Liver INTERNATIONAL

Liver International ISSN 1478-3223

LIVER CANCER

MicroRNA-425-3p predicts response to sorafenib therapy in patients with hepatocellular carcinoma

Valentina Vaira[1], Massimo Roncalli[2], Carlo Carnaghi[3], Alice Faversani[1,4], Marco Maggioni[1], Claudia Augello[1,4], Lorenza Rimassa[3], Tiziana Pressiani[3], Gaia Spagnuolo[2], Luca Di Tommaso[2], Stefano Fagiuoli[5], Elena Rota Caremoli[6], Massimo Barberis[7], Roberto Labianca[6], Armando Santoro[3] and Silvano Bosari[1,4]

1 Division of Pathology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
2 Department of Medical Biotechnologies and Translational Medicine, University of Milan and Division of Pathology, IRCCS Istituto Clinico Humanitas, Rozzano, Milan, Italy
3 Medical Oncology and Haematology Unit, Humanitas Cancer Center, Rozzano, Milan, Italy
4 Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy
5 Division of Gastroenterology and Transplant Hepatology, Department of Medicine, Papa Giovanni XXIII Hospital, Bergamo, Italy
6 Department of Oncology and Haematology, Papa Giovanni XXIII Hospital, Bergamo, Italy
7 Department of Pathology, Papa Giovanni XXIII Hospital, Bergamo, Italy

Keywords
hepatocellular carcinoma – liver – microRNAs – sorafenib

Correspondence
Silvano Bosari, MD, Department of Pathophysiology and Organ Transplants, University of Milan and Division of Pathology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico,
via F. Sforza 35, Milan 20122, Italy
Tel: +39 250 320 179
Fax: +39 255 032 860
e-mail: silvano.bosari@unimi.it

Received 19 February 2014
Accepted 5 July 2014

DOI:10.1111/liv.12636
Liver Int. 2015; 35: 1077–1086

Abstract

Background & Aims: Sorafenib is the standard of care in advanced hepatocellular carcinoma (HCC), however no criteria have been established to select patients likely to benefit from this therapy. In this study, we evaluated the predictive role of microRNAs (miRNAs) in this setting of patients. *Methods:* We profiled 522 miRNA in a series of 26 HCC patients treated with sorafenib (training set) and validated the results in an independent series of 58 patients (validation set). Formalin-fixed paraffin-embedded tumour and cirrhotic liver biopsies were used for RNA extraction and miRNAs profiling with TaqMan Arrays technology. Statistical analyses were used to correlate miRNA levels with clinical outcome, including time to progression (TTP), progression free (PFS), and overall survival. Cell viability and cell motility of HuH-7 or HepG2 HCC cells were tested *in vitro* after transfection with specific miRNA precursor, inhibitor or controls and sorafenib treatment. *Results:* Six miRNAs were significantly associated with clinical variables in the training set and only miR-425-3p could be further validated. Higher levels of miR-425-3p were associated with longer TTP and PFS ($P = 0.0008$; HR = 0.4; 95% CI = 0.2–0.7 and $P = 0.007$; HR = 0.5; 95% CI = 0.3–0.9 respectively). Multivariate analysis confirmed the predictive significance of miR-425-3p. Furthermore, an association between increased miR-425-3p, cell death and reduced cell motility was defined *in vitro* in HCC cell lines treated with sorafenib. *Conclusions:* Assessment of miR-425-3p levels in liver biopsies could help in stratifying patients with advanced HCC for sorafenib treatment. These promising results need to be confirmed in a large prospective study.

In 2008 Llovet *et al.* reported the results of a multicenter double-blind trial that documented the efficacy of the multikinase inhibitor sorafenib in improving overall survival (OS) of patients with advanced hepatocellular carcinoma (HCC) and preserved liver function (1). An equivalent relative treatment benefit was noted in a population from Asia-Pacific (2). Since then, sorafenib has gained routine acceptance in the clinical practice, becoming the standard of care in advanced HCC. However, no clinical or biological criteria have been established to personalize this therapeutic approach to avoid unnecessary adverse events and cost (3, 4). Recently alpha-foetoprotein (AFP) response has appeared as an independent surrogate end point for survival to be evaluated together with radiological response in HCC patients treated with sorafenib (5). Nevertheless, AFP decrease is not a pretreatment characteristic and more studies are required to identify predictive biomarkers of therapy responsiveness.

MicroRNAs (miRNAs), a class of short non-coding RNAs that act as ubiquitous regulators of gene expression, are emerging as key player in physiological or

pathological conditions, including liver cancer (6, 7). Specifically, in HCC aberrant miRNAs expression has been linked to the pathogenesis, growth, and metastatic spread of cancer cells (8, 9). In addition, different stages of hepatocarcinogenesis are characterized by specific miRNAs profiles (10, 11). More recently, miRNAs have been proposed as novel potential diagnostic or prognostic biomarkers in HCC, easily detectable in archival tissue samples, such as formalin-fixed paraffin-embedded specimens, or in body fluids (6, 12, 13). Moreover, miRNAs purification and analysis can be obtained from small amount of tissues, including liver biopsies, thereby providing a valuable tool in the clinical practice to support the management of liver diseases (6, 14).

Some reports also suggest that miRNAs may influence the cellular response to drugs, *in vitro* and *in vivo* (8, 15). In particular miRNA-26 expression level has been shown to be associated with survival and response to adjuvant therapy with interferon-alpha (16).

Therefore, we explored the hypothesis that miRNA levels could influence the response to sorafenib in patients with advanced HCC. Most importantly, we aimed to investigate whether miRNAs could represent a novel class of biomarkers helpful in the clinical practice to stratify HCC patients likely to respond to sorafenib.

Patients and methods

Clinical specimens and cell lines

In this study, we analysed liver tumour tissue samples obtained from patients with advanced HCC treated with sorafenib according to standard medical procedures, i.e. oral dose of 400 mg (two tablets), twice a day, until disease progression, unacceptable toxicity or patient's death. Radiological tumour assessment was performed every eight weeks, conversely patients' compliance to the target therapy was monitored by self-report and pill count. The adherence rates were 99% for both assessments. The Response Evaluation Criteria in Solid Tumours (RECIST) (17) were used to monitor patients' response to sorafenib treatment.

All specimens were obtained before sorafenib therapy. The overall study population consisted in a first series of 26 patients (training set) treated at the Papa Giovanni XXIII Hospital (Bergamo, Italy), and a second independent series of 58 patients (validation set) treated at the Humanitas Clinical and Research Center, Rozzano (Milan, Italy). Matched cirrhotic counterparts were available for 19 and 37 patients of the training and validation sets respectively.

A third cohort of 61 HCC patients surgically resected for curative purposes between 1997 and 2007 that did not receive sorafenib, was used as control series to reduce the possibility that a molecular biomarker was associated to patient's survival and not to sorafenib treatment (18). This study was approved by the Ethical Committee of the Humanitas Clinical and Research Center. Because of the retrospective nature of this study and the use of data anonymization practices, the need for written informed consent was waived.

Study design

To identify prognostic miRNA related to sorafenib treatment, we profiled the expression of ~700 mature miRNAs in the training series. Significant miRNA were internally validated in the training set using the leave-one-out cross validation procedure. miRNA showing a statistically significant correlation to patients 'outcome, were then analysed in the second series of HCC patients (validation set) using univariate or multivariate approaches. The correlations of significant molecular biomarkers to survival times were then analysed in a previously described series of HCC patients that did not experience the target therapy (18) to exclude miRNA merely related to liver cancer disease and not to sorafenib treatment.

MiRNA analysis, *in vitro* modulation and functional analyses (19) of miR-425-3p in HUH-7 or HepG2 HCC cell lines treated with sorafenib or vehicle are described in the Supporting Information.

Statistical analysis

Data from miRNA arrays (training set) was imported in BRB-ARRAYTOOLS software (http://linus.nci.nih.gov/BRB-ArrayTools.html) and filtered to exclude from further analyses miRNAs characterized by a low variability within HCCs (i.e. <20% of expression data have at least a 1.5-fold change in either direction from gene's median value). Five hundred twenty-two miRNAs were available for clinical correlation.

Time to progression (TTP), progression free survival (PFS), and OS intervals were calculated from sorafenib start to clinical events. Global overall survival (OS_G) was calculated from HCC diagnosis until end of follow-up or death.

TTP is defined as the time between sorafenib treatment initiation and tumour progression determined by radiological procedures. In TTP analysis patients' death are censored. PFS is defined as the time from treatment initiation and tumour progression or death.

To identify miRNAs potentially associated to clinical outcomes in HCC patients treated with sorafenib, we analysed the training series using the Survival Analysis Tool (BRB-ARRAYTOOLS software) and miRNA expression values were analysed in continuous. This analysis fits the Cox proportional-hazards model relating survival to each gene, one gene at a time and computes the *P* value for each gene for testing the hypothesis that survival time is independent of the expression level for that gene. Univariate Cox proportional-hazards regression analyses were conducted on the 522 miRNAs that passed the filtering process separately for TTP, PFS or OS using only the training series of patients. To control and limit

the number of false-positive miRNAs in the analysis of the training set, a multivariate permutation test based on 10 000 random permutations was performed together with a maximum accepted false-positive rate of 0.25. Only miRNAs with a permutation *P* values less than 0.05 and simultaneously associated to TTP, PFS and OS were further characterized in the second independent series of HCC patients treated with sorafenib by Cox proportional-hazards regression analysis. When correlations to outcomes were performed for patients' clinical features, these parameters were considered as discrete (categorical) variables. Statistical dependence between variables was assessed using the Spearman's rank correlation coefficient (MEDCALC software, Mariakerke, Belgium).

Independent associations of molecular (miRNA) or clinical variables to patients' survival were determined by multivariate analyses (MEDCALC software). Multivariate analyses were performed for the validation cohort of patients considering only molecular or clinicopathological covariates that showed a significant *P* value at univariate test. The Kaplan–Meier method was used to plot survival curves when patients were categorized in two groups based on the variable. For miRNAs, cut-offs to categorize patients in the low- or high-expressors group were generated in the training set using receiver operating characteristics (ROC) curves and the non-arbitrary criterion derived from the Youden index (J, MEDCALC software). The J index is defined as sensitivity plus specificity minus 1. We calculated the J index on the training series of patients and then we applied it as cut-off on the validation set to consistently test the predictive effect of the identified miRNAs on different patients' series. When we considered patients' outcome, disease status was considered a fixed characteristic and therefore outcome variables were dichotomized as 0, censored or 1, event. Difference in survival curves was computed using the Log-Rank test (MEDCALC software). ROC curves were also used to compare the sensitivity and specificity for survival prediction of the variable as described (20). Variations in miRNA expression between groups of samples were analysed by *t*-test and results are expressed as mean ± SD (GRAPHPAD software version 4, La Jolla, CA, USA). Functional experiments were performed three times in duplicate. Differences in samples' groups were assessed using *t* or chi-squared test. Two-sided *P* values <0.05 were considered statistically significant.

Bioinformatics analysis to search for possible interaction between miR-425-3p and sorafenib were conducted using the web-based algorithm Pharmaco miR, The miRNA Pharmacogenomics Database (http://www.pharmaco-mir.org) using as input the miRNA and the drug, and as search engines for all miR-gene association VerSe, miRecords, miRTarBase and TargetScan, whereas for gene-drug association VerSe and PharmGKb databases were queried. Specifically, as type of intersection, we extended the search to all possible miRNA associated to sorafenib ('all associations' option on the query tool), and not just miR-425-3p ('overlapping associations' option on the query tool) to increase the chance of potential interaction detection.

The miRPath web-based algorithm (21) was used to identify potentially regulated signalling pathway by selected miRNAs. To identify significantly enriched pathways, we queried the system for predicted and experimentally verified targets using TarBase v6 search engine. Then we applied the 'Pathways Intersection' option, which provides a list of pathways with statistically significant results ($P < 0.01$) for all the selected miRNAs. In this option only pathways significantly targeted by all selected miRNAs are included.

Results

Patients' clinicopathological characteristics of the training and validation sets are described in Table S2. Clinical features of the surgically resected HCC patients that did not experience sorafenib treatment are summarized in Table S3. One patient from the training set was excluded from this study because of accidental death, and two patients from the validation set were excluded because of poor RNA quality. For the training set, median PFS was 4.5 months (range: 0.4–14.3 months), and median OS was 9 months (range: 3–14.5 months). At the last follow-up (March 2011), five patients were alive and three of them had stable disease.

For the validation set, median PFS was 4.4 months (range: 1.4–25.5 months), and median OS was 12 months (range: 2–45 months). At the last follow-up (January 2012), three patients were alive and two of them had stable disease. No significantly differences in survival times between the two cohorts could be evidenced ($P = 0.42$ and $P = 0.06$ for TTP and OS respectively). Since our study did not evaluate objective response rate for sorafenib treatment in HCC patients, but surrogate endpoints as TTP or PFS, we did not reanalysed clinical data of the HCC patients' series according to modified RECIST (mRECIST) criteria. Indeed a previous study reported that TTP was similarly assessed by the two criteria (22).

The low density array analysis performed on the training set ($n = 25$) provided a panel of 522 miRNAs with sufficient inter-samples expression variation. This panel was used to obtain miRNAs significantly associated to clinical end points in HCC patients.

According to the statistical design, we evaluated the association between clinical end points and miRNA levels using as threshold for significant correlation a $P < 0.05$ at Cox proportional-hazards model followed by permutation tests. Thirty-five miRNAs were associated with TTP (Table S4), and PFS (Table S5), whereas 25 miRNAs were associated with OS (Table S6). Among these short lists, a set of seven miRNAs (underlined in grey in Tables S4–S6) was identified because simultaneously altered at all clinical end point (including miR-425-3p, miR-155, miR-10b or miR-579), specific for

TTP or PFS (miR-629 and miR-101), or exclusive for OS (miR-185).

The panel of these seven selected miRNAs was then analysed in an independent series of patients (validation set, $n = 56$; Table 1). miR-425-3p was confirmed as significantly associated with TTP and PFS end points, whereas no other miRNAs could be validated in this set of patients (Table 1).

According to miR-425-3p levels, higher miRNA expression (as determined by ROC analysis; Fig. S2A,B) was associated with improved results in terms of TTP and PFS in the validation cohort ($P = 0.002$; HR = 0.4; 95% CI = 0.2–0.8 and $P = 0.0008$; HR = 0.4; 95% CI = 0.2–0.7 respectively; Fig. 1A,B) confirming preliminary findings obtained in training set of patients (Fig. S2C,D). Low levels of miR-425-3p expression detected in a subset of HCCs (circled in Fig. S2B) were not associated to any clinical characteristic including the aetiology of liver disease. Conversely, this subset of patients displayed among others elevated expression of miR-17, miR-19b-3p and miR-92a-3p (Fig. S2E). These miRNAs belong to the oncogenic miR-17/92 cluster which is responsible for controlling a number of cancer-related pathways as documented by our *in silico* analysis (Fig. S2F,G) and by numerous scientific evidences also in liver cancer (23).

We then analysed clinical variables to determine significant correlation with TTP, PFS and OS (Table 2). Patient' performance status (PS) was significantly correlated to prognosis at all clinical end points whereas the number of nodules (No of nodules) was significantly associated to OS (Table 2 and Fig. S3A–D). When statistical dependence among clinical features was investigated, PS at treatment was correlated to nodule size and patients' age (Spearman $\rho = 0.4$ and -0.3, respectively; Table S7) but not to miR-425-3p levels (Table S7). The number of nodules was not associated with other clinical characteristics or with the miRNA (Table S7).

Finally, multivariate analysis (Table 3) showed that miR-425-3p was an independent prognostic factor for

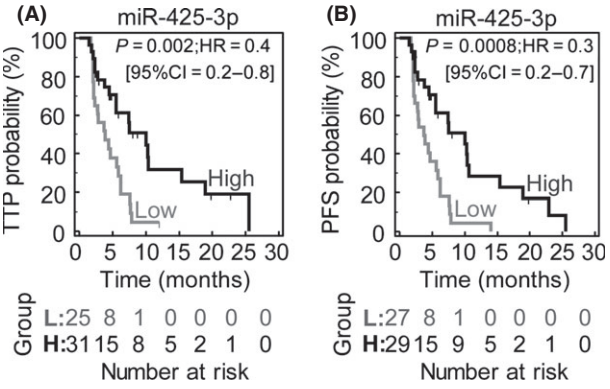

Fig. 1. Kaplan–Meier survival curves of TTP or PFS in HCC patients treated with sorafenib. Time to progression (TTP, A), or progression free (PFS, B) survival curves of the validation patients' series ($n = 56$) according to the miR-425-3p levels in each case. miRNA levels above or below the J Index (Figs S2A and S2B) define 'high' or 'low' expressors respectively. *P* values are from Log-Rank test. 95% CI, 95% confidence interval; HR, hazard ratios.

TTP and PFS end points ($P = 0.002$ and $P = 0.0012$ respectively). When ROC curves were generated to compare sensitivity and specificity of survival prediction, miR-425-3p showed a better ability to predict outcome than did performance status (Fig. S4A,B). No variable was independently associated to patients' OS (Table 3).

To identify whether miRNAs could be bona fide predictors of sorafenib response and not HCC prognostic factors, miR-425-3p expression was related to OS_G or to clinicopathological features. MiR-425-3p was not associated with disease characteristic (Table S8 and Fig. S5), nor its expression was related to OS_G in either the training or the validation series ($P = 0.5$, HR = 1.3, 95% CI = 0.5–3.5 or $P = 0.5$, HR = 0.8, 95% CI = 0.4–1.7 respectively) or in the previously described series of untreated surgically resected patients (data not shown) (6, 18).

To provide a suitable tool to select HCC patients that could benefit from sorafenib treatment, we also investigated miR-425-3p levels in cirrhotic parenchyma

Table 1. Association of the seven selected miRNA to clinical outcome endpoints* in the validation series of HCC patients treated with Sorafenib ($n = 56$)

	Clinical endpoint								
	TTP			PFS			OS		
miRNA	*P*	HR	95% CI of HR	*P*	HR	95% CI of HR	*P*	HR	95% CI of HR
miR-10b	0.24	0.68	0.4–1.3	0.51	0.82	0.5–1.5	0.40	1.28	0.7–2.2
miR-101	0.15	0.63	0.3–1.2	0.16	0.65	0.4–1.2	0.18	0.68	0.4–1.2
miR-155	0.62	1.17	0.6–2.2	0.54	1.21	0.6–2.2	0.87	1.05	0.6–1.8
miR-185	0.81	0.93	0.5–1.7	0.96	1.02	0.6–1.8	0.29	0.74	0.4–1.3
miR-425-3p	0.003	0.37	0.2–0.7	0.001	0.34	0.2–0.6	0.12	0.63	0.3–1.1
miR-579	0.42	0.77	0.4–1.4	0.33	0.74	0.4–1.4	0.46	0.82	0.4–1.4
miR-629	0.74	1.12	0.6–2.2	0.90	1.04	0.5–1.9	0.85	1.06	0.6–1.8

*Cox proportional-hazards regression model.

CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; TTP, time to progression.

Table 2. The relevance of the indicated clinical covariates on clinical outcomes was tested on the validation set of HCC patients treated with sorafenib (n = 56) by Cox proportional-hazards regression analysis

	Clinical endpoint								
	TTP			PFS			OS		
Covariate	*P*	HR	95% CI (HR)	*P*	HR	95% CI (HR)	*P*	HR	95% CI (HR)
Age	0.90	0.9	0.5–1.8	0.99	1.0	0.6–1.8	0.22	1.4	0.8–2.5
Liver disease	0.12	2.0	0.8–4.6	0.08	2.1	0.9–4.7	0.83	0.9	0.4–1.9
AFP (>200)	0.95	1.0	0.5–1.9	0.92	1.0	0.5–1.9	0.06	1.7	0.9–3.0
BCLC	0.5	0.6	0.3–1.5	0.69	0.9	0.4–1.6	0.45	0.8	0.4–1.4
Presence of Metastases	0.58	0.8	0.4–1.5	0.83	0.9	0.5–1.7	0.56	0.8	0.4–1.4
Previous therapy	0.69	1.0	0.4–2.2	0.56	1.3	0.5–2.6	0.73	1.1	0.5–2.3
PS at treatment (0,1 vs. 2)	0.009	4.1	1.4–11.9	0.01	4.0	1.4–11.6	0.04	3.0	1.0–8.4
Gender	0.24	1.8	0.6–4.4	0.13	2.0	0.8–4.6	0.99	1.0	0.3–2.5
Vascular invasion	0.31	1.4	0.7–2.7	0.32	1.4	0.7–2.7	0.12	1.6	0.8–2.9
Child–Pugh	0.36	0.5	0.1–2.1	0.45	0.6	0.1–2.0	0.93	1.0	0.3–2.7
Edmonson	0.36	1.41	0.7–2.9	0.34	1.42	0.6–2.9	0.51	1.23	0.6–2.3
No of nodules	0.06	3.22	0.9–10.9	0.06	3.15	0.9–10.6	0.002	7.69	2.0–28.4
Nodule size	0.52	0.78	0.37–1.6	0.30	0.68	0.3–1.4	0.21	0.68	0.3–1.2

AFP, alpha-foetoprotein; BCLC, Barcelona Clinic Liver Cancer staging classification; CI, confidence interval; HR, hazard ratio; OS, overall survival; PS, Performance status; PFS, progression free survival; TTP, time to progression.

Table 3. Multivariate Cox proportional-hazards regression analysis was performed in the validation cohort of patients for TTP, PFS or OS clinical endpoints

Analysis	Covariate	*P*	HR	95% CI of HR
TTP	PS at treatment	0.009	4.5	1.5–13.9
	miR-425-3p*	0.002	0.4	0.1–0.7
PFS	PS at treatment	0.008	4.6	1.5–14.1
	miR-425-3p*	0.0012	0.3	0.1–0.7
OS	PS at treatment	0.8	1.2	0.1–13.9
	No of nodules	0.08	6.5	0.8–54.4

*miRNAs expression was analysed as dichotomous variable using the ROC generated cut-off (J = 0.95; Fig. S2A).
CI, confidence interval; HR, hazard ratio; OS, overall survival; PS, ECOG performance status; PFS, progression free survival; TTP, time to progression.

associated with HCC and we generated a miRNA expression ratio between matched cirrhotic parenchyma and the tumour tissue (n = 56). In patients with stable disease (SD, n = 18), miR-425-3p levels were higher in tumour tissue than in surrounding parenchyma (mean levels in HCC = 2.1; mean levels in C = 0.9; Fig. 2A). Conversely, miR-425-3p was over-expressed in cirrhotic tissues compared to matched HCC tissues in patients who experienced disease progression during sorafenib treatment (mean levels in HCC = 1.7; mean levels in C = 2.3; P = 0.012 by paired t-test, Fig. 2A). Indeed, patients with progressive disease had a significantly lower miR-425-3p ratio compared to SD patients (miR-425-3p ratio = HCC/C; P = 0.004; Fig. 2B). When TTP was investigated in this subset of patients, a ratio >1 was significantly associated with better prognosis (P = 0.03; HR = 1.9; 95% CI: 1–3.6; Fig. 2C).

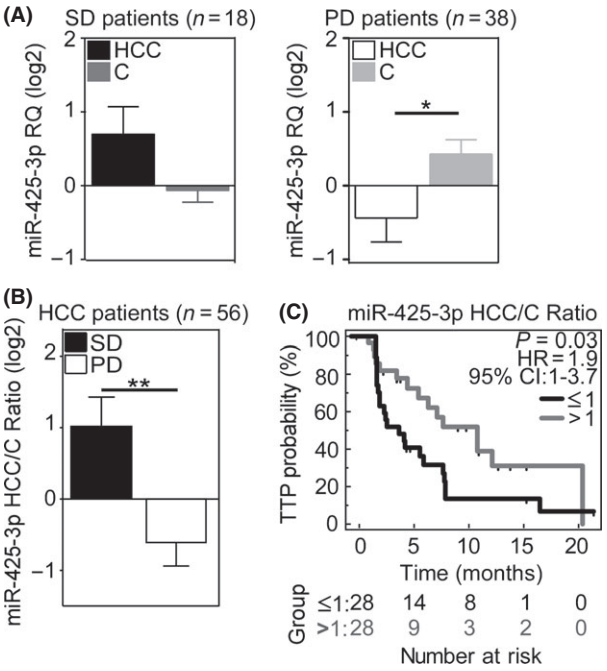

Fig. 2. miR-425-3p ratio is a suitable tool to identify hepatocellular carcinoma (HCC) patients which may better benefit from sorafenib therapy. (A) miR-425-3p expression was evaluated in cirrhotic parenchyma of HCC patients with stable (SD, right panel) or progressed disease (PD, left panel) by qPCR. Data were log2 transformed and expressed as mean values ± SEM. *P = 0.012 from paired t-test. RQ, Relative quantity. (B) A ratio between miR-425-3p levels in HCC and matched cirrhotic counterparts was computed for each patients treated with sorafenib (n = 56). Data are expressed as log2-transformed values. Bars represent mean ± SEM. **P = 0.0049. (C) Kaplan–Meier curves were generated to compare HCC patients according to miR-425-3p ratio values (>1 vs. ≤1). P values are from Log-Rank test. 95% CI, 95% confidence interval; HR, hazard ratios.

Lastly, miR-425-3p functional role in sorafenib treatment response was analysed in HCC cell cultures. HuH-7 or HepG2 cells were incubated with a suboptimal dose of sorafenib (5 μM) in order to prevent excessive cell death because of combination of drug and transfection (Fig. S6A,B) or the induction of a resistant phenotype (24).

MiR-425-3p was less expressed in HuH-7 cells compared to HepG2, although the difference was not significant and sorafenib incubation marginally affected miR-425-3p expression in both cell lines (Fig. 3A). Interestingly, the drug treatment induced a significant increase in the miRNA content in HuH-7 derived

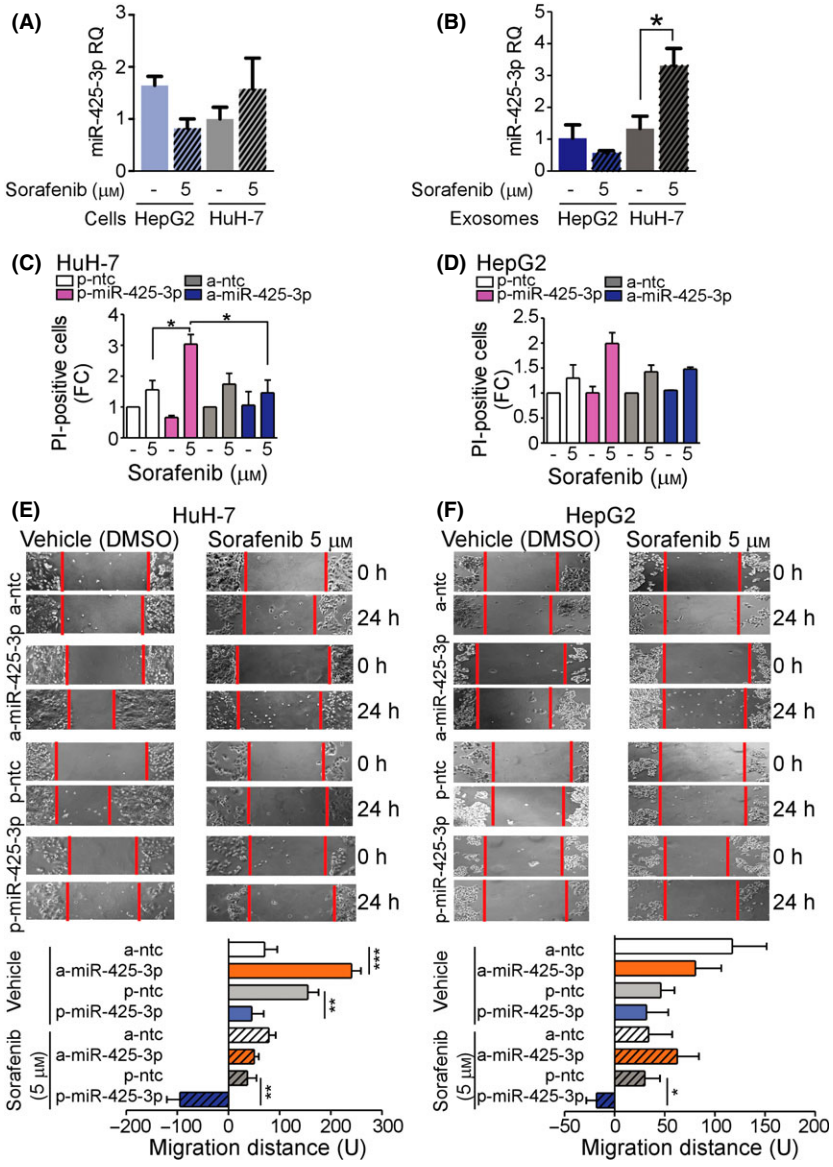

Fig. 3. Re-expression of miR-425-3p in presence of sorafenib sensitizes liver cancer cell lines to apoptosis and reduces cell motility. (A) HepG2 or HUH-7 cells were treated with sorafenib for 48 h and the expression of miR-425-3p was assessed in cell lines (A) or in exosomes secreted in the culture media (B). *P = 0.04 by Mann–Whitney test. (C, D) Quantification of PI-positive cells. HuH-7 (B) or HepG2 (C) cells were transfected with precursor miR-425-3p (p-miR-425-3p), antagonist miR-425-3p (a-miR-425-3p) molecules or corresponding control oligonucleotites (p-ntc or a-ntc respectively) and incubated with 5 μmol/L of sorafenib. After 24 h, cell viability was investigated by propidium iodide (PI) staining and flow cytometry analysis. The percentage of dead cells is expressed as a ratio (fold change, FC) of samples transfected with miR-425-3p mimics and/or incubated with sorafenib relative to corresponding control transfected specimens (p-ntc or a-ntc respectively). *$P < 0.05$ by Student's t-test. (D, E) Cell motility was investigated using a wound-healing assay after miR-425-3p expression modulation and sorafenib treatment in HuH-7 (D) or HepG2 (E) cells. Lower panels, quantification of cell migration. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by Student's t-test. Bars, mean ± SD of three independent experiments.

Liver International (2015)

exosomes (Fig. 3B). Then, HuH-7 or HepG2 cells were transfected with miR-425-3p precursor (p-miR-425-3p), inhibitor (a-miR-425-3p) or control oligonucleotides (p-ntc or a-ntc, respectively; Fig. S6C), and treated with sorafenib.

Treatment of pre-miR-425-3p transfected HuH-7 cells with sorafenib resulted in increased cell death compared to mock-transfected samples, as shown by PI-staining and flow cytometry analysis (Fig. S6D). In absence of sorafenib, miR-425-3p levels did not affect cell viability in either HuH-7 or HepG2 cells (Fig. 3C, D). Conversely, adding sorafenib to the culture media of HuH-7 or HepG2 cells transfected to over-express miR-425-3p, increased cell death in both HCC cell lines with HuH-7 cells being most significantly affected (Fig. 3C, D). MiR-425-3p expression affected also HuH-7 (Fig. 3E) and HepG2 (Fig. 3F) cell motility. Indeed, depletion of the miRNA increased directional HuH-7 cell migration in a wound-healing assay (Fig. 3E). Conversely, miR-425-3p over-expression hampered cell motility per se in HuH-7 cell line (Fig. 3E), or completely inhibited migratory capacities of HuH-7 or HepG2 cells treated with sorafenib (Fig. 3E,F). In parallel, a bioinformatics analysis was performed to disclose potential interactions between miR-425-3p and sorafenib activity or metabolism through a combined search for miR-425-3p predicted gene target and genes potentially associated to the drug, but no interaction could be identified (Table S9).

Discussion

Improvements in cancer patients' stratification for target therapy can lead to longer patients' survival, preventing unnecessary treatments, serious side effects and saving costs.

Scarce and inconsistent biomarkers predictive of sorafenib response or clinical benefit are currently available in the clinical practice for the management of advanced HCC (3). Further, these data could not be confirmed by subsequent studies (SHARP study) (25). Recently, a study from Arao and coworkers preliminary identified amplification of FGF3/4 as a positive prognostic factor for HCC patients treated with sorafenib, whereas Personeni and colleagues suggested decreased AFP levels as an indicator of response (5, 26). In this scenario, we looked for novel biomarkers predictive of sorafenib response in HCC patients.

Our data suggests that miR-425-3p is a novel prognostic biomarker predictive of extended TTP or PFS, and independent from clinical variables or aetiology of liver disease in patients with advanced HCC treated with sorafenib. These results, documented in two independent cohorts of patients, provide the scientific rationale for the evaluation of miR-425-3p levels in liver biopsies to identify HCC patients that could benefit from sorafenib therapy.

Further, this study shows the suitability to quantify differential miR-425-3p expression between cirrhosis and HCC in individual patients (miR-425-3p Ratio). If confirmed in larger cohorts of patients, this test could become a molecular tool useful to help in stratifying HCC patients for sorafenib therapy.

Very limited data are available about miR-425-3p functions *in vitro* and *in vivo* and its deregulation in human diseases. To the best of our knowledge, only two studies reported on miR-425-3p deregulation in human tissues (27, 28), showing reduced miR-425-3p levels in the plasma of patients affected by colorectal cancer (27) and increased miR-425-3p expression in serum from patients suffering from breast cancer (28). Indeed a recent investigation found that miR-191/425 locus was transcriptionally regulated by estrogens, thereby disclosing molecular mechanism of miR-425 deregulation in breast carcinogenesis (29). In this context, our finding about up-regulation of miRNAs belonging to the miR-17/92 oncogenic cluster in HCC patients with very low level of miR-425-3p expression is intriguing. Indeed increased expression of miR-17/92 transcripts in HCC tissues could elicit tumour resistance to the drug, as described for other tumours, limiting the anticancer efficacy of sorafenib in this specific subset of HCC patients. Future studies aimed at understanding the molecular mechanism underpinning miR-425-3p regulation by miR-17/92 miRNAs should shed light on a novel mechanism adopted by HCC cells to resist to sorafenib.

Finally, bioinformatics search of potential correlations between miR-425-3p and sorafenib metabolism or activity did not reveal any mechanism of interactions.

Our results from *in vitro* modulation of miR-425-3p levels in HuH-7 and HepG2 liver cancer cell lines were consistent with clinical observations of higher miR-425-3p levels detected in HCC patients, who better responded to sorafenib treatment. In agreement with clinical data, miR-425-3p levels were induced by sorafenib incubation in HuH-7 cells-derived exosomes, and this cell line was more sensitive to cell death after incubation with the drug. The involvement of extracellular vesicles in modulating HCC response to sorafenib has recently emerged (30) providing a potential novel strategy to interfere with HCC chemoresistance.

Further, our results show that forced expression of miR-425-3p in HCC cells incubated with sorafenib determined increased cell death and reduced cell motility compared to controls or to liver cancer cells silenced for miR-425-3p expression. These results should prompt further studies aimed to identify genes and pathways affected by miR-425-3p, and their derangements in HCC.

Beyond the well-established role of miRNAs in modulating gene expression in physiological or pathological conditions, practical applications of miRNAs as diagnostic or prognostic biomarkers are becoming significant especially in cancer patients (31, 32), and their use

in therapeutic applications is under investigation (33–35). Considering the pleiotropic effects of miRNA on multiple signalling pathways modulation, many investigators surmised that miRNAs could predict or even modify the response to chemotherapy and radiation therapy (for a review, see 36).

Nevertheless, few translational studies were performed in human cancers. MiR-200b or miR-143 was identified as potential indicators of PFS in colorectal cancer patients treated with a combination of cetuximab and chemotherapy (37). MiR-10b levels measured in fine needle aspirates of patients with pancreatic ductal carcinoma were inversely correlated with response to neoadjuvant therapy, or to surgical resectability (38). Finally, in HCC patients, miR-26 was shown to modulate sensitivity to interferon-mediated therapy (16).

Our study has limitations in terms of its retrospective nature and the limited number of patients investigated. However, it is worth of note that the patient population is a non-selected subgroup of advanced HCC treated with sorafenib according to clinical practice. Additional prospective series of patients will be needed to confirm our results and to translate miR-425-3p evaluation into standard medical practice. Furthermore, the mechanisms beyond miR-425-3p effects on sorafenib need to be fully clarified.

We acknowledge that mRECIST introduced a novel method to evaluate response to sorafenib therapy in HCC patients since late 2010. Despite mRECIST revealed to be more accurate than RECIST in assessing objective response rate for sorafenib treatment in HCC patients, surrogate endpoints as TTP or the disease control rate, were equally assessed by the two criteria (22). In line with this observation, a previous study by Edeline and collaborators (39) concluded that no significant differences could be appreciated for stable disease or progressive disease determination by the two criteria, either in terms of frequency, median patients' survival times or median TTP. Furthermore mRECIST recommendations indicate that it should not be adopted in patients previously treated with locoregional or systemic therapies because these treatments could alter HCC lesions visualization at CT/MRI (40). Given all this background, the point that we did not evaluate objective response as surrogate endpoint to patients' OS, nor we related SD or PD to OS, we believe that the use of RECIST instead of mRECIST does not affect our miRNA study.

In conclusion, our data show that elevated expression of miR-425-3p in tumour cells is a novel marker of better prognosis in patients with advanced HCC treated with sorafenib and suggest that the evaluation of miR-425-3p in addition to current clinical parameters could improve stratification of patients suitable for sorafenib.

Our results warrant larger, prospective investigations to validate the innovative tool of miR-425-3p in this setting.

Acknowledgments

The Authors thank Laura Porretti (Fondazione IRCCS Ca' Granda, Milan, Italy) for providing HUH-7 cells. The funding sources had no roles in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Financial Support: This study has been supported by grants to SB from Fondazione Berlucchi (Grant program 2009), Fondazione Cariplo (2010-0846) and by Ministero Salute '5×1000' program; to MR from AIRC (2010-10247).

Conflict of interest: AS is a member of Bayer advisory board and has received honoraria for lecturing from Bayer. The rest of the authors do not have any disclosures to report.

References

1. Llovet JM, Ricci S, Mazzaferro V, *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–90.
2. Cheng AL, Kang YK, Chen Z, *et al.* Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25–34.
3. Bruix J, Raoul JL, Sherman M, *et al.* Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. *J Hepatol* 2012; **57**: 821–9.
4. Llovet JM, Peña CE, Lathia CD, *et al.* Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2012; **18**: 2290–300.
5. Personeni N, Bozzarelli S, Pressiani T, *et al.* Usefulness of alpha-fetoprotein response in patients treated with sorafenib for advanced hepatocellular carcinoma. *J Hepatol* 2012; **57**: 101–7.
6. Augello C, Vaira V, Caruso L, *et al.* MicroRNA profiling of hepatocarcinogenesis identifies C19MC cluster as a novel prognostic biomarker in hepatocellular carcinoma. *Liver Int* 2012; **32**: 772–82.
7. Toffanin S, Hoshida Y, Lachenmayer A, *et al.* MicroRNA-based classification of hepatocellular carcinoma and oncogenic role of miR-517a. *Gastroenterology* 2011; **140**: 1618–28.
8. Bai S, Nasser MW, Wang B, *et al.* MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem* 2009; **284**: 32015–27.
9. Borel F, Konstantinova P, Jansen PL. Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma. *J Hepatol* 2012; **56**: 1371–83.
10. Llovet JM, Chen Y, Wurmbach E, *et al.* A molecular signature to discriminate dysplastic nodules from early hepatocellular carcinoma in HCV cirrhosis. *Gastroenterology* 2006; **131**: 1758–67.
11. Wang XW, Heegaard NH, Orum H. MicroRNAs in liver disease. *Gastroenterology* 2012; **142**: 1431–43.
12. Villanueva A, Hoshida Y, Toffanin S, *et al.* New strategies in hepatocellular carcinoma: genomic prognostic markers. *Clin Cancer Res* 2010; **16**: 4688–94.

13. Giordano S, Columbano A. MicroRNAs: new tools for diagnosis, prognosis, and therapy in hepatocellular carcinoma? *Hepatology* 2013; **57**: 840–7.
14. Hoshida Y, Villanueva A, Kobayashi M, *et al.* Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 1995–2004.
15. Zhou C, Liu J, Li Y, *et al.* microRNA-1274a, a modulator of sorafenib induced a disintegrin and metalloproteinase 9 (ADAM9) down-regulation in hepatocellular carcinoma. *FEBS Lett* 2011; **585**: 1828–34.
16. Ji J, Shi J, Budhu A, *et al.* MicroRNA expression, survival, and response to interferon in liver cancer. *N Engl J Med* 2009; **361**: 1437–44.
17. Therasse P, Arbuck SG, Eisenhauer EA, *et al.* New guidlines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Insitute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205–16.
18. Augello C, Caruso L, Maggioni M, *et al.* Inhibitors of apoptosis proteins (IAPs) expression and their prognostic significance in hepatocellular carcinoma. *BMC Cancer* 2009; **9**: 125.
19. Vaira V, Faversani A, Dohi T, *et al.* miR-296 regulation of a cell polarity-cell plasticity module controls tumor progression. *Oncogene* 2012; **31**: 27–38.
20. Oikonomopoulou K, Li L, Zheng Y, *et al.* Prediction of ovarian cancer prognosis and response to chemotherapy by a serum-based multiparametric biomarker panel. *Br J Cancer* 2008; **99**: 1103–13.
21. Vlachos IS, Kostoulas N, Vergoulis T, *et al.* DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res* 2012; **40**: W498–504.
22. Lencioni R. New data supporting modified RECIST (mRECIST) for hepatocellular carcinoma. *Clin Cancer Res* 2013; **19**: 1312–4.
23. Mogilyansky E, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ* 2013; **20**: 1603–14.
24. Ou DL, Shen YC, Yu SL, *et al.* Induction of DNA damage-inducible gene GADD45beta contributes to sorafenib-induced apoptosis in hepatocellular carcinoma cells. *Cancer Res* 2010; **70**: 9309–18.
25. Abou-Alfa GK, Schwartz L, Ricci S, *et al.* Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2006; **24**: 4293–300.
26. Arao T, Ueshima K, Matsumoto K, *et al.* FGF3/FGF4 amplification and multiple lung metastases in responders to sorafenib in hepatocellular carcinoma. *Hepatology* 2013; **57**: 1407–15.
27. Wang Q, Huang Z, Ni S, *et al.* Plasma miR-601 and miR-760 are novel biomarkers for the early detection of colorectal cancer. *PLoS ONE* 2010; **7**: e44398.
28. Zhao H, Shen J, Medico L, *et al.* A pilot study of circulating miRNAs as potential biomarkers of early stage breast cancer. *PLoS ONE* 2010; **5**: e13735.
29. Di Leva G, Piovan C, Gasparini P, *et al.* Estrogen mediated-activation of miR-191/425 cluster modulates tumorigenicity of breast cancer cells depending on estrogen receptor status. *PLoS Genet* 2013; **9**: e1003311.
30. Takahashi K, Yan IK, Kogure T, Haga H, Patel T. Extracellular vesicle-mediated transfer of long non-coding RNA ROR modulates chemosensitivity in human hepatocellular cancer. *FEBS Open Bio* 2014; **4**: 458–67.
31. Liu N, Chen NY, Cui RX, *et al.* Prognostic value of a microRNA signature in nasopharyngeal carcinoma: a microRNA expression analysis. *Lancet Oncol* 2012; **13**: 633–41.
32. Paranjape T, Heneghan H, Lindner R, *et al.* A 3′-untranslated region KRAS variant and triple-negative breast cancer: a case-control and genetic analysis. *Lancet Oncol* 2011; **12**: 377–86.
33. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov* 2010; **9**: 775–89.
34. Kim M, Kasinski AL, Slack FJ. MicroRNA therapeutics in preclinical cancer models. *Lancet Oncol* 2012; **12**: 319–21.
35. Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M. microRNAs in cancer management. *Lancet Oncol* 2012; **13**: e249–58.
36. Hummel R, Hussey DJ, Haier J. MicroRNAs: predictors and modifiers of chemo- and radiotherapy in different tumour types. *Eur J Cancer* 2010; **46**: 298–311.
37. Mekenkamp LJ, Tol J, Dijkstra JR, *et al.* Beyond KRAS mutation status: influence of KRAS copy number status and microRNAs on clinical outcome to cetuximab in metastatic colorectal cancer patients. *BMC Cancer* 2012; **12**: 292.
38. Preis M, Gardner TB, Gordon SR, *et al.* MicroRNA-10b expression correlates with response to neoadjuvant therapy and survival in pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2011; **17**: 5812–21.
39. Edeline J, Boucher E, Rolland Y, *et al.* Comparison of tumor response by Response Evaluation Criteria in Solid Tumors (RECIST) and modified RECIST in patients treated with sorafenib for hepatocellular carcinoma. *Cancer* 2012; **118**: 147–56.
40. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis* 2010; **30**: 52–60.

Supporting information

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

Fig. S1. Three- and two-reference small RNAs were used to build an internal reference (normalization factor) for target miRNA relative quantification in two of the patients' sets (see Methods for details).

Fig. S2. Identification of miR-425-3p as a predictive marker for sorafenib therapy.

Fig. S3. Kaplan–Meier survival curves of HCC patients treated with sorafenib according to clinical variables.

Fig. S4. Comparison of sensitivity and specificity for survival prediction by miRNA levels or clinical variables in HCC patients treated with sorafenib.

Fig. S5. miR-425-3p levels are not correlated with aetiology of liver disease. HCCs from the training and the validation cohorts were grouped according to the aetiology of liver disease and miR-425-3p expression was analysed ($P = 0.92$ by Kruskal–Wallis test).

Fig. S6. miR-425-3p and sorafenib modulation in liver cancer cell lines.

Table S1. Applied Biosystems identification code (ABI ID, Life Technologies Inc.) of the seven selected miRNAs analysed in HCC samples.

Table S2. Baseline patients' characteristics.

Table S3. Baseline patients' characteristics of the cohort of HCC patients that did not experienced sorafenib treatment (control set) (6, 18).

Table S4. miRNAs significantly associated to time to progression (TTP) in the training set of HCC patients treated with sorafenib.

Table S5. miRNAs significantly associated to progression free survival (PFS) endpoint in the training set of HCC patients treated with sorafenib.

Table S6. miRNAs significantly associated to overall survival (OS; timeframe: from therapy start to end of follow-up) in the training set of HCC patients treated with sorafenib.

Table S7. Statistical dependence between clinical or molecular (miR-425-3p) variables was analysed using the Spearman's rank correlation coefficient (ρ) in the validation cohort.

Table S8. The seven selected miRNAs were tested for differential expression in samples classes according to the indicated clinical variable in the two patients' cohorts.

Table S9. Bioinformatics analysis of potential interactions of miRNAs with sorafenib was performed using the web-based database Pharmaco miR (http://www.pharmaco-mir.org) and miR-425-3p and sorafenib as input of the search.