RESEARCH ARTICLE
ONCOLOGY

Diseases & Research

Determination of mRNA Targets of miR-376c within the RNA-Induced Silencing Complex

Ines Stevic[1], Heidi Schwarzenbach[1,#]

[1] Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg 20246, Germany

Background: The focus of the present study was to establish an easy combined method based on the MirTrap system together with a mRNA array to efficiently define target mRNAs which can be bound by miR-376 within the RNA-induced silencing complex (RISC) as well as to deduce the biological relevance of this miRNA in modulating cancer-associated signaling pathways. The importance of this study is to analyze miR-376/mRNA pairs which are imbedded in RISC, thus in their natural environment using the MirTrap system. The subsequent application of a mRNA array, instead to use the elaborate next generation sequencing approach, eases the identification of mRNA targets that can be bound by a miRNA within RISC.
Methods: MRNA target screening was carried out by the MirTrap system. The breast cancer cell line MDA-MB-468 was transfected with plasmids encoding for miR-376c and RISC. MiR-376c bound to mRNAs and integrated in RISC were immuno-precipitated with an antibody against RISC and identified by a mRNA array containing 89 different mRNAs. Western blot were performed to verify the repression of these target mRNAs by miR-376c.
Results: We found 56 potential mRNA targets for miR-376c within RISC. Particularly strong binding activities of miR-376c were to the matrix metalloproteinase MMP9, the apoptosis inhibitor BCL2 and the cell cycle inhibitor CDKN1A/p21. Western blotting showed a slight and strong repression of protein expression of BCL2 and p21, respectively. The different strength of protein repression is possibly owing to the overexpression of RISC.
Conclusion: Our findings show a variety of potential target mRNAs for miR-376c within RISC with different binding activities. MiR-376c is able to bind tumor suppressive and oncogenic mRNAs.
Keywords: Breast cancer; MirTrap system; microRNAs; mRNA targets; RNA-induced silencing complex.

Introduction

MicroRNAs (miRNAs) belong to the family of small non-coding RNAs and are about 20 nucleotides long. They influence the translation of numerous mRNAs, and consequently participate in a variety of signaling pathways. Their main function is the complementary binding to the 3′- or 5′-untranslated region (3′ or 5′ UTR) of their target mRNA, leading to degradation of their target mRNA or inhibition of the translation of the mRNA into protein[1]. This RNA interference occurs in the RNA-induced silencing complex (RISC)[2]. Since miRNAs have binding affinity to hundreds of mRNAs, they exhibit complex functions that regulate a broad aspect of cellular processes[3,4]. Once miRNAs are secreted by cells, they are either integrated in exosomes and/or are associated with proteins, such as AGO2, which protect them from RNase degradation. The released miRNAs can then be transported by exosomes from cell to cell where they can exert their inhibitory influence on protein expression in the recipient cells or circulate as cell-/exosome-free molecule in the bloodstream[4,5].

In our recent study, we detected that miR-376c was specifically enriched in exosomes of triple-negative breast cancer patients, but not in exosomes of human epidermal growth factor receptor 2 (HER2)-positive patients, suggesting that its oncogenic traits may be disseminated across a subtype of breast cancer cells. Moreover, after neoadjuvant therapy, the elevated concentrations of exosomal miRNA-376c were downregulated to normal levels indicating that changes in its levels may reflect cancer status[6]. These findings prompted us for looking for target mRNAs of miRNA-376c that possibly contribute to the pathogenesis of cancer. Identification of mRNA target molecules for miRNAs is therefore important to understand the biological role of miRNAs in modulating cancer-associated signaling pathways. In addition, the knowledge of mRNA target molecules allows developing miRNA therapeutics and assessing their impact on the different cell processes.

To identify the mRNA targets for miR-376c, we used the MirTrap system which imbeds the miRNA/mRNA pair into RISC which can then be pulled down using an antibody against this complex. Applying an mRNA array, we identified 56 mRNAs that are possibly bound by miR-376c within RISC. The mRNAs mostly enriched within RISC were matrix metallopeptidase 9 (MMP9) and the apoptosis inhibitor BCL2.

Materials and Methods

Cell culture

The MDA-MB-468 cell line was obtained from ATCC (American Type Culture Collection) and authenticated by the Leibniz In-

Correspondence: Heidi Schwarzenbach. E-mail: hschwarzenbach@me.com. Address: Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistraße 52, Hamburg 20246, Germany.
Received 3 April 2023; Revised 23 May 2023; Accepted 2 June 2023; Available Online 3 July 2023
DOI: 10.54457/DR.202302002
© The Author(s) 2023. This is an open access article under the CC BY 4.0 license (https://creativecommons.org/licenses/by/4.0/).

stitute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), Braunschweig, on 30/07/2013 and 14/06/2013, respectively. Single aliquots were frozen in a liquid nitrogen tank and a new one of this cell line was used for this study. The cells were cultured in DMEM supplemented with 10% FCS (fetal calf serum; PAA, Laboratories, Cölbe, Germany), under standard conditions (37 °C, 5% CO_2, humidified atmosphere), and regularly tested for mycoplasma contamination (Minerva Biolabs, Berlin, Germany).

Design of miR-376c

Synthetic miR-376c oligos were designed with 3' overhangs, a non-complementary nucleotide near the 3' end to ease the incorporation into RISC, and phosphorylated 5' ends (Sigma-Aldrich, Munich). Both, 5' and 3' oligo strands were solved in resuspension buffer containing 20 nM NaCl, 10 mM Tris pH 8.0 to a final concentration of 200 µM. Hundred µM hybridized oligos was incubated on a peqSTAR 96 Universal Gradient (Isogen Life science, Utrecht, Netherlands) under following conditions: 95 °C for 2 min, 10 min slope to 37 °C, 37 °C for 60 min, 5 min slope to 25 °C. Annealing of the oligos was confirmed on a 3% agarose gel (Thermo Fisher Scientific, Massachusetts).

Transfection

The MirTrap system (Takara Clonetech, Shiga, Japan) was performed following the company's protocol. Briefly, transfection of MDA-MB-468 cells was carried out with a miR-132 (control) or the designed miR-376c, a pMirTrap Control and a pMirTrap vector. Tube 1 was prepared with 15 µl (500 pmoles) miR-132 or miR-376c, 60 µl (30 µg) pMirTrap vector, 20 µl (10 µg) pMirTrap control vector and 515 µl Xfect reaction buffer, and tube 2 with 12 µl Xfect polymer and 528 µl Xfect reaction buffer. Sixty µl Xfect miRNA transfection polymer was added to the tubes. Both tubes were mixed and incubated for 20 min, to allow formation of nanoparticles complex. MDA-MB-468 cells were transfected with miR-132 or miR-376c and incubated at 37 °C for 24 hours. The cells were lysed with 1 ml cold lysis/wash buffer and incubated on ice for 10 min. Then, lysates were incubated on ice for 10 min and centrifuged at 250 g for 30 min at 4 °C. Fifty µl of supernatant was stored at −80 °C till the time of RNA isolation and labelled with "Before IP".

Immunoprecipitation

Forty µl of immunoprecipitation anti-DYKDDDDK beads from the MirTrap system (Takara, Clonetech) were washed twice with 500 µl lysis/wash buffer containing protease inhibitor cocktail, 10 µl 1 M DTT and 25 µl RNase inhibitor. After centrifugation at 100 x g for 1 min, the bead pellet was incubated with 350 µl lysis/wash buffer, 50 µl 10 mg/ml tRNA solution and 50 µl 10 mg/ml BSA solution for 3 hours at 4 °C and washed twice with 500 µl lysis/wash Buffer. After 2-hour incubation at 4 °C, the beads bound to RISC complex and the tube was labelled with "After IP".

RNA extraction

The Nucleo spin RNA XS kit (Macherey-Nagel, Düren, Germany) was carried out according to the manufacturer's instructions. Briefly, 150 µl lysis buffer and 3 µl TCEP were added to the samples "Before IP", while 200 µl lysis buffer and 4 µl TCEP were added to samples "After IP". After incubation for 2 min and centrifugation at 13,400 g, the supernatants were precipitated with 5 µl proteinase K and 200 µl 70% ethanol on NucleoSpin RNA columns. The columns were washed with 100 µl MDB, supplemented with 25 µl rDNase reaction mix and incubated at room temperature for 15 min. After washing with buffers RA2 and RA3, RNA was eluted in 10 µl RNase-free H_2O, and the RNA concentration was measured on a Qubit (Invitrogen by life technologies).

One Step SYBR RT PCR

The One Step SYBR RT PCR kit (Takara Clonetech) was used to examine the binding of the control miR-132 to its known mRNA targets. MiRNA PCR reactions were carried out for each of the three sets of primers AcGFP1 (known mRNA target-positive control), hPlod3 (mRNA target-negative control) and hGAPDH (internal control), and contained 12.5 µl One Step SYBR RT-PCR buffer 4, 21.0 µl PrimeScript 1 step enzyme mix, 1.0 µl (10 µM) PCR forward primer, 1.0 µl (10 µM) PCR reverse primer. Two µl diluted (1:10) samples "Before IP" and undiluted samples "After IP" were added to the PCR reaction which was carried out on a qPCR CFX96 device (BioRad, Munich, Germany) under following conditions: 1 cycle: 42 °C for 5 min, 95 °C for 10 s; 40 cycles: 95 °C for 5 s, 60 °C for 30 s.

Reverse transcription and mRNA array-based PCR

For identification of mRNA targets of miRNA-376c, the RT² Profiler™ PCR Array Human Breast Cancer kit containing 89 mRNAs (Qiagen, Hilden, Germany) was used. Isolated RNA from samples "Before IP" and "After IP" were reverse transcribed into cDNA using the RT PreAMP cDNA Synthesis kit (Qiagen). Briefly, 8µl RNA was mixed with 2 µl buffer GE and added to 10 µl reverse transcription mix. After incubation at 42 °C for 30 min, 5 µl cDNA mixed with 20 µl pre-amplification mix was pre-amplified on a qPCR CFX96 device (BioRad, Munich, Germany) under following conditions: 1 cycle: 95 °C for 10 min; 12 cycles: 95 °C for 5 s, 60 °C for 2 min. The pre-amplified 102 µl RNA was mixed with 1275 µl 2x RT2SYBR Green Mastermix and 1173ul RNase-free water and loaded to the array card. The real time PCR was carried out using RT2 Profiler PCR array on a qPCR CFX96 device (BioRad, Munich, Germany) and cycling conditions were: 1 cycle: 95 °C for 10 min; 40 cycles: 95 °C for 15 s, 60 °C for 1 min.

Evaluation of the data

In order to reveal whether the target mRNA (AcGFP1) is bound by miR-132 as well as the target mRNAs which are bound by miR-376c, the average ΔCt was calculated and normalized by GAPDH. The fold enrichment was determined by dividing the $2^{\Delta Ct}$ value of the sample "After IP" by the $2^{\Delta Ct}$ value of the sample "Before IP" using MS Excel.

Western blot

The MDA-MB-468 cell line was seeded in a 6-well plate with 200,000 cells per well and transfected the next day. MiR-376c

mimics, AllStars negative control siRNA, miR-376c inhibitors and miScript inhibitor negative control (Qiagen, Hilden, Germany) were dispensed in serum-free culture medium to a final concentration of 15 nM for mimics and 75 nM for inhibitors per well. For transfection, 0.75 µl or 12 µl of HiPerFect transfection reagent (Qiagen) was added to the wells. A mock control and untransfected cells were also included in each analysis. Cells were harvested 48 hours after transfection and lysed in RIPA buffer (Merck Darmstadt, Germany). In order to estimate the enough protein amounts for performing a Western blot, the protein concentrations were measured using the DC Protein Assay Kit (BioRad, Munich, Germany) at a wavelength of 650 nm on a spectrophotometric plate reader (Tecan, Mannedorf Switzerland). A standard curve of 0, 0.625, 1.25, 2.5, 5 and 10 mg/ml BSA (bovine serum albumin; Sigma Aldrich Chemie, Munich, Germany) was excuted by the double-dilution method. Twenty-five µl lysed cells and BSA (Sigma Aldrich Chemie) standard protein samples, all solved in RIPA buffer (Merck), were added to 96-well plates according to the manufacturer's instructions. The protein concentrations were calculated using a linear equation of y = mx + n and applying the linear regression method. Thirty µg of proteins were electrophoretically separated and blotted onto a PVDF membrane (Millipore, Billerica, MA, USA) which was subsequently incubated with antibodies specific for BCL2 (Cell signaling, Danvers, MA, USA), Ki67 (LS Bio Seattle, Seattle, WA, USA), E-cadherin (CDH1; BD Biosciences, Franklin Lakes, NJ, USA) and p21 (CDKN1A, Cell signaling) overnight. Discovery of the specific proteins was carried out using a peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) and a chemiluminescence ECL detection solution (Sigma-Aldrich, St.Louis, MO, USA).

Results

Binding affinity of miR-376c to its target mRNAs in RISC using the MirTrap system

To identify the target mRNAs which miR-376c bind to and its binding affinity to these mRNAs within RISC, we carried out the MirTrap system and used mRNA array cards containing 89 different mRNAs. The MirTrap system is a relatively new assay which identifies the target mRNAs bound by a miRNA directly in RISC. This meets more the reality, because the binding to and the following translational inhibition of mRNAs by miRNAs occurs within RISC. We found 56 potential mRNA targets out of 89 mRNAs which could be bound by miR-376c within RISC. Table 1 summarizes these potential target mRNAs together the fold enrichment within RISC and their functions. This Table also shows that miR-376c is able to bind both oncogenic and tumor suppressive mRNAs. For example, miR-376c had the strongest binding affinity to MMP9 and BCL2 with an enrichment of 1018 and 927, respectively, and the weakest affinity to MLH1 involved in DNA repair and actin beta (ACTB) involved in invasion with an enrichment of 10 and 6, respectively.

Validation of mRNA targets using Western blot

We chose BCL2 (927-fold), Ki67 (572-fold), E-cadherin (CGH1, 158-fold) and p21 (CDKN1A, 112-fold) from Table 1 to check the inhibition of these mRNAs by miR-376c on a Western blot.

As shown on the slightly thinner and thicker band on the Western blot, transfection of MDA-MB-468 cells with mimics and inhibitors of miR-376c reduced and increased the protein expression of BCL2, respectively. However, this effect was not prominent (Fig. 1A). In contrast, transfection of these cells with mimics of miR-376c reduced the protein expression of p21 very strongly, as detected by the weak band on the Western blot, whereas inhibition of miR-376c had no effect on the protein expression of p21 (Fig. 1B). Finally, miR-376c had no effect on E-cadherin (Fig.1C) and Ki67 (Fig. 1D). Possibly, the expression of E-cadherin (Fig. 1C) and Ki67 (Fig. 1D) was too high in MDA-MB-468 cells, to see an effect by miR-376c.

Discussion

In the present study, we detected that miR-376c is able to bind target mRNAs of tumor suppressor genes and oncogenes within RISC. This miRNA had binding affinity to 56 mRNAs out of 89 selected mRNAs using the MirTrap system. We selected four target mRNAs from these 56 potential target mRNAs and carried out validation studies. Western blot showed that miR-376c could suppress the protein expression of BCL2[7], p21[8], but not of E-cadherin[9] and Ki67[10].

Various studies have shown that miR-376c is involved in regulating cell proliferation, migration, cell cycle and apoptosis in different cancer types, such as neuroblastoma[11], gastric cancer[12] and hepatocellular carcinoma[13]. Upregulated levels of miR-376c were detected in plasma and serum of breast cancer patients (regardless of the subtypes)[14]. In our previous study[6], we performed PCR-based microRNA array analyses to quantify miRNAs in exosomes of a large cohort of breast cancer patients with the subtypes of HER2-positive and triple-negative receptor. We found that miR-376c was significantly enriched in exosomes of breast cancer patients with triple-negative receptor status, a particularly aggressive tumor type[15], as compared with HER2-positive patients and healthy women. Moreover, the elevated levels of exosomal miR-376c were downregulated to normal levels after neoadjuvant therapy, indicating that this miRNA may be discharged from the primary tumor into the blood circulation to some extent, and the changes in the levels may directly reflect cancer status. These findings prompted us to search target mRNA molecules for miR-376c.

Notably, most cancer cells do not undergo apoptosis. This particular feature provides cancer cells a survival advantage over normal cells. The apoptosis inhibitor BCL2 is involved in this process and highly upregulated in many cancer types[16]. Performing the MirTrap assay, we found a 927-fold enrichment of BCL2 within RISC which was bound by miR-376c. However, we only observed a weak downregulation of the protein expression of BCL2 on a Western blot. This difference between the high enrichment of BCL2 within the miR-376-bound RISC and the weak inhibitory effect on the protein expression of BCL2 by miR-376c could be explained by the use of two different assays. Besides, it is well-known that repression of translation of mRNAs by miRNAs takes place in a competent environment, the RISC[17]. However, the Western blot was carried out with proteins derived

Table 1. Potential mRNA targets of miR-376c.

mRNAs Targeted by miR-376c	Fold Enrichment	Function
MMP9 (matrix metalloproteinase)	1018	metastasis
BCL2 (B cell lymphoma/leukemia)	927	apoptosis inhibitor
VEGFA (vascular endothelial growth factor)	669	angiogenesis
ABCB1 (P-glycoprotein)	633	drug pump
CDKN2A (cyclin-dependent kinase inhibitor)	595	cell cycle inhibitor
Ki-67	572	cell proliferation
ATM (ataxia telangiectasia mutated)	491	DNA repair
IGFBP3 (insulin-like growth factor binding protein)	302	cell proliferation
BRCA2 (breast cancer)	285	DNA repair
PTGS2 (prostaglandin)	280	apoptosis
CDKN1C (cyclin-dependent kinase inhibitor)	256	cell cycle inhibitor
MYC	252	cell proliferation
ERBB2 (HER2)	250	cell proliferation
IL6 (interleukin)	215	pro- and anti-inflammatory
EGFR (epidermal growth factor receptor)	195	cell proliferation
TMS1 (target of methylation induced silencing)	182	apoptosis
IGF1R (insulinl like growth factor receptor)	164	cell proliferation, differentiation
CDH1 (E-cadherin)	158	metastasis
CSF1 (colony stimulating factor 1)	156	development of macrophages and osteoclasts
MAPKB (mitogen-activated protein kinase)	154	proliferation, differentiation, apoptosis
BRCA1	153	DNA repair
NME1 (nucleoside diphosphate kinase)	151	metastasis suppressor
PRDM2 (methyltransferase PR domain containing)	145	histone/protein methyltransferase
THBS1 (Trombospondin 1)	129	angiogenesis inhibitor
CCNE1 (cyclin)	116	cell cycle progression
CDKN1A (cyclin-dependent kinase inhibitor)	112	cell cycle inhibitor
KRT19 (kreratin)	97	circulating tumor cells
ID1 (inhibitor of differentiation)	96	cell-cycle progression, migration
MUC1 (mucin)	93	circulating cancer cells
NR3C1 (nuclear receptor)	92	glucocorticoid receptor, inflammatory
CTSD (cathepsin)	88	peptidase
MAPK3 (mitogen-activated protein kinase)	80	invasion, metastasis
B2M (beta-2-microglobulin)	79	immune activation
HPRT1 (hypoxanthine phosphoribosyltransferase)	77	generation of purine nucleotides
KRT8 (keratin)	72	cellular structural integrity
MAPK1 (mitogen-activated protein kinase)	67	cell proliferation
SLC39A6 (solute carrier)	66	metabolism, differentiation
AKT1 (serine/threonine kinase protein kinase)	64	cell survival, cell cycle progression, growth
TGFB1 (tumor growth factor)	49	metastasis
CDK2 (cyclin-dependent kinase)	47	cell progression
PTEN (phosphatase and tensin homolog)	46	cell growth, proliferation, migration inhibitor
EGF (epidermal growth factor)	41	angiogenesis, metastasis
MGMT (O-6-methylguanine-DNA methyltransferase)	41	DNA repair
SFRP1 (secreted frizzled related protein)	39	proliferation, migration, invasion inhibitor
RPLPO (ribosomal protein)	37	cell cycle arrest
GRB7 (growth receptor bound protein)	34	cell proliferation, migration activator
BAD (Bcl-2 agonist of cell death)	33	apoptosis
TB53	30	DNA repair
CTNNB1 (catenin beta)	28	cell growth and adhesion
XBP1 (X-box binding protein)	26	cell proliferation, oxidative stress
KRT18 (keratin)	25	cell viability, migration, invasion
KRT5 (keratin)	23	cell viability, migration, invasion
CCND1 (cyclin)	22	cell proliferation
SFN (sulforaphane)	16	growth arrest, apoptosis
MLH1	10	DNA repair
ACTB (actin beta)	6	invasion, metastasis

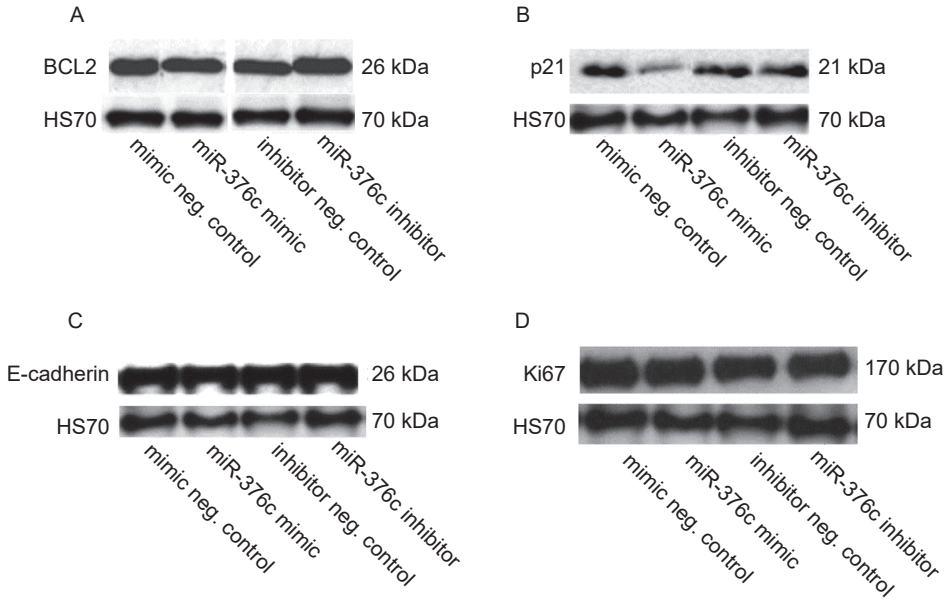

Fig. 1 Impact of miR-376c on protein expression of BCL2, p21, E-cadherin and Ki67.
A. Proteins were extracted from MDA-MB-468, transfected with mimics or inhibitors of miR-376c as well as with controls, and analyzed by Western blot using antibodies specific for BCL2.
B. p21.
C. E-cadherin.
D. Ki67.

from breast cancer cells that overexpressed miR-376c, but not RISC. Possibly, the MirTrap assay is more sensitive than Western blotting. It is unknown whether the binding of miR-376c to BCL2 in the cells transfected with miR-376c mimics or inhibitor, but not transfected with the plasmid encoding for RISC is dependent from cellular RISC and/or independent of it, but the overexpression of RISC performed by the MirTrap system could explain these data. While we only observed a weak decrease in BCL2 protein by miR-376c in breast cancer cells, Tu et al. detected in the gastric adenocarcinoma cell line SGC-7901, that downregulation of miR-376c significantly increased the expression levels of BCL-2 whereas its overexpression decreased the levels of BCL-2[12].

Using the MirTrap system, we detected a 112-fold enrichment of p21/CDKN1A within miR-376c-bound RISC. This strong binding activity was also in line with the significant repression of protein expression of p21/CDKN1A mediated by miR-376c as detected on the weak band of our Western blot. As far, as we know, no literature exists on the binding of miR-376c to p21/CDKN1A. A dual behavior was reported for p21/CDKN1A[18]. This cyclin-dependent kinase inhibitor was initially referred to be a tumor suppressor protein because it was recognized as the main mediator of p53-dependent cell cycle arrest caused by DNA damage[19]. Conversely, it has been proposed that p21/CDKN1A may also function as an oncogene because it can inhibit apoptosis[20]. That miR-376c is also involved in these processes was demonstrated in multiple neuroblastoma cell lines. Ectopic expression of miR-376c in these cells led to a significant inhibition of cell viability and G1-cell cycle arrest by reducing the expression of cyclin D1[11].

Applying the MirTrap assay, we also detected a 572-fold enrichment of Ki-67 and 158-fold enrichment of E-cadherin within miR-376c-bound RISC. However, Western blot showed no inhibitory effect of miR-376c on Ki-67 and E-cadherin. As above described, the different data could possibly be related to the expression level of RISC. To date, interactions of miR-376c with Ki-67 which is a proliferation marker and regulates cell cycle progression in human cells[21] as well as with E-cadherin have not been described. The cadherin family has been documented to play an essential role in mediating cell-to-cell adhesion and in breast cancer metastasis[22]. Overexpression of E-cadherin in MDA-MB-468 cells resulted in a more proliferative and less migratory character in vitro, whereas knockdown of E-cadherin displayed more migratory and invasive and less proliferative features taking longer to form tumors[23].

Using the MirTrap system, we detected a variety of mRNA targets for miR-376c which we have not further assessed on a Western blot. However, only a few of them have been reported in the literature. For example, a transwell migration assay revealed that miR-376c significantly reduced EGF-dependent cell migration in HuCCT1, a human intrahepatic cholangiocarcinoma cell line[24]. We found a 41-fold enrichment of EGF within miR-376c-bound RISC. In melanoma cells, stable over-expression of miR-376c led to a significant decrease in migration in IGF1R protein expression. A luciferase reporter assay indicated that the 3'UTR of IGF1R is a target of miR-376c[25]. We found a 164-fold enrichment of IGF1R within miR-376c-bound RISC.

In conclusion, we show a variety of tumor suppressors and oncogenes which may be potential mRNA target molecules for miR-376c using the MirTrap system. The strong binding activity of miR-376c to p21 whithin RISC also lead to a strong repression of protein expression of p21, as observed on a Western blot. In future, further single analyses including functional cell culture experiments and immunological assays are planned, to verify the MirTrap assay data.

Abbreviations

ATCC, American Type Culture Collection; EGF, epidermal growth factor; FCS, fetal calf serum; HER2, human epidermal growth factor receptor 2; IGF1R, insulin like growth factor 1 receptor; MMP9, matrix metalloproteinase 9; miRNAs, microRNAs; RISC, RNA-induced silencing complex, UTR, untranslated region; and see also Table 1.

Funding

This work was supported by the Wilhelm Sander Stiftung, Munich, Germany.

Acknowledgements

We thank Ms. Bettina Steinbach for her excellent technical assistance.

Authors' contributions

HS contributed to the study concept and design, HS, IS were responsible for data acquisition, HS, IS were responsible for data analysis and manuscript drafting. All authors approved the final version of the manuscript.

References

[1] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell, 2009, 136(2): 215-233.

[2] Gregory RI, Chendrimada TP, Cooch N, et al. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell, 2005, 123(4): 631-640.

[3] Berindan-Neagoe I, Monroig Pdel C, Pasculli B, et al. MicroRNAome genome: a treasure for cancer diagnosis and therapy. CA Cancer J Clin, 2014, 64(5): 311-336.

[4] Schwarzenbach H, Nishida N, Calin GA, et al. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol, 2014, 11(3): 145-156.

[5] Schwarzenbach H, Gahan PB. MicroRNA shuttle from cell-to-cell by exosomes and its impact in cancer. Noncoding RNA, 2019; 5(1).

[6] Stevic I, Muller V, Weber K, et al. Specific microRNA signatures in exosomes of triple-negative and HER2-positive breast cancer patients undergoing neoadjuvant therapy within the GeparSixto trial. BMC Med, 2018, 16(1): 179.

[7] Siddiqui WA, Ahad A, Ahsan H. The mystery of BCL2 family: Bcl-2 proteins and apoptosis: an update. Arch Toxicol, 2015, 89(3): 289-317.

[8] Kreis NN, Louwen F, Yuan J. The Multifaceted p21 (Cip1/Waf1/CDKN1A) in Cell Differentiation, Migration and Cancer Therapy. Cancers, 2019, 11(9).

[9] Wong TS, Gao W, Chan JY. Transcription regulation of E-cadherin by zinc finger E-box binding homeobox proteins in solid tumors. Biomed Res Int, 2014, 2014: 921564.

[10] Li LT, Jiang G, Chen Q, et al. Ki67 is a promising molecular target in the diagnosis of cancer (review). Mol Med Rep, 2015, 11(3): 1566-1572.

[11] Bhavsar SP, Lokke C, Flaegstad T, et al. Hsa-miR-376c-3p targets Cyclin D1 and induces G1-cell cycle arrest in neuroblastoma cells. Oncol Lett, 2018, 16(5): 6786-94.

[12] Tu L, Zhao E, Zhao W, et al. hsa-miR-376c-3p Regulates Gastric Tumor Growth Both In Vitro and In Vivo. Biomed Res Int, 2016, 2016: 9604257.

[13] Wang Y, Chang W, Chang W, et al. MicroRNA-376c-3p Facilitates Human Hepatocellular Carcinoma Progression via Repressing AT-Rich Interaction Domain 2. J Cancer, 2018, 9(22): 4187-96.

[14] Cuk K, Zucknick M, Madhavan D, et al. Plasma microRNA panel for minimally invasive detection of breast cancer. PloS one, 2013, 8(10): e76729.

[15] Feng Y, Spezia M, Huang S, et al. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes Dis, 2018, 5(2): 77-106.

[16] Radha G, Raghavan SC. BCL2: A promising cancer therapeutic target. Biochim Biophys Acta Rev Cancer, 2017, 1868(1): 309-14.

[17] Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat Struct Mol Biol, 2012, 19(6): 586-93.

[18] Zohny SF, Al-Malki AL, Zamzami MA, et al. p21(Waf1/Cip1): its paradoxical effect in the regulation of breast cancer. Breast Cancer, 2019, 26(2): 131-7.

[19] Caldon CE, Daly RJ, Sutherland RL, et al. Cell cycle control in breast cancer cells. J Cell Biochem, 2006, 97(2): 261-74.

[20] Karimian A, Ahmadi Y, Yousefi B. Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. DNA Repair (Amst), 2016, 42: 63-71.

[21] Sun X, Kaufman PD. Ki-67: more than a proliferation marker. Chromosoma, 2018, 127(2): 175-86.

[22] Li DM, Feng YM. Signaling mechanism of cell adhesion molecules in breast cancer metastasis: potential therapeutic targets. Breast Cancer Res Treat, 2011, 128(1): 7-21.

[23] Hugo HJ, Gunasinghe N, Hollier BG, et al. Epithelial requirement for in vitro proliferation and xenograft growth and metastasis of MDA-MB-468 human breast cancer cells: oncogenic rather than tumor-suppressive role of E-cadherin. Breast Cancer Res, 2017, 19(1): 86.

[24] Iwaki J, Kikuchi K, Mizuguchi Y, et al. MiR-376c down-regulation accelerates EGF-dependent migration by targeting GRB2 in the HuCCT1 human intrahepatic cholangiocarcinoma cell line. PloS one, 2013, 8(7): e69496.

[25] Zehavi L, Avraham R, Barzilai A, et al. Silencing of a large microRNA cluster on human chromosome 14q32 in melanoma: biological effects of mir-376a and mir-376c on insulin growth factor 1 receptor. Mol Cancer, 2012, 11: 44.