

MicroRNA-125a-5p Is a Downstream Effector of Sorafenib in Its Antiproliferative Activity Toward Human Hepatocellular Carcinoma Cells

This is the peer reviewed version of the following article:

Original:

Potenza, N., Mosca, N., Zappavigna, S., Castiello, F., Panella, M., Ferri, C., et al. (2017). MicroRNA-125a-5p Is a Downstream Effector of Sorafenib in Its Antiproliferative Activity Toward Human Hepatocellular Carcinoma Cells. JOURNAL OF CELLULAR PHYSIOLOGY, 232(7), 1907-1913 [10.1002/jcp.25744].

Availability:

This version is available <http://hdl.handle.net/11365/1006973> since 2017-05-16T13:47:37Z

Published:

DOI: <http://doi.org/10.1002/jcp.25744>

Terms of use:

Open Access

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. Works made available under a Creative Commons license can be used according to the terms and conditions of said license.

For all terms of use and more information see the publisher's website.

(Article begins on next page)

MicroRNA-125a-5p is a downstream effector of sorafenib in its antiproliferative activity toward human hepatocellular carcinoma cells[†]

Nicoletta Potenza,¹ Nicola Mosca,¹ Silvia Zappavigna,² Filomena Castiello,¹ Marta Panella,¹ Carmela Ferri,² Daniela Vanacore,² Antonio Giordano,³ Paola Stiuso,² Michele Caraglia,^{2*} and Aniello Russo^{1*}

¹Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "L. Vanvitelli", Italy

²Department of Biochemistry, Biophysics and General Pathology, University of Campania "L. Vanvitelli", Italy

³Sbarro Institute for Cancer Research and Molecular Medicine and Center of Biotechnology, College of Science and Technology, Temple University, Philadelphia, USA

*Correspondence to: Aniello Russo, Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "L. Vanvitelli", Caserta, Italy, Via Vivaldi 43, 81100, Italy. Tel. +39 0823 274569; Fax: +39 0823 274571; Email: aniello.russo@unina2.it
Michele Caraglia, Department of Biochemistry, Biophysics and General Pathology, University of Campania "L. Vanvitelli", Via L. De Crescchio, 7 80138 Naples, Italy. Phone +390815665871, Fax +390815665863, email: michele.caraglia@unina2.it or michele.caraglia@fastwebnet.it

Running head: Sorafenib upregulates miR-125a-5p

Keywords:

- RNA interference
- miR-125a
- hepatocellular carcinoma
- sirtuin-7
- Cell Cycle
- P21
- P27
- C-Raf
- Sorafenib

Conflict of interest: All the authors declare no conflict of interest

Contract grant sponsor: Regione Campania; Contract grant number: L5/2002

[†]This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jcp.25744]

Received 28 November 2016; Revised 15 December 2016; Accepted 15 December 2016

Journal of Cellular Physiology

This article is protected by copyright. All rights reserved

DOI 10.1002/jcp.25744

Abstract

Sorafenib is an antitumor drug for treatment of advanced hepatocellular carcinoma. It acts as a multikinase inhibitor suppressing cell proliferation and angiogenesis. Human microRNA-125a-5p (miR-125a) is endowed with similar activities and is frequently downregulated in hepatocellular carcinoma. Looking for a potential microRNA-based mechanism of action of the drug, we found that sorafenib increases cellular expression of miR-125a in cultured HuH-7 and HepG2 hepatocellular carcinoma cells. Upregulation of the microRNA inhibited cell proliferation by suppression of sirtuin-7, a NAD(+)-dependent deacetylase, and p21/p27-dependent cell cycle arrest in G1. Later, recruitment of miR-125a in the antiproliferative activity of sorafenib was inquired by modulating its expression in combination with the drug treatment. This analysis showed that intracellular delivery of miR-125a had no additive effect on the antiproliferative activity of sorafenib, whereas a miR-125a inhibitor could counteract it. Finally, evaluation of other oncogenic targets of miR-125a revealed its ability to interfere with the expression of matrix metalloproteinase-11, Zbtb7a proto-oncogene, and c-Raf, possibly contributing to the antiproliferative activity of the drug. This article is protected by copyright. All rights reserved

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at post-transcriptional level by affecting both translation and stability of complementary mRNAs (Ambros, 2004). MiRNAs play crucial roles in a variety of physiological processes, such as development and cell differentiation (Bartel, 2004; Wienholds and Plasterk, 2005). Several studies have also demonstrated that altered miRNA profiles are linked to pathological conditions, including several types of cancers (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Grammatikakis et al., 2013). Downregulation of the microRNA biosynthesis enzyme Dicer in cancer cells leads to dysregulation of miRNA biogenesis and increased tumor progression (Kitagawa et al., 2013; Rupaimoole et al., 2014). On the other hand, differentiating cells often exhibit increased Dicer expression (O'Rourke et al., 2007; Kawase-Koga et al., 2009; Potenza et al., 2009). In this field, a growing body of evidence indicates that miRNAs can function as either tumor suppressors by down-regulating oncogenic targets, or tumor promoters by negatively regulating oncosuppressor proteins (Di Leva et al., 2014). In the case of hepatocellular carcinoma (HCC), deregulation of miRNA expression has been described for its establishment, growth, and metastatic activity (Negrini et al., 2014). HCC is the third cause of tumor-related mortalities and the fifth most common cancer worldwide (Parkin et al., 2005). Surgery is currently the only curative treatment, but those patients who have advanced stage disease, or recurrent tumor, are treated pharmacologically (Schwartz et al., 2007).

Sorafenib is an oral drug acting as a multikinase inhibitor and represents the standard of care for advanced HCC (Llovet et al., 2008; Cheng et al., 2009). The molecule is endowed with antiproliferative and antiangiogenic properties that suppress tumor growth but its mechanism of action has not been fully elucidated yet (Liu et al., 2006; Wilhelm et al., 2006). On the other hand, various studies highlight changes in miRNA expression profiles in response to sorafenib and other therapeutics (Zhou et al., 2011; Lv et al., 2015; Peveling-Oberhag et al., 2015; Stiuso et al., 2015),

and some reports suggest that miRNAs may modulate the cellular response to the drugs (Bai et al., 2009; Shimizu et al., 2010).

MiR-125a-5p (miR-125a) regulates the expression of several target proteins that are involved in cell proliferation, apoptosis, angiogenesis, and migration (Potenza and Russo, 2013). In breast cancer, several studies support a tumor suppressive role for miR-125a and a germline mutation in the sequence of miR-125a has been linked with the incidence of the tumor (Scott et al., 2007; Guo et al., 2009; Li et al., 2009). In HCC, miR-125a is frequently down-regulated and *in vitro* studies have shown that it inhibits cell proliferation, angiogenesis and cell migration through the down-regulation of sirtuin-7 (SIRT7), a NAD(+)-dependent deacetylase, vascular endothelial growth factor A (VEGF-A), and matrix metalloproteinase-11 (MMP11), respectively (Bi et al., 2012; Kim et al., 2013).

Given the antiproliferative activities of sorafenib and miR-125a toward HCC cells and other similarities in their biological effects, we hypothesized that the miRNA may be involved in the mechanism of action of the drug.

Materials and Methods

Reporter plasmids

The c-Raf DNA segment targeted by miR-125a was obtained by chemical synthesis of complementary oligodeoxynucleotides containing a downstream *NotI* site and upstream *XhoI* and *EcoRV* sites. Sequences were 5'-TCGAGATATCTCCGCATCTCAGCCCTCTCAGGGAGC-3' and 5'-GGCCGCTCCCTGAGAGGGCTGAGATGCGGAGATATC-3' for sense and antisense strand, respectively. The two oligonucleotides were annealed, 5'-phosphorylated with T4 polynucleotide kinase, and ligated into *XhoI* and *NotI* sites of psiCheck-2 vector (Promega). Screening of recombinant clones was performed by digestion with *EcoRV*. Control plasmid with inverted target sequence was obtained using the following oligodeoxynucleotides: 5'-TCGAGATATCAGGGACTCTCCCGACTCTACGCCTGC-3' and 5'-GGCCGCAGGCGTAGAGTCGGGAGAGTCCCTGATATC-3'.

Cell cultures and transfections

Human hepatocarcinoma cell lines HepG2 and HuH-7 were cultured in RPMI 1640 and DMEM respectively, containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 100 µg/ml streptomycin. The day before transfection, cells were trypsinized and seeded in medium without antibiotics. Transfections were then performed with Lipofectamine2000, using 50nM MiRidian mir-125a-5p mimic, miRIDIAN miR-125a-5p hairpin inhibitor, or their control molecules with unrelated sequences (Dharmacon, Lafayette, CO). Mature miRNA sequences were: 5'-UCCCUGAGACCCUUUAACCUGUGA-3' for hsa-miR-125a-5p, 5'-UCACAACCUCCUAGAAAGAGUAGA-3' for microRNA mimic negative control, 5'-UUGUACUACACAAAAGUACUG-3' for microRNA mimic negative control #2. Negative controls were non-targeting mimics whose sequences are based on *C. elegans* microRNAs with minimal sequence identity in mammals. For luciferase reporter assays, miRNA mimic and inhibitor

were transfected along with 200 ng or 50 ng of reporter plasmids in HepG2 and HuH-7, respectively.

Luciferase reporter assay

Assay was performed 48 h after transfection. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Briefly, cells were washed with phosphate buffered saline and lysed chemically. Lysates were then centrifuged to remove cellular debris and 20 μ l of the supernatants were loaded into an automated luminometer (Turner Biosystems). Then the instrument performed a sequential auto-injection of 100 μ l of Luciferase Assay Reagent II (substrate for firefly luciferase) and 100 μ l of Stop and Glow Reagent (stop solution for firefly luciferase containing the substrate for Renilla luciferase). The mean of the luciferase activities measured for 10 s each were used to calculate ratios between Renilla and firefly luciferases.

Cell proliferation assay and flow cytometry

Cells were plated in 96-well microtiter plates and their growth was evaluated by quadruplicate analysis with MTT assay, as detailed below. 50 μ l of 1mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) were mixed with 200 μ l of medium and added to the well. After one hour of incubation at 37 °C, the medium was removed and the purple formazan crystals produced in the viable cells were solubilized in 100 μ l of dimethyl sulfoxide and quantitated by measurement of absorbance at 570 nm with a plate reader. Flow cytometry was performed with a FACScan equipment from Becton Dickinson. Cells were seeded in 100-mm plates at the density of 1×10^6 cells/plate, treated with the test molecule for 72 h, collected, and stained in a propidium iodide solution (50 μ g PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) for 30 minutes at 4 °C in the dark. Flow cytometry analysis was performed using a FACScan flow cytometer (Becton Dickinson). To evaluate cell cycle, propidium fluorescence was collected as FL2 (linear scale) by the ModFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least

20,000 events for each point were analyzed in at least three different experiments giving an SD less than 5%.

RNA purification and real-time PCR

Total RNA was purified from cultured cells using the miRNeasy Mini Kit (Qiagen); the RNA concentration was determined spectrophotometrically with NanoDrop 2000c (ThermoScientific). MiR-125a was quantified along with RNU6B (reference gene) by RT-qPCR with TaqMan® miRNA assays from Applied Biosystems according to the manufacturer's protocol. The expression levels of transcripts targeted by miR-125a were determined by RT-qPCR with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). In particular, 200 ng of RNA were retrotranscribed by Transcriptor High Fidelity cDNA Synthesis Sample kit (Roche) using random examer primers; 1 µl of the cDNA product was then used to amplify the target sequences along with GAPDH as reference. Primers were: GAPDH, 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGTGATGGGATTT-3'; ERBB3, 5'-CCCTGCCATGAGAACTGCAC-3' and 5'-TCACTGTCAAAGCCATTGTCAGAT-3'; SIRT7, 5'-GTCTGCATGAGCAGAAGCTG-3' and 5'-GGAACGCAGGAGGTACAGAC-3'; VEGF-A, 5'-GGAGGAAGGAGCCTCCCTC-3' and 5'-TGTTCTGTCTGATGGTGTGATGG-3'; c-Raf, 5'-GCAATGAAGAGGCTGGTAGC-3' and 5'-GGAGCAGCTCAATGGAAGAC-3'. Primers for ERBB2, MMP11, and Zbtb7a were from the literature (Bi et al., 2012; Hojo et al., 2015). The expression levels of miR-125a and its targeted transcripts were normalized to their respective reference genes by using the $2^{-\Delta Ct}$ method (Marra et al., 2013). Comparison of the data sets was performed by Student's t-test and a value of $p < 0.05$ was considered significant.

Protein extraction and western blotting

Cells were lysed with 1% Triton X-100, 0.5% sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 10 mM Na₂HPO₄, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, 0.025 units/ml

aprotinin. Lysates were clarified by centrifugation; 40 µg protein samples were then separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare) (Esposito et al, 2012; Vitiello et al, 2008). Blots were treated with antibodies against p21, p27, SIRT7, and γ -tubulin from Cell Signaling Technology and Santa Cruz Biotechnology. Immunoreactive bands were detected with the horseradish peroxidase conjugated anti-rabbit antibody (Santa Cruz Biotechnology) and Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

Results

Antiproliferative activity of miR-125a toward hepatocarcinoma cells

Several studies attribute to miR-125a an anti-proliferative activity (for a review see Potenza and Russo, 2013). In breast cancer it counteracts cell proliferation by down-regulation of ERBB2, ERBB3 and/or HuR (Scott et al., 2007; Guo et al., 2009). In hepatocellular carcinoma (HCC) Hep3B and SNU-449 cells, ectopic expression of miR125a inhibits cell proliferation through targeting of SIRT7 and subsequent arrest of cell cycle in G1 phase (Kim et al., 2013). To gain new insights into this interesting activity, we transfected another HCC cell line, HuH-7, with miR-125a mimic or control miRNAs with unrelated sequences and cell proliferation was evaluated by MTT assay. In this system, the miRNA inhibited cell growth by 35 % (Fig. 1a). Ctrl-miRNA was selected as control for the subsequent experiments. FACS analyses of treated cells showed that miR-125a had no effects on apoptosis, autophagy and cellular senescence (data not shown). In contrast, it affected cell cycle by increasing the portion of cells in G1 phase (Fig. 1b). Further analyses by western blot then revealed that p21 and p27, two regulators of G1/S cell cycle progression, were up-regulated and SIRT7 was down-regulated (Fig. 1c). Overall, these data suggest that miR-125a counteracts HuH-7 cell proliferation by downregulating SIRT7 and subsequent p21- and p27-dependent cell cycle arrest in G1.

MicroRNA 125a mediates the antiproliferative activity of sorafenib

In a previous study we have showed that sorafenib inhibits proliferation of HuH-7 and HepG2 cells with IC₅₀ values of 5 and 6 μ M, respectively (Stiuso et al., 2015). We then considered the possibility that sorafenib may exert this activity by recruiting miR-125a. To test this hypothesis, the effect of sorafenib on miR-125a expression was evaluated. HepG2 and HuH-7 cells were treated with sorafenib at concentrations equal to their IC₅₀s and miR-125a expression was quantitated by

RT-qPCR. These analyses revealed an increased miR-125a expression, compared to the untreated cells, with an up-regulation at 72 h of 3.2- and 3.4-fold for HepG2 and HuH-7, respectively (Fig. 2).

Sorafenib treatment of HuH-7 cells was then combined with the modulation of miR-125a activity. Treated cells were transfected with either miR-125a mimic or miR-125a inhibitor and cell proliferation was evaluated. As shown in Fig. 3a, both sorafenib and the miRNA were able to inhibit cell growth but the combination of the two molecules didn't yield any additive effects. Conversely, the inhibition of miR-125a counteracted the antiproliferative activity of sorafenib (Fig. 3b). These data strongly suggest that miR-125a is a downstream effector in the antiproliferative pathway triggered by sorafenib.

Since miR-125a inhibitor antagonized the effect of sorafenib on cell growth, the mechanism underlying this activity was further investigated. First, cell cycle progression was examined using flow cytometry. As shown in Figure 1b, treatment with sorafenib induced a significant accumulation of cells in the G0/G1 and G2/M phases and a decrease of cells in S phase when compared to control; miR-125a mimic potentiated the effects of sorafenib by additionally increasing the cells in G2/M phase and reducing cell accumulation in S phase, while the inhibitor antagonized the G1/S block induced by sorafenib ($p < 0.05$).

p21 plays a key role as cell cycle checkpoint regulator by inhibiting cell cycle progression and blocking the transition from G1 to S phase (Xiong et al., 1993). Previous studies showed that sorafenib induced a significant p21 down-regulation in several human carcinoma cell lines and this downregulation occurred independently from RAF/MEK/ERK signalling pathway (Dasmahapatra et al., 2007; Inoue et al., 2011). Also in our experimental system, sorafenib significantly reduced the expression of p21 (Fig. 1c). However, when miR-125a mimic was combined with sorafenib, the reduction of p21 was largely abrogated whereas the inhibitor potentiated its downregulation. On the other hand, sorafenib increased p27 expression and this effect was enhanced by miR-125a mimic and antagonized by miR125a inhibitor. Overall, these data indicate that miR-125a contributes to

the antiproliferative pathway triggered by sorafenib because it attenuates the downregulation of p21 and enhances the upregulation of p27, thus potentiating cell cycle arrest.

Other oncogenic targets of miR-125a

MiR-125a is known to interfere with expression of ERBB2 and ERBB3 in human breast cancer cell lines (Scott et al., 2007); it also downregulates SIRT7, MMP11 and VEGF-A in hepatocellular carcinoma cell lines (Bi et al., 2012; Kim et al., 2013); finally, a recent report shows its ability to suppress the expression of Zbtb7a proto-oncogene in lung cancer cells (Hojo et al., 2015). We then tested the ability of miR-125a to downregulate these targets in our experimental system. HuH-7 cells were transfected with miR-125a mimic and the expression of target genes was evaluated by RT-qPCR. This analysis showed that expression of MMP11, Zbtb7a and SIRT7 was reduced by 31-39%, whereas ERBB2 and ERBB3 and VEGF-A were unchanged (Fig. 4). We then used MiRanda and Targetscan to predict other potential targets of miR-125a and were intrigued by the finding that both programs indicated c-Raf as a possible new target gene. Its expression in HuH-7 cells was then evaluated following transfection of the miRNA mimic revealing a 32% inhibitory effect (Fig. 4). To rule out the possibility that the effect of miR-125a on c-Raf expression was indirect, we validated the miRNA/mRNA interaction by a luciferase reporter assay. The genomic DNA segment of c-Raf containing the putative binding site for miR-125a (Fig. 5A) was cloned in psiCheck-2 vector within the 3-UTR region of the *Renilla* luciferase gene. Following transfection of the construct in HuH-7, luciferase activity was reduced by the endogenous miRNA by 33% (Fig. 5B). Transfection of miR-125a mimic further increased inhibition to 75%. Similar results were obtained with HepG2 cells. Overall, these results suggest that c-Raf is a direct target of miR-125a in hepatocarcinoma cell lines and that its downregulation may contribute to the antiproliferative activity of sorafenib.

Discussion

HCC is among the most common malignancies worldwide, with more than 500,000 new cases diagnosed each year (Parkin et al., 2005). Sorafenib is used to treat HCC patients with an advanced stage of disease or with malignant progression after locoregional therapy (Llovet et al., 2008; Cheng et al., 2009). Sorafenib is also effective against a variety of other tumors such as renal cell carcinoma, breast cancer, colon cancer, non-small cell lung cancer, and melanoma (Wilhelm et al., 2008) and there are over 400 clinical trials involving this drug (<http://www.clinicaltrials.gov>). Sorafenib is known to inhibit the activity of several cellular kinases: the serine/threonine kinases c-Raf (Raf-1) and B-Raf; platelet derived growth factor receptors; VEGF receptors; the cytokine receptor c-KIT; the receptor tyrosine kinases Flt-3 and RET; the Janus kinase/signal transducer and activator of transcription (JAK/STAT); the mitogen-activated protein kinases MEK and ERK (de La Coste et al., 1998; Hwang et al., 2004; Avila et al., 2006; Carlomagno et al., 2006; Wilhelm et al., 2008). Recent data also indicate that sorafenib induces changes in miRNA expression profiles (Bai et al., 2009; Shimizu et al., 2010; Lv et al., 2015). Considering a potential involvement of miRNAs in the antiproliferative activity of sorafenib, we focused our attention on miR-125a. This miRNA is implicated in the hepatocyte/hepatitis B virus interaction, with hepatitis B being one of the most important risk factors for HCC development (Potenza et al., 2011; Coppola et al., 2013; Mosca et al., 2014; Federico et al., 2005); miR-125a has functional targets with oncogenic potential in HCC and is frequently down-regulated in HCC tissues (Bi et al., 2012; Kim et al., 2013; Potenza and Russo, 2013). In addition, in breast cancer miR-125a plays an antitumor role, and the same activity has been suggested for gastric, lung and ovarian cancers (Scott et al., 2007; Nam et al., 2008; Guo et al., 2009; Li et al., 2009; Wang et al., 2009; Nishida et al., 2011).

In this study, we first inquired the antiproliferative activity of miR-125a toward HuH-7 cells (Fig. 1) and found that the miRNA counteracts cell proliferation by downregulation of SIRT7 and subsequent p21- and p27-dependent cell cycle arrest in G1, as already reported for other HCC cell lines (Bi et al., 2012; Kim et al., 2013). Then, the effect of sorafenib on miR-125a expression was

evaluated, revealing a kinetic of accumulation of the miRNA from 24h to 72h of treatment with the drug (Fig. 2). Later, the involvement of miR-125a in the antiproliferative activity of sorafenib was verified by modulating its expression in combination with sorafenib treatment. The data revealed that intracellular delivery of miR-125a had no additive effect on the antiproliferative activity of sorafenib, whereas a miR-125a inhibitor could counteract it (Fig. 3).

To better understand the mechanisms underlying this effect, cell cycle analysis was performed. As shown in Figure 1b, sorafenib blocked G1/S transition; miR-125a mimic potentiated this effect whereas the inhibitor antagonized it. Moreover, miR-125a mimic partially prevented the down-regulation of p21 induced by sorafenib while the inhibitor additionally downregulated it in combination with the drug. On the other hand, sorafenib increased p27 expression and this effect was potentiated by miR-125a mimic and antagonized by miR125a inhibitor. The effects on p21 expression were related to SIRT7 levels whereby SIRT7 inhibits transcriptional activation of p21(WAF1/Cip1); in fact, sorafenib increased SIRT7 expression, while miRNA attenuated the up-regulation of SIRT7 induced by sorafenib. SIRT7 is a direct target of miR-125a; in our case, mimic reduced SIRT7 expression, as expected but miR-125a inhibitor did not increased its expression probably because these cells express low endogenous miRNA levels and correspondingly high target gene expression so the effect of the inhibitor on target gene expression are strictly related to endogenous miRNA levels. However, these results show that miR-125a contributes to the antiproliferative pathway triggered by sorafenib because it attenuates the downregulation of p21 expression and enhances p27 upregulation induced by sorafenib by potentiating cell cycle arrest.

In previous studies it has been shown that sorafenib was able to block cancer cells in G1 phase of cell cycle (Guan and He, 2011) and cell cycle arrest occurred in parallel with the inactivation of p-MEK and p-ERK and cyclin D1 down regulation. In fact, we found that sorafenib inhibited pERK1/2 activation and this effect was abrogated by miR-125 inhibitor (data not shown). On the other hand, it has been reported that sorafenib decreased p21 levels in several cancer cells (Huynh et al., 2009; Inoue et al., 2011; Dasmapathra et al., 2007). Since p21 is a potent inhibitor of

cyclin-dependent kinases that acts by inhibiting cell cycle progression and blocking the transition from G1 to S phase (Liu and Lozano, 2005), miR125-a likely enhances the antitumor effects of sorafenib by attenuating p21 downregulation that was induced by sorafenib. Increased p21 levels could inhibit cell growth by inducing cell cycle arrest while miR-125a inhibitor could antagonize these effects by additionally reducing p21 levels and activating Raf/MEK/ERK pathway.

We finally found that miR-125a interferes with the expression of MMP11, Zbtb7A proto-oncogene and c-Raf, possibly contributing to the antiproliferative activity of sorafenib. Since sorafenib is a specific c-RAF inhibitor (Ambrosini et al., 2008), and miR125a induced upregulation of cell cycle inhibitor p21^{eip1/waf1}-and reduced c-Raf expression, the dual inhibition of cell cycle progression and c-Raf activation coherently combine to explain the antiproliferative activity of the drug.

Overall, this study contributes to the understanding of the mechanism of action of sorafenib, an antitumor drug for treatment of advanced hepatocellular carcinoma, highlighting a miRNA-based pathway that supports its antiproliferative activity. This knowledge may also be useful for the development of new therapeutic strategies for HCC based on other treatments able to boost the cellular reservoir of miR-125a.

References

- Ambros V. (2004). The functions of animal microRNAs. *Nature* 431:350-355.
- Ambrosini G, HS Cheema, S Seelman, A Teed, EB Sambol, S Singer and GK Schwartz. (2008). Sorafenib inhibits growth and mitogen-activated protein kinase signaling in malignant peripheral nerve sheath cells. *Mol Cancer Ther* 7(4):890-896.
- Avila MA, C Berasain, B Sangro and J Prieto. (2006). New therapies for hepatocellular carcinoma. *Oncogene* 25(27):3866-3884.
- Bai S, MW Nasser, B Wang, SH Hsu, J Datta, H Kutay, A Yadav, G Nuovo, P Kumar and K Ghoshal. (2009). MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem* 284:32015-32027.
- Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-297.
- Bi Q, S Tang, L Xia, R Du, R Fan, L Gao, J Jin, S Liang, Z Chen, G Xu, Y Nie, K Wu, J Liu, Y Shi, J Ding and D Fan. (2012). Ectopic expression of MiR-125a inhibits the proliferation and metastasis of hepatocellular carcinoma by targeting MMP11 and VEGF. *PLoS One* 7(6):e40169.
- Carlomagno F, S Anaganti, T Guida, G Salvatore, G Troncone, SM Wilhelm, M Santoro. (2006). BAY 43-9006 inhibition of oncogenic RET mutants. *J Natl Cancer Inst* 98(5):326-334
- Calin GA and CM Croce CM. (2006) MicroRNA signatures in human cancers. *Nat Rev Cancer* 6: 857-866.
- Cheng AL, YK Kang, Z Chen, CJ Tsao, S Qin, JS Kim, R Luo, J Feng, S Ye, TS Yang, J Xu, Y Sun, H Liang, J Liu, J Wang, WY Tak, H Pan, K Burock, J Zou, D Voliotis and Z Guan. (2009). 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* 10:25-34.
- Coppola N, Potenza N, M Pisaturo, N Mosca, G Tonziello, G Signoriello, V Messina, C Sagnelli, A Russo and E Sagnelli. (2013). Liver microRNA hsa-miR-125a-5p in HBV chronic infection: correlation with HBV replication and disease progression. *PLoS One* 8(7):e65336.

- Dasmahapatra G, N Yerram, Y Dai, P Dent and S Grant. (2007). Synergistic interactions between vorinostat and sorafenib in chronic myelogenous leukemia cells involve Mcl-1 and p21CIP1 down-regulation. *Clin Cancer Res* 13(13):4280–4290.
- de La Coste A, B Romagnolo, P Billuart, CA Renard, MA Buendia, O Soubrane, M Fabre, J Chelly, C Beldjord, A Kahn and C Perret. (1998). Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc Natl Acad Sci USA* 95(15):8847-8851.
- Di Leva G, M Garofalo and CM Croce. (2014). MicroRNAs in cancer. *Annu Rev Pathol* 9:287-314.
- Esposito F, Boscia F, Gigantino V, Tornincasa M, Fusco A, Franco R, Chieffi P. (2012). The high-mobility group A1-estrogen receptor β nuclear interaction is impaired in human testicular seminomas. *J Cell Physiol*. 12:3749-55.
- Esquela-Kerscher A and FJ Slack. (2006). Oncomirs-microRNA with a role in cancer. *Nat Rev Cancer* 6:259-269.
- Federico A, Tuccillo C, Terracciano F, D'Alessio C, Galdiero M, Finamore E, D'Isanto M, Peluso L, Del Vecchio Blanco C, Loguercio C. (2005). Heat shock protein 27 expression in patients with chronic liver damage. *Immunobiology*. 10:729-35.
- Grammatikakis I, M Gorospe and K Abdelmohse. (2013). Modulation of Cancer Traits by Tumor Suppressor microRNAs. *Int J Mol Sci*. 14:1822-1842.
- Guan YS and Q He. (2011). Sorafenib: activity and clinical application in patients with hepatocellular carcinoma. *Expert Opin Pharmacother*. 12:303-313.
- Guo X, Y Wu and RS Hartley. (2009). MicroRNA-125a represses cell growth targeting HuR in breast cancer. *RNA Biol* 6:575-583.
- Hojo N, N Tatsumi, N Moriguchi, A Matsumura, S Morimoto, J Nakata, F Fujiki, S Nishida, H Nakajima, A Tsuboi, Y Oka, N Hosen, S Hayashi, H Sugiyama and Y Oji. (2015). A Zbtb7a proto-oncogene as a novel target for miR-125a. *Mol Carcinog*. doi: 10.1002/mc.22446.

- Huynh H, JW Lee, PK Chow, VC Ngo, GB Lew, IW Lam, HS Ong, A Chung and KC Soo. (2009). Sorafenib induces growth suppression in mouse models of gastrointestinal stromal tumor. *Mol Cancer Ther* 8(1):152-159.
- Hwang YH, JY Choi, S Kim, ES Chung, T Kim, SS Koh, B Lee, SH Bae, J Kim and YM Park. (2004). Over-expression of c-raf-1 proto-oncogene in liver cirrhosis and hepatocellular carcinoma. *Hepatol Res* 29(2):113-121.
- Inoue H, SH Hwang, AT Wecksler, BD Hammock and RH Weiss. (2011). Sorafenib attenuates p21 in kidney cancer cells and augments cell death in combination with DNA-damaging chemotherapy. *Cancer Biol Ther* 12(9):827–836.
- Kawase-Koga Y, G Otaegi and T Sun (2009). Different timings of Dicer deletion affect neurogenesis and gliogenesis in the developing mouse central nervous system. *Dev Dyn.* 238: 2800-2812
- Kim JK, JH Noh, KH Jung, JW Eun, HJ Bae, MG Kim, YG Chang, Q Shen, WS Park, JY Lee, J Borlak and SW Nam. (2013). Sirtuin-7 oncogenic potential in human hepatocellular carcinoma and its regulation by the tumor suppressors MiR-125a-5p and MiR-125b. *Hepatology* 57:1055-1067.
- Kitagawa N, H Ojima, T Shirakihara, H Shimizu, A Kokubu, T Urushidate, Y Totoki, T Kosuge, S Miyagawa and T Shibata (2013). Downregulation of the microRNA biogenesis components and its association with poor prognosis in hepatocellular carcinoma. *Cancer Sci* 104: 543-551
- Li W, R Duan, F Kooy, SL Sherman, W Zhou and P Jin. (2009). Germline mutation of microRNA-125a is associated with breast cancer. *J Med Genet* 46:358-360.
- Liu G and G Lozano. (2005). p21 stability: linking chaperones to a cell cycle checkpoint. *Cancer Cell* 7(2):113–114.
- Liu L, Y Cao, C Chen, X Zhang, A McNabola, D Wilkie, S Wilhelm, M Lynch and C Carter. (2006). Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces

tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 66:11851-11858.

Llovet JM, S Ricci , V Mazzaferro , P Hilgard, E Gane, JF Blanc, AC de Oliveira, A Santoro, et al. (2008). SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 359:378-390.

Lv JF, L Hu, W Zhuo, CM Zhang, HH Zhou and L Fan. (2015). Epigenetic alterations and cancer chemotherapy response. *Cancer Chemother Pharmacol*. doi: 10.1007/s00280-015-2951-0

Marra L, Cantile M, Scognamiglio G, Perdonà S, La Mantia E, Cerrone M, Gigantino V, Cillo C, Caraglia M, Pignata S, Facchini G, Botti G, Chieffi S, Chieffi P, Franco R. (2013). Deregulation of HOX B13 expression in urinary bladder cancer progression. *Curr Med Chem*. 6:833-9.

Mosca N, F Castiello, N Coppola, MC Trotta, C Sagnelli, M Pisaturo, E Sagnelli, A Russo and N Potenza. (2014). Functional interplay between hepatitis B virus X protein and human miR-125a in HBV infection. *Biochem Biophys Res Commun* 449:141-145.

Nam EJ, H Yoon, SW Kim , H Kim , YT Kim , JH Kim, JW Kim and S Kim. (2008). MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* 14:2690–2695.

Negrini M, L Gramantieri, S Sabbioni and CM Croce. (2011). microRNA involvement in hepatocellular carcinoma. *Anticancer Agents Med Chem*. 11:500-521.

Nishida N, K Mimori, M Fabbri, T Yokobori, T Sudo, F Tanaka, K Shibata, H Ishii, Y Doki and M Mori. (2011). MicroRNA-125a-5p is an independent prognostic factor in gastric cancer and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab. *Clin Cancer Res* 17(9):2725-2733.

O'Rourke JR, SA Georges, HR Seay, SJ Tapscott, MT McManus, DJ Goldhamer, MS Swanson and BD Harfe BD (2007). Essential role for Dicer during skeletal muscle development. *Dev Biol*. 2007: 359–368

- Parkin DM, F Bray, J Ferlay and P Pisani. (2005). Global cancer statistics, 2002. *CA Cancer J. Clin* 55:74-108.
- Peveling-Oberhag J, C Döring, S Hartmann, N Filmann, A Mertens, A Piiper, E Herrmann, ML Hansmann, S Zeuzem, J Trojan and MW Welker. (2015). Feasibility of global miRNA analysis from fine-needle biopsy FFPE material in patients with hepatocellular carcinoma treated with sorafenib. *Clin Sci (Lond)* 128:29-37.
- Potenza N and A Russo. (2013). Biogenesis, evolution and functional targets of microRNA-125a. *Mol Genet Genomics*. 288:381-389.
- Potenza N, Papa U, Mosca N, Zerbini F, Nobile V, Russo A (2011) Human microRNA hsa-miR-125a-5p interferes with expression of hepatitis B virus surface antigen. *Nucleic Acids Res* 39: 5157-5163.
- Potenza N, U Papa and A Russo A (2009). Differential expression of Dicer and Argonaute genes during the differentiation of human neuroblastoma cells. *Cell Biol Int*. 33: 734-738
- Rupaimoole R, SY Wu, S Pradeep, C Ivan, CV Pecot, KM Gharpure, AS Nagaraja, GN Armaiz-Pena, M McGuire, B Zand, HJ Dalton, J Filant, JB Miller, C Lu, NC Sadaoui, LS Mangala, M Taylor, T van den Beucken, E Koch, C Rodriguez-Aguayo, L Huang, M Bar-Eli, BG Wouters, M Radovich, M Ivan, GA Calin, W Zhang, G Lopez-Berestein and AK Sood (2014). Hypoxia-mediated downregulation of miRNA biogenesis promotes tumour progression. *Nat Commun* 5: 5202
- Schwartz M, S Roayaie and M Konstadoulakis. (2007). Strategies for the management of hepatocellular carcinoma. *Nat Clin Pract Oncol* 4:424-432.
- Scott GK, A Goga, D Bhaumik, CE Berger, CS Sullivan and CC Benz. (2007). Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. *J Biol Chem* 282:1479-1486.
- Shimizu S, T Takehara, H Hikita, T Kodama, T Miyagi, A Hosui, T Tatsumi, H Ishida, T Noda, H Nagano, Y Doki, M Mori and N Hayashi. (2010). The let-7 family of microRNAs inhibits Bcl-

xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. *J Hepatol* 52:698-704.

Stiuso P, N Potenza, A Lombardi, I Ferrandino, A Monaco, S Zappavigna, D Vanacore, N Mosca, F Castiello, S Porto, R Addeo, SD Prete, F De Vita, A Russo and M Caraglia. (2015). MicroRNA-423-5p Promotes Autophagy in Cancer Cells and Is Increased in Serum From Hepatocarcinoma Patients Treated With Sorafenib. *Mol Ther Nucleic Acids* 4:e233. doi: 10.1038/mtna.2015.8

Vitiello M, D'Isanto M, Finamore E, Ciarcia R, Kampanaraki A, Galdiero M. (2008). Role of mitogen-activated protein kinases in the iNOS production and cytokine secretion by *Salmonella enterica* serovar Typhimurium porins. *Cytokine*. 3:279-85.

Wang G, W Mao, S Zheng and J Ye. (2009). Epidermal growth factor receptor-regulated miR-125a-5p-a metastatic inhibitor of lung cancer. *FEBS J* 276:5571–5578.

Wienholds E and RH Plasterk. (2005). MicroRNA function in animal development. *FEBS Lett* 579:5911-5922.

Wilhelm SM, L Adnane, P Newell, A Villanueva, JM Llovet and Lynch M. (2008). Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* 7(10):3129-3140 .

Wilhelm S, C Carter , M Lynch, T Lowinger, J Dumas , RA Smith, B Schwartz, R Simantov and S Kelley. (2006). Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* 5:835-844.

Xiong Y, GJ Hannon, H Zhang, D Casso, R Kobayashi and D Beach. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* 366:701–704.

Zhou C, J Liu , Y Li, L Liu , X Zhang, CY Ma, SC Hua, M Yang and Q Yuan. (2011). microRNA-1274a, a modulator of sorafenib induced a disintegrin and metalloproteinase 9 (ADAM9) down-regulation in hepatocellular carcinoma. *FEBS Lett* 585:1828-1834.

Figure legends

Fig. 1. Antiproliferative activity of miR-125a. HuH-7 cells were transfected with miR-125a mimic, miR-125a inhibitor, or their control RNA molecules with unrelated sequences. After 72 hours, (a) cell growth was estimated by MTT assay, (b) cell cycle phase distribution was defined by FACS analysis, and (c) expression of p21, p27, and SIRT7 was evaluated by western blot (c). **p<0.01.

Fig. 2. Up-regulation of miR-125a by sorafenib. HepG2 (a) and HuH-7 (b) cells were treated with sorafenib and miR-125a expression was evaluated by RT-qPCR at the indicated times (solid bars) in comparison with untreated controls (empty bars). Probability values at Student's t-test were *p<0.05, **p<0.01, ***p<0.001.

Fig. 3. Effect of miR-125a on the antiproliferative activity of sorafenib. HuH-7 cells were transfected with miR-125a mimic (a) or miR-125a inhibitor (b) in the presence (solid bars) or absence (empty bars) of sorafenib. After 72 hours, cell growth was evaluated by MTT assay. Probability values at Student's t-test were *p<0.05, **p<0.01, ***p<0.001.

Fig. 4. Functional targets of miR-125a. HuH-7 cells were transfected with 50 nM miR-125a mimic and the expression of its targets was evaluated after 48 h by RT-qPCR.

Fig. 5. Validation of c-Raf as a direct target of miR-125a. a) Predicted base-pairing between miR-125a and the 3'-UTR of c-Raf mRNA. b) Cultured hepatocellular carcinoma cells were transfected with the luciferase reporter plasmid psiCheck-2 containing the c-Raf gene 3'-UTR segment harboring the putative miR-125a binding site (WT) or a control DNA with inverted sequence (I). Luciferase activity was measured 48 h after transfection.

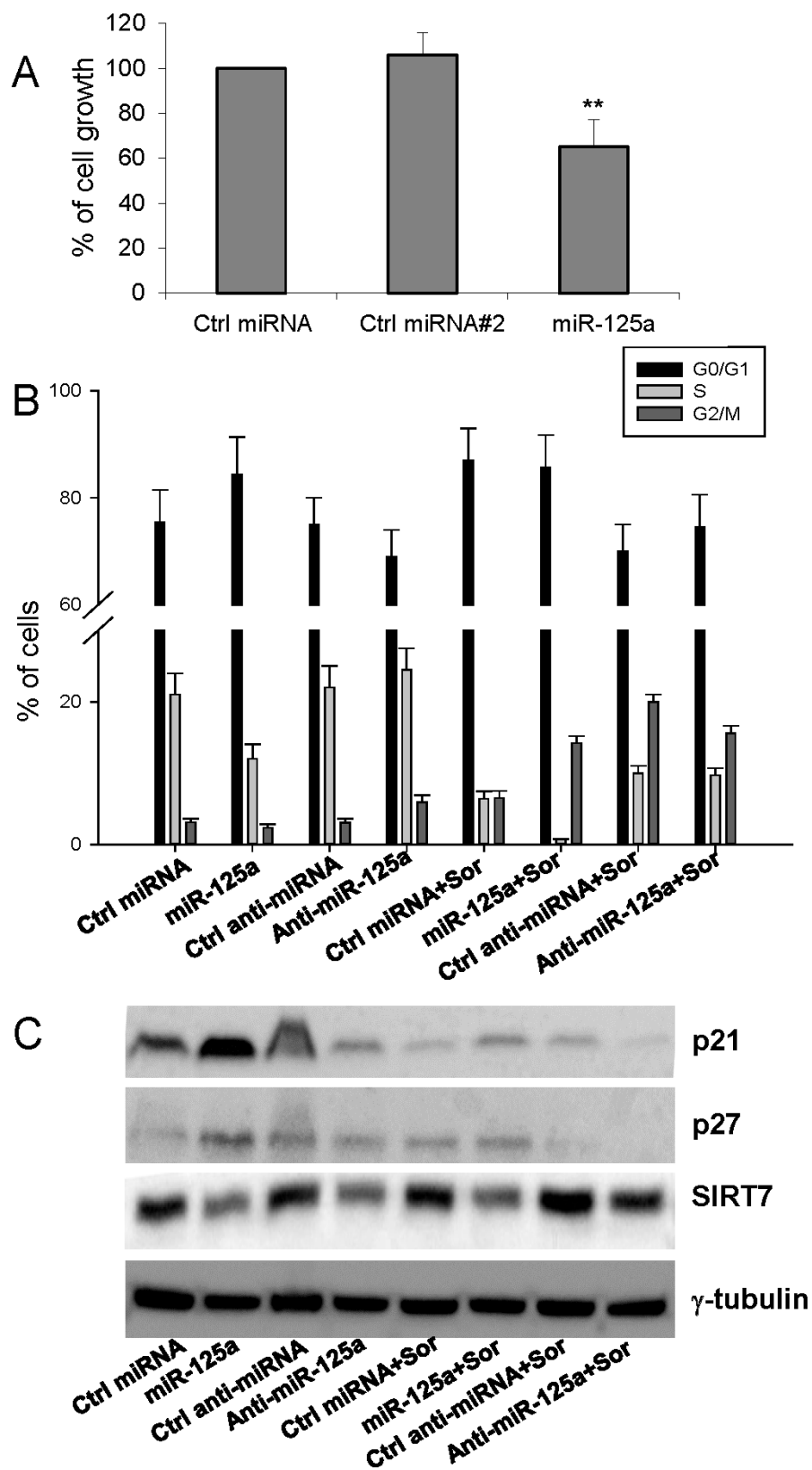


Figure 1

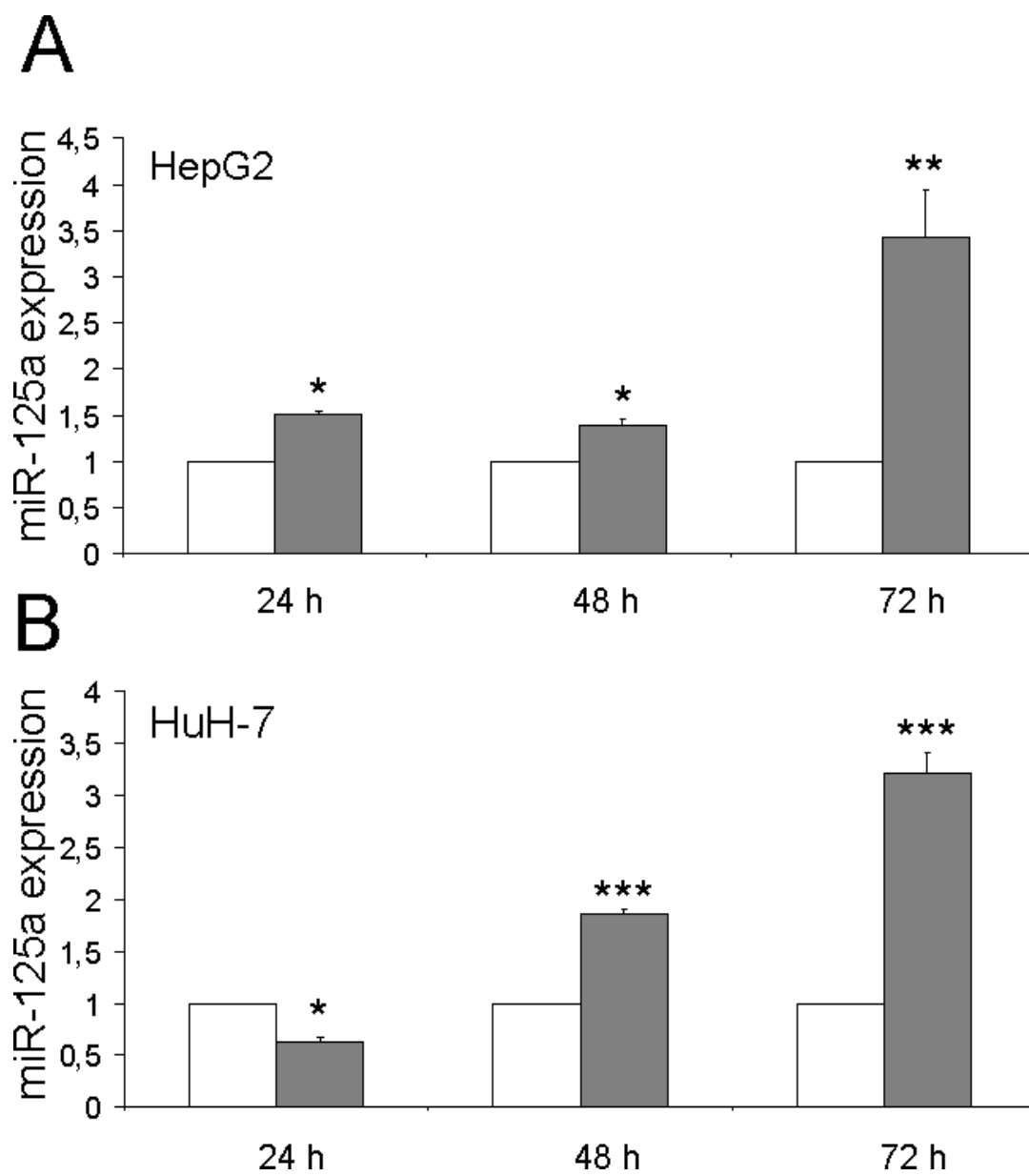


Figure 2

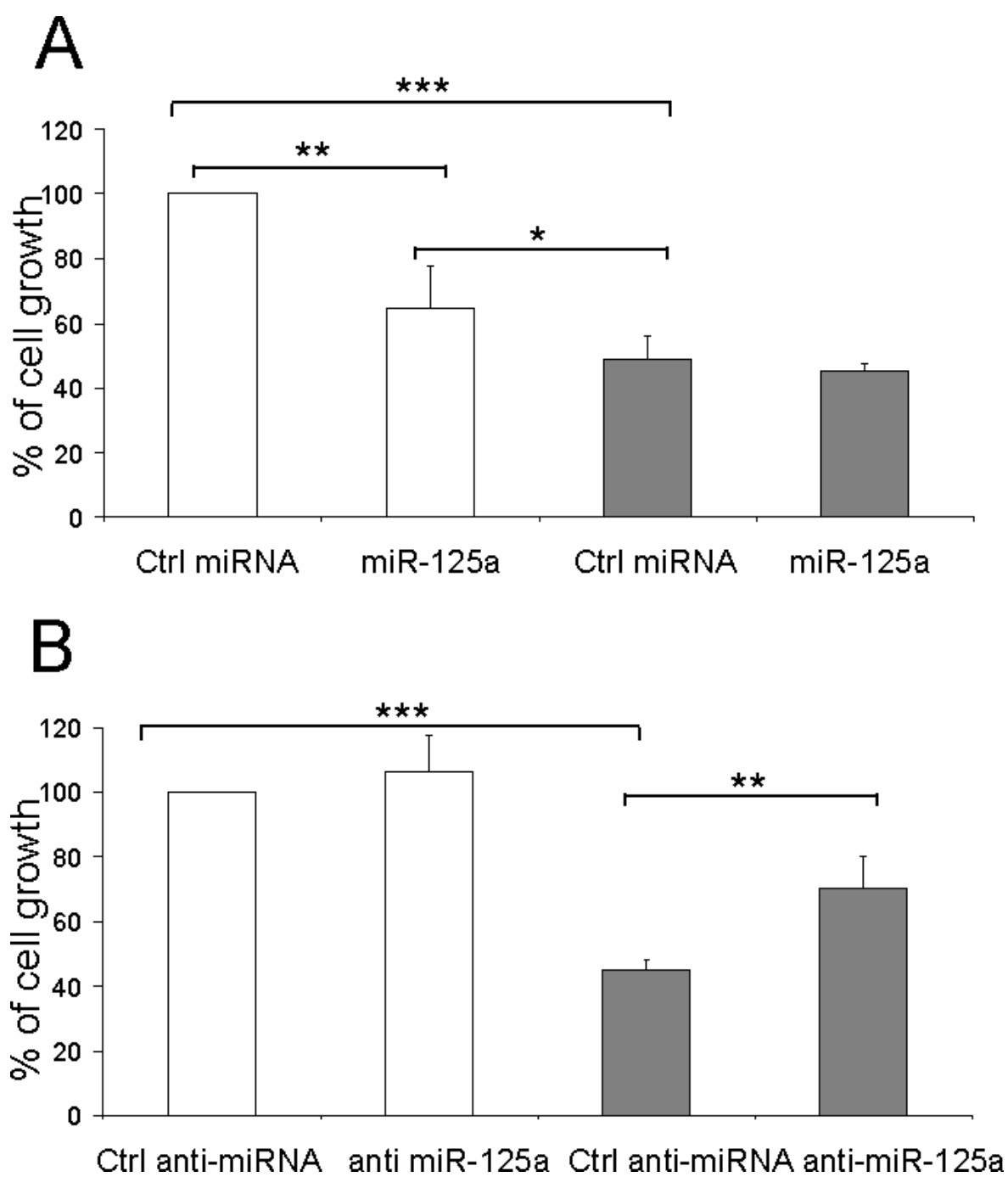


Figure 3

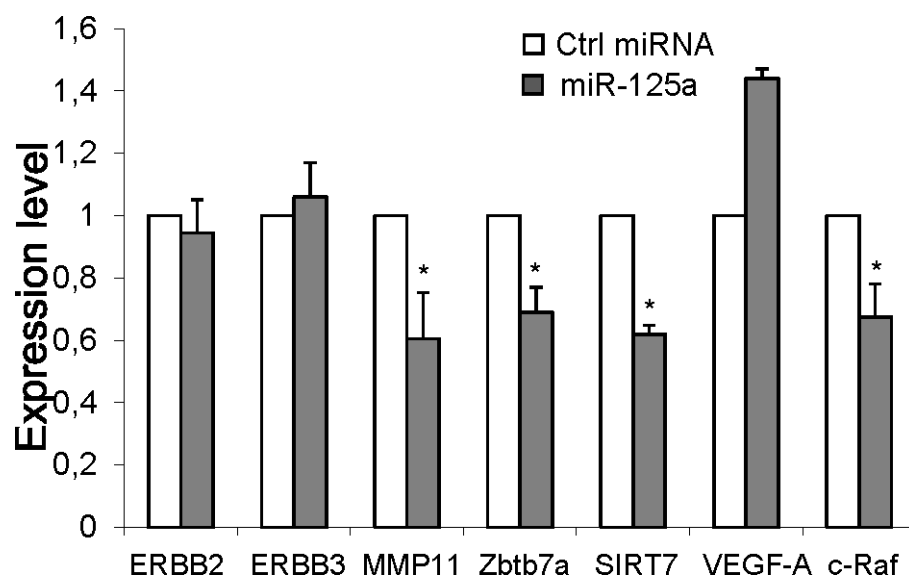


Figure 4

A

Position 625-649 of c-Raf 3'UTR 5' UCCGCA--UCU-CA-GCCCUCUCAGGGA 3'
 | | : | | | | | | | | | |
 miR-125a 3' A-GUGUCCA-AUUUCC--CAGAGUCCCU 5'

B

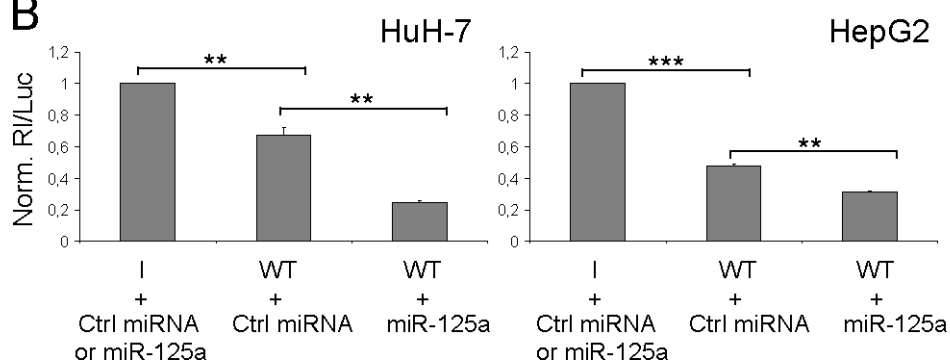


Figure 5