-miR-4525 | 2.42 | 1.23 | 2.28 | .0281 |
| hsa-miR-23a-5p | 4.91 | 3.78 | 2.19 | .0405 |
| hsa-miR-548q | 5.2 | 4.17 | 2.05 | .0021 |
| hsa-miR-3150a-5p | 1.01 | 2.11 | −2.15 | .0482 |
| hsa-miR-125b | 7.82 | 9.8 | −3.95 | .0316 |
| hsa-miR-100 | 7.04 | 9.04 | −4.02 | .0353 |
| hsa-miR-3201 | 1.62 | 4.2 | −5.95 | .0009 |

Abbreviations: CR: complete responder; FC: fold change; PRPD: partial responder and progressive disease.

### Identification of miRNA Biomarker Candidates for Curative Treatments

We used the qRT-PCR assays to confirm the expression of 2 selected miRNA candidates from the miRNA array study (miR-370 and miR-3201). We assessed the miRNA expression in other 80 blood samples from HCC patients collected at T0 (n = 35), T1 (n = 24), and T2 (n = 21). Among the selected miRNAs, only miR-3201 was confirmed as statistically significant in the differential expression between CR and PRPD at all considered time points with a reduction of 23% in CR ($P = .026$) (Table 3 and Figure 2a). Indeed, the expression of miR-3201 was always higher in patients not responding to therapy compared to responders, although not significant ($P = .06$) (Figure 2b), with the highest difference observed at T2. In addition, there was no difference in the miRNA expression when comparing resection *versus* RF at all considered time points (resection: $1.45 \pm 0.82$ *vs* RF: $1.30 \pm 0.72$, $P = .59$), before (T0: $1.21 \pm 0.63$ *vs* $1.44 \pm 0.76$, $P = .35$), and after the treatments (T1 + T2: $1.68 \pm 0.95$ *vs* $1.20 \pm 0.68$, $P = .15$).

To validate the discriminatory potential of miR-3201 between CR and PRPD at all considered times, the area under the curve (AUC) was determined with a ROC analysis (Figure 2c). MiR-3201 showed an AUC = 0.69 (95% CI:

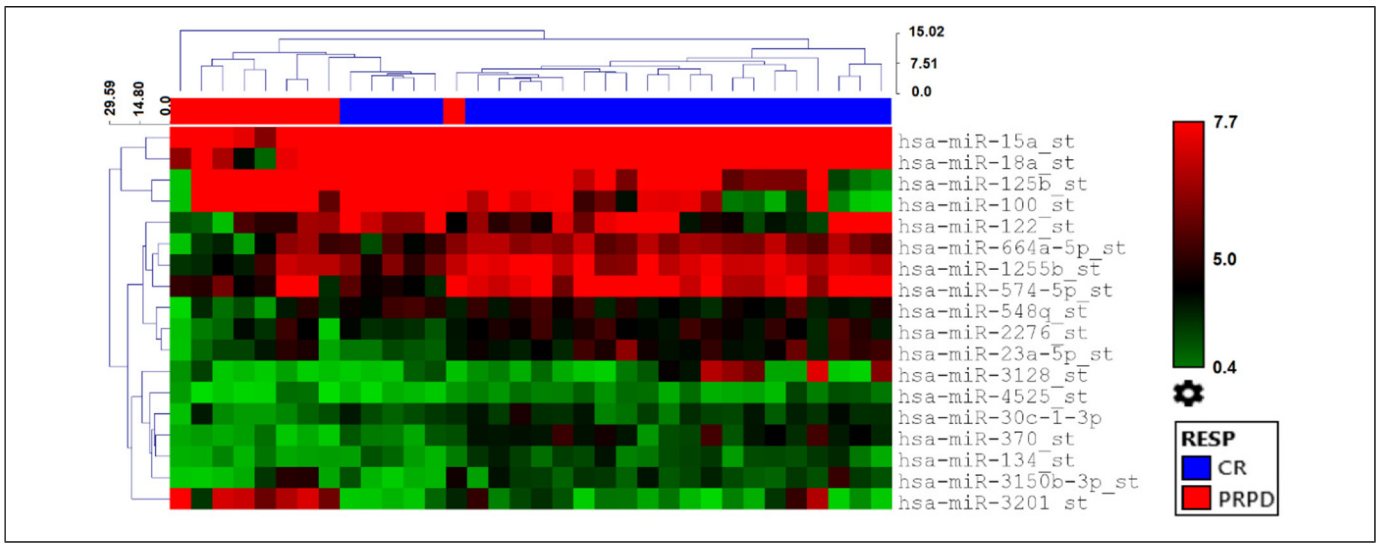


**Figure 1.** Heatmap with the pseudo-color scale underneath the differentially expressed miRNAs between CR (blue) and PRPD (red) in the group of patients treated with curative therapies, at all considered time points (T0, T1, and T2). Unsupervised hierarchical clustering was used to order samples and miRNAs and the $log_2$-transformed microarray signal was considered. The sample tree with optimized leaf ordering was drawn using Euclidean distances and average linkages for cluster-to-cluster distance.
Abbreviations: CR: complete responder; PRPD: partial responder and progressive disease.

0.52-0.81, $P = .0036$), with a sensitivity and specificity of 71% and 70%, respectively, at a cut-off determined at ≥1.52.

### *Higher Levels of miR-3201 are Associated With longer Survival in HCC Patients*

Cut-off value determined by ROC curve analysis (1.52) was used to compare the survival time of patients with low *versus* high miRNA expression. Kaplan–Meier survival analysis demonstrated that low expression of miR-3201 was significantly associated with longer OS in patients, at all considered time points ($P = .0006$), with a hazard ratio (HR) of 2.61 (95% CI: 1.29-5.29) (Figure 3), corresponding to CR patients that have lower levels of the circulating miRNA. Indeed, the OS rates at 24 months were 85% for CR and 46% for PRPD patients, 70% and 42% at 48 months, and 55% and 22% at 72 months.

**Table 3.** Differentially Expressed miRNAs Between CR and PRPD Patients of the Validation Cohort at all Considered Time Points

| ID | CR Avg. ± SD (linear) | PRPD Avg. ± SD (linear) | FC (linear) | *P* value |
|---|---|---|---|---|
| hsa-miR-370 | 0.58 ± 0.42 | 0.54 ± 0.49 | 1.07 | .450 |
| hsa-miR-3201 | 1.30 ± 0.73 | 1.69 ± 0.64 | 0.77 | .026 |

Abbreviations: CR, complete responder; FC: fold change;PRPD: partial responder and progressive disease.

## Discussion

Hepatic resection and RF are considered curative treatments for HCC, showing a 5-year OS rate ranging from 60% to 70% for resection and ~60% for RF.[16,18–22] However, there is a portion of patients that have a relapse. In this context, MiRNAs can represent helpful prognostic biomarkers.

In 2017, Cho et al. suggested circulating miR-26a and miR-29a as prognostic biomarkers predicting poor DFS and LT-free survival in Hepatitis B virus (HBV)-related HCC

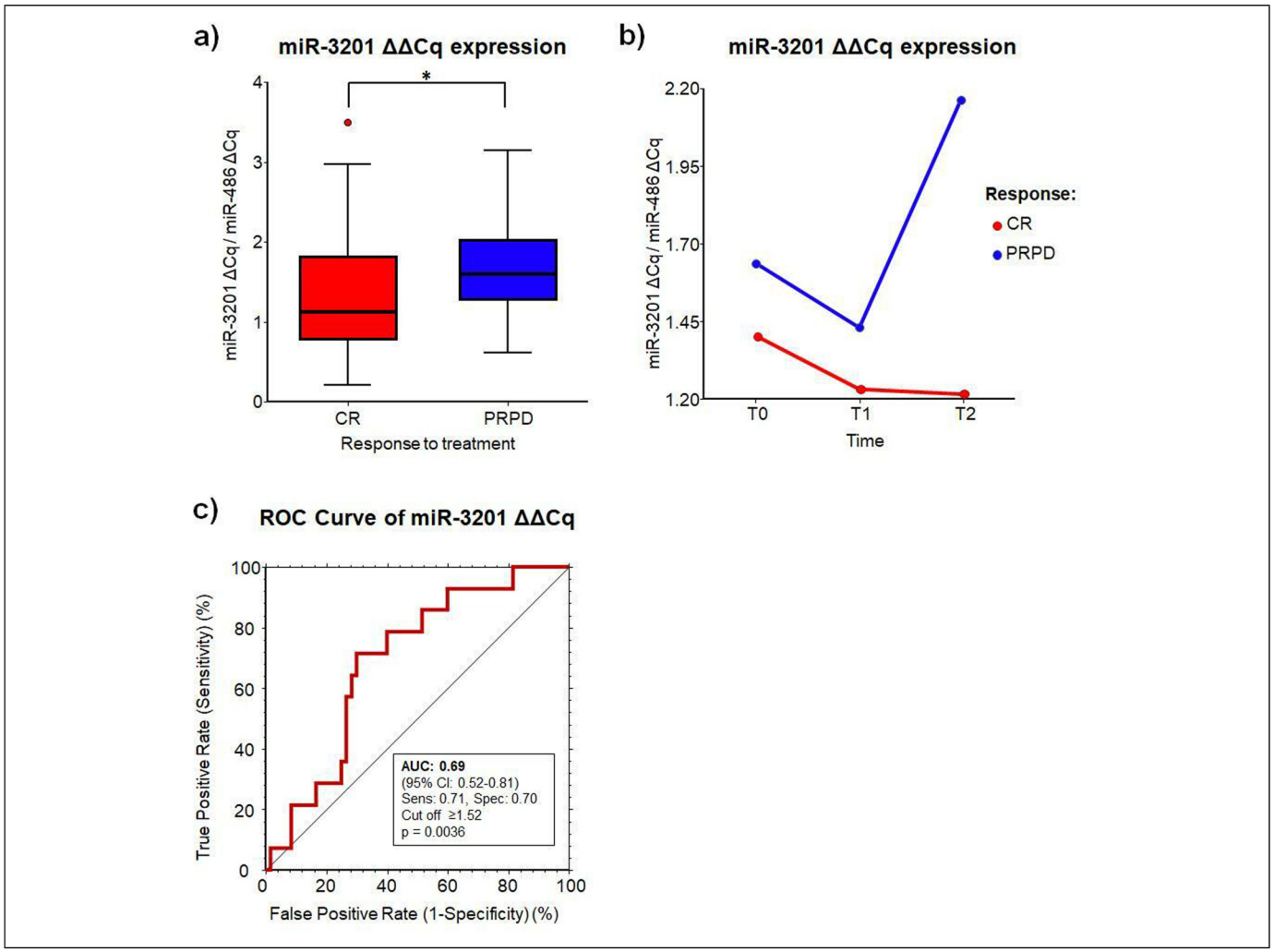


**Figure 2.** MiR-3201 expression in HCC blood samples. (a) Mean ΔΔCq expression of miR-3201 in CR and PRPD. (b) Mean ΔΔCq expression of miR-3201 in CR and PRPD *versus* time. (c) Receiver operating curve (ROC) analysis of miR-3201 when considering all times.
Abbreviations: CR: complete responder; PRPD: partial responder and progressive disease.

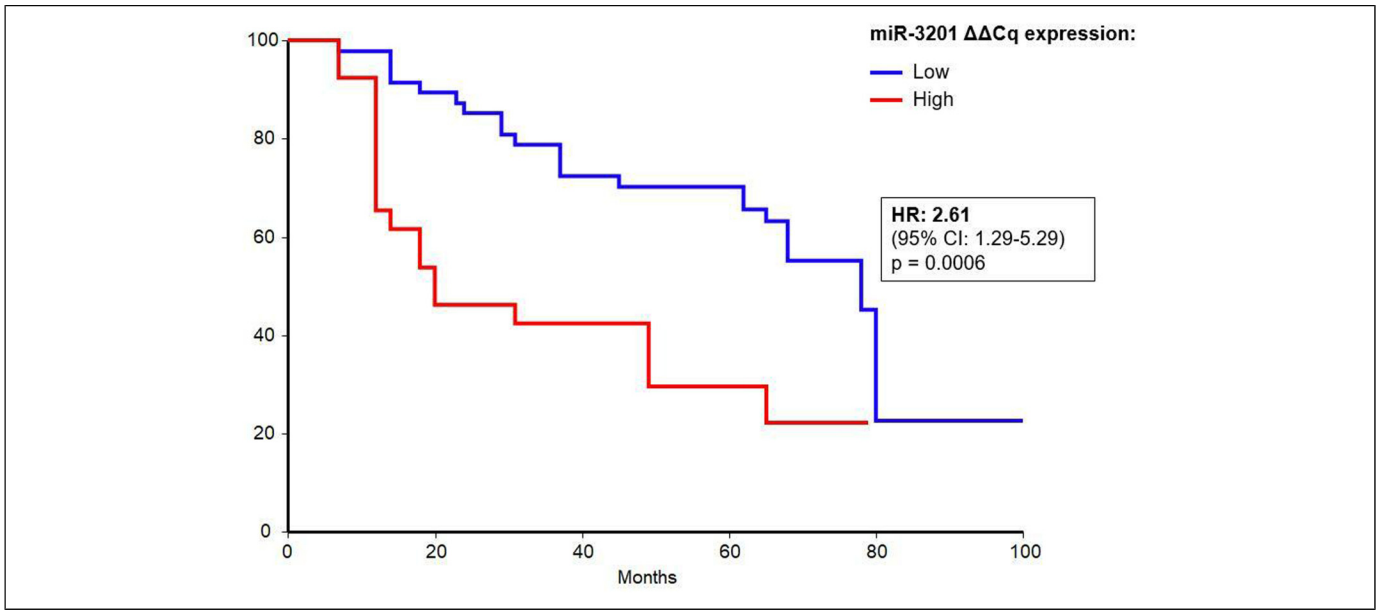


**Figure 3.** Kaplan–Meier survival analysis by log-rank test for blood miR-3201.
Abbreviation: CI: confidence interval; HR: hazard ratio.

patients undergoing curative treatments (resection or RF).[31] Indeed, pre-treatment low plasma levels of miR-26a and miR-29a were significantly associated with poor LT-free survival.[31] More recently, Yokota et al. identified exosomal miR-638 as a prognostic biomarker for resected patients.[32] Patients with low levels of miR-638 showed longer DFS, having a 2-year DFS rate of 77.4% when compared with 47.1% of high miRNA expressing group.[32] In our recent study, we identified serum miR-4443, miR-4530, and miR-4454 as biomarkers to predict therapy response to curative treatments with a sensitivity and specificity of 72% and 75%, respectively.[33] In addition, higher serum levels of miR-4454 (HR = 2.79, $P = .029$) and miR-4530 (HR = 2.97, $P = .011$) were associated with longer OS. All these studies investigated circulating miRNAs in serum and plasma, providing evidence to the potential use of circulating miRNAs as valuable biomarkers for HCC patients' management. However, to our knowledge, there is a lack of studies exploring the value of circulating miRNAs in whole blood, which may provide additional information to clinicians.

In this study, we investigated the potential of blood miRNAs as biomarkers of therapy response in a group of 52 patients treated with hepatic resection or RF. The blood samples were collected at different time points: at the time of diagnosis (T0), 1 month after therapy (T1), and 6 months after therapy (T2). The initial discovery phase, performed on 13 patients, identified 19 differently expressed miRNAs between CR and PRPD patients with miR-370 and miR-3201 being the top-scoring miRNAs. The subsequent validation confirmed the potential of miR-3201 as a biomarker of response to hepatic resection and RF.

The blood levels of miR-3201 were significantly decreased in patients responding to therapy at all the considered time points ($P = .026$), showing an increasing trend of expression over time in PRPD patients. Moreover, ROC curve analysis evidenced the interesting role of miR-3201 in discriminating CR from PRPD patients with a sensitivity and specificity of 71% and 70%, respectively (AUC = 0.69), thus suggesting its potential use as a noninvasive biomarker to assess the response to curative treatments. In addition, blood miR-3201 showed a relevant performance in identifying patients with longer OS. Indeed, in the Kaplan–Meier analysis, patients with low expression of miR-3201 have a significantly higher OS, at all the considered time points, thus suggesting the potential of miR-3201 as a noninvasive prognostic biomarker for the survival of patients treated with hepatic resection and RF.

The function of miR-3201 is still controversial. MiR-3201 expression was significantly reduced in tissues of recurrent epithelial ovarian cancer,[34] while it was significantly increased in the serum of melanoma patients when compared with healthy volunteers.[35] In addition Su et al. showed a correlation between serum miR-3201 upregulation and higher OS in pancreatic cancer suggesting the involvement of miR-3201 in different pro-carcinogenic pathways.[36] However, all these studies underline the still unclear role of this miRNA in the tumor. Indeed, both the biological function of miR-3201 and its role as a biomarker need to be deeply studied.

## Conclusions

Considering the percentage of patients not reaching a complete response at 6 months after therapy, there is still a margin for the improvement of the patient selection and management during the follow-up to increase patients' life expectancy. In this regard, biomarkers in biofluids may be of particular interest for the stratification of patients responding to curative therapies.

In our study, we showed a possible role for blood miR-3201 as a biomarker for HCC patients undergoing curative treatments.

The limitation of our study consists of the restricted number of enrolled patients. However, the repeated measurements of this miRNA at 3 different time points showed a consistency in the differential expression between CR and PRPD patients, as well as in the identification of patients with longer OS. Despite this limitation, the study provides new hints for an extensive study confirming the potential of miR-3201 as a blood biomarker in HCC.

## Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Institutional Review Board Statement

The study was approved by the regional ethical committee (Comitato Etico Regionale Unico FVG, No. 14/2012 ASUITS).

## Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

## Data Availability

The datasets used during the current study are available from the corresponding author upon reasonable request.

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