RESEARCH ARTICLE

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Circulating MiRNA-373 as a Predictor of Response to Super-Selective Transarterial Chemoembolization Bridging Therapy in Hepatocellular Carcinoma Patients Awaiting Liver Transplantation

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Abstract

Background: Super selective transarterial chemoembolization (TACE) has emerged as a bridging therapy for early hepatocellular carcinoma (HCC) patients awaiting liver transplantation. This study aimed at assessing the expression profiles of circulating MiR-210 and MiR-373 as potential predictors of response to TACE bridging therapy in a group of Egyptian HCC cases on top of chronic hepatitis-C infection, awaiting liver transplantation. **Methods:** Fifty-three HCC cases awaiting liver transplantation referred for TACE, were followed up for three months, resulting in forty-five responders and eight non-responders based on modified response evaluation criteria in solid tumors (mRECIST). Circulating pre TACE MiR-210 and MiR-373 expressions were determined using reverse transcription quantitative polymerase chain reaction. **Results:** Circulating pre TACE MiR-373, but not MiR-210, was significantly higher in non-responders than responders. Receiver operating characteristics (ROC) curve analysis of MiR-373, pre-TACE tumor volume, inflammatory score, and albumin bilirubin (ALBI) grade revealed highest sensitivity for pre-TACE tumor volume (cutoff>11.49 cm^3) and highest specificity for pre-TACE MiR-373 (cutoff>1.46-fold change). Multivariate logistic regression revealed pre TACE MiR-373 as a significant independent predictor of TACE response after adjusting for pre TACE tumor volume. **Conclusion:** Circulating pre-TACE MiR-373 could assist as a noninvasive predictor marker of response to TACE bridging therapy in early HCC patients awaiting liver transplantation.

Keywords: Hepatitis-C virus- ALBI grade- inflammatory score- mRECIST- responders- circulating MiR-373

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm and the third most frequent cause of cancer-related death worldwide (Ghouri et al., 2017). Egypt ranks the third and the 15th most populous country in Africa and worldwide, respectively (Ezzat et al., 2021). The hepatitis-C virus (HCV) prevalence in the age group (15–59 years) has declined from 14.7% in 2008 to 10.6% in 2015 (Waked et al., 2020). However, the disease burden and complications such as cirrhosis, the most important risk factor in developing liver cancer, continues to grow considerably due to the increased survival rate of cirrhotic patients (Rashed et al., 2020).

The Barcelona Clinic Liver cancer (BCLC) system is currently the only staging system that includes an integrated assessment of liver disease (Llovet et al., 1999). Despite the significant improvement in the clinical staging systems, the integration of molecular data into these algorithms remains hypothetical according to the European Association for the Study of the Liver EASL (Peter R Galle, 2018). Molecular profiling of HCC allowed a better understanding of HCC pathophysiology and facilitated developing novel molecular targeted therapies for tumors previously considered as therapy-refractory (Radwan et al., 2022).

Transarterial chemoembolization (TACE) is considered the standard treatment for intermediate-stage (BCLC stage B) HCC in patients with preserved liver function (Piscaglia and Ogasawara, 2018). The role of TACE has extended to become an efficient and safe treatment for resectable early-stage HCC with an overall survival rate similar to

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that of hepatic resection (Zhang et al., 2021). The modified response evaluation criteria in solid tumors (mRECIST), is used to assess TACE response. The mRECIST, unlike RECIST, depends on tumor viability (viable enhancing lesions) rather than shrinkage of tumor size (Lee et al., 2020).

To date no single marker has been agreed upon as a surrogate prognostic marker to predict the prognosis of HCC patients who underwent TACE (Ho et al., 2017). Albumin bilirubin (ALBI) grade has been recently introduced as a simple and objective prognostic marker in assessing liver functional reserve for HCC patients (Khalid et al., 2019). Gutkowski et al., (2013), have calculated the inflammatory score to assess inflammation in all HCC patients as helpful tools that guide HCC prognosis (Gutkowski et al., 2013).

Carcinogenesis is a complex multistep process that involves genetic, epigenetic, and biochemical changes, with special reference to MicroRNAs (miRNAs). MiRNAs are a family of small non-protein-coding RNAs approximately 17–25 nucleotides in length involved in post-transcriptional gene regulation through inhibition of protein translation or mRNA degradation (O'Brien et al., 2018).

The tumor microenvironment (TME) promotes cancer progression in many aspects. Hypoxia as a hallmark of the TME serves a function in tumor growth, metastasis and recurrence. Hypoxia regulated miRNAs (HRMs) are differentially expressed in response to hypoxia, participating in the cellular adaptation to the hypoxic microenvironment (Nakayama and Kataoka, 2019).

The hypoxia-regulated MiR-210 is upregulated in cancer cells allowing their adaptation to neoplastic stress which was demonstrated by the increased metastatic potential in HCC cells, and the increased cell growth, survival, genome destabilization, and angiogenesis in other human tumor models. Moreover, MiR-210 was found to be a regulator of multiple functions acting as both oncogene and tumor suppressor under different conditions in different types of cancer (Ji et al., 2018). MiR-373 is another potent hypoxia HRM that is induced in a hypoxia inducible factor (HIF)-1α-dependent manner, by targeting both RAD23B and RAD52 which are involved in repair pathways; thus impairs the DNA damage repair, resulting in genetic instability (Wei et al., 2015). It also participates in cell proliferation, apoptosis, senescence, meso/endoderm differentiation, migration, and invasion (Wei et al., 2015).

Notably, non-coding miRNAs are stable in most body fluids which offer an opportunity to study the role of circulating miRNAs as diagnostic, prognostic and treatment response markers (Ajit, 2012). This study was carried out to assess the expression profiles of circulating MiR-210 and MiR-373as potential predictors of response to TACE bridging therapy in Egyptian HCC patients awaiting liver transplantation.

Materials and Methods

Subjects

A minimum sample size (calculated using Medcalc Program version 14.8.1. according to (Hanley and McNeil, 1982) of 45 patients was found to be required to designate an area under the receiver operating characteristic curve (AUROC) of 0.80 as statistically significantly different from a null hypothesis AUROC of 0.5 (meaning no discriminating power), with a power of 90% and at a significance level of 0.05. Patients were recruited from the Intervention Radiology Department clinic, Alexandria University between April 2017 and May 2018.

The study was conducted on seventy-three subjects divided into fifty-three HCV infected HCC patients, referred for TACE as a bridging therapy, presenting with nodular HCC lesions [up to 3 lesions, up to 6cm size], BCLC (0, A, B) and Child-Pugh A till B8, and twenty healthy volunteers (reference group). Patients with portal vein thrombosis, biliary invasion, BCLC (C, D), hepatitis B viral infection, renal failure, heart failure, chest diseases, peripheral vascular disease, and other types of malignancies were excluded. After approval of the institutional ethical committee, written informed consents were obtained from all participants enrolled in the study. A retrospective review of records was done for patients to confirm HCV infection and the absence of exclusion criteria.

The TACE was carried out in a cath-lab under local anesthesia, beginning with a femoral arterial puncture, followed by introducing a sheath into the vessel, and 5 fr primary catheter over a guidewire. The celiac or superior mesenteric artery was super-selectively catheterized, followed by injecting a contrast medium, and a 3-D rotational angiogram was performed to identify all potential tumor feeders. A micro-catheter coaxial system was then inserted where the catheter was guided to super-selectively catheterize each tumor feeder individually. Once properly positioned, injection of the chemo-emulsion mixture consisting of Lipiodol and doxorubicin (adryablastina 50 mg) was carried out till saturation of the tumor feeder, followed by permanent embolic polyvinyl alcohol particles (PVA). The procedure was repeated till complete tumor devascularization or depletion of the maximum allowable doses of chemoemulsion mixture.

Before and three months after completing the scheduled TACE sessions, a triphasic computed tomographic (CT) scan was done for all HCC cases. The response to TACE was assessed based on the mRECIST criteria. The mRECIST has recently been adopted as a response evaluation method specifically tailored to HCC (Lencioni and Llovet, 2010). It is based on follow up measurement of only the enhanced (viable) portion of HCC target lesions, offering an improved prognostic value for predicting overall survival (Jeon et al., 2018) compared to previous criteria for tumor response adopted by WHO (Miller et al., 1981) or RECIST (Therasse et al., 2000), both of which are based on size and number of target lesions of any solid tumors regardless of their viability. Consequently, tumor necrosis induced by treatment can be misinterpreted as living tumor.

To be selected as a target lesion for mRECIST, an HCC lesion should meet all of the three following criteria; it can be accurately measured in at least one dimension as 1 cm or more, suitable for repeated measurement and

finally must show intra-tumoral arterial enhancement on contrast-enhanced magnetic resonance imaging (MRI) or CT. The selected target lesions were followed up for three months after TACE and accordingly our patients were first classified into four subtypes based on mRECIST; those showing complete response (CR) with disappearance of any intra-tumoral arterial enhancement in all target lesions, those showing partial response (PR) with at least a 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of the diameters of target lesions, those showing progressive disease (PD) with an increase of at least 20% in the sum of the diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started and finally those showing stable disease (SD) defined as any cases that do not qualify for either progressive disease or partial response. Then, our patients were finally classified into two major sub-groups, the responders' sub-group, which included all patients showing either CR or PR, and the non-responders' sub-group, which included all patients showing either SD or PD.

Laboratory work up and detection of MiRNAs

Laboratory investigations were carried out for both the reference group and HCC cases before the ssATCE. Following an overnight fasting period, ten milliliters (mL) of whole venous blood were collected from every participant. Samples were collected under completely aseptic technique. 2 mL of EDTA blood for complete blood picture on the Advia 2120 automated cell counter (Siemens Healthineers, USA). Three mL of serum were used for the determination of levels of creatinine, albumin, bilirubin, C-reactive protein and activity of aspartate aminotransferase on the Olympus AU480 clinical chemistry analyzer (Beckman Coulter Inc, Brea CA, USA). Serum alpha fetoprotein was performed using an electrochemiluminescence (ECL) immunoassay on the Cobas 6000 modular analytical system platform (Roche Diagnostics, GmbH, D-68305 Mannheim, Germany). Prothrombin time and INR calculation were determined from citrated plasma using a fully automated CA-1500 Sysmex coagulation analyzer (Siemens Healthineers, USA). Calculations of the inflammatory score and ALBI grade score were done for HCC cases only according to the following equations: [17.0 + 0.0049 AST (U/L) – 3.40 total albumin (g/dl) + (total bilirubin (mg/dl) x – 0.4128) + 0.2527 CRP (mg/L)] and (log10 bilirubin x 0.66) + albumin x -0.085) where bilirubin was expressed in µmol/L and albumin in g/L respectively.

Total RNA Extraction (including miRNA) from EDTA plasma

Genomic analysis was carried out on EDTA plasma which was collected on ice and stored at -80^{O}C immediately after processing until extraction. RNA extraction was done using the miRNeasy Mini Kit (QIAGEN cat.no.217004, Maryland, USA) according to the manufacturer's instructions after decontamination of all surfaces with RNAZap. The concentration and purity of extracted RNA were determined at 260, 280, and 230 nm using NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, USA). The A260:A280 ratio was greater than 2 and the A260:A230 ratio was greater than 1.7.

Reverse Transcription (RT) and relative quantification of mature miRNA by RQ-PCR

Reverse Transcription (RT) of RNA was carried out using the miRNA miScript II RT Kit (QIAGEN, Maryland, USA) according to the manufacturer's protocol, in a conventional thermal cycler (Biometra) (Analytik Jena AG, Germany). The samples in duplicates were incubated at 37°C for 60 minutes then the reaction was terminated by heating at 95°C for 5 minutes to inactivate the reverse transcriptase (Bustin and Mueller, 2005). The resulting clone deoxyribonucleic acid (cDNA) served as the template for real time polymerase chain reaction (RQ-PCR). Both MiR-210 and MiR-373 were detected using SYBER Green based RQ-PCR. The analysis was carried out on the Rotor gene Q thermal cycler (QIAGEN, Maryland, USA) using miRNA-specific miScript Primer Assay (forward primer) and the miScript SYBR Green PCR Kit, which contained the miScript Universal Primer (reverse primer) and QuantiTect SYBR® Green I PCR master mix (QIAGEN, Maryland, USA) as follows; a hot start Taq DNA Polymerase activation step for 15 minutes at 95°C, followed by a 3 step cycling of 15 seconds denaturation at 94°C, 30 seconds annealing at 55°C and 30 seconds extension at 70ºC for a total of 40 cycles. A no template control consisting of nuclease-free water instead of the cDNA was included with each PCR run. (Table 1). The relative quantitation of each miRNA was calculated according to the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$), with PCR efficiency of 100%, and presented as the fold change normalized to the reference/ housekeeping gene (RNU6) of the average reference subjects (Arya et al., 2005).

Statistical analysis

Data were coded and fed to the Statistical Package for Social Science (SPSS) program version 20.0 (SPSS, Inc., Chicago, IL) (Kirkpatrick and Feeney, 2013). Kolmogorov-Smirnov test of normality revealed significance in the distribution of the variables, so the non-parametric statistics were adopted. Numerical data were described using the median, minimum, and maximum and inter-quartile range. Mann-Whitney U test was used for comparison between two independent groups, while the Wilcoxon Signed Ranks Test was used for comparison between two related groups. Receiver operating characteristics (ROC) curve analysis was performed using MedCalc software version 14.0 (MedCalc Software, Ostend, Belgium) for variables that showed statistical significance by univariate analysis (Schoonjans, 2017). Based on the coordinates of the ROC curves, the best cut off values were deduced using Youden's index. An alpha error was set to 5% with a significance level of 95% (except for multiple comparisons). Multivariate Logistic regression analysis was carried out to assess the relationship between response status (outcome) and plasma MiR-373 as a predictor after adjusting for the

pre-TACE tumor volume as a possible confounding factor.

Results

This study was conducted on a final number of fifty-three adult HCV infected HCC Egyptian patients from a total number of 70 HCC cases (Figure 1). Assessment of the response to TACE was performed after a three-month follow-up period. Patients were classified based on the mRECIST criteria into two groups: forty-five responders (group I) and eight non-responders (Group II).

The pre- and post- TACE tumor volumes were significantly higher in non-responders compared to responders (p=0.001 and p<0.001 respectively), while paired comparison showed a significant reduction in tumor volume following TACE in responders only (p<0.001) (Table 2). According to the Child-Pugh score, 45 cases had Child A score, and 8 cases had Child B score. According to the BCLC staging, 40 cases (75%) staged A, and 13 cases (25%) were stage B.

The pre-TACE laboratory workup done for responders, and nonresponders revealed non-remarkable findings, apart from a significantly lower median value for serum albumin in nonresponders compared to responders (p<0.001) (Table 2). The inflammatory score in responders was significantly higher compared to nonresponders (p<0.001) (Table 2). As regards the ALBI grade, 11 cases had grade 1 (<-2.6), 38 cases had grade-2 (>-2.6 to <-1.39), leaving only 4 cases with grade-3 (>-1.39). Only seven among the 38 cases with ALBI grade 2 and 1 among the 4 cases

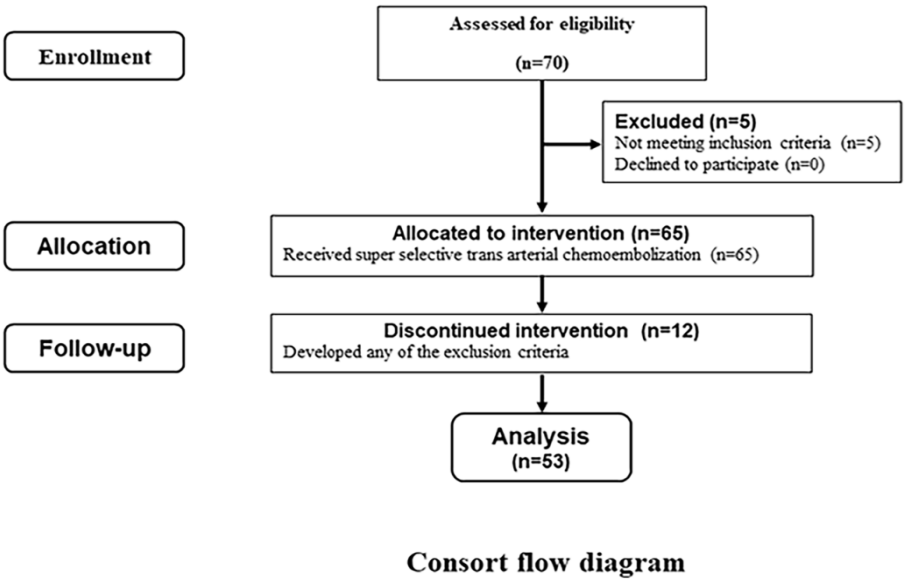

Figure 1. Consort Flow Diagram

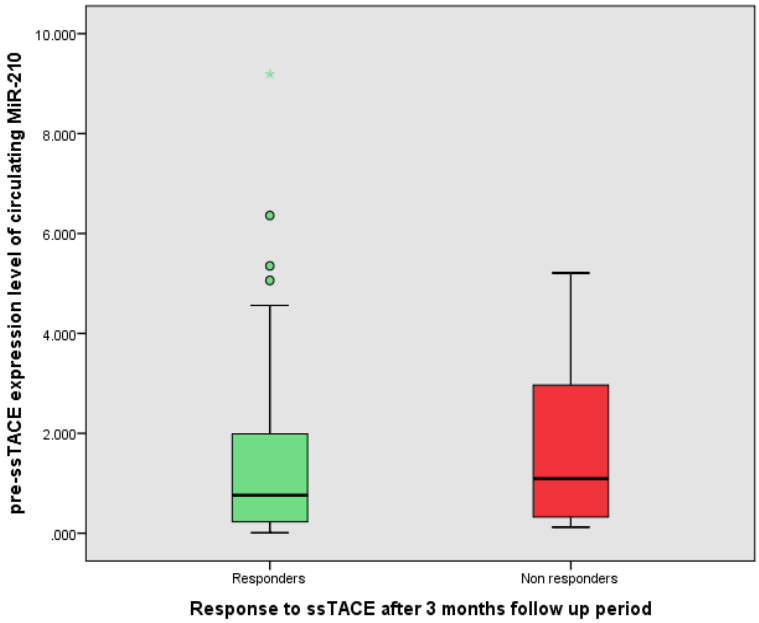

Figure 2. Box and Whisker Plot of Pre-TACE Expression Level of Circulating MiR-210 (Cir MiR-210 (RQ) among HCC Cases Classified into Responders and Non-responders to TACE after 3 Months Follow up Period

Table 1. Details of Names and Volumes of Reagents and Nucleic Acids for Each miRNA Sample Used in Reverse Transcription and Real Time Quantitative Polymerase Chain Reaction

Items	Volume (µL)
Reverse transcription reaction	
Template RNA	Variable (45 ng/tube)
5x miRNA RT Buffer (HiSpec Buffer)	4
miScript Reverse Transcriptase Mix	2
10x miScript Nucleics Mix	2
Ribonuclease-free water to a final volume of	20
Real time quantitative polymerase chain reaction	
2x QuantiTect SYBR Green PCR master mix	12.5
10x miScript Universal Primer	2.5
10x miScript Primer Assay for MiR-210 and MiR-373	2.5
Ribonuclease (RNase)-free water	2.5
Template cDNA (Diluted 1:6 with RNase free water)	5
Total volume per PCR tube	25

with ALBI grade-3 constituted the nonresponders group. Its median value was significantly higher in nonresponders compared to responders (p=0.006) (Table 2).

The expression level (RQ) of circulating MiR-210 and MiR-373 revealed a significantly higher median value for MiR-373 in nonresponders (median RQ value=1.93 [0.140–3.460]) compared to responders (median RQ value=0.370 [0.010– 4.690]) (p=0.017), but not for the MiR-210 (median RQ value for nonresponders=1.09 [0.120–5.210] versus median RQ value for responders=0.760 [0.010–9.190]) (p=0.628) (Figures 2 and 3).

Receiver operating characteristics (ROC) curves analyses were performed for variables that showed statistical significance by univariate analysis (inflammatory score, Pre-TACE tumor volume, RQ MiR-373 and ALBI grade) to evaluate their discriminatory power to classify HCC patients into TACE responders and nonresponders. The mRECIST, based on Tri phasic CT, was considered the gold standard. Taken into consideration that pre-TACE tumor volume had the highest sensitivity (100%) and MiR-373 had the highest specificity (84.4%) among the studied discriminators, a series approach starting with pre-TACE tumor volume followed by MiR-373 (RQ) was performed in order to improve the overall specificity. MiR-373 (RQ) expression level was done as a confirmatory test at a cut off value of >1.46 only for nonresponders (n=23) by pre-tumor volume at a cut off value of >11.49 cm^3, achieving an overall sensitivity of 75% and a specificity of 97.8% (Figure 4, Table 3).

Multivariate logistic regression modeling for results among all HCC patients that performed TACE revealed circulating MiR-373 (RQ) as a significant independent predictor of response to TACE (after adjusting for pre-TACE tumor volume). The odds of being a nonresponder was increased by a factor of 2.054 (105.4%) for every unit increase in the relative quantitation of circulating MiR-373 (p=0.027, adjusted Odds ratio=2.054, 95%CI= 1.0841–3.890) (Table 4).

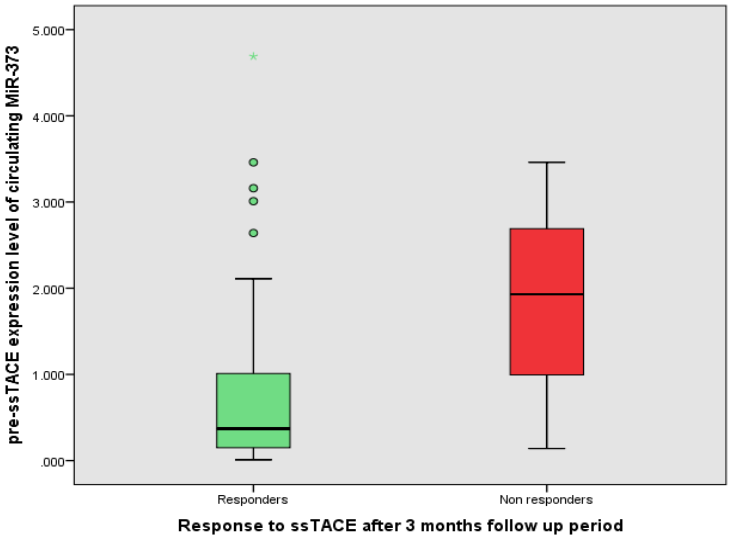

Figure 3. Box and Whisker Plot of Pre-TACE Expression Level of Circulating MiR-373 (Cir MiR-373 (RQ) among HCC Cases Classified into Responders and Non-responders to TACE after 3 Months Follow up Period

Table 2. Age, Gender, Tumor Volume, Selected Hematological and Biochemical Variables, Prothrombin Time, International Normalized Ratio and Calculations Compared among the Studied Groups

Variable (Units)	Responders (n=45)	Non responders (n=8)	Test of Sig.	p-value
Age (years)	55 (45 – 76)	60 (53 – 69)	$Z_{(MW)}$=1.830	0.067
Gender (Male (%)/ Female (%))	32 (71.1) / 13 (28.9)	6 (75) / 2 (25)	χ^2(Y) =0	0.99
Pre-TACE tumor vol (cm^3)	8.18 (1.15 – 179.50)	30.06 (14.13 – 87.07)	$Z_{(MW)}$=3.047	0.001*
Post-TACE tumor vol (cm^3)	2.14 (0.0042 – 69.42)	23.85 (2.5711 – 77.91)	$Z_{(MW)}$=3.481	<0.001*
Hemoglobin (g/dl)	12.8 (8.7 – 15.5)	11.7 (9.8 – 13.8)	$Z_{(MW)}$=1.777	0.075
Red blood cell count (x10^9/L)	4.42 (3.16 – 6.92)	4.24 (3.71 – 5.06)	$Z_{(MW)}$=1.056	0.297
Platelets count (x10^6/L)	112 (53 – 296)	83 (45 – 165)	$Z_{(MW)}$=1.528	0.128
Total leucocytic count (x10^6/L)	4.45 (2.10 – 9.80)	3.26 (2.40 – 6.00)	$Z_{(MW)}$=1.750	0.08
Prothrombin Time (seconds)	15.30 (11.80 – 20.90)	13.95 (13.00 – 18.10)	$Z_{(MW)}$=0.201	0.855
International Normalized Ratio	1.30 (1.00 – 1.78)	1.19 (1.12 – 1.41)	$Z_{(MW)}$=0.201	0.855
Creatinine (mg/dL)	0.89 (0.50 – 1.40)	0.91 (0.72 – 1.19)	$Z_{(MW)}$=0.461	0.652
Albumin (g/dL)	3.70 (2.70 – 4.50)	3.35 (2.60 – 3.60)	$Z_{(MW)}$=3.529	<0.001*
Total Bilirubin (mg/dL)	1.27 (0.45–2.70)	1.18 (0.52 – 2.80)	$Z_{(MW)}$=0.211	0.836
Direct Bilirubin (mg/dL)	0.59 (0.20 – 2.00)	0.60 (0.19 – 1.91)	$Z_{(MW)}$=0.261	0.798
Aspartate aminotransferase (U/L)	58 (30 – 160)	42 (30 – 89)	$Z_{(MW)}$=1.256	0.214
C-reactive protein (mg/L)	3.7 (1.7 – 7.5)	3.4 (2.3 – 4.8)	$Z_{(MW)}$=0.298	0.779
Pre-TACE-Alpha fetoprotein (ng/ml)	13.7 (4.4 – 311.4)	79.8 (7.0 – 190)	$Z_{(MW)}$=1.516	0.135
Post TACE Alpha fetoprotein (ng/ml)	6.3 (2.7–120.8)	18.65 (2.8 – 250)	$Z_{(MW)}$=0.634	0.533
Inflammatory Score	30.33 (26.59 – 33.29)	28.98 (26.10 – 30.04)	$Z_{(MW)}$=3.354	<0.001*
ALBI grade	-2.26 (-3.24 – -1.22)	-1.98 (-2.43 – -1.21)	$Z_{(MW)}$=2.64	0.006
RQ of MiR-210	0.760 (0.010-9.190)	1.09 (0.120-5.210)	$Z_{(MW)}$= 0.485	0.628
RQ of MiR-373	0.370 (0.010-4.690)	1.93 (0.140-3.460)	$Z_{(MW)}$=-2.386	0.017*

Median, (min.-max.); χ^2, Chi Square; Z (MW), Mann-Whitney U test statistic; *, statistically significant (p<0.05)

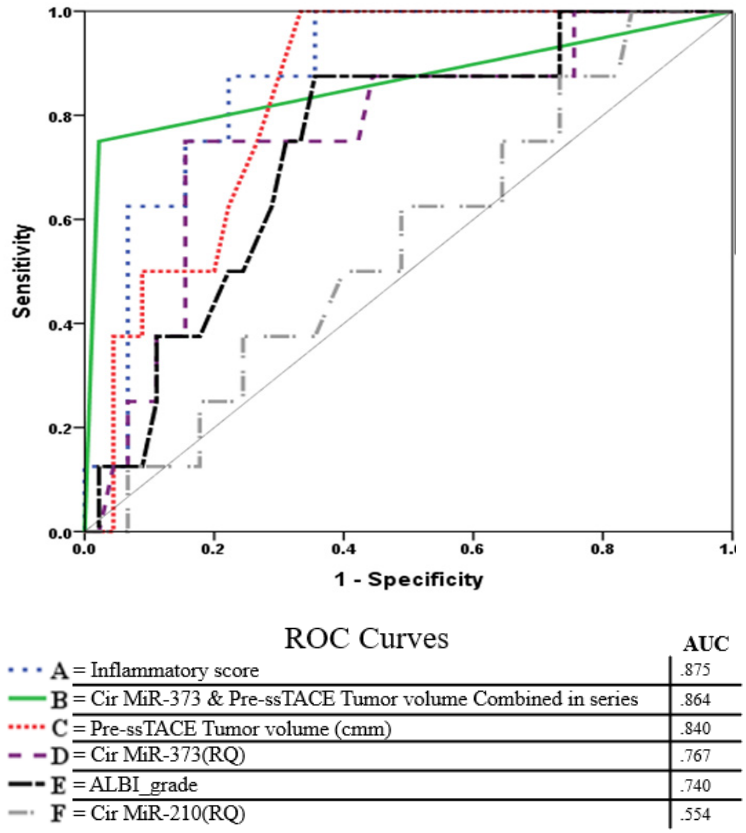

Figure 4. Multiple ROC Curves Comparing Performances of Pre-TACE Tumor Volume, Inflammatory Score, MiR-373, ALBI Grade and Finally Combining Pre-TACE Tumor Volume in Series with Cir MiR-373 (RQ) as Discriminators of Response to TACE

Table 3. Diagnostic Performances of Pre-TACE Tumor Volume, Inflammatory Score, MiR-373, ALBI Grade and Finally Combining Pre-TACE Tumor Volume in Series with Cir MiR-373 (RQ) for Discrimination of Response to TACE

Classifier	AUC (95% CI)	Cut-off value (YI)	Sensitivity % (95% CI)	Specificity % (95% CI)	LR +ve (95% CI)	LR -ve (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Pre-TACE tumor volume (cm^3)	0.84 (0.714-0.926)	>11.49	100 (63.1-100.0)	66.67 (51.0-80.0)	3 (2-4.5)	0	34.8 (16.4-57.3)	100 (88.4-100)
Inflammatory score	0.875 (0.755-0.950)	<29.75	87.5 (47.3-99.7)	77.78 (62.9-88.8)	3.94 (2.1-7.2)	0.16 (0.03-1.0)	41.2 (18.4-67.1)	97.2 (85.5-99.9)
Cir MiR-373 (RQ)	0.767 (0.630-0.872)	>1.46	75 (34.9-96.8)	84.44 70.5-93.5	4.82 (2.2-10.6)	0.3 (0.09-1.0)	46.2 (19.2-74.9)	95 (83.1-99.4)
ALBI grade	0.796 (0.663-0.894)	>-2.11	87.5 (47.3-99.7)	73.33 58.1-85.4	3.28 (1.9-5.7)	0.17 (0.03-1.1)	36.80% (16.3-61.6)	97.10% (84.7-99.9)
Pre-TACE Tumor volume combined in series with Cir MiR-373 (RQ)	0.864 (0.742- 0.943)	**	75 (34.9-96.8)	97.78 (88.2-99.9)	33.75 (4.7-244.2)	0.26 (0.08-0.8)	85.7 (45.3-97.7)	95.7 (86.9-98.7)

LR +ve, -ve, positive and negative likelihood ratio; PPV, NPP, positive and negative predictive values; AUC, Area under the ROC curve; RQ, relative quantitation; YI, Youden index; 95% C.I., 95% confidence interval; ** series approach starting with pre-TACE tumor volume at cutoff>11.49 ccm followed by MiR-373 (RQ) at cutoff>1.46(RQ)

Table 4. Results of Multivariate Logistic Regression Analysis for Circulating MiR-373 (RQ) as a Predictor of Response to TACE after Adjusting for Pre-TACE Tumor Volume

	P-value	AOR	95% C.I. for AOR	
			Lower	Upper
Pre TACE tumor volume (ccm)	0.066	1.0192	0.9987	1.0401
Cir MiR-373 (RQ)	0.027	2.0535	1.0841	3.8896
Constant	0	23.996		

Cir MiR-373 (RQ), relative quantitation of circulating MiR-373; B, Standardized Coefficient; S.E, Standard error; df, Degree of freedom; W, Wald statistics AOR, Adjusted Odds ratio; 95% C.I., 95% confidence interval

Discussion

In our study, we chose MiR-210 and MiR-373 because both miRNAs are HRMs induced in response to hypoxia in a HIF-1α dependent manner (Ji et al., 2018). Additionally, MiR-373 is a proviral host miRNA that is upregulated by HCV, facilitating viral immune evasion through inhibiting the JAK/STAT pathway of interferon signaling in hepatocytes (Mukherjee et al., 2015). HCV infection is reported to stimulate a virus-induced pseudo-hypoxic response. Furthermore, hypoxia promotes HCV replication, while inhibiting HIF-1α activity reduced viral replication (Wilson et al., 2014).

MiR-210 plasma expression level did not significantly differ between responders and nonresponders. This could be related to the tumor entity and the cellular specificity. Our data was supported by El-Halawany et al., (2015), who studied the expression of MiR-210 among a selected panel of 94 miRNAs in formaldehyde-fixed, paraffin-embedded archival hepatic tissue samples collected before treatment from 15 Egyptian patients diagnosed with advanced HCC. They reported no statistically significant difference in MiR-210 expression between responders and nonresponders, yet reported an increase in MiR-210 expression in HCC samples compared with healthy subjects. The latter finding was not evident in our study because unlike their study, our study was carried out among early HCC cases. Moreover, the sample used in our MiR-210 determination was plasma and not tissue as in their study. A meta-analysis studying the prognostic value of MiR-210 in various carcinomas revealed that MiR-210 upregulation was mostly correlated with poor prognosis; however, data concerning the prognostic value of MiR-210 in HCC superimposed on HCV infection was lacking in this study (Liu et al., 2017b).

The present work demonstrated a significantly higher expression level of plasma MiR-373 in nonresponders than responders, conferring a poor prognostic ability for this miRNA. Wu et al., (2011) found that MiR-373 was upregulated in human HCC tissues compared to normal liver tissues. MiR-373 functions as an oncogene facilitating the proliferation of HCC cell lines by down-regulating the tumor suppressor gene coding for protein phosphatase-6C protein. This gene is considered a direct target for MiR-373 and predicted to have a MiR-373-binding site in its 3' untranslated region. Cairo et al., (2010) reported an upregulation of miR-371-3 cluster in hepatoblastoma, in cooperation with other deregulated miRNAs. Hence, conferring stem cell-like characteristics to cancer cells, and creating a MiR signature that could be used to discriminate not only aggressive tumors but also invasive HCCs.

Doxorubicin resistance has emerged as an obstacle that limits the treatment opportunities of HCC patients treated with doxorubicin-based TACE. Many mechanisms that result in doxorubicin resistance have emerged,

including multiple molecular targets, signaling pathways, and miRNAs (Cox and Weinman, 2016). There is no consensus on a direct mechanism by which MiR-373 could achieve doxorubicin-resistance in TACE. However, MiR-373 is interrelated with several mechanisms involved in doxorubicin action, including activation of the PI3K/AKT-Rac1-JNK pathway (Liu et al., 2017a).

An inflammatory score was calculated to account for the level of HCV induced inflammation in the studied subjects. It revealed a significantly higher median value in responders compared to nonresponders. Despite serum C-reactive protein being one of the score parameters, it did not show any significant difference between both groups. Therefore, it remains evident that the hepatic component of the score was the main drive of the statistically significant difference. It could be explained by the two opposing roles of the immune system, both protecting against tumor development and promoting tumor growth in cancer referred to as immuno-editing. A continuous debate is whether the tumor microenvironment is pro- or anti-inflammatory and whether the infiltrating immune cells promote tumor growth or act as immune-surveillance to combat tumor progression (Ostrand-Rosenberg, 2008). Chew et al., (2010) reported that inflammatory tumor microenvironment was associated with a superior survival in HCC patients. The vigorous immune activation may be promoted by local signals sensed by toll-like receptors expressed by immune cells within the tumor itself. Therefore, inflammation is a detrimental factor during carcinogenesis and exerts a protective effect in the control of well-established tumors.

The ALBI grade had a significantly higher median value in responders compared to nonresponders. This was in concordance with the results of Ho et al., (2017), who studied the prognostic value of eight noninvasive liver reserve markers. They concluded that ALBI grade had a superior prognostic power in HCC patients undergoing TACE. Furthermore, Oh et al., (2017) reported that the ALBI grade was clinically more useful in predicting the prognosis of patients with early-stage HCC who underwent radiofrequency ablation.

Although plasma MiR-373 showed a positive relation with serum alpha fetoprotein in our HCC cases, yet the latter did not show any significant differences in pre and post TACE median values in responders and nonresponders. The use of alpha fetoprotein as a prognostic marker to assess response to treatment in HCC has multiple limitations, including the heterogeneous nature of its elevations that can undergo several orders of magnitude; nearly up to half of HCC lesions may not produce alpha fetoprotein. In addition, it may not normalize completely even after complete elimination of the tumor (Kim et al., 2011).

The ROC curve analyses conducted for the statistically significant parameters by univariate analysis in discriminating responders from nonresponders to TACE showed the highest specificity with plasma MiR-373 and highest sensitivity with pre-TACE tumor volume. The series approach was adopted with plasma MiR-373 as a confirmatory tool for the 23 HCC cases deemed non-responsive to TACE by pre-TACE tumor volume alone. This improved the overall specificity in discriminating responders from nonresponders to TACE.

Circulating MiR-373 has survived the multivariate analysis' adjustment for the confounding effect of pre-TACE tumor volume. Subsequently, it proved to be a significant independent predictor of response status to TACE. The odds of being a nonresponder was increased by a factor of 2.054 (105.4%) for every unit increase in the relative quantitation of circulating MiR-373 after adjusting for pre-TACE tumor volume.

The relatively high number of dropouts among HCC cases who did not comply with the scheduled follow up plan may constitute a limitation for this study.

In conclusion, the results of our study highlights the value of determining circulating MiR-373 as a predictor marker of response to TACE bridging therapy in early HCC patients awaiting liver transplantation, tailoring the selection of TACE candidates based on their pre TACE MiR-373 expression level. Further multicentric studies on a larger population of HCC patients focusing on the prognostic value of hypoximirs as predictors of response to TACE therapy are highly recommended.

Author Contribution Statement

All the authors contributed equally in the submitted work.

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Ethics approval

The study was conducted after approval of the Medical Research Institute's Ethical committee.

Animal Research (Ethics)

Not Applicable.

Consent to participate

After approval of the institutional ethical committee, written informed consent was obtained from all participants enrolled in the study.

Consent for publication

The authors gave their consent for the publication of identifiable details, which can include figure (s) and/or details within the text ("Material") to be published.

Data Availability

The data are available upon request.

Plant Reproducibility

Not applicable as the study does not contain any plant.

Conflicts of interests

The authors declare no conflicts of interest.

References

Ajit SK (2012). Circulating microRNAs as biomarkers, therapeutic targets, and signaling molecules. *Sensors (Basel)*, **12**, 3359-69.

Arya M, Shergill IS, Williamson M, et al (2005). Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn*, **5**, 209-19.

Bustin SA, Mueller R (2005). Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin Sci (Lond)*, **109**, 365-79.

Cairo S, Wang Y, de Reyniès A, et al (2010). Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer. *Proc Natl Acad Sci U S A*, **107**, 20471-6.

Chew V, Tow C, Teo M, et al (2010). Inflammatory tumour microenvironment is associated with superior survival in hepatocellular carcinoma patients. *J Hepatol*, **52**, 370-9.

Cox J, Weinman S (2016). Mechanisms of doxorubicin resistance in hepatocellular carcinoma. *Hepat Oncol*, **3**, 57-9.

El-Halawany MS, Ismail HM, Zeeneldin AA, et al (2015). Investigating the pretreatment miRNA expression patterns of advanced hepatocellular carcinoma patients in association with response to TACE treatment. *Bio Med Res Int*, **2015**, 649750.

Ezzat R, Eltabbakh M, El Kassas M (2021). Unique situation of hepatocellular carcinoma in Egypt: A review of epidemiology and control measures. *World J Gastrointest Oncol*, **13**, 1919-38.

Ghouri YA, Mian I, Rowe JH (2017). Review of hepatocellular carcinoma: Epidemiology, etiology, and carcinogenesis. *J Carcinog*, **16**, 1.

Gutkowski K, Hartleb M, Kacperek-Hartleb T, et al (2013). Laboratory-based scoring system for prediction of hepatic inflammatory activity in patients with autoimmune hepatitis. *Liver Int*, **33**, 1370-7.

Hanley JA, McNeil BJ (1982). The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology*, **143**, 29-36.

Ho S-Y, Liu P-H, Hsu C-Y, et al (2017). Prognostic role of noninvasive liver reserve markers in patients with hepatocellular carcinoma undergoing transarterial chemoembolization. *PLoS One*, **12**, e0180408-e.

Jeon MY, Lee HW, Kim BK, et al (2018). Reproducibility of European association for the study of the liver criteria and modified response evaluation criteria in solid tumors in patients treated with sorafenib. *Liver Int*, **38**, 1655-63.

Ji J, Rong Y, Luo C-L, et al (2018). Up-Regulation of hsa-miR-210 promotes venous metastasis and predicts poor prognosis in hepatocellular carcinoma. *Fronti Oncol*, **8**.

Khalid MA, Achakzai IK, Hanif FM, et al (2019). To determine the prognostic value of the albumin-bilirubin grade (ALBI) in patients underwent transarterial chemoembolization for unresectable hepatocellular carcinoma. *Gastroenterol Hepatol Bed Bench*, **12**, 110-5.

Kim KW, Lee JM, Choi BI (2011). Assessment of the treatment response of HCC. *Abdom Imaging*, **36**, 300-14.

Kirkpatrick L, Feeney B (2013). A Simple Guide to IBM SPSS Statistics for Version 20.0, Cengage Learning, Belmont, California, Wadsworth.

Lee JS, Choi HJ, Kim BK, et al (2020). The modified response evaluation criteria in solid tumors (RECIST) yield a more accurate prognoses than the RECIST 1.1 in hepatocellular carcinoma treated with transarterial radioembolization. *Gut Liver*, **14**, 765-74.

Lencioni R, Llovet JM (2010). Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis*, **30**, 52-60.

Liu Y, Cheng Z, Pan F, et al (2017a). MicroRNA-373 promotes growth and cellular invasion in osteosarcoma cells by activation of the PI3K/AKT-Rac1-JNK pathway: The Potential Role in Spinal Osteosarcoma. *Oncol Res*, **25**, 989-99.

Liu Y, Wang Y, Xu Q, et al (2017b). Prognostic evaluation of microRNA-210 in various carcinomas: Evidence from 19 studies. *Medicine (Baltimore)*, **96**, e8113.

Llovet JM, Brú C, Bruix J (1999). Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis*, **19**, 329-38.

Miller AB, Hoogstraten B, Staquet M, et al (1981). Reporting results of cancer treatment. *Cancer*, **47**, 207-14.

Mukherjee A, Di Bisceglie AM, Ray RB (2015). Hepatitis C virus-mediated enhancement of microRNA miR-373 impairs the JAK/STAT signaling pathway. *J Virol*, **89**, 3356-65.

Nakayama K, Kataoka N (2019). Regulation of gene expression under hypoxic conditions. *Int J Mol Sci*, **20**, 3278.

O'Brien J, Hayder H, Zayed Y, et al (2018). Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. F*ront Endocrinol (Lausanne)*, **9**, 402.

Oh IS, Sinn DH, Kang TW, et al (2017). Liver function assessment using Albumin–Bilirubin grade for patients with very early-stage hepatocellular carcinoma treated with radiofrequency ablation. *Dig Dis Sci*, **62**, 3235-42.

Ostrand-Rosenberg S (2008). Immune surveillance: a balance between protumor and antitumor immunity. *Curr Opin Genet Dev*, **18**, 11-8.

Peter R Galle AF, Josep ML, et al (2018). EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol*, **69**, 182-236.

Piscaglia F, Ogasawara S (2018). Patient selection for transarterial chemoembolization in hepatocellular carcinoma: Importance of Benefit/Risk Assessment. *Liver Cancer*, 7, 104-19.

Radwan NH, Abdelkhalik HA, Elgayar DF, et al (2022). Study the role of cell free DNA in the diagnosis of hepatocellular carcinoma, an Egyptian Study. *Asian Pac J Cancer Biol*, **7**, 3-8.

Rashed W, Kandeil M, Mahmoud M, et al (2020). Hepatocellular Carcinoma (HCC) in Egypt: A comprehensive overview. *J Egypt Natl Canc Inst*, **32**, 1-22.

Schoonjans F (2017). MedCalc manual: Easy-to-use statistical software, Ostend, Belgium, MedCalc Software.

Therasse P, Arbuck SG, Eisenhauer EA, et al (2000). New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst*, **92**, 205-16.

Waked I, Esmat G, Elsharkawy A, et al (2020). Screening and treatment program to eliminate Hepatitis C in Egypt. *N Engl J Med*, **382**, 1166-74.

Wei F, Cao C, Xu X, et al (2015). Diverse functions of miR-373 in cancer. *J Transl Med*, **13**, 162.

Wilson GK, Tennant DA, McKeating JA (2014). Hypoxia inducible factors in liver disease and hepatocellular carcinoma: current understanding and future directions. *J Hepatol*, **61**, 1397-406.

Wu N, Liu X, Xu X, et al (2011). MicroRNA-373, a new regulator of protein phosphatase 6, functions as an oncogene in hepatocellular carcinoma. *Febs J*, **278**, 2044-54.

Zhang YJ, Chen MS, Chen Y, et al (2021). Long-term outcomes of transcatheter arterial chemoembolization combined with radiofrequency ablation as an initial treatment for early-stage hepatocellular carcinoma. *JAMA Netw Open*, **4**, e2126992.